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“MiR-34c may protect lung cancer cells from paclitaxel-induced apoptosis”

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Abstract

MicroRNAs (miRNAs) constitute a class of small non-coding RNAs that negatively regulate gene expression at post-transcriptional level in a sequence specific manner. They are involved in many biological processes, including cell proliferation, apoptosis and differentiation, and are considered as promising new therapeutic targets for cancer. However, the identity of miRNAs involved in apoptosis and their respective targets remain largely unknown. Given the elevated complexity of miRNA regulation of gene expression, we performed a functional screening as an alternative strategy to identify those miRNAs that in lung cancer cells may interfere with the apoptotic process. To this aim we generated a derivative of the non-small cell lung carcinoma A549 cell line in which caspase-8, a critical upstream initiator of apoptosis, can be activated by the administration of the small dimerizer drug AP20187.

We found a number of miRNAs that may rescue cell viability from caspase-8 activation. They included miRNAs already described as oncogenic such as miR-17, miR-135, miR-520, but also some miRNAs such as miR-124-1 and miR-34c for which a tumor suppressive role has been instead described or expected. Among them, miR-34c-5p markedly increased resistance to paclitaxel induced apoptosis. We demonstrate that Bmf (Bcl-2 modifying factor) is a target of miR-34c-5p and that its silencing, together with that of c-myc, a known target of miR-34c-5p, contributes to resistance to apoptosis induced by paclitaxel via p53 downregulation.
1. Background

MiRNAs are a class of short endogenous non-coding RNAs that act as key regulators of cell proliferation and apoptosis through the control of gene expression either inhibiting translation or triggering degradation of multiple target mRNAs (Bartel 2009). MiRNAs have been found deregulated in different malignancies, implicating them as oncogenes or tumor suppressors and thus considered as promising new therapeutic targets for cancers (Farazi et al. 2011; Garofalo and Croce 2011). Indeed, the selective induction of cell death by oligonucleotide-based drugs is a challenging goal for a rationale approach to novel therapeutic strategies in cancer treatment.

Programmed cell death, or apoptosis, is mediated through two major pathways, the death receptor pathway and the mitochondrial pathway. In the death receptor pathway, stimulation of death receptors leads to the formation of a death-inducing signaling complex which includes the initiator caspases such as caspase-8 that drives its activation through self-cleavage and then activates several downstream effectors including caspase-9 and the ultimate effector caspase-3 (Ashkenazi and Dixit 1998). In the mitochondrial pathway, stimuli such as drugs, radiation, infectious agents and reactive oxygen species transmit the death signals to mitochondria through the activation of BID, a pro-apoptotic member of the Bcl-2 family (Hengartner 2000). Caspase-8-mediated cleavage of BID thus provides integration of the death-receptor with the mitochondrial pathway.

Identifying miRNAs that selectively regulate the expression of proteins involved in apoptosis could be helpful in the development of new tools for the diagnosis and the treatment of cancer (Inui et al. 2010).
1.1 MiRNAs

1.1.1 The discovery of miRNAs

MiRNAs were first discovered in mutant larvae of the nematode *Caenorhabditis elegans* as stRNA (small temporal RNA) involved in regulating the transition from one stage to another during development. Later it became clear that they represent the prototype of a large family of small RNAs called miRNAs, highly conserved during evolution. In larvae of *C. elegans* it was observed that the mutation in *lin-4* gene caused defects in temporal control during the post-embryonic development (Wightman et al. 1993). *Lin-4* encodes for a small RNA that binds the mRNA of the gene *lin-14* and silences its expression. During post-embryonic development, *C. elegans* undergoes four larval stages, from L1 to L4. The first stage of larval development, L1, is regulated by the transcription factor *lin-14* that promotes the expression of key genes in this phase and represses that of L2-specific genes. The levels of transcription factor *lin-14* are high at the beginning of the phase L1 and then decrease for the action of *lin-4* that blocks the translation of the messenger of *lin-14* by allowing the L2-specific gene expression. Following these observations thousand miRNAs have been identified in different species of animals and plants that regulate many biological processes such as proliferation, differentiation and programmed cell death.

1.1.2 MiRNAs: structure and biogenesis

MiRNAs are small (19-23 nucleotides) non-coding RNA molecules, widely conserved through the evolution, that negatively regulate gene expression at post-transcriptional level in a sequence specific manner. They bind a specific site in the 3’UTR of a target mRNA allowing to degradation of the messenger if there is a perfect complementarity between the miRNA and its 3’UTR, or translational repression in case of not perfect complementarity.

MiRNAs are first transcribed into long primary miRNAs (pri-miRNAs) by polymerase II or, in few rare cases, by polymerase III. Typically, pri-miRNAs display a 33 bp stem and a terminal loop structure with flanking segments. A single pri-miRNA may contain a "cluster" of different miRNAs or a single miRNA and can contain from 200 to several thousands of nucleotides (Lagos-Quintana et al. 2003; Cai et al. 2004). Primary miRNAs processing begins in the nucleus where an RNAse III enzyme, Drosha, removes the flanking segments and 11 bp of the stem region, inducing the conversion of pri-miRNAs
into precursor miRNAs (pre-miRNAs) (Lee et al. 2003). Pre-miRNAs are 60-70 nt long hairpin RNAs with 2-nt overhangs at the 3’ end. They are transported into the cytoplasm and subsequently processed by Dicer, a cytoplasmic endonuclease RNAse III enzyme, to create a double strand structure containing the mature miRNA and its complementary that will be degraded. The mature miRNA is then incorporated into the RISC (RNA-induced silencing complex) which facilitates the interaction between the miRNA and its target mRNA and function as an endonuclease that cuts the target mRNA, or, for steric hindrance, can block the binding of mRNA with the complex of protein synthesis (Figure 1) (Krol 2010).

Figure 1. Schematic representation of miRNA processing pathway

The pri-miRNA, transcribed by the polymerase II, is first clave by the RNAse Drosha in a pre-miRNA of ~ 70 nucleotides. The pre-miRNA is then exported into the cytoplasm by Exportin-5 and processed by the RNAse III Dicer. Mature single strand
miRNA is incorporated into the RISC (RNA-induced silencing complex) which facilitates the interaction between the miRNA and its target mRNA.

1.1.3 MiRNAs and cancer

Recent studies have shown that miRNAs are involved in different biological processes and pathological states, particularly in the development of cancer since they can act as “oncomirs” or “tumor suppressors” (Croce 2009). Thus, miRNAs represent a new class of promising diagnostic and prognostic biomarkers as well as new targets for cancer therapy (Fabbri 2010; Bartels et al. 2009). They are more useful tools than proteins and mRNAs, since a great fraction of them are regulatory molecules. Furthermore, being shorter than mRNAs, they are less vulnerable to degradation by ribonucleases and, because miRNAs are not structural modified, they are easier to detect than proteins. To date altered expression levels of miRNAs have been linked to various cancers; expression studies of various tumor types has revealed specific alterations in miRNA profiles, where some miRNAs are overexpressed and others strongly repressed, depending on the cancer type, disease stage and response to the treatment (Kitade and Akao 2010; Volinia et al. 2006; Esquela-Kerscher and Slack 2006; Lu et al.. 2005; Goga and Benz 2007).

The first evidence for miRNAs involvement in human cancer comes from a study by Calin et al (2002), examining a recurring deletion at chromosome 13q14 to search for a tumor suppressor gene involved in chronic lymphocytic leukemia (CLL). In this study was found that the region of deletion encodes two miRNAs, miR-15a and miR-16-1. Subsequent investigations have confirmed the involvement of these two miRNAs in the pathogenesis of CLL (Calin et al. 2005; Cimmino et al. 2005). Furthermore, Constinean et al reported that a miRNA by itself can induce a neoplastic disease (Costinean et al. 2006). In fact, by using a transgenic mouse model, they demonstrated that overexpression of miR-155 in B cells induce lymphoma pre-B leukemia.

Several other miRNAs deregulated in different human cancer types have been reported. For example, it has been demonstrated that let-7 family contains miRNAs regulating the RAS family of oncogenes (Johnson et al. 2005). Petrocca et al. (2008) showed that the miR-106b-25 cluster plays a key role in gastric cancer interfering with proteins involved both in cell cycle and apoptosis. In other studies, miR-155 was found overexpressed in Hodgkin lymphoma, pediatric Burkitt lymphoma and diffuse large B-cell Lymphoma (Eis et al. 2005; Kluiver et al. 2005; Metzler et al. 2004); miR-143 and miR-145 were significantly downregulated in colon cancer tissue compared with colonic mucosa (Michael et al. 2003); miR-21 was overexpressed in many
tumors (Volinia et al. 2006), including glioblastoma (Chan et al. 2005), cholangiocarcinoma (Meng et al. 2006), multiple myeloma cells (Loffler et al. 2007) and breast cancer (Si et al. 2007; Zhu et al. 2008).

Moreover, studies that investigated the expression of the entire microRNAome in various human solid tumors and hematologic malignancies have revealed differences in miRNA expression profiling between neoplastic and normal tissues (Calin et al. 2005, Ciafrè et al. 2005, Pallante et al. 2006, Weber et al. 2006). These studies show that each neoplasia has a distinct miRNA signature.

MiRNAs have an important role also in tumor metastasis. Indeed, for example miR10-b was found highly expressed in metastatic breast cancer cells (Ma et al. 2007) while miR-139 suppresses Metastasis of Hepatocellular Carcinoma (Wong et al. 2011). In addition a variety of tumor-specific miRNA expression profiles are highly predictive for the response to the therapy (Hummel et al. 2010).

More recently, many evidences are emerging that tumor-derived miRNAs are present and detectable in serum, plasma, urine and other human body fluids. Because of their abundance, tissue specificity and relative stability, circulating miRNAs hold a great promise as noninvasive or minimally invasive biomarkers in cancer (Kosaka et al. 2010; Laterza et al. 2009).

1.1.4 MiR-34 family

MiR-34 family is an evolutionarily conserved miRNA family including three different members in vertebrates: miR-34a, miR-34b, and miR-34c. MiR-34a is encoded by its own transcript on the human chromosome 1p36, whereas miR-34b and miR-34c are generated by the processing of a bicistronic transcript from the chromosome 11q23. In mice, miR-34a is ubiquitously expressed with the highest expression in brain, whereas miR-34b/c is mainly expressed in lung tissues. It has been reported that the tumor suppressor gene TP53, one of the genes most commonly mutated in cancer, acts as a transcriptional factor to induce the expression of a set of miRNAs including miR-34 family, following DNA damage and oncogenic stress (Chang et al. 2007; He et al. 2007a; Raver-Shapira et al. 2007; Tarasov et al. 2007; Bommer et al. 2007; Corney et al. 2007). Moreover, it has been demonstrated that miR-34c negatively regulate c-myc expression, which in turn activates the deacetylase protein Sirt1, that positively regulates p53 activity, thus establishing a positive feedback loop leading to cell cycle arrest. Still now how this loop is attenuated remains unknown.
Several converging evidences demonstrated that miR-34 members mediate p53 action negatively regulating cell cycle and thus acting as “bona fide” tumor suppressor genes (Hermeking 2010; Liu et al. 2011; Li et al. 2009; He et al. 2007b). In some cases, ectopic expression or delivery of synthetic miR-34 mimetics resulted in cell cycle arrest or senescence. In other cases, the output was apoptosis, with the response being reduced by inhibition or depletion of miR-34. (Yamakuchi et al. 2008; Rokhlin et al. 2008).

However there are also cases in which a different role for miR-34 has been demonstrated or suggested. Indeed, it has been recently shown that the cognate miRNA, miR-34a, may confer resistance to bortezomib-induced apoptosis by downregulating p53, and this survival and p53 downregulation depend on the expression levels of c-myc (Dacic et al. 2010; Lee et al. 2011; Sotillo et al. 2011). Furthermore it has also been reported that in some tumor miR-34c is surprisingly upregulated (Katada et al. 2009; Dacic et al. 2010). Therefore, the role of miR-34 family is still not completely clear and probably achieving these very different outcomes may depend on the wide spectrum of miR-34 regulatory targets that are differently expressed in a given cell type or cell conditions.
1.2 NSCLC

Lung cancer is the leading cause of cancer-related death in the world, with non-small-cell lung cancer (NSCLC) accounting for 80% of all cases (Greenlee et al. 2001). NSCLC is divided further into adenocarcinoma, squamous cell carcinoma (SCC), and large cell carcinoma histologies (Breuer et al. 2005). All share similar treatment approaches and prognoses but have distinct histologic and clinical features. Adenocarcinoma is the most frequent non-small cell lung cancer in the United States, representing 35-40% of all lung cancers. It originates from the bronchial mucosal glands and is the subtype observed most commonly in persons who don’t smoke. This type may manifest as multifocal tumors in a bronchoalveolar form. Bronchoalveolar carcinoma is a distinct subtype of adenocarcinoma with a classic manifestation as an interstitial lung disease on chest radiograph. Bronchoalveolar carcinoma arises from type II pneumocytes and grows along alveolar septa. This subtype may manifest as a solitary peripheral nodule, multifocal disease, or a rapidly progressing pneumonic form.

SCC accounts for 25-30% of all lung cancers. Whereas adenocarcinoma tumors are peripheral in origin, SCC is found in the central parts of the lung. The classic manifestation is a cavitary lesion in a proximal bronchus. This type is characterized histologically by the presence of keratin pearls and can be detected with cytological studies because it has a tendency to exfoliate.

Large cell carcinoma accounts for 10-15% of lung cancers, typically manifesting as a large peripheral mass on chest radiograph; it appears to be decreasing in incidence because of improved diagnostic techniques.

Histologically, this type has sheets of highly atypical cells with focal necrosis, with no evidence of keratinization (typical of SCC) or gland formation (typical of adenocarcinomas).

Types of lung cancer in the United States, as well as in many other countries, have also changed in the past few decades: the frequency of adenocarcinoma has risen, and that of SCC has declined. Both exposure (environmental or occupational) to particular agents and individual susceptibility to these agents contribute to the risk of developing lung cancer.

The main exposure agents are smoking, occupational exposure to asbestos, radon and outdoor air pollution. In addition, preexisting nonmalignant lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and tuberculosis have all been shown to be associated with increased lung cancer rates.

Most lung carcinomas are diagnosed at an advanced stage, conferring a poor prognosis. Indeed lung cancer is very insidious, and it may produce no symptoms until the disease is well advanced. Approximately 7-10% of patients...
with lung cancer are asymptomatic, and their cancers are diagnosed incidentally after a chest radiograph performed for other reasons. The need to diagnose lung cancer at an early and potentially curable stage is thus necessary, and for this purpose microRNAs represent very promising and innovative targets.
1.3 Programmed cell death

Apoptosis is an evolutionary conserved process of programmed cell death that occurs in various physiological and pathological situations (Hengartner 2000).

In mammals apoptosis proceeds through two distinct pathways: the mitochondrial pathway, also called intrinsic, and the death receptor pathway, also called extrinsic (Figure 2).

These two pathways, following specific initiator caspases activation, converge both in the activation of the same effector downstream caspases. Effector caspases are cysteiny l aspartate proteases, which proteolyze different cellular proteins and activate the enzyme CAD (caspase activated DNAs e) that degrades cellular DNA. This process finally cause cellular demolition associated with characteristic features, such as chromatin condensation and plasma membrane blebbing (Hengartner 2000; Salvesen and Dixit 1997; Shi 2002).

Figure 2. Scheme of extrinsic and intrinsic apoptotic pathways
In the extrinsic apoptotic pathway signals from death receptors activate the initiator caspase-8 which then cleaves and activates downstream effector caspases, such as caspase-3. In the mitochondrial pathway pathogenic infection, growth factor deprivation and a broad range of cytotoxic stresses lead to permeabilization of the outer mitochondrial membrane. Some proapoptotic proteins are so released, including cytochrome c which directly triggers caspase-3 activation through formation of cytochrome c/Apaf-1/caspase-9-containing apoptosome complex.

1.3.1 Extrinsic apoptotic pathway

In the extrinsic pathway apoptosis activation is a consequence of the stimulation of death receptors of the tumor necrosis factor (TNF) superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5). These stimulations lead to the activation of the initiator caspase-8 which then cleaves and activates downstream effector caspases, such as caspase-3 (Walczak and Krammer 2000).

TNF receptors superfamily includes more than 20 proteins with a wide range of biological functions: regulation of cell death and survival, differentiation and immune regulation (Walczak and Krammer 2000; Ashkenazi 2002).

All these receptors share similar cysteine-rich extracellular domains and a cytoplasmic domain of about 80 amino acids called ‘death domain’, which plays a crucial role in transmitting the death signal from the cell’s surface to intracellular signaling pathways.

Stimulation of death receptors results in receptor trimerization, clustering of the death domains and recruitment of adaptor molecules such as Fas-associated death domain (FADD) through homophilic interaction mediated by the death domain (Walczak and Krammer 2000). Fas-associated death domain in turn recruits caspase-8 to the activated receptor to form the death-inducing signaling complex (DISC). Oligomerization of caspase-8 upon DISC formation drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases, including caspase-3, -6 and -7. For the extrinsic signaling pathway, two distinct prototypic cell types have been identified (Scaffidi et al. 1998). In type I cells, caspase-8 is activated upon death receptor ligation at the DISC in quantities sufficient to directly activate downstream effector caspases such as caspase-3 (Scaffidi et al. 1998). In type II cells, however, the amount of active caspase-8 generated at the DISC is insufficient to activate caspase-3 and a mitochondrial amplification loop, through BID cleavage and activation, is necessary for full activation of effector caspases (Scaffidi et al. 1998).
1.3.2 Intrinsic apoptotic pathway

Intrinsic pathway can be activated in response to pathogenic infection, growth factor deprivation and different cytotoxic stresses, including DNA damage or hypoxia.

This pathway is regulated by the complex interactions of >15 proteins belonging to one pro-survival and two pro-apoptotic sub-groups of the Bcl-2 family.

The pro-survival Bcl-2 family members - Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl1 and Boo/Diva – share an amino acid sequence homology across four Bcl-2 homology (BH) domains and fold to form similar 3D structures (Muchmore et al. 1996; Lessene et al. 2008; Youle and Strasser 2008). The pro-apoptotic Bcl-2 family members, instead, can be divided into two distinct sub-groups. The so-called “multi-BH domain” pro-apoptotic Bcl-2 proteins, including Bax and Bak, that share all four BH domains plus the transmembrane region, and the pro-apoptotic BH3-only subgroup, comprising Bim/Bod, Puma/Bbc3, Bid, Noxa/Pmaip1, Bad, Hrk/DP5, Bmf and Bik/Blk/Nbk that share sequence similarity with each other and the wider Bcl-2 family only across the BH3 domain.

Caspases activation is closely linked to permeabilization of the outer mitochondrial membrane by pro-apoptotic members of the Bcl-2 family (Green and Kroemer 2004). Upon disruption of the outer mitochondrial membrane, a set of proteins normally found in the space between the inner and outer mitochondrial membranes is released, including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G (Saelens et al. 2004). Once in the cytosol, these apoptotic proteins trigger the execution of cell death by promoting caspases activation or by acting as caspase-independent death effectors (Saelens et al. 2004). The release of cytochrome c from mitochondria directly triggers caspase-3 activation through formation of cytochrome c/Apaf-1/caspase-9-containing apoptosome complex.
2. Aims of the study

Much of the effort in oncology is devoted to identify new effective molecular targets for cancer diagnosis and therapy. The selective induction of cell death by drugs or cytokines in cancer treatment is a challenging goal for a rationale approach to novel therapeutic strategies. However tumor cells often retain the ability to evade drug-induced death signals through the activation of antiapoptotic mechanisms, making some patients unresponsive toward the therapeutic approaches commonly used. Therefore identifying molecular basis of this resistance seems to be of primary importance.

MiRNAs are small non-coding RNAs that negatively regulate gene expression and are involved in the control of many biological processes, such as cell proliferation and apoptosis. Since miRNAs have been found deregulated in different malignancies, acting as oncogenes or tumor suppressors, they are considered new promising therapeutic targets for cancers (Farazi et al. 2011; Garofalo and Croce 2011). Identifying miRNAs that selectively regulate the expression of proteins involved in apoptosis could be helpful in the development of new tools for diagnosis and the treatment of cancer. However, the potential for combinatorial regulation of gene expression by miRNAs makes it difficult to understand what target molecules are involved and what is their coordinate mechanism of action.

To this aim we developed a “functional selection-based screening” as an alternative approach to high-throughput screening to identify those miRNAs able to rescue cells from apoptosis in lung cancer cells. This approach has the advantage to identify unique functional combinations of miRNA molecules without any assumption about the targets involved. Since a pivotal upstream event in the death receptor pathways is the activation of caspase-8, the screening has been based on the use of an engineered lung cancer cell line in which the activation of caspase-8 has been placed under the control of a dimerizing agent named AP20187. Here we show that this is an unbiased approach able to uncover unpredicted effects of miRNA expression as in the case of miR-34c, a p53 effector miRNA (Chang, et al. 2007; He, et al. 2007), that revealed to be able to antagonize paclitaxel-induced apoptosis, with obvious fall-outs in the design of miRNA-based new therapeutic agents for cancer treatment.
3. Materials and Methods

3.1 Cell Cultures
Human NSCLC A549 and H460 (American Type Culture Collection) were grown in RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Corp. Saint Louis, MO, USA), 2mM L-glutamine and 100 U/ml penicillin–streptomycin. A549-FK, clone Cl-K and clone Cl-T were cultured in the same medium supplemented with 5 µg/ml puromycin, while A549 stably silenced for p53 were cultured in medium supplemented with 1 µg/ml puromycin.

3.2 Immunoprecipitation and immunoblotting
Total cell lysates were prepared in JS buffer (50 mM Hepes ph 7.5, 150 mM NaCl, 1%Glicerol, 1%Triton X-10, 1.5 mM MgCl₂, 5 mM EGTA, 1 mM Na3VO4 and protease and phosphatase inhibitors) and then boiled in SDS/β-mercaptoethanol sample buffer. 40 or 30 µg samples were loaded into 15% or 12% polyacrylamide gels and the proteins were separated by electrophoresis and then blotted onto PVDF membranes (Millipore, Billerica, MA, USA) by electrophoretic transfer. The membranes were then blocked with 5% dried milk in TBS containing 0,1% Tween 20 and incubated at 4°C overnight with the following primary antibodies: anti-caspase 3, anti-caspase 9, anti-BID, anti-Bcl-XL, anti-Bmf, anti-Acetyl-p53 (Lys382), anti-cyclin D1 (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-procaspase-3 and anti-procaspase-9 (Abcam plc, Cambridge, UK), anti-c-myc (9E10), anti-p53 (DO-1), anti-Chk1 (G-4), anti-cyclin B1 (H-433), anti-SIRT1 (H-300) (Santa Cruz, CA, USA), anti-α-tubulin (Sigma-Aldrich Corp. Saint Louis, MO, USA) and anti-MDM2 (mAB 2A10) (Calbiochem).

For Bax activation analysis total cell lysates were, instead, prepared in 1% Chaps buffer (5mM MgCl₂, 137 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Chaps, 20 mM Tris-HCl ph 7.5 and protease inhibitors. 500 µg proteins were immunoprecipitated with anti-Bax antibody (6A7; BD Pharmingen) at 4°C for 2 h. Immunoprecipitates were captured by a Protein A/G PLUS-Agarose (Santa Cruz, CA, USA) in lysis buffer at 4°C overnight. Immunoprecipitates were then recovered by centrifugation and washed three times in 1% Chaps buffer. Immunoprecipitates and total extracts (40 µg) were separated on 12% polyacrylamide gel. After SDS-PAGE, proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and then blocked with 5% dried milk in PBS-0,1% Tween 20. Membranes were then incubated with primary anti-Bax (N20) (Santa Cruz, CA, USA) and peroxidase (HRP)-conjugated
secondary antibodies in 10% dried milk in PBS containing 0.1% Tween 20 and detected with ECL Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ).

3.3 Cell proliferation and cell death analysis

Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO₂ incubator. AP20187 (ARIAD Pharmaceuticals, Inc., Cambridge, MA, USA) was used for 3 h, 6 h and 24 h at 10 nM. Cell viability was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega BioSciences Inc. San Luis Obispo, CA, USA), according to the manufacturer’s protocol. Metabolically active cells were detected by adding 20 µl of MTT to each well. After 20 min of incubation, the plates were analyzed in an Absorbance Microplate Reader (ELx800 BioTek Instruments, Inc.). Apoptosis was assessed using propidium iodide assay followed by flow cytometric analysis and caspase-3 and -9 fluorimetric assays. For propidium iodide assay, 24 h after transfection, cells were seeded in triplicate in 96-well plates at 3.6×10³ cells per well and grown overnight at 37°C in a 5% CO₂ incubator. Then cells were treated with 10 nM AP20187 (ARIAD Pharmaceuticals, Inc., Cambridge, MA, USA) for 6 h or with 100 nM Paclitaxel (Sigma-Aldrich Corp. Saint Louis, MO, USA) for 24 h. The next day analysis of DNA content by propidium iodide (Sigma-Aldrich Corp. Saint Louis, MO, USA) incorporation was performed in permeabilized cells by flow cytometry. For caspase-3 and -9 fluorimetric assays, 50 µg of total cell lysates were incubated for 1 h at 37°C with DEVD-AFC or LEHD-AFC substrate respectively, according to the manufacturer’s protocol (BioVision Inc. Mountain View, California). After incubation samples have been read in a fluorimeter equipped with 400 nm excitation filter and 505 nm emission filter. For cell cycle analysis cells transfected with 70 nM of miRNA precursor hsa-miR-34c-5p or a scrambled molecule were recovered 48h and 72h post-transfection, fixed in 70% ethanol, stained with 50 µg/ml propidium iodide (Sigma-Aldrich Corp. Saint Louis, MO, USA) and analyzed by FACS (Fluorescence-activated cell sorter).
3.4 Cell transfections

The day before transfection cells were seeded in 10% FBS medium without antibiotics. All transfections were performed in serum-free Opti-MEM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. To alter miRNA level cells were transfected with 70 nM of miRNA precursor has-miR-34c-5p, has-miR-34c-3p, has-miR-124, has-miR-124*, has-miR-362-5p, has-miR-362-3p and Negative Control #1 (Ambion) or 50 nM of LNA anti-miR-34c-5p, anti-miR-34c-3p and anti-miR negative control inhibitor (Exiqon). To alter c-myc and Bmf level cells were transfected with 60 nM siRNA c-myc (Sigma-Aldrich Corp. Saint Louis, MO, USA) and 120 nM siRNA Bmf (Santa Cruz, CA, USA).

For p53 stable gene silencing, A549 cells were transfected in 100 mm dishes with 10 µg of shRNAp53 or shRNAcntrl (Open Biosystems). For stable clone selection RPMI medium supplemented with 1 µg/ml puromycin was used.

3.5 Luciferase assays

For target validation the two Bmf 3' UTR sequences were PCR amplified using the following primers: BMF S1 Fw: 5'-CTAGAAGGATTTTCTGGCCCAGCCTGCT-3', BMF S1 Rw: 5'-TCTAGATTTCCTCGCCCTGACAAACTTG-3'; BMF S2 Fw: 5'-TCTAGAAGGATCAAGGTGGTACAGGCACAGC-3'; BMF S2 Rw: 5'-TCTAGATACCTCCTGAGTTTCTGTGGAGGA-3'; BMF S3 Fw: 5'-TCTAGAATTGCTTTAAGACCCACGAGCTTCG-3'; BMF S3 Rw: 5'-TCTAGACACTGTCCTGGCTTCTCTCATTCA-3'; BMF S4 Fw: 5'-TCTAGATGGGGCTACTTCTCCAAAGAAGAAG-3'; BMF S4 Rw: 5'-TCTAGAGAGGGAGAAACTATGTATGCACCCAT-3' and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega BioSciences Inc. San Luis Obispo, CA, USA). We deleted a sequence in S2 and S3 miRNA-binding site by using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) using the following primers: BMF S2 mut Fw 5'-CTGGGGAGGCTGGAAGCTGACACTGTC-3' BMF S2 mut Rw 5'-GACCCCGTCCACCTTGAGCTGACAGC-3'; BMF S3 mut Rw: 5'-GTTTCTTACTTCTGACCCCTCAGACAGCAGC-3'; S3 mut Rw: 5'-TGCTGGAGGTTGAGCATGGAAGTAAGAACC-3'.

The megakaryoblastic cell line, MEG-01, as widely used efficient recipient was use for luciferase assays. Cells were cotransfected with 1.2 µg of each generated plasmid and 400 ng of a Renilla luciferase expression construct pRL-TK (Promega BioSciences Inc. San Luis Obispo, CA, USA) with Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post-transfection and assayed with Dual Luciferase Assay (Promega BioSciences Inc. San Luis
Obispo, CA, USA) according to the manufacturer’s instructions. Three independent experiments were performed in triplicate.

For p53 promoter activity analysis cells were transfected with Lipofectamine 2000 (Invitrogen) with 500 ng of pLuc-MDM2 expression vector (kindly provided by S. Soddu), 900 ng of miRNA precursor hsa-miR-34c-5p or Negative Control #1 (Ambion), together with 40 ng of pCMV-Renilla in 6 well plates. Luciferase and Renilla activity were assessed with Luciferase assay system and Renilla Luciferase assay system (Promega BioSciences Inc. San Luis Obispo, CA, USA) 48 h after the transfection. The Luciferase activity was normalized for the Renilla activity. All the experiments were performed three times in triplicate and the mean±s.d. was reported.

3.6 qRT-PCR analysis

Reverse transcription reaction was done starting from 1 µg total RNA and using miScript Reverse Transcription Kit (QIAGEN, Milan Italy), according to the manufacturer’s protocol. The expression of mature hsa-miR-34c-5p, has-miR-34c-3p, hsa-miR-362-5p, hsa-miR-362-3p, hsa-miR-124, hsa-miR-124* and of U6 RNA, as housekeeping gene, was assayed using the miScript SYBR Green PCR Kit (QIAGEN) and Real-time PCR was done in triplicate for each case. MiRNA expression was measured using Ct (threshold cycle). The ΔΔCt method for relative quantization of gene expression was used to determine miRNA expression levels. The ΔCt was calculated by subtracting the Ct of U6 RNA from the Ct of the miRNA of interest. The ΔΔCt was calculated by subtracting the ΔCt of the reference sample (A549-FK cells not transduced with miRNAs) from the ΔCt of each sample. Fold change was generated using the equation 2-ΔΔCt.
4. Results and Discussion

4.1 Identification of protective miRNAs by functional selection

In order to identify miRNAs able to rescue cells from caspase-8 induced cell death we adopted a functional selection screening based on a FKBP caspase-8 inducible chimera (puro-DD-FKC8) (Carlotti 2005; Chang and Yang 2003). The human non small cell lung cancer (NSCLC) A549 cells have been transduced with a retroviral vector with inserted the caspase-8 chimera and the most responsive cell clone, the A549-FK, was then transduced with a pooled miRNA library and subjected to selection for resistance to caspase-8 induction of cell death, as described in Figure 3a.

In out of 16 cell clones analyzed we found integrated miRNAs already described as oncogenic such as miR-17, miR-135 (Nagel et al. 2008), miR-520 (Huang et al. 2008), but also some miRNAs such as miR-124-1 and miR-34c for which a tumor suppressive role has been instead described or expected. The most frequent integrated miRNA was miR-34c that was as well the only miRNA that was found not only in combination with other miRNAs but also alone in two independent cell clones, thus suggesting to be sufficient by its own to confer resistance to caspase-8 activation (Figure 3b). Other miRNAs were always found to be present as a combination of at least two different molecules (not shown). By qRT-PCR we thus evaluated in all cell clones the expression levels of transduced miRNAs (as compared to non-transduced control cells) and assessed that, with the only exception of miR-645 in the clone Cl-Y, all transduced miRNAs are expressed in the corresponding surviving clones (not shown). In the case of miR-34c we assessed the expression levels of both the -5p and -3p strands in all clones analyzed (Figure 3c).
Figure 3. Functional selection of miRNAs

(a) Schematic representation of the functional selection procedure. A549-FK cells were infected with a lenti-miR pooled library containing 330 human pre-miRNAs. In infected cells apoptosis was then induced by treatment with AP20187 dimerizer (10
nM, 15 nM and 25 nM) for 3h or 6h. Surviving clones were amplified and integrated miRNAs were identified by sequencing. (b) Relative abundance of transduced miRNAs in cell clones resulting from the screening. Only miRNAs found in at least two independent cell clones are reported. With the exception of miR-34c all miRNAs were always found only as a combination of multiple miRNAs. (c) Relative expression of miR-34c-5p and miR-34c-3p in clones where miR-34c was found alone (Cl-T and Cl-8) or in combination with other transduced miRNAs: in Cl-H (miR-17, miR-34a, miR-34c, miR-520e, miR-644), Cl-Y (miR-34c, miR-346), Cl-1 (miR-34a, miR-34c, miR-346), Cl-2 (miR-34a, miR-34c, miR-135a-2, miR-320a, miR-345, miR-346, miR-362), Cl-K (miR-34c, miR-124-1, miR-362), Cl-12 (miR-34a, miR-34c, miR-135a-2, miR-320a, miR-346), Cl-17 (miR-34c, miR-345 and miR362), Cl-53 (miR-34a, miR-34c, miR-362).

4.2 miR-34c-5p protects cells from capase-8 induced apoptosis

Given the high frequency of integration of miR-34c in surviving clones, we wondered if, in our system, forced over-expression of the most expressed isoform, miR-34c-5p, might protect cells from caspase-8 induced apoptosis. As determined by MTT assay (Figure 4a) all clones transduced with miR-34c are resistant to 10 nM AP20187 regardless if it was present alone (in two clones, Cl-T; Cl-8) or in combination with other miRNAs (in clones Cl-H; Cl-K; Cl-Y; Cl-1; Cl-2; Cl-12; Cl-17; Cl-53) (see legend to Figure 3c). In A549-FK cells, caspase-8 dimerization activates caspase-3 (Figure 4b, lanes 1-3) through cleavage of the BH3-only protein BID (Figure 4c), that leads to activation of Bax (Figure 4d) and caspase-9 (Figure 4b middle panel) (Jost et al. 2009). In contrast to parental A549-FK, both cell clones analyzed (Cl-T and Cl-K), in which miR-34c was integrated either alone (Cl-T) or together with miR-124-1 and miR-362 (Cl-K), behave resistant to AP20187-induced apoptosis (Figure 4b-d). Resistance is unlikely due to the occurrence of new clonal mutations impairing caspsases that are indeed activated following cisplatin treatment (Figure 4e). Conversely, transfecting the Cl-T and Cl-K with a miR34c-5p inhibitor sensitizes cells to AP20187-induced cell death with a consequent increase of caspase-3 and caspase-9 activity (Figure 4f and not shown).
Figure 4. Cell clones expressing miR-34c-5p are protected from AP20187 induced apoptosis

(a) MTT assay for resistance to AP20187 of cell clones that express miR-34c. A549-FK (Cntl) cells and individual cell clones were plated in triplicate in 96-well plates and treated with 10 nM AP20187 for 3 h. Values are expressed as the per cent of the untreated control; (b) parental A549-FK (lanes 1-3) or Cl-K (transduced with miR-
34c, miR-362 and miR-124-1) or Cl-T (transduced with miR-34c) were treated for the times indicated with AP20187 (10 nM) and levels of caspase-3 and caspase-9 cleaved products were analyzed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. (c) the same as in (b) except that levels of BID cleaved products were analyzed. (d) A549-FK, Cl-K or Cl-T were treated for 3 h with AP20187 (10 nM) or left untreated and cell lysates analyzed by immunoblot with anti Bax N-20 antibody. Lysates were either first immunoprecipitated with anti Bax6A7 antibody, specific for the active conformation of Bax (upper panel) or directly used for immunoblot analysis (lower panel). Intensity of bands was measured by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition. Values were each normalized for the corresponding values in the direct blot and are expressed as the per cent of the intensity measured in the second lane (labeled with asterisk). (e) Parental A549-FK cells or Cl-K or Cl-T were treated for 24 h with cisplatin (50µg/ml) and levels of caspase-3 cleaved products were analyzed by immunoblot. The blots (in b,c,d,e) are representative of at least four independent experiments. (f) A549-FK or Cl-T cells were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either anti-miR-34c-5p inhibitor sequence or a corresponding scrambled sequence was transfected and cell lysates analyzed for caspase-3 (upper panel) and caspase-9 (lower panel) activity as measured by hydrolysis of Ac-DEVD-AFC or Ac-LEHD-AFC respectively.

Further, upon AP20187 treatment, the presence of miR-34c-5p, but not of miR-34c-3p (not shown), makes the cells more resistant to caspase-8 induced cell death, reducing the per cent of apoptotic cells from 70% (of scrambled control) to around 30% (Figure 5a). Consistently, resistance to apoptosis conferred by miR-34c-5p is accompanied by the lack of activation of caspase-9 and of its effector, caspase-3, with an increase in the overall cell survival (Figure 5 b-d). These data indicate that while in a proliferating cell population miR-34c-5p may induce an increase in apoptosis (Chang et al. 2007; He et al. 2007), in the presence of a strong acute pro-apoptotic signal, as the dimerization of caspase-8, it may, in contrast, elicit a clear protective effect.
Figure 5. miR-34c-5p interferes with AP20187 induction of apoptosis

(a) A549-FK cells (Cntrl) cells were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic sequence or a corresponding scrambled sequence was transfected. The per cent of cells in the pre-G1 fraction was determined by FACS analysis as a measure of apoptotic cells. (b, c) A549-FK cells were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic or a corresponding scrambled sequence was transfected and caspase-9 (b) and caspase-3 activity (c) analyzed as in Figure 4f. (d) A549-FK cells (Cntrl) cells were treated with AP20187 (10 nM) as indicated. Where indicated two different scrambled sequences, miR-34c-5p, miR-34c-3p mimic sequences or both miRNAs together were transfected and cell viability was determined by MTT assay as per cent of corresponding untreated cells (see legend to Figure 4a).

This effect is unlikely due to the occurrence of new clonal critical mutations in the apoptotic pathway, indeed, 1): in two analyzed cell clones, Cl-K and Cl-T, caspase-8 sensitivity is restored by antagonizing miR-34c-5p action with a specific antimiR; 2) apoptosis is readily activated by treating the same cell clones with an unrelated proapoptotic drug like Cisplatin; and 3) transfecting miR-34c-5p mimic protects the A549-FK cells by AP20187-induced cell death.
4.3 miR34-c-5p protects cells from paclitaxel induced apoptosis

We thus determined whether miR-34c-5p has antiapoptotic function toward the broadly used chemotherapeutic agents. As shown in Figure 6a, treating parental A549 cells with paclitaxel for 24 h (100 nM) induces caspase-3 and caspase-9 activation that were partially reverted by transfecting cells with miR-34c-5p mimic (left panel) but not with miR-34c-3p mimic (right panel). Consistently, transfecting miR-34c-5p in A549 induces a clear reduction in the percent of paclitaxel-induced apoptosis (Figure 6b). On the other hand, antagonizing with specific LNA-based miR inhibitors the miR34c-5p, but not the miR34c-3p, rescued the sensitivity to paclitaxel-induced apoptosis in Cl-K cells (Figure 6c). A similar protective effect against paclitaxel is elicited by miR-34c in A549-FK, but not in the more sensitive H460 NSCLC cells (not shown). Further, differently from what observed with paclitaxel, transfecting miR-34c-5p mimic doesn’t reduce the levels of active caspase-3 and caspase-9 neither upon TRAIL (200 ng/ml) nor cisplatin (50 µg/ml) treatments but, at least in transient, miR-34c-5p may cooperate with cisplatin (Figure 6 d-e). Taken together these results well support the notion that the protective potential of miR-34c may be only unveiled in a cell context and pathway specific manner.
Figure 6. miR-34c-5p protects from paclitaxel-induced apoptosis

(a) A549 cells were transfected with either miR-34c-5p mimic sequence (left panel) or miR-34c-3p (right panel) and treated for 24 h with paclitaxel (100 nM). Levels of caspase-3 and caspase-9 cleaved products were analyzed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments. (b) A549 cells (Cntrl) cells were treated for 24 h with paclitaxel (100 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic sequence or a corresponding scrambled sequence was transfected. The per cent of cells in the pre-G1 fraction was determined by FACS.
analysis as a measure of apoptotic cells. (c) Cl-K was transfected with LNA anti-miR-34c-5p, anti-miR-34c-3p or anti-miR negative control inhibitor. Levels of caspase-3 and caspase-9 cleaved products were analyzed by immunoblot compared to A549-FK control cells. Filters were hybridized with anti α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments. (d,e) A549 cells transfected with either miR-34c-5p mimic sequence or a corresponding scrambled sequence were treated with TRAIL (d) or cisplatin (e) for times and concentrations indicated. Levels of caspase-3 and caspase-9 cleaved products were analyzed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments.

4.4 Targets of miR-34c-5p

In an attempt to understand the molecular events elicited by high levels of miR-34c-5p on apoptosis, and thus what are the target proteins involved, we first used a small antibody array to analyze the relative expression of a selected number of apoptosis related proteins in A549-FK cells transfected with miR-34c-5p (not shown). Since, using this assay, no major changes were observed we thus decided to take advantage of the information coming from the functional screening by assuming that the pathways involved may be shared between the different miRNAs selected for their ability to rescue A549-FK cells from caspase-8 DD-FKC8 chimera-induced cell death. We thus combined the bioinformatics prediction (Diana-microT3) for targets common to those miRNAs transduced and expressed in three independent cell clones together with miR-34c-5p : i.e. in Cl-17 (miR-345 and miR362), in Cl-K (miR-362 and miR-124-1), in Cl-Y (miR-346). Among the candidate targets considered, even if with different score values (see legend to Figure 7), Bmf (Bcl2-modifying factor) was predicted to be a common target for all of miRNAs analyzed. In the 3’ UTR of Bmf two recognition sites are predicted for miR-34c-5p, one at position 737-765, and one at position 2283-2311 (Diana-microT3) (Figure 7a). Moreover, in the 3’ UTR of Bmf are also predicted by Diana-microT3 two recognition sites for miR-34c-3p, the mature form of the microRNA that doesn’t seem to protect cells from AP20187- and paclitaxel-induced apoptosis. Therefore, at first we determined whether Bmf might be a direct target of miR-34c-5p. As shown in Figure 7b, transfecting miR-34c-5p either in A549-FK or in parental A549 cells resulted in a decrease of Bmf protein (of approximately 60%). Furthermore, in two miR-34c infected cell clones analyzed, that over express miR-34c-5p either alone (Cl-T) or in combination with other miRNAs (Cl-K), the levels of Bmf are reduced as compared to parental A549-FK cells (Figure 7c). Conversely, by using specific LNA based inhibitors to antagonize the miR34c-5p and -3p in clone T we rescued the expression of BMF (Figure 7d). To prove a direct interaction between miR-34c-5p and Bmf mRNA, two
Bmf 3′-UTR sequences (BMF S1 and BMF S2), which include each of the two potential target sites for miR-34c-5p, were fused downstream of the luciferase gene. The two constructs containing either BMF S1 or BMF S2, either individually or in combination, were cotransfected into MEG-01 cells together with miR-34c-5p, or a scrambled miRNA as negative control. As shown in Figure 7e, luciferase activity of BMF S2 was decreased of around 30% by cotransflecting miR-34c-5p. In contrast luciferase activity of both the BMF S1 and a deletion mutant of BMF S2 are poorly affected by miR-34c-5p cotransfection. Taken together these data indicate that miR-34c-5p may decrease Bmf expression by recognizing a binding site on its 3′ UTR starting at position 2283. As mentioned above, we identified BMF as promising target for miR-34c-5p based on the assumption that common messengers should be targeted by miRNAs found in independent cell clones together with miR-34c. In order to verify this assumption we determined whether miR-362 and miR-124 may affect as well Bmf levels. As shown transfecting either miR-362 or miR-124 decrease Bmf levels (Figure 7f). Further we determined by luciferase assay whether miR-362 interacts with the predicted sites for the miR-362-5p and -3p strand in the Bmf 3′ UTR (BMF S3 and S4 respectively). As shown in Figure 7g, luciferase activity of BMF S3 was decreased of around 60% by cotransfecting miR-362-5p, and at much lower extent miR-362-3p. In contrast, luciferase activity of the BMF S3 mutant is poorly affected by miR-362-5p cotransfection. These results well support the working hypothesis that miRNAs for common targets may be preferentially selected during the functional screening.
Figure 7. Bmf as target of miR-34c-5p

(a) Bmf 3’UTR contains two predicted miR-34c binding sites. In the figure is shown the alignment of the two seed regions (S1 and S2) of miR-34c with Bmf 3’UTR. The sites of target mutagenesis (deletion) are indicated by red dashes and red XXX. (b) A549 or A549-FK cells were transfected with either miR-34c-5p or control scrambled sequence and cell lysates analyzed for the expression of Bmf. (c) A549-FK, Cl-K and Cl-T cell lysates were analyzed for the expression of Bmf. Intensity of bands was measured as in Figure 4. In the insert are reported the miRNAs transduced in the Cl-K and Cl-T and their relative (as compared to A549-FK control cells) expression levels.
Values below the blot (in b and e) indicate signal levels of Bmf relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 4. (d) Cl-K was transfected with LNA anti-miR-34c-5p, anti-miR-34c-3p or anti-miR negative control inhibitor and levels of Bmf were analyzed compared with that of A549-FK control cells. (e) pGL3-Bmf luciferase constructs, containing a wild type (left side of the histogram) or mutated (right side of the histogram) Bmf 3’ UTRs, are transfected into MEG01 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results. The predicted recognition site of BMF at position 737-765 (S1) is less conserved (only bosTau2 genome) than that at position 2283-2311 (S2) that is conserved in bosTau2 and rat (rn4). The predicted free energies were calculated and are: -15.1 kJ for BMF S1 and -24.2 kJ for BMF S2, (f) A549 cells were transfected with miR-124, miR-124*, miR-362-5p, miR-362-3p or a control scrambled sequence and Bmf protein levels were analyzed at indicated times by immunoblot. (g) Bmf 3’UTR contains predicted binding sites for miR-362-5p and miR-362-3p. In the figure is shown the alignment of the two seed regions (S3 for miR-362-5p and S4 for miR-362-3p) of miR-362-5p and miR-362-3p with Bmf 3’UTR. The sites of target mutagenesis (deletion) are indicated by red dashes and red XXX. pGL3-Bmf luciferase constructs, containing wild type (either for miR-362-5p and miR-362-3p) or mutated (for miR-362-5p) Bmf 3’ UTRs, are transfected into MEG01 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results.

Diana Lab: miTG score for Bmf (miR-34c, 5.72; miR-124, 10.89; miR-362-5p, 3; miR-362-3p, 2.11; miR-345, 5.0; miR-346, 2).

For all the blots shown anti α-tubulin was used to confirm equal loading. The blots are representative of at least four independent experiments.

4.5 Mechanism of action

In order to understand if the decrease of Bmf levels by miR-34c-5p is sufficient to cause protection to apoptosis, we transfected A549-FK cells with either the miR-34c-5p or with an siRNA for Bmf and analyzed the activation of caspases-9 and -3 after forced dimerization of caspase-8. Interfering with Bmf expression, either by miR-34c-5p or by Bmf specific siRNA protects the cells from AP20187-induced caspases activation even if the Bmf siRNA is less effective than miR-34c-5p mimic (Figure 8a). Intriguingly, miR-34c, belongs to a family of evolutionarily conserved miRNAs (miR-34a, miR-34b, and miR-34c), whose expression has been shown to be under the transcriptional control of the tumor suppressor protein p53 and known to be implicated in the negative control of the cell cycle, senescence and apoptosis (Corney et al. 2007; Bommer et al. 2007; He et al. 2007; Hermeking 2010). Therefore, with the aim to reconcile this with the protective effect of miR-34c-5p on paclitaxel-induced apoptosis we asked whether c-myc, a known target of miR-34c (Cannell and
Bushell 2010), may as well participate to inhibit apoptosis. Indeed, c-myc, acting on the protein deacetylase Sirt1, regulates p53 acetylation, and thus its activity (van Leeuwen and Lain 2009). As expected, transfecting A549-FK cells either with miR-34c-5p, or with a c-myc specific siRNA, resulted in a partial decrease of c-myc protein levels (Figure 8b). Interestingly, attenuation of c-myc expression protects cells from AP20187 and most importantly from paclitaxel-induced caspase-3 activation (Figure 8c). In order to determine whether c-myc downregulation mediates miR-34c-induced protection, we depleted A549 cells of c-myc by transfecting a specific siRNA. As shown in Figure 8d, we found that miR-34c-5p become poorly efficient to further increase protection over that induced by c-myc silencing. Even though, the contribution to protection of the passenger -3p strand remains to be elucidated, we show that the miR-34c-3p mimic we used decreases the Bmf levels, but is less effective on c-myc (Figure 8e). This suggests that c-myc is a critical target of miR-34c-5p that may act by protecting cells from paclitaxel-induced apoptosis.

Paclitaxel is a microtubule destabilizer and cell cycle integrity has been shown to be required for sensitization (Chabavier et al. 2006; Zachos et al. 2007). We thus determined whether perturbation of c-myc levels by miR-34c-5p affects cell cycle thus providing an explanation for resistance. As assessed by FACS analysis, cell cycle is not appreciably impaired by the transfection of miR-34c-5p (Figure 8f). Accordingly, as determined by immunoblot, no drastic changes were observed upon transfection of the miR-34c-5p, neither in cyclins B1, D1 nor in Chk1 levels (Figure 8g). Similarly, cyclin B1 induction following treatment with paclitaxel is not impaired by miR-34c-5p thus indicating that resistance is unlikely attributable to abnormal mitotic checkpoint response (Figure 8h). On the other hand, the levels of cyclin D1, that mediates p53-dependent cell cycle arrest, were increased by paclitaxel, while increasing is impaired in the presence of miR-34c-5p (Figure 8h).
Figure 8. c-myc mediates miR-34c-5p effects

(a, b) A549-FK cells transfected with the control scrambled sequence, the miR-34c-5p mimic and a Bmf siRNA (a), or a c-myc siRNA (b) and the levels of caspase-3 and caspase-9 products were analyzed after AP20187 treatment by immunoblot as indicated. (c) A549 cells were transfected with a c-myc siRNA or a control siRNA and the levels of caspase-3 cleaved products were analyzed by immunoblot after treatment with paclitaxel as indicated. Values below the blots a, b, c indicate signal levels relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 4.
(d) A549 cells were cotransfected with a c-myc siRNA and a scrambled sequence or miR-34c-5p mimic as alternative. Levels of caspase-3 cleaved products, c-myc and Bmf were analyzed by immunoblot. (e) A549 cells were transfected with miR-34c-3p mimic or with a scrambled sequence and levels of Bmf and c-myc were analyzed by immunoblot 72h post-transfection. The target prediction algorithm (Diana-microT\textsuperscript{3}) predicts Bmf but not c-myc as target of miR-34c-3p. (f,g) A549 cells were transfected with miR-34c-5p mimic or with a scrambled sequence and 48h or 72h post-transfection cells were recovered, stained with 50 µg/ml propidium iodide and analyzed by FACS (f), or cell lysates were extracted for cyclin B1, cyclin D1 and Chk1 analysis by immunoblot (g). (h) A549 cells transfected with miR-34c-5p mimic or with a scrambled sequence were treated with paclitaxel as indicated and cyclin B1, cyclin D1 and Chk1 levels were evaluated by immunoblot analysis. For all the blots shown anti α-tubulin was used to confirm equal loading. The blots are representative of at least four independent experiments.

We then wondered whether transfecting miR-34c-5p may act on the activity of p53, monitoring p53 acetylation that is essential for maximal p53-dependent apoptosis (Sykes et al. 2006; Tang et al. 2006; Mellert et al. 2011). As shown in Figure 9a in untreated, exponentially growing, A549 cells the levels of acetylated p53 are very low and are strongly induced by paclitaxel treatment (24 h). Interestingly, transfecting miR-34c-5p counteracts paclitaxel by reducing the induction of acetylated p53 levels to less than 2 fold over basal. The interference of miR-34c-5p on the levels of acetylated p53 is well mirrored by the corresponding decrease in the paclitaxel-induced expression of total p53. This suggests that miR-34c-5p, by regulating p53 synthesis or stability, may indirectly act on its activity. To confirm that miR-34c-5p may interfere with the induction p53 activity by paclitaxel we performed luciferase assays transfecting a construct expressing the luciferase reporter gene under the transcriptional control of the p53-responsive human MDM2 promoter. As shown in Figure 9b transfecting miR-34c-5p abolishes the induction of p53 activity by paclitaxel as assessed by the corresponding levels of luciferase activity. Consistently, miR-34c-5p abolishes as well the induction of the MDM2 protein and decreases the levels of SIRT-1, a known direct target of miR-34c (Yamakuchi et al. 2008) (Figure 9c). Therefore, in the attempt to understand how MDM2 is implicated in the attenuation of p53 response to paclitaxel we inhibited MDM2 activity. Treating A549 cells with Nutlin-3a, a direct inhibitor of MDM2, increases the levels of p53, likely by increasing its stability. At difference of what happens with paclitaxel-induced p53, transfecting miR-34c-5p in the absence of MDM2 doesn’t result in the attenuation of p53 (Figure 9d, upper panel). Conversely, we show that following inhibition of MDM2 the balance between miR-34c-5p and p53 levels cannot be interfered by antagonizing miR-34c-5p with a specific LNA based inhibitor (Figure 9d, lower panel).
Based on these results the question arises of why miR-34c-5p is unable to protect cells from cisplatin. Indeed, as shown in Figure 9e both cisplatin and paclitaxel-induced activation of caspase-3 is reduced in stable A549 cells silenced for p53 (Figure 9e). However, at difference of paclitaxel, the cisplatin-induced levels of p53 are unaffected by miR-34c-5p (Figure 9f).

Taken together these data indicate that transfecting miR-34c-5p in the presence of a proapoptotic insult, as paclitaxel, may protect cells from apoptosis likely by interfering with the levels of p53 likely by tuning the levels of c-myc and suggest that miR-34c-5p-induced p53 attenuation depends on MDM2 activity.
Figure 9. miR-34c-5p attenuates p53 activity

(a) A549 cells were treated with paclitaxel 100 nM for 24h. Cell lysates of untransfected cells (Cntrl) and of cells transfected with a control scrambled sequence or with miR-34c-5p mimic were analyzed with anti-acetyl–p53 and anti-p53 antibodies. Values below the blot indicate signal levels of acetyl–p53 and p53 relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 4.
(b) A549 cells were transfected with a reporter construct containing the promoter region of MDM2 gene fused to luciferase. Luciferase activity was measured after treatment with Paclitaxel in cells in which the reporter gene is transfected together with miR-34c-5p mimic or with a scrambled sequence as control. (c) A549 cells were treated with paclitaxel and either scrambled transfected or miR-34c-5p mimic transfected cells were analyzed for MDM2 and SIRT-1 protein expression. Filters were hybridized with anti α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments. (d) A549 cells were transfected with a control scrambled sequence and with miR-34c-5p mimic (upper panel) or with an LNA anti-miR negative control inhibitor and an anti-miR-34c-5p inhibitor (lower panel). After treatment with 10 µM Nutlin-3a for 24h p53 protein levels were analyzed by immunoblot compared to untransfected cells (Cntrl). Filters were hybridized with anti α-tubulin to confirm equal loading. (e) p53 stable silenced A549 cells (shRNAp53) or mock stable transfected cells (shRNAcntrl) were treated with Paclitaxel or cisplatin as indicated and levels of caspase-3 and caspase-9 cleaved products were analyzed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. (f) A549 cells were transfected with either miR-34c-5p mimic or with a scrambled sequence and treated for 24 h with cisplatin (50 µg/ml). Levels of p53 were analyzed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. The blots are representative of at least three independent experiments.
5. Conclusions

In order to identify miRNAs implicated in the pro-apoptotic pathway we engineered a NSCLC derived cell line to induce caspase-8 dependent cell death. By using a functional selection we identified miRNAs whose expression may protect cells to undergo apoptosis. Eleven different miRNAs have been selected that if expressed, either alone or in combination, confer resistance to caspase-8 dimerization. Among these miRNAs, miR-34c revealed to be of particular interest. MiR-34c belongs to a conserved miRNA family consisting of three members: miR-34a, miR-34b and miR-34c that are under the direct positive control of p53 (Chang et al. 2007; He et al. 2007a; Raver-Shapira et al. 2007; Tarasov et al. 2007; Bommer et al. 2007; Corney et al. 2007). Several converging evidence demonstrated that miR-34 members mediate p53 action to negatively regulate cell cycle thus acting as “bona fide” tumor suppressor genes (Hermeking 2010; Liu et al. 2011; Li et al., 2009; He et al. 2007b). Though exceptions exist (Dacic et al. 2010; Lee et al. 2011), miR-34a/b/c have been consistently found poorly expressed in several tumor and tumor derived cell lines (He et al. 2007a; Corney et al. 2010).

In apparent contradiction with its tumor suppressive potential, here we demonstrate that forced expression of miR-34c may confer resistance to caspase-8-induced apoptosis. Indeed, in A549-FK cells, the drug induces the activation of the mitochondrial pathway through the activation of several pro-apoptotic members of the Bcl2 family, including BID, Bmf, and Bax, that are clearly impaired by miR-34c ectopic expression. Furthermore, we demonstrated that miR-34c is also able to protect cells from paclitaxel-induced apoptosis. Recently it has been reported (Sotillo et al. 2011) that miR-34a, belonging to the same family of miR-34c, may confer resistance to bortezomib-induced apoptosis by downregulating p53 in a c-myc dependent way. Indeed, c-myc is a key regulator of cell proliferation since its induction sustains cell proliferation and transformation. However, in concert with p53, elevated levels of c-myc may sensitize tumor cells to proapoptotic stimuli and promote cell death (Maclean et al. 2003; Cannell et al. 2010; Murphy et al. 2008; Soucek and Evan, 2010; Allen et al., 2011).

In an attempt to understand what are the critical targets of miR-34c, we assumed that, given the protocol of functional selection used, some of these targets should be common to multiple miRNAs selected and found in combination with miR-34c. Based on this assumption, we identified among the genes with predicted consensus sites for miR-34c, the proapoptotic protein Bmf. Indeed, attenuation of Bmf expression, even poorly, protects cells from caspase-8-induced apoptosis and thus is a promising target candidate of miR-34c, likely by directly targeting one of the predicted consensus sites in its 3’ UTR at position 2283.

According with recent reports that indicate c-myc as target of miR-34a/c (Cannell and Buschell 2010; Christoffersen et al. 2010; Kress et al. 2011) here
we show that c-myc is downregulated by miR-34c-5p and that its downregulation seems by its own to be sufficient to mediate much of the protective effects of miR-34c, thus proving that in A549 cells downregulation of c-myc is critical to mediate survival. On the other hand, our results indicate that the effects of miR-34c involve, besides c-myc, the MDM2 and p53 loop, the link between c-myc and MDM2 remains to be determined since A549 cells carry a deletion of the MDM2 regulatory protein, p14/Arf (Xie et al. 2005). Indeed, miR-34c attenuates the paclitaxel-dependent induction of p53 by a mechanism that is unlikely determined by a decrease in the extent of p53 acetylation since the levels of the deacetylase SIRT-1 (a target of miR-34c) are as well decreased. Our results rather show that inhibiting MDM2 with Nutlin-3a induces p53 expression in a way insensitive to the intracellular levels of miR-34c thus indicating that MDM2 activity could be required for attenuation. Therefore, a plausible explanation of the fact that miR-34c is unable to attenuate the levels of cisplatin-induced p53, while the silencing of p53 confers resistance to both cisplatin and paclitaxel, may likely rely on the genotoxic action of cisplatin that, at difference of paclitaxel, causes MDM2 inhibition. On the other hand, it has been shown that active p53 directly induces miR-34c expression that in turn indirectly increases p53 activity thus establishing a positive feedback loop leading to cell cycle arrest (Corney et al. 2007; Yamakuchi et al. 2008; Yamakuchi et al. 2009; Hermeking 2010). How this loop is attenuated is still unknown. In light of our results, it seems reasonable to hypothesize that increased levels of miR-34c may contribute to switch off the loop and thus interfering with paclitaxel-induced cell cycle arrest.

These results can be relevant both for the understanding of molecular mechanisms that governs the interplay between miR-34c, p53 and c-myc to control cell proliferation and apoptosis and for the development of targeted specific new therapeutic agents.
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Aptamers as Innovative Diagnostic and Therapeutic Agents in the Central Nervous System

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Abstract: Aptamers are short non-naturally occurring single stranded DNA or RNA able to bind tightly, due to their specific three-dimensional shapes, to a multitude of targets ranging from small chemical compounds to cells and tissues. Since their first discovery, aptamers became a valuable research tool and show great application to fundamental research, drug selection and clinical diagnosis and therapy.

Thanks to their unique characteristics (low size, good affinity for the target, no immunogenicity, chemical structures that can be easily modified to improve their in vivo applications), aptamers may represent a valid alternative to antibodies particularly for the treatment of neurological disorders that urgently need modalities for drug delivery through the blood brain barrier. Aptamers have excellent potential as reagents for the targeted delivery of active drug substances, either through direct conjugation to the aptamer, or through their encapsulation in aptamer-coated vesicles.

We will review here the recent and innovative methods that have been developed and the possible applications of aptamers as inhibitors or tracers in neurological disorders and brain cancer.

Keywords: Alzheimer's disease; Aptamers; Blood-brain barrier; brain cancer; Central nervous system; Parkinson's disease; Prion diseases; SELEX.

INTRODUCTION

Anomalous protein misfolding and aggregation, with an accompanying "toxic gain of function" occurs in several neurodegenerative conditions including Prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease and is central to their pathogenesis. For example, in AD, misfolded amyloid beta peptide 1-42, a proteolytic product of amyloid precursor protein metabolism, accumulates in the neuronal endoplasmic reticulum and extracellularly as plaques. In PD cases there is abnormal accumulation of alpha-synuclein in neuronal cell bodies, axons, and synapses. Prion diseases, as well, are caused by a transmissible agent that is able to induce abnormal folding of normal cellular prion proteins in the brain, leading to brain damage and the characteristic signs and symptoms of the disease. Furthermore, in the case of brain cancers, the complex heterogeneity of malignant cells make it difficult, to distinguish between even close tumor types with high accuracy.

Hence, finding specific ligands capable of detecting and measuring the altered pattern of gene expression and to discriminate between different disease phenotypes, is a strategic and plausible objective for the diagnosis and therapy of important diseases.

Furthermore, the presence of the tight blood-brain barrier (BBB) has turned out to be the major constraint to any strategy of safe, non-invasive delivery of therapeutics to the brain, either of viral or non-viral origin and enlarge the need to find new therapeutic and diagnostic modalities for central nervous system-related pathologies. To date, antibody-based approaches have been developed for in vivo applications but, in most cases, adequate sensitivity has not yet been reached; they show toxicity in vivo and are not able to efficiently cross the BBB.

Promising alternative approach to antibodies is now represented by single-stranded nucleic acid ligand molecules, named aptamers. They are isolated from combinatorial libraries by a selection procedure, the Systematic Evolution of Ligands by EXponential enrichment (SELEX) technology [1, 2], which, in the last few years, has yielded several high-affinity ligands and potential antagonists of disease associated proteins [3-6]. Unlike other small noncoding RNAs either natural or artificial (antisense, ribozymes, siRNAs, miRNAs) that inhibit gene expression, aptamers can be raised against a variety of targets and act by directly binding the protein target without interfering with its expression. Indeed, they assume three-dimensional shapes that dictate high-affinity binding to a given target thus frequently modulating its function [5-7]. They display the following unique properties that render them promising tools for the treatment of a multitude of diseases: (i) ability to discriminate between different conformations of the same target proteins; (ii) capability to quantify the level of expression of the altered versus the physiologic forms; (iii) low molecular weight and chemical structures ideal for in vitro and in vivo purposes; and finally, for therapeutic applications they (iv) may interfere with the altered target product.
Here we will discuss the properties of aptamers as suitable tools to detect disease-associated biomarkers in vivo and on the possible applications of aptamers as diagnostic/therapeutic agents in central nervous system related pathologies.

**APTAMER PRODUCTION**

The starting point of the SELEX technology for the aptamer production is the synthesis of a high complexity library of single-stranded oligonucleotides (RNA, DNA or modified RNA), as schematized in Fig. (1). Randomisation of a sequence stretch from 22 up to 100 nucleotides in length is used to create an enormous diversity of possible sequences, which in consequence generate a vast array of different conformations with different binding properties, from which ligands are isolated which may stably interact with specific target molecules by forming highly specific contacts. The SELEX method includes steps of (i) incubating the library with the target molecule under conditions favourable for binding; (ii) partitioning unbound nucleic acids from those bound specifically to the selector molecule; (iii) dissociating the nucleic acid-protein complexes; and (iv) amplifying the nucleic acids pool enriched for specific ligands. After the final round of the aptamer selection process, the PCR products are cloned into a vector and sequenced to allow for the identification of the best binding sequences and for screening of conserved sequences and structural elements indicative of potential binding sites. A typical aptamer library has a complexity of $10^{14}$, which is of several orders of magnitude higher than that of the phage display libraries used to screen for antibodies. As a result, SELEX usually requires eight or more rounds of screening to isolate aptamers with nanomolar affinities.

The broad utility of aptamers derives both from their versatility, with high selectivity and sensitivity, and their ease of screening and production. They are entirely chemically synthesized, thus avoiding complex manufacturing processes using cell-based (eukaryotic or prokaryotic) expression systems that are required for antibodies production. The dissociation constants of RNA aptamers/target molecules are in the nanomolar/picomolar range. Values are therefore better than those determined for protein/protein interactions detected by phage display experiments, and are comparable to dissociation constants measured in antigen/antibody interactions. The size of aptamers (8-15 kDa) is between that of a single chain antibody fragment and a small peptide and thanks to their structure and low molecular weight are not immunogenic—a very useful property for reagents that need to be administered repeatedly to the same individual for therapy or diagnostics when studying disease progression.

One particularly attractive feature of these synthetic molecules is that they can support chemical modifications, whilst maintaining their structure and function. Since aptamers, especially RNA-based aptamers, are rapidly degraded by nucleases in whole organisms major efforts have therefore been addressed to improve their stability by a

![Fig. (1). Steps employed in the SELEX process. The single-stranded (ss) DNA library is amplified by Polimerase Chain Reaction (PCR) in order to generate the double-stranded DNA pool that will be transcribed by T7 RNA Polymerase. The pool of RNA molecules with different conformations will be used for the selection process (see text for details).](image-url)
Aptamers as Innovative Diagnostic and Therapeutic Agents

APTAMERS IN DIAGNOSIS AND THERAPY

In 1990, Tuerk and Gold selected aptamers of T4 RNA polymerase by SELEX [1]. In the same year, Ellington and Szostak isolated from a population of random sequence RNA molecules, aptamers specific for a variety of organic dyes [2]. To date, aptamers achieve the specific recognition in many diagnostic applications and are in several clinical studies as therapeutic agents.

For example, nucleic acid aptamers have been reported as useful tools for curing thrombus and inhibiting endometrium hyperplasia and angiogenesis [13-15]. The potential clinical utility of aptamers might be best appreciated considering that, despite being discovered less than two decades ago, an aptamer-based therapeutic (known as pegaptanib or Macugen, marketed by Pfizer) has already received US FDA approval for the treatment of age-related macular degeneration. This is a RNA aptamer that binds to and antagonizes the action of vascular endothelial growth factor, by blocking vascular endothelial growth factor binding to its receptor [16].

Great promise in developing specific molecular probes for disease biomarker discovery and for diagnostic and therapeutic applications is recently represented by the intact cell-based SELEX strategy that allows for the selection of nucleic acid aptamers against living cells [see 17 for a review]. Indeed, cell-SELEX to target proteins expressed on the cell surface, can be used to develop aptamers that are able to bind preferentially to diseased cells compared with normal cells or to discriminate among different phenotypes of the same cell type. This strategy has the advantage of a direct selection of ligands without prior knowledge of the target molecules and enables the identification of aptamers that bind large cell surface-specific markers, in their native conformation. By applying the SELEX technology against whole-living cells in culture, for the first time we succeeded in demonstrating that even by using a target as complex as an intact cell, it is possible to obtain aptamers against even rare antigens if specifically expressed on the target cell [18, 19]. We adopted this strategy to generate nuclease resistant RNA-aptamers specific for PC12 cells expressing the human receptor tyrosine kinase, Ret and selected aptamers that bind specifically to Ret and inhibit its downstream signaling effects [18].

Aptamers to multiple targets have been simultaneously isolated by using red blood cell membrane preparations as target [20]. By using living cells as targets, aptamers able to discriminate cells from distant tumor types like T-cell ALL versus B-cell lymphoma [21] and small lung cancer cells versus large cell lung cancer [22] have been generated.

APTAMERS IN NEURODEGENERATIVE DISEASES

Prion Diseases

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders usually rapidly progressive and always fatal. TSEs essentially comprise Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in bovines. Other encephalopathies have been demonstrated in the Felidae, in mink or certain wild animals, such as deer or elk. These diseases are caused by unconventional transmissible agents that are called prions. A distinguishing feature is the conversion of the normal cellular isoform of the host prion protein (PrPC) into an insoluble, proteinase K-resistant isoform (PrPSc) which is deposited as aggregates in the brain and other tissues. The molecular details of the process by which PrP is converted into the disease-associated homologue are unclear. Investigations of prion isoforms led to the conclusions that variations in disease phenotype are determined by the conformation of PrPSc. Since prion strains replicate, suggesting that PrPSc must act as a template for the refolding of PrP into a second molecule of PrPSc, thus causing an exponential accumulation of the pathogenic protein [23, 24].

Much of the effort in this field is devoted to finding specific reagents able to differentiate the prion isoforms associated with specific neuropathologies or disease phenotypes, and allow differential diagnosis. Currently, antibody-based approaches have been developed but adequate sensitivity has not yet been reached. Furthermore, several agents have been found to interfere with PrPSc biogenesis. They include polyanionic drugs, derivatives of polyene antibiotics or anthracycline and synthetic peptides, porphyrin compounds, soluble lymphotixin beta receptor, lipophilic agents and anti-PrP antibodies. Most of these compounds are highly toxic in vivo, do not discriminate between PrPC and PrPSc and are not able to efficiently cross the BBB. Recently DNA and RNA aptamers have been selected against the prion protein and some of them are able to efficiently discriminate the aberrant from the normal form of the protein (as schematized in Fig. 2).

The group of Famulok [25] selected nuclease-resistant 2'-amino-2'-deoxyxpyrimidine-modified RNA aptamers which recognize a peptide comprising amino acid residues 90-129 of the human prion protein with high specificity. This domain of the prion protein is thought to be functionally important for the conversion of PrP into its pathogenic isoform PrPSc and is highly homologous among prion proteins of various species including mouse, hamster, and man. One of the selected aptamers, named DP7, binds to the full-length human, mouse, and hamster prion protein. Despite the use of the epitope 90-129 from human PrP as target for the selection, the aptamer binds as well the human full-length PrP, thus confirming what has already been observed for others aptamers [see 26, 27 as examples] that have been selected to bind an accessible peptide epitope, and which are also capable of recognizing that epitope when
present in the complete protein. Interestingly, at low concentrations in the growth medium of persistently prion-infected neuroblastoma cells, DP7 aptamer significantly reduced the level of PrPSc accumulation [25].

Furthermore, 2'-fluoro-modified RNA aptamers have been selected on infectious material by using purified, non-proteinase K-treated scrapie-associated fibrils (SAF) that are expected to include all variant PrP conformations and PK sensitivities associated with infectivity of prion [28]. This SAF preparation thus represent an appropriate target to investigate the capacity of PrPSc as well as other proteins that co-purify with it to retrieve high affinity aptamer ligands from a complex combinatorial RNA library. Intriguingly, the best ligand selected on this material, named SAF-93, binds to a site of the prion protein that overlaps with the binding site of DP7 aptamer even if the two ligands do not share any similarity in either the primary or the secondary structures. SAF-93 as well, is able to interfere with the conversion of PrPC into PrPSc in a near physiological cell-free conversion assay, producing about 2-fold inhibition of conversion at a concentration of 40 nM. Furthermore, the aptamer has been modified by reducing its size and by derivatizing it with biotin in order to generate a ligand to be applied in multiple formats for the detection of abnormal forms of PrP in vitro [29]. In addition to its diagnostic use, the molecule may in the future have potential therapeutic application in TSE diseases by inhibiting conversion of PrPC to the pathological PrPSc isoform.

In addition to these promising findings, a panel of DNA aptamers has been identified which binds at different degrees to prions derived from a variety of host species [30, 31].

These aptamers, generated against recombinant human PrPC, specifically bound to PrPC in its native form conformation but did not bind to PrPSc and other neuroproteins. These results indicate that these ligands could be successfully applied to differentiate prion isoforms, helping in the diagnosis of TSEs.

Aptamers are good molecular recognition elements for biosensors. Their conformational change, which is induced by the binding to the target molecule, enables the development of several types of useful detection systems. Recently, a detection system based on the conformational change in a prion-specific DNA aptamer has been developed and its application to simple bound/free separation has been reported [32]. The system is based on the properties of the so-called “capturable aptamers” to change their conformation upon binding to the target molecule and thereby expose a single-strand bearing the complementary sequence to a capture DNA probe immobilized onto the support.

Alzheimer’s Disease

AD is a progressive neurodegenerative disorder leading to the most common form of dementia in elderly people. Central to the pathogenesis of AD is the deposition in the brain of extracellular plaques containing aggregated of β-amyloid (βA) peptide that occurs as 40 and 42 amino acid containing fragments. This peptide is generated by proteolytic processing of the β-amyloid precursor protein by β-secretase (BACE1) and γ-secretase [33]. The longer more hydrophobic βA (1-42) folds much faster into the insoluble β-sheet conformation than the shorter one and forms the core of the plaques, which then act as seeds for the aggregation of the much more abundant βA (1-40) [34]. The amyloid aggregates can be found in fibrillar and diffuse forms. Whereas the amorphous plaques seem not to be pathological, it is known that fibrillar aggregates are neurotoxic [35]. Since the deposition of the βA (1-40) in the brain of patients is a process that starts long before any symptoms are detectable, the development of early diagnosis for AD is of major interest. Therefore, much of the effort in this field is devoted to designig ligands able to identify with high specificity and affinity the amyloid fibrils in order to generate new diagnostic and eventually therapeutic tools.

To date, the development of conformation-sensitive polyclonal, monoclonal or recombinant anti-βA antibodies
allowed the identification of specific conformational epitopes in βA fibrils or in βA oligomers, that are often found to be conformationally related to fibrils or oligomers from unrelated proteins [36, 37]. In this contest, the generation of nucleic acid aptamers specific for βA detection/neutralization seems to be a promising task. A schematic mode of action of the AD-related aptamers is reported in Fig. (3).

Ylera et al. [38] selected RNA aptamers against immobilized βA (1-40) that display affinities in the range of 29-48 nM. Furthermore, the sequence with the lowest dissociation constant, aptamer β55, has been labeled with colloidal gold and applied to visualization of the amyloid fibrils by electron microscopy, thus introducing a new diagnostic approach to detect and mark the fibers in AD [38].

Besides their use for diagnostic and visualization purposes, the aptamers may also have therapeutic potential. Since BACE1 initiates βA generation, it represents a valuable target to interfere with βA production and treatment of AD [39]. To date, small interfering RNA approaches have been reported for β-secretase proteins and in some cases their targeting ameliorates disease neuropathology in animal models [40]. BACE1 is a transmembrane protein characterized by an extracellular domain containing the enzymatic activity, and a short cytoplasmic tail (B1-CT). This tail, containing a single phosphorylation site, serves as a binding site for two proteins, the copper chaperone for superoxide dismutase-1 and the Golgi-localized, γ-ear-containing, ADP ribosylation factor-binding (GGA1) protein. Phosphorylation and GGA1 binding to B1-CT regulate BACE1 transport [41, 42], suggesting that modulation of BACE localization or interference with protein factors that interact with the cytoplasmic domain might also affect β-amyloid precursor protein processing and βA formation. Rentmeister et al. [43] isolated RNA aptamers that selectively target the B1-CT, by using as target of the selection an immobilized 24 aminoacid peptide representing the entire cytoplasmic domain of the BACE1. Among these aptamers, the best binders, S10 and TH14, endowed with a dissociation constant of 360 and 280 nM, respectively, share a structurally identical domain of 27 nucleotides in conserved loop and bulge motifs. Importantly, the S10 aptamer, recognizes cellular authentic full-length BACE1 from HEK269 cells and the binding site is restricted to the membrane-proximal half of the C terminus. Consistently with its selective binding site, S10 interferes with the recruitment of copper-chaperone for superoxide dismutase whereas it does not block the association of B1-CT with GGA1, nor does it inhibit the phosphorylation of B1-CT. The finding that a short aptamer can discriminate binding regions within a 3-kDa peptide suggests that this aptamer may provide a useful tool for elucidating functions associated with this epitope without affecting other important biological activities elicited by the C-terminal half of the BACE1 cytoplasmic domain. In this regard, these inhibitors, provide a rational for further functional investigation in order to develop new therapeutic modalities to block βA formation.

**Parkinson's Disease (PD)**

Considerable effort has been devoted to the search for molecules that might promote neuronal survival, and potentially be of therapeutic value in the treatment of PD.

One such candidate is the glial cell line-derived neurotrophic factor (GDNF) given its potent trophic effects on dopaminergic nigral neurons, indicating that this factor could provide neuroprotection in patients with early PD. In various animal models of PD, GDNF can prevent the neurotoxin-induced death of dopamine neurons and can promote functional recovery [44], and more importantly, the clinical use of GDNF for the treatment of the PD in humans is currently under evaluation [45]. GDNF signals through a multisubunit system in which the glycosyl-phosphatidylinositol-linked protein GFRα1 and the transmembrane tyrosine kinase Ret, function as the ligand-binding and signaling components, respectively. Ret
activation is necessary for GDNF-dependent neuronal cell survival. In vitro studies have shown that following GDNF binding to GFRα1 the resulting complex recruits Ret leading to its activation by dimerisation and autophosphorylation [46]. The expression of the Ret receptor is highly localized in the human substantia nigra to dopaminergic neurons and evidence indicates that Ret is an essential component of the GDNF receptor complex in other populations of neurons in human brain. Ret-like immunoreactivity was observed in many of surviving dopaminergic neurons in substantia nigra of PD cases [47].

Based on these findings, aptamer ligands for Ret receptor could be promising diagnostic/therapeutic tools for PD disease and neurodegeneration. One such candidate appears to be the RNA-aptamer, named D4, that we have generated by applying a whole-cell SELEX strategy, as highly specific ligand of human Ret receptor [18]. D4 is a 93-base 2′-fluoropyrimidine-RNA aptamer that specifically bind the extracellular domain of Ret, displaying a Kd of 30 nM, thus interfering with ligand induced stimulation of its intrinsic tyrosine kinase activity [18].

Works is in progress in our group to reformulate the D4 structure in order to allow the aptamer to cross the BBB and directly target brain neurons expressing the transmembrane tyrosine kinase receptor Ret in PD.

APTAMERS IN BRAIN TUMORS

The hope of success of therapeutic interventions in brain tumors, characterised by a high cellular heterogeneity, largely relies on the possibility to distinguish with high accuracy between tumor cells and normal healthy cells and even between cells of close tumor types. Given the high affinity and specificity of the aptamers, they could be usefully applied as diagnostic/therapeutic tools in cancer.

Aptamers have been isolated against tenasin-C (TN-C), an extracellular matrix protein over-expressed during different cellular processes including tumor growth [48, 49]. Since TN-C levels in tumors are significantly higher than in normal tissue and are correlated with invasiveness [50, 51] these ligands could be developed as clinically useful tumor-targeting agents. Hicke et al. [49] selected RNA aptamers by combining protein-SELEX on purified TN-C with cell-SELEX on TN-C-expressing U251 glioblastoma cells. A 13 kDa-size nuclease-stabilized aptamer, named TTA1, binds to the fibrinogen-like domain of human TN-C with a dissociation constant of 5 nM and does not cross-react with mouse TN-C. Intriguingly, the radio-labelled TTA1 aptamer is able to bind tumor tissue that expresses TN-C but does not bind to tissue that lacks TN-C. Thus, this aptamer has been proposed as a new ligand for targeted delivery of radioisotopes or chemical agents to diseased tissues [49].

In order to select a brain tumor-targeting tool, Blank et al. [52] reported a fluorescence-based SELEX procedure against transformed endothelial cells (EC) as a complex target. Neoangiogenic EC, associated with tumor development, differ from pre-existing quiescent EC by qualitative and quantitative changes in expression of molecular endothelial proteins. Thus, such molecules could serve as molecular addresses differentiating the tumor vasculature from those of the normal brain. Single-stranded DNA aptamers have been generated against a pathologic endothelial target, represented by Adenovirus-12 SV40-transformed YPEN-1 rat endothelial cells. Among the selected aptamers, the aptamer III.1 selectively bound microvessels of rat brain glioblastoma but not the vasculature of the normal rat brain including peritumoral area. The target protein recognised by this aptamer was isolated from endothelial cells by ligand-mediated magnetic DNA affinity purification and identified by mass spectrometry as rat homologue of mouse pigpen, a not widely known endothelial protein whose expression parallels the transition from quiescent to angiogenic phenotypes in vitro. Thus, the selected aptamer can be used as a probe to analyze pathological angiogenesis of glioblastoma and provide important insights for tumor diagnostics.

Neuroblastoma is the most common solid tumor of childhood outside the central nervous system; it is made of neural crest cells that can occur at any point along the sympathetic ganglia or the adrenal medulla [53]. It has been shown that the expression of the neurotrophic factor receptors TrkA and TrkB as well as the receptor for GDNF, Ret, is induced upon retinoic acid (RA) treatment in neuroblastoma cells [54, 55]. Using the D4 aptamer and a truncated Ret protein as specific inhibitors of Ret, we have shown that RA-induced differentiation of human neuroblastoma cells is mediated by a positive autocrine loop that sustains Ret downstream signaling and depends on glial cell-derived neurotrophic factor expression and release. Furthermore, in neuroblastoma cells expressing functional Ret and TrkB receptors, the RA-induced cell differentiation depends on the signaling interplay by TrkB and Ret. Indeed, by promoting Ret phosphorylation in a GDNF independent manner, the BDNF-stimulated TrkB, induces neurite outgrowth and expression of differentiation-specific molecular markers. This is the first report describing the need of a functional transactivation between two unrelated receptor tyrosine kinases to achieve the final cell phenotype, in addition, this work provides important insights on the molecular mechanism of RA action, which might be relevant for the development of biologically based therapeutic strategies [56, 57].

PERSPECTIVES

As discussed above, the number of generated aptamers of potential medical importance in central nervous system-related pathologies is rapidly increasing, and, the possibility to introduce changes in their structure through defined chemical modifications that will enhance their in vivo applications represents a great advantage with respect to the use of antibodies. Due to their relatively small size in comparison to antibodies, the isolated aptamers should be better suited for rapid tissue penetration and blood clearance, two excellent characteristics for contrast agents in imaging. In this regard, a notable example of aptamer plasticity is represented by the use of the DNA aptamer inhibitor of the human neutrophil elastase for in vivo imaging of inflammation [58]. Remarkably, a better signal-to-noise ratio was achieved by the aptamer, labelled with Technetium-99m, compared to the rat anti-elastase antibody.

In addition, it is possible to attach to nucleotides functional groups such as fluorescent dyes or chemically reactive groups allowing aptamers to be conjugated with
enzymes, beads or radiotracers in diagnostic assays [59], or with siRNAs [60] or toxins [61] for therapeutic applications.

Also the development of a safe, efficient, specific, and non-pathogenic system for the delivery of diagnostic/therapeutic aptamers is highly desirable. Indeed, to be successfully applied in vivo aptamers must possess defined molecular properties needed to cross the collagen microfibrillar network of the extracellular matrix, and reach the target tissue or cells and, most importantly, also penetrate the cell membrane. Coupling aptamers to inert large molecules, such as cholesterol or polyethylene glycol, has been used to keep them in circulation anchored to liposome bilayers [62]. For example, with the attempt to develop a therapeutic approach to treat experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis, an RNA aptamer that blocks midkine function has been generated and reformulated in its structure for in vivo application [63]. The administration of a 49-mer RNA aptamer, stabilized with ribose-2' modifications and cholesterol, neutralizes midkine induced expansion of CD4+CD25+ regulatory T cells and reduces the symptoms of experimental autoimmune encephalomyelitis.

More recently, and still in preclinical development, aptamers have begun to find applications at the interface of nanotechnology and medicine in the form of aptamer-nanoparticle conjugates [64, 65]. Aptamers conjugated to nanoparticles resulted in increased targeting and more efficient therapeutics, as well as more selective diagnostics. For example, nanoparticle-aptamer bioconjugates with RNA aptamers have been developed that bind at high specificity to the prostate-specific membrane antigen, a well-known prostate cancer tumor marker that is overexpressed on prostate acinar epithelial cells. This was the first report of targeted drug delivery with nanoparticle-aptamer bioconjugates [66].

APTAMERS AS DELIVERY AGENTS TO CROSS THE BBB

The study of brain drug delivery has lagged behind but is now rapidly progressing and is predicted to become a "hot topic" within the next few years. Currently, it is not practical to administer neurotrophins to humans by invasive procedures such as intracerebroventricular infusion or intracerebral injection, on the other hand, non-invasive intravenous administration of brain neurodiagnostic or neurotherapeutic agents remains a challenge because of the low permeability of the BBB. Indeed, the same mechanisms that protect the brain against intrusive chemicals can also frustrate therapeutic interventions. The anatomical basis of the BBB arises from the special cellular features of the brain capillary endothelial cells which exhibit high-resistance tight junctions acting as zippers that close the inter-endothelial pores, and connect and seal the cells thereby restricting the free movement of substances between the blood and the cerebral interstitial fluid [67]. Although approximately 100% of large molecules do not cross the BBB, the problem is nearly as severe for small-sized drugs since more than 98% of these do not cross the BBB. However, peptides, protein or nucleic acids therapeutics may be delivered to the brain by using a chimeric strategy in which the active drug is conjugated to a transporter molecule. Indeed, the so-called "brain drug-targeting technology" allows the coupling of a molecule that is not normally transported across the BBB to a transport vector that crosses the BBB by means of receptor-mediated transcytosis. Several recent papers describe the possibility to apply the brain drug-targeting technology to the diagnosis or therapy of many brain disorders [68-70]. Peptidimimetic monoclonal antibodies (MAbs) that bind endogenous transport system within the BBB, such as the insulin receptor, the transferrin receptor or the leptin receptor, have been used for targeting neuropeptides or antisense agents through the BBB in vivo.

As an example, human GDNF was re-engineered by fusion of the mature GDNF protein to the carboxyl terminus of the chimeric MAb to the human insulin receptor (HIR). The HIRMAb-GDNF fusion protein is bi-functional, and both components binds the HIR, to trigger receptor-mediated transport across the BBB, and binds the GDNF receptor GFRα1, to activate GDNF neuroprotection pathways behind the BBB [71].

In this context, it appears a challenging goal to reformulate the receptor-mediated approach, and to substitute natural neurotrophins and antibodies with synthetic, small RNA-based aptamers, by designing new and original chimeric molecules. Indeed, with respect to antibodies, non-immunogenic and low size aptamers appear as suitable tools to cross the BBB using receptor-mediated transcytosis and to bind with high affinity to specific brain targets. Furthermore, aptamers may be linked together, thus enabling the construction of a custom designed targeting molecule of choice.

Recently, with ultimate objective of introducing therapeutic enzymes across the blood-brain barrier, RNA-aptamers and DNA-aptamers have been selected to bind to the extracellular domain of the mouse transferrin receptor. After selection, these molecules were modified with biotin and linked to dye-labeled streptavidin to demonstrate their ability to deliver streptavidin to mouse fibroblast cells Ltk- cells. Furthermore, the DNA aptamer was able to deliver a lysosomal enzyme into deficient mouse fibroblasts and correct the defective glycosaminoglycan degradation in these cells [72].

These results support the possibility that peptides, proteins or nucleic acids conjugated to aptamers that bind to the extracellular domain of the transferrin receptor can undergo receptor-mediated transcytosis.

CONCLUSION

With the understanding of molecular mechanisms underlying central nervous system diseases, the use of aptamers as bio-recognition and inhibiting elements offers several advantages over classical methods mainly based on antibodies. Moreover, the enormous diversity of random oligonucleotide libraries can exceed the diversity of antibodies in the mammalian genome by several orders of magnitude. In this regard, numerous aptamers have already been selected against a wide array of proteins, and the possibility of acquiring aptamers against proteomes has been advanced by automation of the in vitro selection procedure. These considerations, explain why now the aptamer-based technology for protein detection is in advanced stages of
development as useful tools in clinical diagnosis and therapy.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Aβ  = Amyloid Beta Peptide
AD  = Alzheimer’s Disease
BACE1 = β-Secretase
B1-CT  = BACE1 Cytoplasmic Tail
BBB  = Blood-Brain Barrier
EC  = Endothelial Cells
GDNF  = Glial Cell-Derived Neurotrophic Factor
GGA1  = γ-Ear-Containing, ADP Ribosylation Factor-Binding
HIR  = Human Insulin Receptor
MAb  = Monoclonal Antibody
PD  = Parkinson’s Disease
PrPC  = Prion Protein
PrPSC  = Prion Protein, K-Resistant Prion Protein
RA  = Retinoic Acid
SAF  = Scrapie-Associated Fibrils
SELEX  = Systematic Evolution of Ligands by Exponential Enrichment
TN-C  = Tenascin-C
TSE  = Transmissible Spongiform Encephalopathy

REFERENCES

Review

Recent Advance in Biosensors for microRNAs Detection in Cancer

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Abstract: MicroRNAs (miRNAs) are short non-protein-coding RNA molecules that regulate the expression of a wide variety of genes. They act by sequence-specific base pairing in the 3’ untranslated region (3’UTR) of the target mRNA leading to mRNA degradation or translation inhibition. Recent studies have implicated miRNAs in a wide range of biological processes and diseases including development, metabolism and cancer, and revealed that expression levels of individual miRNAs may serve as reliable molecular biomarkers for cancer diagnosis and prognosis. Therefore, a major challenge is to develop innovative tools able to couple high sensitivity and specificity for rapid detection of miRNAs in a given cell or tissue. In this review, we focus on the latest innovative approaches proposed for miRNA profiling in cancer and discuss their advantages and disadvantages.

Keywords: microRNA; biomarker; cancer
1. Introduction

MiRNAs are a class of small (19–25 nucleotides) non-coding RNA molecules widely conserved through evolution. MiRNAs are first transcribed into long primary miRNAs (pri-miRNAs) by polymerase II or, in few rare cases, by polymerase III. Typically, pri-miRNAs display a 33 bp stem and a terminal loop structure with flanking segments. Primary miRNAs processing begins in the nucleus where an RNAse III enzyme, Drosha, in complex with other proteins processes the pri-miRNAs, inducing the conversion into precursor miRNAs (pre-miRNAs). Pre-miRNAs are 60–70 nt long hairpin RNAs with 2-nt overhangs at the 3’ end. They are transported into the cytoplasm by exportin-5, a RanGTP-dependent dsRNA-binding protein, and subsequently processed by Dicer, a cytoplasmic endonuclease RNase III enzyme, that generates a miRNA duplex. The functional strand of the mature miRNA is then incorporated into the RISC (RNA-induced silencing complex), a ribonucleoprotein effector containing a catalytic endonuclease core, Argonaute2 (Ago2); Dicer; a dsRNA-binding protein named transactivating response RNA-binding protein (TRBP) and a protein activator of protein kinase R (PACT). The RISC mediates the degradation or the translation inhibition of the target mRNA (Figure 1). To produce the functional mature miRNA the duplex generated after Dicer-mediated cleavage need to be unwound, but to date it remains unclear when (i.e., before or after RISC loading) and how the two strands are separated [1,2].

Figure 1. Scheme of miRNA processing pathway.
Recent studies have shown that miRNAs are involved in different biological processes and pathological states, particularly in the development of several cancers. Altered expression levels of miRNAs have been correlated with cancer type, tumor stage and response to treatments [3]. Thus, miRNAs represent a new class of promising diagnostic and prognostic biomarkers as well as new targets for cancer therapy [4,5].

On this basis, it is important to develop analytical methods for rapid and sensitive identification of miRNAs present in a particular cell or tissue or fluids (such as serum and plasma) samples. A key issue is the ability to distinguish between the precursor and mature form of a miRNA, since the mature form is the functional one and cellular levels of miRNA precursors does not necessarily correspond to cellular concentration of functional miRNAs [4].

To date, several methodologies have been applied to profile miRNAs including Northern blotting, in-situ hybridization, oligonucleotide microarrays, quantitative Reverse-Transcription-Polymerase Chain Reaction (qRT-PCR) and deep-sequencing [6]. Although Northen blot and in-situ hybridization continues to be used as the standard methods, these detection approaches have low sensitivity and generally require many steps, resulting laborious time-consuming procedures that are difficult for routine miRNA analysis.

In this review, we analyze in detail the recent advances in microarray-based detection platforms and next generation methods based on nanotechnologies. Innovative qRT-PCR, amplification and enzymatic-based methods as well as deep sequencing strategies are also discussed. In addition, in the final section we focus on the detection and characterization of circulating miRNAs as cancer biomarkers.

2. Alterations of miRNAs in Cancer

MiRNAs are expressed in a tissue-specific manner and changes in miRNA expression within a tissue type can be correlated with disease status. The tissue concentrations of specific miRNAs have been associated with response to therapy, metastatic potential and other clinical features in various types of cancer [5,7].

The first evidence for miRNAs involvement in human cancer comes from a study by Calin et al. [8], examining a recurring deletion at chromosome 13q14 to search for a tumor suppressor gene involved in chronic lymphocytic leukemia (CLL). This study describes that the region of deletion encodes two miRNAs, miR-15a and miR-16-1. Subsequent investigations have confirmed the involvement of these two miRNAs in the pathogenesis of CLL [9]. Furthermore, Constinean et al. reported that a miRNA by itself can induce a neoplastic disease [10]. In fact, by using a transgenic mouse model, they demonstrated that overexpression of miR-155 in B cells induce lymphoma pre-B leukemia.

Several other miRNAs dysregulated in different human cancer types have been reported. For example, it has been demonstrated that let-7 family contains miRNAs regulating the RAS family of oncogenes [11]. Petrocca et al. [12] showed that the miR-106b-25 cluster plays a key role in gastric cancer interfering with proteins involved both in cell cycle and apoptosis. In other studies, miR-155 was found overexpressed in Hodgkin lymphoma, pediatric Burkitt lymphoma and diffuse large B-cell Lymphoma [13-15]; miR-143 and miR-145 were significantly downregulated in colon cancer tissue compared with colonic mucosa [16]; miR-21 was overexpressed in many tumors [7], including glioblastoma [17], cholangiocarcinoma [18], multiple myeloma cells [19] and breast cancer [20,21].
Moreover, studies that investigated the expression of the entire microRNAome in various human solid tumors and hematologic malignancies have revealed differences in miRNA expression profiling between neoplastic and normal tissues [9,22-24]. These studies indicate that neoplastic tissues may be distinguished by the expression of specific signatures of as few as 20–30 different miRNAs and expression profiles may be highly predictive for the degree of response to several therapeutic agents [25]. Further, being much shorter than mRNAs, miRNAs are less vulnerable to degradation by ribonucleases and, unlike proteins, are not post-synthetic structural modified, and therefore easier to detect.

MiRNAs play a key role also in tumor metastasis. Indeed, for example miR-139 suppresses metastasis of hepatocellular carcinoma [26], while miR10-b was found highly expressed in metastatic breast cancer cells [27] even if its clinical utility is still questioned [28].

More recently, many evidences are emerging that tumor-derived miRNAs are present and detectable in serum, plasma, urine and other human body fluids (Table 1). Because of their abundance, tissue specificity and relative stability, circulating miRNAs hold a great promise as noninvasive or minimally invasive biomarkers in cancer [29,30].

### Table 1. Circulating miRNAs upregulated in cancer.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Body Fluids</th>
<th>Diseases</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155, miR-210, miR-21</td>
<td>Serum</td>
<td>Diffuse large B-cell lymphoma</td>
<td>[31]</td>
</tr>
<tr>
<td>miR-141</td>
<td>Plasma</td>
<td>Prostate cancer</td>
<td>[32]</td>
</tr>
<tr>
<td>miR-25, miR-223</td>
<td>Serum</td>
<td>NSCLC</td>
<td>[33]</td>
</tr>
<tr>
<td>miR-155</td>
<td>Serum</td>
<td>Breast cancer</td>
<td>[34]</td>
</tr>
<tr>
<td>miR-155, miR-21</td>
<td>Plasma (exosomes)</td>
<td>Lung cancer</td>
<td>[35]</td>
</tr>
<tr>
<td>miR-21, miR-141, miR-200 family</td>
<td>Plasma (exosomes)</td>
<td>Ovarian cancer</td>
<td>[36]</td>
</tr>
<tr>
<td>miR-17-3p, miR-92</td>
<td>Serum</td>
<td>Colorectal cancer</td>
<td>[37]</td>
</tr>
<tr>
<td>miR-126, miR-182</td>
<td>Urine</td>
<td>Bladder cancer</td>
<td>[38]</td>
</tr>
<tr>
<td>miR-125a, miR-200a</td>
<td>Saliva</td>
<td>Oral squamous cell carcinoma</td>
<td>[39]</td>
</tr>
</tbody>
</table>

### 3. Methods for miRNA Detection

Various strategies for miRNA detection have been developed. Here we discuss some of the most innovative ones remarking their advantages and limits (Table 2). Moreover we analyze methods used for detection and characterization of circulating miRNAs as new highly promising biomarkers for cancer diagnosis.
<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Throughput</th>
<th>Cost</th>
<th>Relevant Features</th>
</tr>
</thead>
</table>
| **Microarray-based**        |             |             |            |            | • Can be used for clinical diagnosis  
• Can only measure miRNA relative abundance  
• Requires 0.2-2 µg of total RNA  
• Specificity and sensitivity can be improved by LNA modification of probes |
| Microarray                  | Low         | Low         | High       | Relatively High |                                                                                                                                             |
| ENT                         | High        | High        | Medium     | High       | • Capable of identifying miRNAs with < 2 fold difference in expression level  
• Detection limit at fM level  
• Requires sophisticated instruments  
• No amplification and no labeling are required |
| SPRI                        | High        | High        | Medium     | High       | • Detection limit at attomole level  
• Requires sophisticated instruments  
• No amplification and no labeling are required |
| **Nanotechnology-based**    |             |             |            |            |                                                                                                                                                 |
| Gold nanoparticles-based    | High        | High        | Low        | Relatively Low | • Relatively simple  
• Detection limit at fM level  
• Does not require sophisticated instruments  
• No amplification and no labeling are required |
| SERS                        | High        | High        | Low        | High       | • Simple to perform  
• Requires sophisticated instruments  
• No amplification and no labeling are required  
• Detection limit at fM level  
• Complicated data interpretation |
|                      | Stem-loop qRT-PCR | High  | High  | Low   | High  | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Can be multiplex for high-throughput  
|                      |                  |       |       |       |       | • Specific for mature miRNA  
|                      |                  |       |       |       |       | • Requires only few pg of starting RNA  
|                      |                  |       |       |       |       | • Very sensitive to mismatches  
| SYBR Green qRT-PCR  | High  | High  | Low   | High  | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Can be multiplex for high-throughput  
|                      |                  |       |       |       |       | • Specific for mature miRNA  
|                      |                  |       |       |       |       | • Detection limit at fM level  
| miR-Q                | High  | High  | Low   | Low   | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Specific for mature miRNA  
|                      |                  |       |       |       |       | • Detection limit at fM level  
| Poly(A)-Tailed Universal RT | High  | Medium | Low   | Low   | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Requires only few pg of starting RNA  
| Molecular beacons    | Medium | High  | Medium | Low   | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Simple to perform  
|                      |                  |       |       |       |       | • Detection limit at low nM level  
|                      |                  |       |       |       |       | • Can be used for mature miRNA detection  
|                      |                  |       |       |       |       | • Very sensitive to mismatches  
| Amplification-based  | Padlock-probes and rolling-circle amplification | Medium | High  | Low   | Low   | • Requires radioactive labeling  
|                      |                  |       |       |       |       | • Very sensitive to mismatches  
|                      |                  |       |       |       |       | • Requires few ng of starting RNA  
|                      | Bead-based flow cytometry | Medium | High  | High  | Low   | • Can be used for clinical diagnosis  
|                      |                  |       |       |       |       | • Detection limit at pM level  
|                      |                  |       |       |       |       | • Requires sophisticated instruments  
|
**Table 2. Cont.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Enzymatic-based</th>
<th>Deep sequencing-based</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splinted ligation</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>ELONA</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Bioluminescence detection</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>RAKE</td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>Invader assay</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>454 pyrosequencing</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>SOLiD</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Solexa</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

**Enzymatic-based**

- **Splinted ligation**: Requires radioactive labeling, requires from ng to µg of starting RNA.
- **ELONA**: Requires very short time, requires more than 30 ng of starting RNA.
- **Bioluminescence detection**: Simple and rapid, detection limit at fM level.
- **RAKE**: Relatively complex, requires as low as 10 pg of starting RNA.
- **Invader assay**: Can be modified for high throughput, very simple and rapid, able to discriminate between precursor and mature miRNAs, very sensitive to mismatches, requires at least 50 ng of total RNA.

**Deep sequencing-based**

- **454 pyrosequencing**: Allow also to discovery new miRNAs, requires multiple steps, results must be validated by alternative methods, requires at least 2–10 µg of total RNA.
- **SOLiD**: Allow also to discovery new miRNAs, requires multiple steps, results must be validated by alternative methods, requires at least 2–10 µg of total RNA.
- **Solexa**: Allow also to discovery new miRNAs, requires multiple steps, results must be validated by alternative methods, requires at least 2–10 µg of total RNA.
3.1. Microarray-Based Methods

To date the most widely used techniques in literature to study the expression profile of miRNAs in cancer, are based on microarray analysis. These approaches are particularly attractive for miRNA profiling since they allow multiplexed detection of miRNAs [40].

Microarray technologies are based on the hybridization between target molecules and their respective complementary probes. Oligonucleotide probes are immobilized on a support platform through a covalent link and fluorescent labeled miRNAs are hybridized with the array. The specific link between miRNAs and probes generates fluorescent signals that are revealed and quantified as discrete spots on the slide. This technique is very attractive because it allows the analysis of a large number of miRNAs at the same time obtaining a miRNA expression profile of specific cancer samples.

The trickiest steps in microarray analysis are the design of probes used for capture of miRNA molecules and labeling procedure of biological samples. Several modifications in both these steps have been introduced during the last years that have permitted to improve this technique.

The probe design is influenced by a number of matters related to the nature of miRNAs. Indeed, miRNAs are small molecules that represent only a tiny fraction of total cellular RNA with many of them belonging to the same family and differ only by few nucleotides. These characteristics make it difficult to design multiple probes with a suitable melting temperature (Tm), thus optimizing hybridization conditions without compromising specificity. Moreover, because there are often hundreds to thousands of probes in the same miRNA microarray, Tm normalization is absolutely required.

Different strategies have been proposed to overcome these problems. Recently locked nucleic acids (LNA) [41,42] have been used to increase melting temperature, probe affinity for its target and mismatch discrimination. This approach provides high sensitivity and specificity. Otherwise, Baskerville et al. reported a strategy for Tm normalization by adjusting the length of the probes. In this method, appropriate adaptor sequences are linked to either one or both ends of the miRNA molecules and, based on the adaptor sequence, the probe is suitably lengthened or appropriately truncated if the original Tm is either too low or too high [43].

The procedure used for miRNAs labeling is another pivotal step for the success of microarray analysis. Different ways for direct or indirect labeling of miRNAs have been proposed [44]. Indirect methods are based on the labeling of the reverse transcribed miRNA or the RT-PCR product. This increases the labeling stability and sensibility. Direct methods (such as the use of guanine reagents, T4-RNA ligase or chemical labeling) are usually easier to use and help to avoid errors introduced by the reactions of reverse transcription and PCR amplification, even though they require a considerable amount of RNA (in the order of micrograms).

To date, various companies (such as Affymetrix, Inc., Santa Clara CA, USA; Agilent Technologies, Inc., Santa Clara CA, USA; Applied Biosystems, Inc., Foster City CA, USA; Exiqon A/S, Vedbaek Denmark; and Rosetta Genomics, Inc., Rehovot, Israel) provide different microarray platforms for miRNA detection with a great potential applicability in clinical field.
3.2. Nanotechnology-Based Methods

In recent years different strategies based on nanoparticles have been developed for mature miRNA profiling. All these methods are direct approaches able to minimize artifacts due to sample amplification and labeling.

A number of nanotechnology-based approaches proposed in recent years are based on the use of electrical detection techniques. In a first approach Gao Z. and Yang Z. [45] employed the Elettrocatalytic Nanoparticle Tags (ENT) strategy that is based on amplified chemical ligation utilizing an indium tin oxide electrode and isoniazid-capped OsO$_2$ nanoparticle tags. MiRNAs are oxidized with sodium periodate and then hybridized to DNA capture probes on the electrode. The signal is then chemically amplified through a ligation reaction to tag miRNAs with the OsO$_2$ nanoparticles that efficiently catalyze the oxidation of hydrazine resulting in an electrocatalytic activity at 0.10 V. This procedure greatly enhances the sensitivity leading to detect amounts of miRNAs as low as 80 fM. The assay was successfully applied to analyze let-7b, mir-106 and mir-139 in total RNA extracted from HeLa cells reducing the amount of sample needed to nanograms [45]. The same group developed another electrical detection strategy based on a microscopic platform made with interlocking gold and titanium microelectrodes with wells in between. Capture probes of peptide nucleic acid (PNA) are chemically fixed into these wells and hybridized with the target miRNAs. The anionic nature of the miRNA phosphate backbone then catalyzes the formation of polyaniline (PAn) nanowires from a solution of cationic aniline particles, so that the conductance of the deposited PAn nanowires correlates directly with the amount of the captured miRNAs. By using total RNA extracted from different cancer cell lines, the target miRNAs can be quantified in a range from 10 fM to 20 pM with a detection limit of 5.0 fM [46].

More recently Peng et al. [47] further ameliorated this strategy proposing a novel protocol enabling electrical detections with minimal background. In this approach, target miRNAs are tagged with RuO$_2$ nanoparticles that serve as a catalyst for the polymerization of aniline, allowing selective PAn deposition exclusively at the hybridized miRNA strands thus producing a clean background and a high signal-to-noise ratio.

An innovative nanotechnology-based method that utilizes Surface Plasmon Resonance Imaging (SPRI) has been developed by Fang et al.[48]. They described a novel approach that combines surface poly(A) enzyme chemistry and nanoparticle-amplified SPRI measurements for the miRNA detection on LNA microarrays. The target miRNAs are hybridized on LNA microarray and poly(A) tails are added to the miRNAs by poly(A) polymerase reaction. Poli(T) oligo-modified gold nanoparticles (GNPs) are then adsorbed onto poly(A) tails for signal amplification and subsequently detected with SPRI. This methodology can be used to measure miRNAs present in total RNA samples with excellent sensitivity at attomole levels, resulting about 50 times more sensitive than the fluorescence-based microarray [48].

In order to develop a simple read-out and high sensitive method that does not require expensive equipment, Yang et al. [49] proposed a colorimetric approach based on gold nanoparticles. This method utilizes two probes, a biotinylated probe (capture probe) and a gold nanoparticle probe hybridized to the complementary target miRNAs in a sandwich assay format. The complex is then immobilized onto the surface of a streptavidin-coated microplate and the signal of absorbed gold nanoparticles is amplified by silver enhancement and recorded with colorimetric absorbance by a
microplate reader. By this method, distribution of miR-122a/miR-128 in total RNA from mouse brain and liver tissue was detected and synthetic miRNA-122a was quantified with a detection limit of 10 fM miRNA or 2 ng of total RNA [49].

Recently, the Raman enhancing property of GNPs has been exploited to develop a surface-enhanced Raman scattering (SERS)-based assay for miRNAs. However, SERS platforms based on GNPs has proven to be not useful for quantitative diagnostic assays due to low reproducibility [50]. To overcome this problem, alternative SERS enhancing substrates have been proposed. Driskell J. D. et al. examined a silver nanorod (AgNR) array prepared via oblique angle vapor deposition [51-53]. In their approach miRNA sequences are incubated with the silver nanorod array SERS substrate and SERS spectrum was analyzed. Different synthetic miRNAs (such as let-7 miRNAs, miR-16, miR-21, miR-24a, miR-133, miR-218 and miR-224) were analyzed with high specificity [53].

To date the number of new strategies that employ nanomaterials are growing rapidly [54,55], revealing these approaches to be the most promising for the development of miRNAs-based prognostic and diagnostic tools in cancer.

3.3. qRT-PCR-Based Methods

Among the several advantages of qRT-PCR, widely used for gene expression quantization [56,57], are the high level of sensitivity (only few picograms of starting material are needed), accuracy and practical ease that make qRT-PCR a powerful tool for miRNA detection as well.

On the other hand, the main limit to extend this method to miRNA detection is represented by the very short length of mature miRNAs. In fact, the first approach used allowed to detect and quantify precursor molecules rather than mature miRNAs [58].

A stem-loop qRT-PCR based on TaqMan assay was developed by Chen and colleagues from Applied Biosystems and is currently commercialized [59]. This approach, obviously, shows all the advantages of conventional TaqMan qRT-PCR, such as sensitivity (only 25 picograms of starting RNA are needed), but it involves the use of a stem-loop primer during the reverse transcription reaction. Such an approach is specific for mature miRNA identification and allows discriminating between strictly related miRNAs. This method is also better than conventional TaqMan qRT-PCR in terms of reverse transcription efficiency and specificity. To date, stem-loop qRT-PCR is successfully and widely utilized to detect miRNA dysregulation in different cancer types [60-63].

In the same year, Raymond et al. [4] developed a very sensitive (femtomolar concentrations of starting RNA) SYBR Green qRT-PCR for the detection of mature miRNAs using Locked Nucleic Acid (LNA)-modified primers. Both stem-loop and SYBR Green qRT-PCR methods have the disadvantage to be quite costly.

To develop a new cost-effective qRT-PCR approach for mature miRNA detection, Sharbati-Tehrani et al. [64] proposed a highly specific and sensitive method (called miR-Q), which neither requires the use of fluorophore probes, nor LNA-modified oligonucleotides. MiRNAs are first reverse transcribed and simultaneously elongated using a miRNA-specific oligonucleotide with 5’ overhang and then cDNA molecules are amplified using three DNA-oligonucleotides at different concentrations. This approach has been utilized to quantify miRNAs in different cancer cell lines and then for miRNA expression profiling of in vitro-fertilized bovine embryos [64,65]. A very simple and
convenient method is based on Poly(A)-Tailed Universal Reverse Transcription \([66,67]\). In this approach total RNA is first polyadenylated by poly(A) polymerase and then cDNA is synthesized by using a specific primer containing oligo dTs flanked by an adaptor sequence. Finally, the cDNA is amplified using a miRNA-specific primer and a universal primer.

The above approaches are all low throughput methods. However in more recent years these approaches have been modified by high-throughput miRNA profiling. For example, Applied Biosystems Inc. provide TaqMan Low Density Array cards that simultaneously quantifies hundreds of miRNAs by TaqMan qRT-PCR reactions using Megaplex™ stem-loop primer pools for the miRNA reverse transcription step. Furthermore, Signosis Inc. has developed a highly sensitive and specific platform that combine oligo-ligation and SYBR green based qRT-PCR for multiple miRNA detection. These platforms are particularly attractive since they can be used extensively for clinical diagnosis.

An example to simultaneously quantify different miRNAs in the same qRT-PCR reaction is the use of innovative probes, named molecular beacons. Molecular beacons are single-stranded probes with a stem-loop structure that recognize a specific target molecule \([68,69]\). The complementary sequence to the target is in the loop of the molecule, while the stem is formed by the annealing of two complementary sequences with a fluorophore linked to the end of one arm and a quencher linked to the end of the other one. Molecular beacons emit fluorescence only when they hybridize with the target, undergoing a spontaneous conformational reorganization that forces the fluorophore and the quencher to move away from each other. This approach is very sensitive to mismatches and, since probes can be linked with different fluorophores, is also helpful to simultaneously detect different target miRNAs. Molecular beacons have also been modified \([70]\) to specifically quantify mature miRNAs. In this case when the probes hybridize with pre-miRNA or pri-miRNA, its fluorescence is quenched by a guanine in the target sequence, while hybridization of the probe with mature miRNA which has no complementary guanine results in fluorescent emission. This approach has been recently utilized for detection of miRNAs overexpressed during myogenic differentiation \([71]\).

### 3.4. Amplification-Based Methods

Here we discuss some promising miRNA detection strategies that require a PCR amplification step but do not involve real time quantitative analysis.

The padlock-probes and rolling-circle amplification technology was initially developed by Nilsson et al. \([72,73]\) and more recently improved by Jonstrup et al. \([74]\) for detecting and quantifying miRNAs. Padlock probes are linear DNA probes where the terminal sequences are designed to be exactly antisense to the 5’-end and the 3’-end of a specific miRNA. After annealing with the miRNA the padlock-probe is circularized by a DNA ligase and then the miRNA is used as primer for rolling circle amplification. The method is very sensitive to mismatches and has the power to discriminate between closely related miRNAs. Moreover this technology is highly quantitative, specific and very inexpensive, since no specific equipments required. This approach has been successfully used to quantify different miRNAs including miR-16, miR-17-5p, miR-20a, miR-21, miR-27a and miR-92 \([74]\).

Another example of amplification-based approach is the bead-based flow cytometric miRNA expression profiling \([75]\). In this approach miRNAs are first processed with a ligation reaction that adds adapter oligos to both 3’ and 5’ ends and then reverse transcribed with primers complementary to
the adaptor oligos. The resulting cDNA is amplified with biotinylated forward primers, PCR products are hybridized with specific probes on fluorescent beads and finally analyzed by flow cytometry. This method has high accuracy, low costs and is useful for high-throughput miRNA profiling. This technique has been successfully applied to carry out a systematic expression analysis of 217 mammalian miRNAs from 334 samples, including multiple human cancers [75]. In this work the data from the bead-based miRNA profiling allowed to distinguish tumors of different developmental origin and at different stages. Thus bead-based flow cytometric miRNA expression profiling seems to be a very interesting diagnostic tool for cancer, since it is also easy to implement in a routine clinical setting.

3.5. Enzymatic-Based Methods

In this section we analyze examples of miRNA profiling strategies that involve enzymatic activities different from DNA polymerase.

A first promising approach included in this category is the splinted ligation method. It was originally developed by Moore and Query [76] and then Maroney et al. [77,78] adapted the technology to detect and quantify miRNA expression. Splinted ligation method is a sensitive and simple approach that also allows simultaneous processing of multiple samples. This method is based on the use of a couple of oligonucleotides named, the bridge and the ligation oligonucleotide respectively. The bridge oligonucleotide hybridize with the target miRNA and with a 5'-end-radiolabeled ligation oligonucleotide. This allows the formation of a double-stranded structure with a nick on one strand that is ligated by T4 DNA ligase, thus labeling the template miRNAs while the unligated oligonucleotides is treated with a phosphatase to remove the 5'-end labeling. Following the splinted-ligation reaction, labeled miRNAs and any residual-labeled ligation oligonucleotides can be separated and analyzed by denaturing gel electrophoresis. This approach has been successfully validated for different miRNAs including miR-21, miR-1, miR-9, miR-16, miR-20a, miR-21, miR-26a, miR-124a and miR-37 [77,78]. The main disadvantages of this technique are the use of radioactive labeling and the relatively low sensitivity. Other enzymatic-based methods that avoid the use of radioactive labeling have been proposed.

For example, the enzyme-linked oligonucleotide assay (ELONA) was developed by Mora and Getta [79]. This assay is based on the use of miRNA probes and miRNAs labeled at the 3' end with a 31-base oligonucleotide complementary to the outer arms of horseradish peroxidase (HRP)-conjugated DNA dendrimers. MiRNA probes are first spotted onto microtiter plates and then are hybridized with labeled miRNAs. HRP-conjugated dendrimers are finally used as detection molecules for signal amplification. ELONA takes very short experimental time, it is no expensive and it has a high sample throughput, but it has relatively low sensitivity (require more than 30 ng/well of sample). This approach has been successfully used to quantify tissue-specific expression of miR-1 in heart tissue, miR-122 in liver and miR-124a in brain [79].

An alternative to ELONA is the Bioluminescence miRNA detection method [80]. It is a competitive solid-phase hybridization-based method that makes use of the bioluminescent protein Renilla (Rluc) as label. This method is simple, rapid and sensitive with a detection limit in the order of 1 fmol. This approach has been successfully applied for determination of miR-21 in both human breast adenocarcinoma MCF-7 and non tumorigenic epithelial MCF-10A cellular extracts [80].
Other interesting enzymatic-based methods are the RNA-primed Array-based Klenow Enzyme assay (RAKE) [81] and the invader assay [82]. RAKE is a new method for high-throughput miRNA detection that is even more specific than other microarray-based expression profiling platforms. An oligonucleotide with a 5’ spacer, covalently linked onto a glass platform, followed by a antisense probe complementary to the target miRNA that permits forming double stranded hybrids. Following exonuclease I degradation of unbound single stranded oligos, the miRNA bound to the probe is used as primer for Klenow fragment of DNA polymerase I, which catalyzes the addition of biotin-conjugated dATP onto the spacer template with an increase of the signal without any need of PCR amplification of the template. RAKE assay was used to profile miRNAs from normal human adult and fetal brains and from reactive astrocytosis and oligodendroglial tumors. It is also a sensitive method since it requires as low as 10 pg of starting RNA [81].

The invader assay is a detection technology developed by Allawi et al. [82] and based on the use of a structure-specific 5’ nuclease (Cleavase) and a final fluorescence measurement. The miRNA target is first hybridized with a specific probe and an Invader oligonucleotide, designed to form an overlap-flap structure, substrate of the Cleavase. After the cleavage the flap fragment is released and a second overlap-flap structure is formed by the annealing of the flap fragment and a FRET oligonucleotide, linked to a fluorophore and a quencher, to a secondary reaction template. Then the Cleavase separate the fluorophore from the quencher on the FRET oligonucleotide, thus generating a fluorescence signal. This is a very simple and rapid approach (requiring only 2–3 hours of incubation), able to discriminate between precursor and mature miRNAs and also between closely related miRNAs, but it has the disadvantage to be less specific and sensitive than qRT-PCR-based methods (at least 50 ng of total RNA as template are required). Invader assay has been successfully applied for the detection of several miRNAs in cancer [13] and has been also modified for high-throughput miRNA profiling [83,84].

3.6. Deep Sequencing-Based Methods

In recent years innovative deep sequencing technologies, originally used for genomic sequencing [85], have been also applied for simultaneous sequencing of up to millions of miRNA molecules. These include the 454 Genome Sequencer (Roche Applied Science, Basel CH) based on pyrosequencing, the Illumina Genome Analyzer (Illumina Inc., San Diego, CA, USA) based on Solexa technology and the SOLiD platform (Applied Biosystems, Inc., Foster City, CA, USA), a ligation-based sequencing. For all different high-throughput systems small RNA cDNA library preparation is a critical point and the following basic steps are required: (1) total RNA isolation; (2) small RNAs enrichment; (3) 3’ and a 5’ adaptor ligation (platform-specific); (4) reverse transcription; (5) PCR amplification by minimal rounds to avoid library bias; (6) sequencing [86].

In the 454 and in the SOLiD technologies an adaptor-flanked library is amplified by an emulsion multi-template PCR using a single primer pair, corresponding to the adaptor sequences. One PCR primer is 5’-linked to the surface of micron-scale beads, included in the reaction. After PCR amplification, each bead will bear on its surface PCR products corresponding to a single molecule from the template library. These clonally amplified beads can then be used as template for features for 454 or SOLiD sequencing platform.
With the 454 platform beads are randomly deposited on the wells of a microarray and sequenced by pyrosequencing. In this approach in each cycle a single nucleotide is introduced and then a substrate (luciferin, adenosine 5’-phosphosulphate) is added to produce light signal at wells where polymerase drives the incorporation of the nucleotide.

Instead, with the SOLiD platform, beads are used to create a disordered, dense array of sequences and in each sequencing cycle is introduced a partially degenerate population of fluorescently labeled octamers. In this population the label correlates with the identity of the central 2 bp of the octamer. Several such cycles will iteratively interrogate an evenly spaced, discontiguous set of bases.

Finally in the Solexa technology an adaptor-flanked library is amplified by a bridge PCR, in which primers are linked to the surface of a solid substrate by a flexible linker. At the end of the PCR reaction are generated different clonal clusters each containing ~1,000 copies of a single member of the starting library. These clusters are then sequenced. Each sequencing cycle includes the simultaneous addition of a mixture of four fluorescent labeled deoxynucleotides modified with a reversibly terminating moiety at the 3’ position. A modified DNA polymerase drives synchronous extension of primed sequences and then the results are acquired by imaging in four channels.

These strategies allow fast evaluation of absolute miRNA levels and are also able to identify novel miRNAs, but to date they are still costly. In addition, since some errors can be introduced at several steps, limiting the accuracy of the analysis, sequencing results must be validated by alternative methods such as qRT-PCR.

3.7. Methods for Detection and Characterization of Circulating miRNAs

Recently, circulating miRNAs are emerging as very promising biomarkers for cancer [29,30], since they are abundant, tissue specific and relatively stable. Thus, several methods have been recently proposed to detect miRNAs in serum, plasma, urine and other human body fluids (Figure 2). Noteworthy the use of body fluids as biological materials for miRNA assays has the main advantage to be noninvasive or minimally invasive approaches.

High-throughput profiling techniques such as Solexa sequencing, miRNA microarray and bead-based miRNA profiling are effective tools for quantification of circulating miRNAs. However, because of the rather high amounts of serum required, high-throughput techniques are mainly used for initial screening analysis. For circulating miRNA detection a highly promising strategy has been recently developed which combines Solexa sequencing or oligonucleotide microarray with the qRT-PCR [33,87,88] thus exploiting the high specificity and sensitivity of the TaqMan stem-loop qRT-PCR based method.

In addition, to make easier the detection of circulating miRNAs, new techniques have been recently developed. By performing miRNA detection through an electrochemical genosensor, Lusi et al. [89] were able to directly detect miRNAs without the need of PCR and a labeling reaction, with an assay simple, very fast and ultrasensitive (detection limit of 0.1 pmol). Further developing these and other approaches will certainly enable the application of circulating miRNAs as biomarkers for cancer diagnosis.

However, a common drawback of all these approaches remains the lack of a house-keeping miRNA for normalization of circulating miRNAs that, in contrast to tissue or cellular miRNAs, cannot be normalized against U6 since it is present in a very low concentration in serum and plasma [31,90].
Nevertheless, the normalization of the volume of serum or plasma samples has been proposed as an effective way to overcome such problems. The best approach is to normalize experimental miRNA data using spiked-in synthetic, nonhuman mature miRNA from *C. elegans* or plants as control [91].

**Figure 2.** Main methods used for circulating miRNA detection.

4. Conclusions

In the last decade the biologic role of miRNAs as “oncomirs” or “tumor suppressors” has generated an enormous expectation for their use in cancer diagnosis, prognosis and treatment. As a consequence the demand for miRNA profiling strategies continues to increase exponentially.

An effective method for miRNA profiling should: (i) involve easy and rapid experimental protocols; (ii) require minimum sample quantity; (iii) have a high specificity and sensitivity with a large measurement dynamic range from sub-femtomolar to nanomolar concentrations and (iv) have low cost. Even if this ideal technique does not yet exist, all the methods summarized in this review address these issues through various strategies.

Furthermore the detection of circulating miRNAs in serum, plasma, urine and other body fluids is now emerging as a promising novel tool to improve cancer screening. Although the field of circulating RNA research is still in its infancy, they show a great potential since they are stable molecules, easily accessible and they can be collected in a relatively noninvasive manner.
Acknowledgements

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New Insight into Clinical Development of Nucleic Acid Aptamers

CARLA LUCIA ESPOSITO, SILVIA CATUOGNO, VITTORIO DE FRANCISCIS, AND LAURA CERCHIA

Abstract: Nucleic acid-based aptamers have been shown as high-affinity ligands and potential antagonists of disease-associated proteins. Aptamers, isolated from combinatorial libraries by an iterative in vitro selection process, discriminate between closely related targets and are characterized by high specificity and low toxicity thus representing a valid alternative to antibodies to target specific proteins of biomedical interest. Moreover, they are non-immunogenic and can be easily stabilized by chemical modifications thus expanding their therapeutic potential. Here, we will focus on the structural and functional features of aptamers that have entered the clinical development pipeline together with those aptamers holding great potential as therapeutics in preclinical studies. The future perspectives of aptamers as therapeutics will be discussed as well.

Introduction

Innovative targeted therapeutic strategies aim at developing new molecules with high affinity and specificity with suitable pharmacokinetic properties for in vivo applications. In this perspective an emerging attractive class of targeting molecules is represented by single-stranded nucleic acid ligands, named aptamers, being able to bind with high affinity to specific protein or non-protein targets by folding into complex tertiary structures.

Aptamers have a number of important advantages over proteins as therapeutic reagents (Cerchia and de Franciscis, 2010; Missailidis and Hardy, 2009). Most importantly, they are readily synthesized chemically, thus avoiding the use of animal cells, and therefore are easier to characterize than antibodies produced by recombinant means. The SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology for the production of aptamers starts with the synthesis of a nucleic acid library of a large sequence complexity (generally between $10^{13}$ and $10^{15}$ members) followed by the selection for oligonucleotides that are able to bind at high affinity to the target molecule. As schematized in Figure 1, a typical SELEX experiment includes reiterated rounds of: (1) incubating the library with the target molecule; (2) partitioning nucleic acids bound specifically to the target molecule from unbound sequences; (3) dissociating the nucleic acid-protein complexes; and (4) amplifying of the nucleic acids pool enriched for specific ligands. After the final round, the PCR products are cloned and sequenced to subsequently identify the best binding sequences. Due to the fact that the specific, three-dimensional arrangements of a small number of contact points of the aptamer, rather than a general affinity for the sugar-phosphate backbone of the nucleic acid, mediate the target-aptamer interaction. Aptamers can achieve high target selectivity. In addition, the binding characteristics of aptamers can be influenced by the type of the experimental system used for the selection and counter-selection (depletion of aptamers that bind to non-target molecules). As a result, aptamers show binding affinities in the low
nanomolar to picomolar range and are thus comparable to those of antibodies; but different from antibodies, they are sufficiently stable and can be readily modified chemically to further enhance their stability, bioavailability, and pharmacokinetics (Keefe and Cload, 2008). Moreover, aptamer immunogenicity has never been reported (Foy et al., 2007). This property is based on the fact that antibodies to synthetic oligonucleotides are not generally produced and, in addition, the innate immune response against non-self RNAs does not hinder aptamer therapy because 2’-modified nucleotides abrogate Toll-like receptor responses (Yu et al., 2009).

Since their first description in the early 1990s (Ellington and Szostak, 1990; Tuerk and Gold, 1990), aptamers have been generated against a wide variety of targets (Keefe et al., 2010; Thiel and Giangrande, 2009) ranging from small chemical compounds to cells, including parasites and bacteria, tissues, and more recently, by means of in vivo-SELEX, even a tumor implanted in mice (Mi et al., 2010).

In this review we will focus on aptamers generated against targets of therapeutic interest with a major emphasis on those that are currently in clinical development. Their structural and functional features will be discussed and compared to traditional therapies.

**Translating Aptamers to Clinic**

One main advantage of using aptamers for applications in clinic is that nucleic acids can support chemical modifications apt to optimize their in vivo stability and persistence in physiological conditions, whilst maintaining their structure and function.

Since oligonucleotides, especially RNAs, are rapidly degraded by nucleases in whole organisms resulting in a very short half-life in the blood, a variety of approaches have been recruited to improve their stability (Keefe and Cload, 2008). The most effective modifications to circumvent this limitation are the substitutions at the 2’-ribose of the pyrimidines that are mainly affected by serum nuclease degradation. RNA-aptamers containing 2’-fluoro and 2’-amino pyrimidine (2’-F-Py and 2’-NH2-Py, respectively) can be generated by performing the selection in the presence of 2’-modified nucleotides (Chelliserrykattil and Ellington, 2004). Even if pyrimidine-modified aptamers show considerable increase in serum stability in the absence of other modifications, introduction of 2’-O-Metyl purines (Burmeister et al., 2005) and changes in the internucleotide linkages (such as the use of phosphorothioate) and in the nucleobases (for example, the substitution of uridine at position 5) as well as the capping at the oligonucleotide 3’-terminus, have been reported.
Further, the use of locked nucleic acids (LNA), containing a methylene bridge to connect the 2'-O to the 4'-C, increases the stability of base pairing, stabilizing the duplex and enhancing the resistance to nuclease. For example the introduction of LNA-modifications in the anti-tenasin C (TN-C) aptamer significantly improves its plasma stability of approximately 25% and enhances the tumor uptake (Schmidt et al., 2004).

An interesting application of the SELEX process is based on the use of the mirror-image of an intended target molecule (e.g., an unnatural D-amino acid peptide) as target for the selection, followed by the chemical synthesis of the mirror-image of the selected sequence, named spiegelmers (Eulberg and Klussmann, 2003). Since they are enantiomers of natural nucleic acids, they are not recognized by nucleases. Two aptamers in clinical trials (NOX-A12 and NOX-E36) are spiegelmers (see below and Table 1).

Even if the low molecular weight of aptamers allows cost-effective chemical synthesis, low immunogenicity, and good target accessibility, it renders them susceptible to a rapid clearance by renal filtration. To overcome this problem, the most used strategy is to increase the size of the aptamers by conjugation with polyethylene glycol (PEG) (Healy et al., 2004). As an example, 40 kDa PEG conjugation to anti-vascular endothelial growth factor (VEGF) aptamer enhances its half-life from 2 hours to 1 day in rodents (Burmeister et al., 2005) whereas the half-life was 10 days when administered to humans (see prescribing information for Macugen).

As discussed below, the U.S. Food and Drug Administration (FDA)-approved aptamer, Macugen, as well as all the aptamers in clinical trials, except for one (Nu172), possess several of these modifications.

### Aptamers in Clinical Trials

To date, nine RNA and DNA-aptamers have entered the clinical development pipeline for treating a number of diseases in different pathologic states (Table 1).

#### Developing aptamers for age-related macular degeneration (AMD)

The most successful therapeutic application of an aptamer is represented by the RNA-aptamer commercially known as Macugen (or pegaptanib, marketed by Eyetech Pharmaceuticals/Pfizer) for the treatment of exudative AMD, the form of AMD characterized by the formation of a neovascular membrane leaking blood and fluid under the retina with consequent destruction of the macula and loss of vision (Ng et al., 2006). The

<table>
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<th>Table 1. Aptamers in Clinical Trials</th>
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<tr>
<td><strong>Aptamer Name</strong></td>
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<tr>
<td>Macugen (Pegaptanib)</td>
</tr>
<tr>
<td>E10030</td>
</tr>
<tr>
<td>ARC1905</td>
</tr>
<tr>
<td>ARC1779</td>
</tr>
<tr>
<td>NU172</td>
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<tr>
<td>REG-1 (RB006/RB007)</td>
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<tr>
<td>NOX-A12</td>
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<td>NOX-E36</td>
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<td>AS1411 (AGRO0001)</td>
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aptamer, approved by the FDA in December 2004, binds and antagonizes the action of VEGF-165, the VEGF isoform preferentially involved in pathological ocular neovascularization. With the intent to improve the pharmacodynamic and pharmacokinetic properties of this 28-mer aptamer, it was chemically modified with 2′-F-Py and 2′-OMe-Pu, capping, and linkage to a 40 kDa branched PEG molecule (Figure 2), which increases the intravitreal residence time of the molecule (Ng et al., 2006). Clinical effectiveness of Macugen was studied in two controlled, double-masked, randomized studies in patients with exudative AMD. Patients were randomized to receive sham or 0.3 mg (licensed dose), 1 mg, or 3 mg Macugen, administered by intravitreal injections every 6 weeks for 48 weeks. In both of these studies, one year of 0.3 mg Macugen treatment resulted in decreasing of the growth of mean total lesion size and choroidal neovascular network size. Further, patients who were re-randomized to discontinue Macugen after one year lost visual acuity during the second year (Chakravarthy et al., 2006).

Different studies have been recently carried out to assess the clinical effectiveness and cost-effectiveness of Macugen and ranibizumab (Lucentis, Genentech), a monoclonal antibody targeting all isoforms of human VEGF-A and approved in 2006 by the FDA for the treatment of exudative AMD. Both drugs show comparable therapeutic efficacy and mild adverse events, while the economic evaluation varies considerably depending on the methodology for cost-effectiveness used in different studies (Colquitt et al., 2008; Mitchell et al., 2011).

In phase III clinical trials Macugen demonstrated to be effective for diabetic retinopathy treatment, resulting in improved vision and reduced macular edema. Furthermore, Macugen is also a potential candidate for the treatment of highly vascularized tumors, even if the effectiveness of a systemic administration is still unclear.

In addition to the RNA-aptamer Macugen, a DNA-aptamer directed against the platelet-derived growth factor-B (PDGF-B) has been proposed for exudative AMD treatment (Green et al., 1996). This aptamer, named E10030 (Ophthotech Corp./Archemix Corp.), is a 29-mer oligonucleotide containing 2′-F-Py and 2′-OMe-Pu, 5′-conjugation with a 40 kDa PEG molecule, and capping at the 3′-terminus with an inverted nucleotide. In an open-label phase I clinical study, E10030, administered to AMD patients intravitreally as a monotherapy or in combination with ranibizumab, demonstrated to be effective and well tolerated with no evidence of adverse events. As these results are promising, phase II clinical trials are currently being developed.

Although anti-VEGF therapy is the first line of defense against exudative AMD, anti-inflammatory agents may provide an adjunct or alternative mechanism to suppress the inflammatory processes driving AMD progression. Indeed, the anti-complement component 5 (C5) agent ARC1905 RNA-aptamer (Ophthotech Corp./Archemix Corp.) is currently in phase I clinical trial for AMD. It is a 39-mer oligonucleotide chemically modified with 2′-F-Py, 5′-conjugation with
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40 kDa PEG, and 3′-terminus capping with an inverted nucleotide to improve its pharmacokinetic and pharmacodynamic properties (Biesecker et al., 1999). The aptamer works by inhibiting C5, a central component of the complement cascade, which plays multiple roles in innate immunity and inflammatory diseases and whose aberrant activation is implicated in the pathogenesis of both exudative and dry (nonvascular) AMD (Nozaki et al., 2006). A multicenter study in which ARC1905 is administered with ranibizumab by intravitreal injection in patients with exudative AMD is ongoing. In addition, a study investigating ARC1905 in patients with dry AMD was initiated in 2009.

Targeting blood clotting

Among aptamers currently in clinical trials, some are directed against blood clotting factors. The ARC1779 is a DNA-aptamer directed against the A1 domain of von Willebrand factor (vWF) and is able to inhibit its binding to the glycoprotein Ib receptors on platelets, resulting in an antithrombotic effect (Diener et al., 2009). ARC1779 is a 40-mer oligonucleotide chemically modified with a single phosphorothioate linkage to increase target affinity, 5′-conjugation with a 20 kDa PEG molecule to reduce renal filtration, and capping at the 3′-terminus with an inverted nucleotide and 2′-OMe-Pu. It has been developed by Archemix Corp. for the treatment of thrombotic microangiopathies (TMA), a group of diseases that include thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome, caused by increased vWF activity. In phase I clinical trials on healthy volunteers, in which ARC1779 has been administered intravenously at different doses, no serious adverse events were observed and the aptamer resulted in inhibiting plasma vWF activity and platelet function (Gilbert et al., 2007). Currently phase II clinical trials are analyzing the effect of ARC1779 on platelet function and vWF activity in patients with TMA and the eventual adverse reactions. The therapeutic utility of this aptamer is pronounced given the absence of a drug treatment approved for any form of TMA.

Another aptamer that prevents blood clotting is Nu172, a 26-mer DNA aptamer, directed against thrombin with no chemical modifications in its molecular structure. In phase I clinical trials the aptamer resulted in inducing an effective and dose-dependent anticoagulation effect with a favorable safety profile. This aptamer is currently being evaluated in phase II clinical trials by ARCA Biopharma/Archemix Corp. as an anticoagulant for use during acute coronary artery bypass surgery. Being unmodified and unstable in vivo, it has to be administered by continuous intravenous infusion.

REG-1 (RB006/RB007) is a therapeutic agent consisting of an RNA-aptamer (34-mer) directed against the coagulation factor IXa (RB006) and a single stranded RNA oligonucleotide (17-mer) complementary to the 52′-terminal region of RB006 aptamer (RB007). The inhibition of the factor IXa, consequent to RB006 administration, is blocked by RB007 treatment, thus permitting a time-controllable therapy (Rusconi et al., 2004). RB006 is modified with 2′-F-Py, conjugation with 40 kDa PEG, and 3′-terminus capping with an inverted nucleotide while RB007 is modified with 2′-OMe-Pu. REG-1 is being developed by Regado Biosciences/Archemix Corp. Phase I clinical trials showed that it is effective and well-tolerated in both healthy volunteers and patients with stable cardiovascular disease taking oral antplatelet drugs, such as aspirin and clopidogrel (Chan et al., 2008a; Chan et al., 2008b; Dyke et al., 2006). Phase II clinical trials have been completed in patients affected by acute coronary syndromes undergoing percutaneous coronary intervention. In these studies the effectiveness of REG-1 has been compared to that of heparin but results are not yet available.

Development of unnatural enantiomers

NOXXON Pharma developed two anti-chemokine spiegelmers, NOX-A12 and NOX-E36, for the treatment of hematologic tumors and complications of type 2 diabetes, respectively. NOX-A12 is a 45-mer L-RNA spiegelmer conjugated with 40 kDa PEG and is directed against the stromal cell-derived factor-1 α (SDF-1α), a chemokine which attracts and activates immune and non-immune cells that bind to chemokine receptors CXCR4 and CXCR7. There are lines of evidence indicating that the inhibition of this binding could be an effective therapeutic tool for the treatment of hematologic and solid tumors (Hirbe et al., 2010; Kryczek et al., 2007). A phase I single dose study demonstrated that NOX-A12 was safe and well-tolerated up to the highest tested dose (10.8 mg/kg) if administered intravenously to healthy volunteers. Pharmacodynamic analysis from this study also revealed a long lasting and dose dependent mobilization of white blood cells and CD34 positive cells. A phase I multiple ascending dose study in healthy subjects is ongoing to compare the mobilization of hematopoietic stem cells obtained with NOX-A12 alone with that obtained in combination with filgrastim, a granulocyte colony-stimulating factor analog used to stimulate the proliferation and differen-
tiation of granulocytes in neutropenia conditions. The results from this study will establish the basis for further development of NOX-A12 for the treatment of lymphoma patients undergoing autologous hematopoietic stem cell transplantation.

NOX-E36 is a 40-mer L-RNA conjugated with 40 kDa PEG (Maasch et al., 2008; Ninichuk et al., 2008) that interacts with the chemokine ligand 2 (CCL2), a protein involved in the recruitment of monocytes and T cells to sites of injury and inflammation. CCL2 has recently been introduced as an “adipokine” playing an important role in obesity and complications of type 2 diabetes (Rull et al., 2010). In a phase Ia study the aptamer has been administered by both intravenous and subcutaneous injection up to a maximum dose of 2 mg/kg in healthy volunteers. Results of this study have shown that NOX-E36 was safe and well tolerated at all doses tested in both administration routes; furthermore, the drug was able to induce a dose-dependent decrease of peripheral blood monocytes. A phase Ib clinical trial is currently ongoing to establish the effectiveness of NOX-E36 in the treatment of patients affected by type 2 diabetes with multiple complications, including diabetic nephropathy. In this study healthy volunteers or type 2 diabetic patients are treated with NOX-E36 intravenously and the safety, tolerability, pharmacokinetics, and pharmacodynamics of the drug will be evaluated.

Cancer therapeutics

Very interesting for cancer therapy is the AS1411 DNA-aptamer (AGRO001), directed against nucleolin (Bates et al., 2009), a ubiquitous intranuclear and cytoplasmic phosphoprotein implicated in cellular proliferation and often overexpressed on the surface of tumor cells. This aptamer is a 26-mer guanine-rich oligonucleotide (GRO), which in solution folds into quadruplex structures that make it very stable and resistant to degradation by serum enzymes (Dapic et al., 2003). AS1411 was identified among a panel of GROs exposed to cancer cells and screened for antiproliferative activity. Once bound to nucleolin, the AS1411 aptamer is taken into the cancer cell, where it causes cellular death by apoptosis through inhibiting nuclear factor-κB (NF-κB) (Girvan et al., 2006) and Bel-2 (Soundararajan et al., 2008) pathways. In phase I and II clinical trials, conducted by Antisoma/Achremix Corp., AS1411 showed its effectiveness as an anticancer therapy for different solid human malignancies as well as for acute myeloid leukemia (AML), and was well-tolerated by intravenous administration. On the basis of these positive results, the aptamer is currently in a phase Iib trial to evaluate its effectiveness in combination with high-dose cytarabine in patients with relapsed and refractory AML.

Aptamers of Therapeutic Interest Not Yet Investigated in Clinical Trials

The enormous diversity of random oligonucleotide libraries can exceed the diversity of antibodies in the mammalian genome by several orders of magnitude. In this regard, numerous aptamers have already been selected against a wide array of proteins, and the possibility of acquiring aptamers against proteomes has been advanced by automation of the in vitro selection. In this section, we discuss the aptamers that appear as excellent drug candidates for a wide range of human pathologies (Table 2).

Among them, several aptamers now appear as promising molecules to target specific cell-surface signaling receptors of cancer cells in clinical diagnosis and therapy. Dysregulation of these receptor proteins by overexpression or constitutive activation promotes proliferation and/or survival of tumor cells, and thus their inhibition using neutralizing aptamers represents an innovative method to interfere with the growth of many human malignancies. Aptamers against the human transmembrane receptors such as epidermal growth factor receptor 3 (Her3/ErbB3) (Chen et al., 2003), transforming growth factor β type III receptor (TGF-β type III) (Ohuchi et al., 2006), and receptor tyrosine kinase Ret (Cerchia et al., 2005; Vento et al., 2008) have been generated that inhibit their target in vitro. Other examples of aptamers against cancer cell surface epitopes showing promise as anticancer therapeutics include the RNAs against the prostate-specific membrane antigen (PSMA) (Lupold et al., 2002) and the TN-C, an extracellular matrix protein that is overexpressed during different cellular processes including tumor growth (Daniels et al., 2003; Hicke et al., 2001). Both of these aptamers were shown to be effective as imaging agents and delivery tools in preclinical studies in mouse xenograft models of human tumors.

Apart from the therapeutic utility of those aptamers that bind and consequently inhibit the activity of their target proteins, another promising application of aptamers against cell surface molecules is to employ their excellent targeting properties to specifically deliver a drug to a cell or tissue thus increasing the efficacy of a given therapy as well as attenuating the overall toxicity of the drug (Cerchia and de Franciscis, 2010; Zhou and Rossi, 2010). To date the best-characterized aptamers for targeted delivery are the RNAs directed against PSMA.
(Lupold et al., 2002) that were conjugated with nanoparticles (Farokhzad et al., 2004), quantum dots (Chu et al., 2006b), toxin (Chu et al., 2006a), or small interfering RNA (siRNA) (Chu et al., 2006c; Dassie et al., 2009; Wullner et al., 2008) in order to drive these functional molecules toward the surface of prostate cancer cells. Furthermore, the list of aptamers of being able to be internalized by cells and being used as delivery agents for cytotoxic cargos into cancer cells is growing rapidly and now includes those against the extracellular proteins involved in cancer protein tyrosine kinase 7 (PTK7) (Huang et al., 2009), nucleolin (Ko et al., 2009), mucin 1 (MUC1) (Cheng et al., 2009; Ferreira et al., 2009), and epidermal growth factor receptor (EGFR) (Li et al., 2010).

In addition to the inhibitory aptamers, aptamers with agonistic-like activity have also been generated (Table 2) (Dollins et al., 2008; McNamara et al., 2008). McNamara et al. (2008) generated a bivalent molecule by linking two monomeric aptamers against the co-stimulatory T-cell receptor 4-1BB through the addition of 21 complementary nucleotides to the 3′ end of each aptamer. The resulting aptamer was effective to induce dimerization and consequent activation of 4-1BB receptor leading to immune response induction in vitro and tumor regression in vivo.

A major challenge in cancer therapy is to distinguish, with high accuracy, between closely related cell populations or even the same cell population under various growth conditions or insults. In this respect, counter-selection strategies can be adopted during cell-SELEX in the pursuit of aptamers that can bind to and discriminate between distinct cell types. Aptamers that discriminate cells from distant tumor types, like T-cell acute lymphocytic leukemia (ALL) versus B-cell lymphoma (Shangguan et al., 2006) and small lung cancer cells versus large cell lung cancer (Chen et al., 2008), have been generated. Recently, we have developed a differential cell-SELEX to isolate a panel of RNA-aptamers that bind human malignant glioblastoma cells, discriminating them from non-tumorigenic glioblastoma cells (Cerchia et al., 2009).

The great advances in cell-SELEX now offer the opportunity to develop innovative approaches to identify and isolate subpopulations of cancer stem cells, an emerg-

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Therapeutic Indication</th>
<th>Reference</th>
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<tbody>
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<td><strong>Extracellular Proteins</strong></td>
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<td></td>
</tr>
<tr>
<td>Ret</td>
<td>Cancer</td>
<td>Cerchia et al., 2005; Vento et al., 2008</td>
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<tr>
<td>Her3/ErbB3</td>
<td>Cancer</td>
<td>Chen et al., 2003</td>
<td></td>
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<tr>
<td>TGF-β type III</td>
<td>Cancer</td>
<td>Ohuchil et al., 2006</td>
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<tr>
<td>gp120</td>
<td>HIV infection</td>
<td>Dey et al., 2005</td>
<td></td>
</tr>
<tr>
<td>HIV-1 reverse transcriptase</td>
<td>HIV infection</td>
<td>Li et al., 2008</td>
<td></td>
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<tr>
<td>CD4</td>
<td>Immune modulation</td>
<td>Kraus et al., 1998</td>
<td></td>
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<tr>
<td>BACE-1</td>
<td>Alzheimer's disease</td>
<td>Rentmeister et al., 2006</td>
<td></td>
</tr>
<tr>
<td><strong>Intracellular Proteins</strong></td>
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<td></td>
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</tr>
<tr>
<td>NF-κB</td>
<td>Inflammatory disease and cancer drug resistance</td>
<td>Lebruska and Maher, 1999; Mi et al., 2007</td>
<td></td>
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<tr>
<td>E2F</td>
<td>Cancer and cardiovascular disease</td>
<td>Giangrande et al., 2007</td>
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<td>β-catenin</td>
<td>Cancer</td>
<td>Lee et al., 2007</td>
<td></td>
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<tr>
<td><strong>Delivery Tools</strong></td>
<td>PSMA</td>
<td>Cancer</td>
<td>Chu et al., 2006a; Chu et al., 2006b; Chu et al., 2006c; Dassie et al., 2009; Farokhzad et al., 2004; Wullner et al., 2008</td>
</tr>
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<td>PTK7</td>
<td>Cancer</td>
<td>Huang et al., 2009</td>
<td></td>
</tr>
<tr>
<td>MUC1</td>
<td>Cancer</td>
<td>Cheng et al., 2009; Ferreira et al., 2009</td>
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<tr>
<td>EGFR</td>
<td>Cancer</td>
<td>Li et al., 2010</td>
<td></td>
</tr>
<tr>
<td>TN-C</td>
<td>Cancer</td>
<td>Hicke et al., 2001</td>
<td></td>
</tr>
<tr>
<td>gp120</td>
<td>HIV infection</td>
<td>Neff et al., 2011; Zhou et al., 2011</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>HIV infection</td>
<td>Guo et al., 2005</td>
<td></td>
</tr>
<tr>
<td><strong>Agonistic</strong></td>
<td>4-1BB</td>
<td>Cancer</td>
<td>McNamara et al., 2008</td>
</tr>
<tr>
<td>OX-40</td>
<td>Cancer</td>
<td>Dollins et al., 2008</td>
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</table>
ing target of more effective cancer treatments (Guo et al., 2007; Hoffmann et al., 2008).

Intracellular proteins of biomedical interest (Table 2) can be targeted with aptamers (so-called intramers) as well by means of strategies allowing their entry into intracellular compartments (Giangrande et al., 2007; Lebruska and Maher, 1999; Lee et al., 2007; Mi et al., 2007).

A serious effort has been initiated to find aptamers as novel therapeutics for diseases other than cancer, with several groups of aptamers dealing with human immuno-deficiency virus (HIV) infection and neurodegenerative diseases. The aptamers against HIV reverse transcriptase (Li et al., 2008) and glycoprotein gp120 (Dey et al., 2005) reduce the HIV virus infectivity, and the anti-CD4 (Kraus et al., 1998) receptor aptamer modulates the immune response. Both anti-gp120 and anti-CD4 aptamers have been also validated as delivery agents for anti-HIV siRNAs resulting in the simultaneous inhibition of HIV infection and replication (Neff et al., 2011; Guo et al., 2005; Zhou et al., 2011). Among the aptamers against targets whose role has clearly been established for neurological disorders are those against β-secretase BACE-1 (Rentmeister et al., 2006), amyloid fibril constituent Aβ (Rahimi and Bitan, 2010), and prion protein PrP (Sayer et al., 2004).

Future Perspectives

Although the aptamer technology was first described in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990), aptamers’ widespread acceptance in therapeutics and diagnostics is still being realized. Indeed, despite the continuous advances in SELEX technology that now allow to easily generate aptamers against virtually any protein, the list of aptamers in clinical development is still limited. Because only one aptamer has been approved and is in the market, the costs for large scale production and modification of aptamers are still high and are affected by the absence of an adequate technological platform for their development as therapeutics and by a limited number of companies that are engaged in their development. Indeed, to date, it is Archemix Corp., a biopharmaceutical company that leads the development of aptamers as therapeutics. It is the owner of patents of aptamer technology and it collaborates with other pharmaceutical companies (Regado, Antisoma, ARCA Biopharma, and Ophthotech) to develop and commercialize a pipeline of partnered aptamers in the areas of cardiovascular disease, hematology, and oncology.

As discussed in this review, aptamers have a number of advantages in their clinical usage over their antibody counterparts, and it is plausible that the global interest for developing nucleic acid aptamer therapeutics will increase in the next few years. Accordingly, a new technical market research report from BCC Research (Nucleic acid aptamers for diagnostic and therapeutics: global markets, BIO071, www.bccresearch.com) estimated that the aptamer global market value of $236 million in 2010 will grow to nearly $1.9 billion in 2014, for a 4-year compound annual growth rate of 67.5%.

Acknowledgments

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Disclosure

The authors report no conflicts of interest.

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New Insight into Clinical Development of Nucleic Acid Aptamers


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Pharmaceuticals 2011, 4, 1434-1449; doi:10.3390/ph4111434

Review

Coupling Aptamers to Short Interfering RNAs as Therapeutics

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Abstract: RNA-based approaches are among the most promising strategies aimed at developing safer and more effective therapeutics. RNA therapeutics include small non-coding miRNAs, small interfering RNA, RNA aptamers and more recently, small activating RNAs. However, major barriers exist to the use of RNAs as therapeutics such as resistance to nucleases present in biological fluids, poor chemical stability, need of specific cell targeted delivery and easy entry into the cell. Such issues have been addressed by several recent reports that show the possibility of introducing chemical modifications in small RNAs to stabilize the molecular conformation and increase by several fold their integrity, while still preserving the functional activity. Further, several aptamers have been developed as excellent candidates for the specific recognition of cell surface targets. In the last few years, by taking advantage of recent advances in the small RNA field, molecular bioconjugates have been designed that permit specific targeting and may act as cargoes for cell internalization of small RNAs acting on gene expression that will be discussed in this review.

Keywords: aptamer; intracellular delivery; microRNA; small interfering RNA
1. Introduction

Innovative targeted therapeutic strategies aim at developing new molecules with high target affinity and specificity with suitable pharmacokinetic properties for in vivo applications. From this optic short non-coding RNAs were revealed to be attractive molecules. In the last decades significant advances have been attained in the knowledge of molecular mechanisms leading to selective inhibition of gene expression and protein function. However, in order to successfully translate RNA-based therapeutics to the clinic several challenges must be addressed, including appropriate stability in biological fluids, high efficiency and specificity of delivery, durable safety and target selectivity.

Several classes of molecules have been characterized with potential applications as RNA therapeutics in the treatment of human diseases. These include ribozymes, RNA decoys, aptamers, small interfering RNA (siRNA) and microRNA (miRNA) [1].

The discovery of RNA-mediated interference (RNAi) for gene silencing has provided a powerful tool for loss-of-function studies and therapeutic opportunities [2,3]. RNA interference is a natural process of gene specific silencing that occurs in organisms ranging from plants to mammals as a defense against viruses. si/miRNAs are formed from longer precursor molecules as short double-stranded RNAs (dsRNAs) of 20–24 base pairs [4]. One strand that directs silencing is the guide strand while the other strand, named the passenger, is degraded. In the cytoplasm, the RNA-induced silencing complex (RISC) drives the guide strand of the dsRNA to hybridize with the target mRNA to prevent translation or induce degradation depending on the degree of complementarity [5]. Base pairing between siRNAs and their targets generally shows full complementarity whereas, with the exception of the 2–8 bases seed region at the 5’ terminus, miRNAs usually show partial complementarity with their targets. miRNAs have the capacity to target multiple genes simultaneously and regulate important biological processes including, transcription, cell cycle, cell growth, proliferation and apoptosis. They have been shown to be involved in the pathogenesis of diverse diseases including cancer, stroke, diabetes, diseases of the liver, kidney, and cardiovascular system as well as neurodegenerative and infectious diseases [6-8]. On the other hand siRNAs are the best characterized RNA-based reagents that have been developed for several disease including cancer, kidney, ocular, retinal and metabolic disorders.

As a difference with siRNAs and miRNAs, the function of ribozymes and aptamers doesn’t involve the formation of the RISC. The hammerhead small ribozymes are nucleolytic oligonucleotides that recognize and excise a given target RNA molecule [9]. Aptamers constitutes an emerging attractive class of therapeutic molecules able to tightly bind to specific protein or non-protein targets by folding into complex tertiary structures [10,11].

Recognition by toll-like receptors (TLRs) in immune cells represents a major obstacle to the use of RNA-based therapeutics. However, immune recognition the immune response of single stranded siRNAs (ss-siRNA) or ds-siRNAs by TLRs can be bypassed by the replacement of only uridines with their 2’-fluoro, 2’-deoxy, or 2’-O-methyl modified counterparts without reducing their silencing potency [12-15]. In addition, immunogenicity has been found to be either absent or limited when 1,000-fold higher doses of a nucleic acid aptamer than would be required clinically were administered to monkeys [16]. This property depends on the fact that antibodies to synthetic oligonucleotides are not generally produced and, in addition, the innate immunity response against non-self RNAs does not hinder aptamer therapy because 2’-modified nucleotides abrogate TLRs responses [17].
A major impediment to the clinical development of RNA drugs is the lack of an appropriate and high efficiency in vivo delivery strategy to guarantee intracellular target accessibility and specificity of delivery. The use of viral vectors, despite their high efficiency, has been impaired greatly due to the associated mutagenicity or oncogenesis, several host immune responses, and high cost of production. Therefore, non-viral vectors continue to draw significant attention despite their low efficacy.

2. RNA-Based Therapies

Currently, the list of oligonucleotides of therapeutic interest is growing rapidly with over one hundred clinical trials and two therapeutic oligonucleotides that have been already approved by U.S. Food and Drug Administration (FDA) and marketed, the Vitravene antisense antiviral and the Macugen RNA-based aptamer. Two classes of therapeutic oligonucleotides have predominantly been developed: siRNA and aptamers, and several of them are currently in clinical trials (Table 1).

<table>
<thead>
<tr>
<th>Name</th>
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<th>Clinical Stage</th>
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<td>Pachyonychia congenita</td>
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<td>Non-arteritic anterior ischaemic optic neuropathy</td>
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<td>AGN211745</td>
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<td>VEGFR1</td>
<td>AMD Choroidal neovascularization</td>
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<td>RTP801</td>
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<td>β2 adrenergic receptor</td>
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<td>PLK1</td>
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<tr>
<td>FANG</td>
<td>Gradalis</td>
<td>Furin</td>
<td>Solid tumours</td>
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<td>RRM2</td>
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<td>NCT00672542</td>
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<td>Hypercholesterolaemia</td>
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<td>RSV in volunteers</td>
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<td>vWF</td>
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<td>NU172</td>
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<td>Thrombin</td>
<td>Acute coronary artery bypass surgery</td>
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<td>Factor IXa</td>
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<td>NOXXON Pharma</td>
<td>SDF-1α</td>
<td>Lymphoma patients (undergoing autologous stem cell transplantation)</td>
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<td>NOXXON Pharma</td>
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<td>Antisoma/Archemix Corp.</td>
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<td>AML</td>
<td>Phase II</td>
</tr>
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</table>

| **Aptamers**    |                                 |                                 |                                   |                |
| NOX-A12         | NOXXON Pharma                  | SDF-1α                           | Lymphoma patients (undergoing autologous stem cell transplantation) | Phase I        |
| NOX-E36         | NOXXON Pharma                  | CCL2                             | Type 2 diabetes and diabetic Nephropathy | Phase I        |
| AS1411 (AGRO001)| Antisoma/Archemix Corp.        | Nucleolin                        | AML                               | Phase II       |

#### 2.1. siRNAs

Recently, the use of RNAi-based gene silencing has been demonstrated in humans for treatment of several diseases, as discussed in multiple recent reviews [18]. We report here only few not exhaustive examples of the possible therapeutic applications.

Various clinical studies have explored the direct tissue delivery of siRNA into the eye for macular degeneration in humans. Among the growth factors implicated in the age-related macular degeneration (AMD) process, the vascular endothelial growth factor (VEGF) has been shown to be a major inducer of choroidal neovascularization [19]. Several studies have recently addressed the silencing of VEGF [20,21] or the VEGF receptor 1 (VEGFR1) [22] by RNA interference (RNAi) using either intravitreous/periocular injection of siRNA or using adenovirus backbones to allow stable endogenous transgene expression of short hairpin (sh)RNAs resulting in a potent reduction of VEGF or VEGFR1.

Silencing of gene expression by RNAi has been extensively studied to develop innovative cancer therapeutic strategies. Indeed, many of the siRNAs are in different stages of development for the treatment of different kind of tumors. For examples, among the siRNA therapeutics for the treatment of solid tumors, CALAA-01 and Atu027, targeting the M2 subunit of ribonucleotide reductase and protein kinase N3, respectively, are in Phase I, whereas, FANG against Furin is in Phase II. Further, SPC2996 against BCL-2 is in Phase II for treatment of chronic myeloid leukemia.
As shown in Table 1, the number of possible applications of RNAi therapeutics are growing rapidly and now include also viral infections, respiratory, brain, skin and metabolic diseases.

In recent studies, given the strong impact of siRNAs for therapeutic applications, a great effort is focused on the optimization of the efficacy of the siRNAs through relatively minor chemical and structural modifications to canonical siRNA. The final aim is to improve loading of the guide strand into the RNAi machinery and reduce off-target effects and competition with endogenous miRNAs.

The group of Rossi [23-26] has reported pioneering studies demonstrating that Dicer substrate interfering RNA (dsiRNA) are more potent than classical synthetic 21-mer siRNAs, showing more robust formation of a high molecular weight complex known to contain Dicer and TRBP (two primary members of the RISC-loading complex).

2.2. Aptamers

Aptamers are short single-stranded DNAs or RNAs that like antibodies, bind with high affinity to specific targets by folding into complex tertiary structures. They have some important advantages over antibodies and other protein-based reagents as therapeutics. A number of these advantages stem from the fact that aptamers are generated by an iterative in vitro evolution procedure named Systematic Evolution of Ligands by EXponential enrichment (SELEX) avoiding the use of animals or cells. In addition, aptamers can be readily chemically modified to enhance their bioavailability and pharmacokinetics [27-29]. Further, as discussed above, another important advantage of RNA aptamers over proteins is the fact that RNA is much less immunogenic than proteins [16].

The list of aptamers against important therapeutic targets is growing rapidly and some of them have already entered the clinical pipeline (see Table 1) for the treatment of different diseases [30-32]. The most successful therapeutic application of an aptamer is represented by Macugen (or pegaptanib, marketed by Eyetech Pharmaceuticals/Pfizer), an RNA-aptamer that binds and antagonizes the action of VEGF. The aptamer has been fully approved by the FDA in December 2004 for the treatment of exudative AMD. In order to translate this aptamer into the clinic, it has been chemically modified with 2'-fluoropyrimidines (2'-F-Py), 2'-O-Me-purines (2'-O-Me-Pu) and polyethylene glycol (PEG) to generate a better therapeutic agent [33,34].

Many other aptamers, not yet approved by the FDA, are currently in clinical trials. For example other two aptamers, named E10030 and ARC1905, are in Phase II and I of clinical trials for the treatment of AMD, respectively. E10030 is a DNA-aptamer directed against the platelet-derived growth factor-B (PDGF-B) chemical modified with 2'-F-Py and 2'-OMe-Pu and PEG [35]; while ARC1905 is a RNA-aptamer targeting the complement component 5 (C5) containing 2'-F-Py and PEG [36,37].

Furthermore, different aptamers targeting blood-clotting factors seems to be effective anticoagulant agents. The ARC1779 is a DNA-aptamer directed against the A1 domain of von Willebrand factor, currently in phase II clinical trials for the treatment of thrombotic microangiopathies (TMA) [38,39]; while Nu172 is a chemical unmodified DNA-aptamer directed against thrombin, currently in phase II clinical trials to evaluate its potential use as an anticoagulant during acute coronary artery bypass surgery.

Particularly interesting is REG-1, an aptamer targeting the coagulation factor IXa. This is the first case of a modulator-controlled aptamer able to provide a time-controllable therapy. REG-1 is a
two-part therapeutic agent, consisting of an RNA aptamer specific for the coagulation factor IXa (RB006) and a single stranded RNA oligonucleotide complementary to the RB006 aptamer (RB007). Aptamer inhibition of the factor IXa by RB006 is structurally disrupted by administration of the antidote complementary strand RB007. The REG-1 aptamer-antidote therapy has been tested in Phase I and II clinical trials with promising results as an anticoagulation therapy to prevent clot formation during cardiac surgery [40].

Moreover, different aptamers for cancer therapy are also in clinical trials. NOX-A12 is an L-RNA spiegelmer directed against the stromal cell-derived factor-1α (SDF-1α), a chemokine which attracts and activates immune and non-immune cells that bind to chemokine receptors CXCR4 and CXCR7. This aptamer is in Phase I clinical trials for the treatment of hematologic tumors. The AS1411 aptamer, instead, showed effectiveness for the treatment of acute myeloid leukaemia (AML) in phase I and II clinical trials. AS1411 is a DNA-aptamer, directed against nucleolin [41], a protein often overexpressed on the surface of cancer cells. This DNA aptamer is part of the guanine-rich oligonucleotide class of aptamers that form G-quartets, a structural element that exhibits antiproliferative activity. Nucleolin has many functions, so inhibiting this protein with AS1411 affects a variety of signaling pathways, including NF-κB [42] and Bcl-2 [43].

Apart from the aptamers mentioned above, many other aptamers are not yet developed in clinic but target molecules of high therapeutic interest thus appearing as excellent drug candidates for a wide range of human pathologies [30].

2.3. miRNAs

Although the clinical development of miRNAs has not yet been realized, they are attractive candidates as prognostic biomarkers and therapeutic targets in different diseases including cardiovascular disease and cancer. In addition, the use of complementary antisense oligonucleotides has been developed for miRNA silencing in research and therapy. Antisense inhibitors act by competing for miRNA binding to the proper sites on target mRNAs and include small synthetic RNAs, antagomir, and modified RNA oligonucleotides, as locked nucleic acid (LNA) [44].

Cardiovascular disease is the leading cause of death in industrialized nations. Several miRNAs have been recently implicated in cardiomyocyte hypertrophy, increased fibrosis and apoptosis during heart failure [45]. Using mice with induced cardiac hypertrophy it has been recently shown that miR21 is upregulated in heart fibroblasts, increases the extracellular signal-related kinases (ERKs)-mitogen-activated protein kinase (MAPK) activity and regulates cell survival and growth factor secretion. Cardiac hypertrophy and fibrosis can be attenuated and even prevented by the administration of a specific antagomir that suppresses miR-21 levels and reduces cardiac ERK-MAPK activity [46]. On the other hand, has been shown that miR-199a expression is sensitive to low oxygen levels and is rapidly downregulated in cardiac myocytes to undetectable levels, thus rapidly resulting in increased levels of mRNA target, hypoxia-inducible factor (Hif)-1alpha. Conversely, restoring miR-199a levels during hypoxia inhibits Hif-1alpha expression, reduces apoptosis and protects the cells from hypoxic injury [47]. Cardiac remodeling can be as well prevented by the administration of an inhibitory antagomir for the cardiac-specific miR-208a thus improving the overall survival of treated rats [48]. All together, these studies indicate the potential of RNA-based therapies for cardiovascular diseases.
Expression of several miRNAs has been shown to be deregulated in many cancer types. Further, based on their involvement in basic cellular functions, miRNAs may act as oncogenes (oncomirs) or tumor suppressor as critical players in cell transformation [49-51].

For example, it has been demonstrated that the let-7 family contains miRNAs regulating the RAS family of oncogenes [52]. Petrocca et al. [53] showed that the miR-106b-25 cluster plays a key role in gastric cancer interfering with proteins involved both in cell cycle and apoptosis. In other studies, miR-155 was found overexpressed in Hodgkin lymphoma, pediatric Burkitt lymphoma and diffuse large B-cell Lymphoma [54-56]; miR-143 and miR-145 were significantly downregulated in colon cancer tissue compared with colonic mucosa [57]; miR-21 was overexpressed in many tumors [49], including glioblastoma [58], cholangiocarcinoma [59], multiple myeloma cells [60] and breast cancer [61,62]. Moreover, studies that investigated the expression of the entire microRNAome in various human solid tumors and hematologic malignancies have revealed differences in miRNA expression profiling between neoplastic and normal tissues [63-66]. miRNAs play a key role also in tumor metastasis. Indeed, for example miR-139 suppresses metastasis of hepatocellular carcinoma, while miR10-b was found highly expressed in metastatic breast cancer cells [67,68] even if its clinical utility is still questioned [69].

3. RNA-Based Bioconjugates Molecules for siRNA Delivery

Potent sequence selective gene inhibition by siRNA ‘targeted’ therapeutics promises the ultimate level of specificity, but siRNA therapeutics is hindered by poor intracellular uptake, thus efficient delivery strategies remains the main challenge for their clinical development [70].

In this respect a promising application of aptamers is to use them to deliver a variety of secondary reagents, including therapeutic siRNAs, specifically to a targeted cell population (Table 2) [71,72].

<table>
<thead>
<tr>
<th>Aptamer composition</th>
<th>Target</th>
<th>Cargos/targeted delivery</th>
<th>Therapeutic Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA, 2’-F-Py</td>
<td>PSMA</td>
<td>siRNA,Toxin, QDs, nanoparticles and chemotherapeutics</td>
<td>Prostate cancer therapy</td>
</tr>
<tr>
<td>RNA, 2’-F-Py</td>
<td>gp120</td>
<td>siRNA</td>
<td>HIV infection</td>
</tr>
<tr>
<td>RNA, 2’-F-Py</td>
<td>CD4</td>
<td>siRNA</td>
<td>HIV infection</td>
</tr>
<tr>
<td>RNA</td>
<td>EGFR</td>
<td>Au NPs</td>
<td>Cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>PTK7</td>
<td>Doxorubicin, Au-Ag NPs</td>
<td>Cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>Mucin 1</td>
<td>QDs, photodynamic therapy agents</td>
<td>Cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>Nucleolin</td>
<td>QDs</td>
<td>Cancer</td>
</tr>
</tbody>
</table>

This means that aptamers function as specific recognition ligands to target cells, which is especially significant given the whole cell-SELEX strategy to target specifically cell surface epitopes [73]. Once delivered, the secondary reagents would then impart their therapeutic effect to this subset of cells within the treated individual. Because non-targeted cells would not be exposed to the secondary reagent, the potential for unwanted side-effects such as death of normal cells is substantially reduced.

The cell-SELEX method allows for the generation of aptamers against cell surface targets by replicating the native conformation and glycosylation pattern of the extracellular regions of proteins.
Recently, multiple groups have reported selections using living cells as the target to identify receptor-specific aptamers and those that bind to a specific cell type [73,74]. Some of these aptamers have been used as delivery cargos to target cells giving the cell-type specific expression of cell surface proteins on cell populations of therapeutic value.

In the aptamer-based delivery approach, the last goal is to develop an aptamer to the extracellular portion of such a protein and then use the aptamer to deliver the secondary reagent to the targeted cell population via binding the targeted protein on the surface of the targeted cell type. Because this binding in some cases also results in the endocytosis of the aptamer/secondary reagent complex, this approach can be used to deliver reagents such as siRNAs that depend on delivery to intracellular compartments for their proper function (Figure 1).

**Figure 1.** Aptamers as delivery agents. Aptamers that bind to cell surface receptors can be used to deliver siRNA to target cells.

To date, the best-characterized aptamers for targeted delivery are the two 2′-F-Py-RNA aptamers (A9 and A10) that have been generated against the extracellular domain of the prostate-specific membrane antigen (PSMA) [75]. These aptamers bind with high affinity to the acinar epithelial cells of prostate cancer tissue. They have been used to deliver not only siRNA, but also nanoparticles, quantum dots (QDs) and toxins to prostate cancer cells [73]. Different approaches in which PSMA-aptamer has been linked to siRNAs have been reported (Figure 2).

A first study reports the non-covalent conjugation of siRNA with A9 aptamer via a streptavidin connector [76]. The 27mer Dicer substrates targeting laminin A/C and GAPDH genes and the RNA aptamers were chemically conjugated with biotin. Thus, two biotinylated siRNAs and two aptamers were non-covalently assembled via a streptavidin bridge. The resulting conjugates were incubated with PSMA-positive LNCaP cells without any further preparation, and were taken up within 30 min. The inhibition of gene expression was mediated by the aptamers and as efficient as observed with conventional lipid-based reagents.
**Figure 2.** Anti-PSMA aptamer-siRNA chimeras. (a) The RNA duplex and RNA aptamers are chemically conjugated with biotin. Thus, two biotinylated siRNAs and two aptamers are non-covalently assembled via streptavidin; (b) The 3’ end of the aptamer is extended to contain the nucleotide sequence that is complementary to the antisense strand of the siRNA, and the chimera is formed by annealing the aptamer to the siRNA antisense strand; (c) optimized chimeras in which the aptamer portion of the chimera is truncated, and the sense and antisense strands of the siRNA portion are swapped. A two-nucleotide 3’-overhang and a PEG tail are added to the chimera; (d) the 3’-terminus of the aptamer is conjugated to the sense strand of the siRNA, followed by a 10-mer loop sequence and then by the antisense strand of the siRNA.

In the same year, McNamara et al. [77] described the generation of the anti-PSMA A10 aptamer-siRNA chimeras. The 3’ end of the aptamer was extended to contain the nucleotidic sequence complementary to the antisense strand of siRNA targeting the polo-like kinase 1 (PLK1) and BCL-2 survival genes, and the chimera was formed by annealing the aptamer to the siRNA antisense strand. The resulting chimeras were effective in silencing target genes and inducing cell death specifically in PSMA-positive cancer cells.

In addition, the PSMA-siRNA chimeric molecule has been further modified for in vivo application [78]. The aptamer portion of the chimera was truncated, and the sense and antisense strands of the siRNA portion were swapped. A two-nucleotide 3’-overhang and a PEG tail were added
to the chimera. The modified chimera was able to inhibit prostate cancer xenograft growth when administered systemically.

To date several groups have adapted the covalent assembly approach to aptamer-mediated siRNA delivery [72]. In these studies, the anti-PSMA A10 aptamer has been conjugated to siRNAs against eukaryotic elongation factor (EEF)2 [79] and two key components of the nonsense-mediated mRNA decay (NMD) [80]. In addition, since short hairpin RNAs (shRNAs), like miRNA precursors are better substrates for Dicer, Ni et al. [81] linked a shRNA against the DNA-activated protein kinase (DNA-PK) to a truncated A10 aptamer (A10-3) generating a single intact nuclease-stabilized 2’ fluoro-modified pyrimidine molecule. The 3’-terminus of the A10 aptamer was conjugated to the passenger (sense) strand of the siRNA, followed by a 10-mer loop sequence and then by the guide or silencing (antisense) strand of the siRNA.

Rossi and colleagues have extensively characterized the HIV glycoprotein gp120 as a target for aptamer-mediated siRNA delivery [82-84]. In these studies, an inhibitory RNA aptamer targeting the HIV envelope protein gp 120, has been used to deliver attached anti-HIV tat/rev siRNAs into HIV infected cells via binding to envelope expressed on the cell surface, resulting in internalization of the aptamer and delivery of a dicer substrate siRNA to RISC. In vivo delivery of the aptamer and aptamer-siRNA conjugates into a humanized mouse model for HIV infection suppressed HIV replication and completely protected T-cells from HIV mediated T-cell killing.

With the development of the conjugation strategies, the list of aptamers against surface epitopes that are being used as delivery agents is growing rapidly and now includes those against PTK7 [85,86], nucleolin [87], mucin 1 [88,89], and EGFR [90] the have been used to deliver not only siRNA, but also nanoparticles, quantum dots (QDs), toxin and chemiotherapeutics to target cells (see Table 2).

4. Market and Perspectives

Even if only one nucleic acid aptamer has been approved and is on the market, aptamers hold an extraordinary potential in drug development and it is plausible that the global interest for their development will increase in the next few years. Accordingly, a new technical market research report, from BCC Research [91], estimated that the global aptamer market value of $236 million in 2010 will grow to nearly $1.9 billion in 2014, for a 4-year compound annual growth rate of 67.5%.

To date, Archemix Corp. is a leading biopharmaceutical company in the development of aptamers as therapeutics. It is the owner of the aptamer technology patent and it collaborates with other pharmaceutical companies (Regado, Antisoma, ARCA Biopharma and Ophthotech) to develop and commercialize a pipeline of partnered aptamers in the cardiovascular disease, hematology and oncology areas.

Moreover, the development of aptamers as delivery agents for therapeutic RNAs can have a considerable impact on aptamer market in the near future. Indeed, intracellular delivery has been a key challenge for RNA modalities and the potential of bringing together the properties of aptamers and microRNA therapeutics will allow to overcome this limitation and open further potential for RNA-based therapeutics.

Recently Archemix Corp. started a collaboration with miRagen Therapeutics Inc., a biopharmaceutical company focused on developing innovative microRNA-based therapeutics for cardiovascular and
muscle disease, for the development of conjugated aptamer-microRNA molecules capable of intracellular delivery and subsequent microRNA targeting. Combining aptamers and microRNA therapeutics has the potential to solve the intracellular delivery challenge for certain RNA-based therapeutic approaches. In this perspective, even if aptamer-miRNA chimeras have not been already described in literature, it is plausible that the approaches discussed in this review for aptamer-siRNA conjugation could be as well adapted to generate aptamer-miRNA molecules of fundamental therapeutic value.

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MiR-34c may protect lung cancer cells from paclitaxel-induced apoptosis

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Running Title: anti-apoptotic effects of miR-34c

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Abstract

MicroRNAs (miRNAs) constitute a class of small non-coding RNAs that negatively regulate the expression of their target genes. They are involved in many biological processes, including cell proliferation, apoptosis and differentiation, and are considered as promising new therapeutic targets for cancer. However, the identity of miRNAs involved in apoptosis and their respective targets remain largely unknown. Given the elevated complexity of miRNA regulation of gene expression, we performed a functional screening as an alternative strategy to identify those miRNAs that in lung cancer cells may interfere with the apoptotic process. To this aim we generated a derivative of the non-small cell lung carcinoma A549 cell line in which caspase-8, a critical upstream initiator of apoptosis, can be activated by the administration of the small dimerizer drug AP20187.

We found a number of miRNAs that may rescue cell viability from caspase-8 activation. They included miRNAs already described as oncogenic such as miR-17, miR-135, miR-520, but also some miRNAs such as miR-124-1 and miR-34c for which a tumor suppressive role has been instead described or expected. Among them, miR-34c-5p markedly increased resistance to paclitaxel induced apoptosis. We demonstrate that Bmf (Bcl-2 modifying factor) is a target of miR-34c-5p and that its silencing, together with that of c-myc, a known target of miR-34c-5p, contributes to resistance to apoptosis induced by paclitaxel via p53 downregulation.

Key words: A549, p53, AP20187
Introduction

MicroRNAs (miRNAs) are a class of short endogenous non-coding RNAs that act as key regulators of cell proliferation and apoptosis through the control of gene expression either inhibiting translation or triggering degradation of multiple target mRNAs (Bartel, 2009). MiRNAs have been found deregulated in different malignancies, implicating them as oncogenes or tumor suppressors and thus considered as promising new therapeutic targets for cancers (Farazi et al., 2011; Garofalo and Croce, 2011). Indeed, the selective induction of cell death by oligonucleotide-based drugs is a challenging goal for a rationale approach to novel therapeutic strategies in cancer treatment.

Programmed cell death, or apoptosis, is mediated through two major pathways, the death receptor pathway and the mitochondrial pathway. In the death receptor pathway, stimulation of death receptors leads to the formation of a death-inducing signaling complex which includes the initiator caspases such as caspase-8 that drives its activation through self-cleavage and then activates several downstream effectors including caspase-9 and the ultimate effector caspase-3 (Ashkenazi and Dixit, 1998). In the mitochondrial pathway, stimuli such as drugs, radiation, infectious agents and reactive oxygen species transmit the death signals to mitochondria through the activation of BID, a pro-apoptotic member of the Bcl-2 family (Hengartner, 2000). Caspase-8-mediated cleavage of BID thus provides integration of the death-receptor with the mitochondrial pathway.

Identifying miRNAs that selectively regulate the expression of proteins involved in apoptosis could be helpful in the development of new tools for the diagnosis and the treatment of cancer. However, the potential for combinatorial regulation of gene expression by miRNAs makes it difficult to understand which are the targets involved and what are their coordinate mechanisms of action (Inui, et al., 2010).

Therefore, as alternative to high-throughput screening, here we developed a functional selection-based screening to identify those miRNAs able to rescue cells from apoptosis in lung cancer cells. This approach has the advantage to identify unique functional combinations of miRNA molecules without any assumption about the targets involved. Since a pivotal upstream event in the death receptor pathways is the activation of caspase-8, the screening has been based on the use of an engineered lung cancer cell line in which the
activation of caspase-8 has been placed under the control of the dimerizing agent AP20187. Here we show that this is an unbiased approach able to uncover unpredicted effects of miRNA expression. Indeed, miR-34c, a p53 effector miRNA (Chang, et al., 2007; He, et al., 2007), revealed to be able to antagonize paclitaxel-induced apoptosis, with obvious fall-outs in the design of miRNA-based new therapeutic agents for cancer treatment.
Results

Identification of protective miRNAs by functional selection

In order to identify miRNAs able to rescue cells from caspase-8 induced cell death we adopted a functional selection screening based on a FKBP caspase-8 inducible chimera (puro-DD-FKC8) (Carlotti 2005; Chang and Yang 2003). The human non small cell lung cancer (NSCLC) A549 cells have been transduced with a retroviral vector with inserted the caspase-8 chimera. The most responsive cell clone, the A549-FK, was then transduced with a pooled miRNA library and subjected to selection for resistance to caspase-8 induction of cell death, as described in Supplementary Information and in Figure S1a.

In out of 16 cell clones analysed we found integrated miRNAs (see SI for more details) already described as oncogenic such as miR-17, miR-135 (Nagel et al., 2008), miR-520 (Huang et al., 2008). However, we also found some miRNAs such as miR-124-1 and miR-34c for which a tumor suppressive role has been instead described or expected. The most frequent integrated miRNA was miR-34c that was as well the only miRNA that was found not only in combination with other miRNAs but also alone in two independent cell clones, thus suggesting to be sufficient by its own to confer resistance to caspase-8 activation (Figure S1b). The other miRNAs were always found to be present as a combination of at least two different molecules (not shown). By qt-RT-PCR we thus evaluated in all cell clones the expression levels of transduced miRNAs (as compared to non-transduced control cells) and assessed that, with the only exception of miR-645 in the clone Cl-Y (see Supplementary Information), all transduced miRNAs are expressed in the corresponding surviving clones (not shown). In the case of miR-34c the expression levels of both the -5p and -3p strands were analysed (see Figure S1c).

MiR-34c-5p protects cells from capase-8 induced apoptosis

Given the high frequency of integration of miR-34c in surviving clones, we wondered if, in our system, forced over-expression of the most expressed isoform, miR-34c-5p, might protect cells from caspase-8 induced apoptosis. As determined by MTT assay (Figure 1a) all clones transduced with miR-34c are resistant to 10 nM AP20187 regardless if it was present alone (in two clones, Cl-T; Cl-8) or in combination with other miRNAs (in clones Cl-H; Cl-K; Cl-Y; Cl-1; Cl-2; Cl-12; Cl-17; Cl-53) (see legend to Figure S1 c). In A549-
FK cells, caspase-8 dimerization activates caspase-3 (Fig 1b, lanes 1-3) through cleavage of the BH3-only protein BID (Figure 1c), that leads to activation of Bax (Figure 1d) and caspase-9 (Figure 1b middle panel) (Jost et al., 2009). In contrast to parental A549-FK, both cell clones analysed (Cl-T and Cl-K), in which miR-34c was integrated either alone (Cl-T) or together with miR-124-1 and miR-362 (Cl-K), were resistant to AP20187-induced apoptosis (Figure 1b-d). Resistance is unlikely due to the occurrence of new clonal mutations impairing caspases that are indeed activated following cisplatin treatment (Figure 1e). Conversely, transfecting cell clones with a miR34c-5p inhibitor sensitizes cells to AP20187-induced cell death with a consequent increase of caspase-3 and caspase-9 activity in both Cl-T (Figure 1f) and in Cl-K (not shown). Further, upon AP20187 treatment, the presence of miR-34c-5p, but not of miR-34c-3p (not shown), makes the cells more resistant to caspase-8 induced cell death, reducing the per cent of apoptotic cells from 70% (of scrambled control) to around 30% (Figure 2a). Consistently, resistance to apoptosis conferred by miR-34c-5p is accompanied by the lack of activation of caspase-9 and of its effector, caspase-3, with an increase in the overall cell survival (Figure 2 b-d). These data indicate that while in a proliferating cell population miR-34c-5p may induce an increase in apoptosis (Chang, et al., 2007; He, et al., 2007), in the presence of a strong acute pro-apoptotic signal, as the dimerization of caspase-8, it may, in contrast, elicit a clear protective effect.

**miR34-c-5p protects cells from paclitaxel induced apoptosis**

We thus determined whether miR-34c-5p has antiapoptotic function toward broadly used chemotherapeutic agents. As shown in Figure 3a, treating parental A549 cells with 100 nM paclitaxel for 24 h induces caspase-3 and caspase-9 activation that were partially reverted by transfecting cells with miR-34c-5p mimic (left panel) but not with miR-34c-3p mimic (right panel) . Consistently, transfecting miR-34c-5p in A549 induces a clear reduction in the per cent of paclitaxel-induced apoptosis (Figure 3b). On the other hand, antagonizing with specific LNA-based miR inhibitors the miR34c-5p, but not the miR34c-3p, rescued the sensitivity to paclitaxel-induced apoptosis in Cl-K cells (Figure 3c). A similar protective effect against paclitaxel is elicited by miR-34c in A549-FK, but not in the more sensitive H460 NSCLC cells (not shown). Further, differently from what observed with paclitaxel, transfecting miR-34c-5p mimic doesn’t reduce the levels of
active caspase-3 and caspase-9 neither upon TRAIL (200 ng/ml) nor cisplatin (50 µg/ml) treatments. However, at least in transient, miR-34c-5p may cooperate with cisplatin (Figure 3d-e). Taken together these results well support the notion that the protective potential of miR-34c may be only unveiled in a cell context and pathway specific manner.

**Targets of miR-34c-5p**

In an attempt to understand the molecular events elicited by high levels of miR-34c-5p on apoptosis, and thus which are the target proteins involved, we first used a small antibody array to analyze the relative expression of a selected number of apoptosis related proteins in A549-FK cells transfected with miR-34c-5p (not shown). Since, using this assay, no major changes were observed we thus decided to take advantage of the information coming from the functional screening by assuming that the pathways involved may be shared between the different miRNAs selected for their ability to rescue A549-FK cells from caspase-8 DD-FKC8 chimera-induced cell death. We thus combined the bioinformatic prediction (Diana-microT³) for targets common to those miRNAs transduced and expressed in three independent cell clones together with miR-34c: i.e. in Cl-17 (miR-345 and miR362), in Cl-K (miR-362 and miR-124-1), in Cl-Y (miR-346). Among the candidate targets considered, even if with different score values (see legend to Figure 4), Bmf (Bcl2-modifying factor) was predicted to be a common target for all of miRNAs analysed. In the 3’ UTR of Bmf two recognition sites are predicted for miR-34c-5p, one at position 737-765, and one at position 2283-2311 (Diana-microT³) (Figure 4a). Therefore, we determined whether Bmf might be a direct target of miR-34c-5p. As shown in Figure 4b, transfecting miR-34c-5p either in A549-FK or in parental A549 cells resulted in a decrease of Bmf protein (of approximately 60%). Furthermore, in two miR-34c infected cell clones analysed, that overexpress miR-34c-5p either alone (Cl-T) or in combination with other miRNAs (Cl-K), the levels of Bmf are reduced as compared to parental A549-FK cells (Figure 4c). Conversely, by using specific LNA based inhibitors to antagonize the miR34c (either -5p and -3p) in Cl-T we rescued the expression of BMF (Figure 4d). To prove a direct interaction between miR-34c-5p and Bmf mRNA, two Bmf 3’-UTR sequences (BMF S1 and BMF S2), which include each of the two potential target sites for miR-34c-5p, were fused downstream of the luciferase gene. The two constructs containing either BMF S1 or BMF S2, either
individually and in combination, were cotransfected into MEG-01 cells together with miR-34c-5p, or a scrambled miRNA as negative control. As shown in Fig 4e, luciferase activity of BMF S2 was decreased of around 30% by cotraskanfecting miR-34c-5p. In contrast luciferase activity of both the BMF S1 and a deletion mutant of BMF S2 are poorly affected by miR-34c-5p cotransfection. Taken together these data indicate that miR-34c-5p may decrease Bmf expression by recognizing a binding site on its 3’ UTR starting at position 2283. As mentioned above, we identified Bmf as promising target for miR-34c-5p based on the assumption that common messengers should be targeted by miRNAs found in independent cell clones together with miR-34c. In order to verify this assumption we determined whether miR-362 and miR-124 may affect as well Bmf levels. As shown transfecting either miR-362 or miR-124 decreases Bmf levels (Figure 4f). Further we determined by luciferase assay whether miR-362 interacts with the predicted sites for the miR-362-5p and -3p strand in the Bmf 3’ UTR (BMF S3 and S4 respectively). As shown in Figure 4g, luciferase activity of BMF S3 was decreased of around 60% by cotransfecting miR-362-5p. In contrast, luciferase activity of the BMF S3 mutant is poorly affected by miR-362-5p cotransfection. These results well support the working hypothesis that miRNAs for common targets may be preferentially selected during the functional screening.

**Mechanism of action**

In order to understand if the decrease of Bmf levels by miR-34c-5p is sufficient to cause protection to apoptosis, we transfected A549-FK cells with either the miR-34c-5p or with an siRNA for Bmf and analysed the activation of caspases-9 and -3 after forced dimerization of caspase-8. Interfering with Bmf expression either by miR-34c-5p and, even if at less extent, by Bmf specific siRNA protects the cells from AP20187-induced caspases activation (not shown). Further, to understand whether Bmf mediates the effects of miR-34c-5p we silenced Bmf in the A549 cells with a specific shRNA and determined the residual ability of miR-34c-5p to elicit protection from paclitaxel-induced caspase-3 activation. As compared to Bmf silenced cells, overexpression of miR-34c-5p was able to further reduce caspase-3 activation thus indicating that Bmf doesn’t fully mediate miR-34c-5p protective effects (Figure 5a). Intriguingly, miR-34c, belongs to a family of evolutionarily conserved miRNAs (miR-34a, miR-34b, and miR-34c), whose expression has been shown to be under the transcriptional control of the tumor suppressor protein p53 and known to be implicated in the
negative control of the cell cycle, senescence and apoptosis (Corney et al., 2007; Bommer et al., 2007; He et al., 2007; Hermeking 2010). Therefore, with the aim to reconcile this with the protective effect of miR-34c-5p on paclitaxel-induced apoptosis we asked whether c-myc, a known target of miR-34c (Cannell and Bushell, 2010), may as well participate to inhibit apoptosis. Indeed, c-myc, acting on the protein deacetylase Sirt1, regulates p53 acetylation, and thus its activity (van Leeuwen and Lain, 2009). As expected, transfecting A549-FK cells either with miR-34c-5p, or with a c-myc specific siRNA, resulted in a partial decrease of c-myc protein levels (Fig 5b). Interestingly, attenuation of c-myc expression protects cells from AP20187 and most importantly from paclitaxel-induced caspase-3 activation (Figure 5c). In order to determine whether c-myc downregulation mediates miR-34c-induced protection, we depleted A549 cells of c-myc by transfecting a specific siRNA. As shown in Figure 5d, we found that miR-34c-5p become poorly efficient to further increase protection over that induced by c-myc silencing. Even though, the contribution to protection of the passenger -3p strand remains to be elucidated, we show that the miR-34c-3p mimic we used may decrease the Bmf levels, but it is less effective on the regulation of c-myc levels (Figure 5e). Together these results suggest that c-myc is a critical target of miR-34c that may act by protecting cells from paclitaxel-induced apoptosis.

Paciltaxel is a microtubule destablizer and cell cycle integrity has been shown to be required for sensitization (Chabalier et. al. 2006; Zachos et al., 2007). We thus determined whether perturbation of c-myc levels by miR-34c-5p affects cell cycle thus providing an explanation for resistance. As assessed by FACS analysis, cell cycle is not appreciably impaired by the transfection of miR-34c-5p (Figure 5f). Accordingly, as determined by immunoblot, no drastic changes were observed upon transfection of the miR-34c-5p, neither in cyclins B1, D1 nor in Chk1 levels (Figure 5g). Similarly, induction of cyclin B1 levels by treatment with paclitaxel was not impaired by miR-34c-5p thus indicating that resistance is unlikely attributable to an abnormal mitotic checkpoint response (Figure 5h). On the other hand, the levels of cyclin D1, that mediate p53-dependent cell cycle arrest (Del Sal et al., 1996), were increased by paclitaxel, while in the presence of miR-34c-5p such increase was impaired (Figure 5h).

We then wondered whether transfecting miR-34c-5p may act on the activity of p53, monitoring p53 acetylation that is essential for maximal p53-dependent apoptosis (Sykes et al., 2006; Tang et al., 2006; Mellert et al., 2011). As shown in Fig 6a in untreated, exponentially growing, A549 cells the levels of
acetylated p53 are very low and are strongly induced by paclitaxel treatment (24 h). Interestingly, miR-34c-5p overexpression counteracts paclitaxel effects by reducing the induction of acetylated p53 levels to less than 2 fold over basal. The interference of miR-34c-5p on the levels of acetylated p53 is well mirrored by the corresponding decrease in the paclitaxel-induced expression of total p53. This suggests that miR-34c-5p, by regulating p53 synthesis or stability, may indirectly act on its activity. To confirm that miR-34c-5p may interfere with paclitaxel-induced p53 activity we performed luciferase assays transfecting a construct expressing the luciferase reporter gene under the transcriptional control of the p53-responsive human ubiquitin ligase MDM2 (mouse/human double minute 2) promoter. As shown in Figure 6b miR-34c-5p overexpression abolishes the induction of p53 activity by paclitaxel as assessed by the corresponding levels of luciferase activity. Consistently, miR-34c-5p abolishes the induction of the MDM2 protein and decreases the levels of the deacetylase SIRT-1, that is in turn a known direct target of miR-34c (Yamakuchi et al., 2008) (Figure 6c). Therefore, in the attempt to understand how MDM2 is implicated in the attenuation of p53 response to paclitaxel we inhibited MDM2 activity. The treatment of A549 cells with Nutlin-3, a direct inhibitor of MDM2, increases the levels of p53, likely by increasing its stability. At difference of what happens with paclitaxel-induced p53, transfecting miR-34c-5p in the absence of MDM2 didn’t result in the attenuation of p53 (Figure 6d, upper panel). Conversely, we show that following inhibition of MDM2 the balance between the levels of miR-34c-5p and p53 cannot be interfered by antagonizing miR-34c-5p with a specific LNA based inhibitor (Figure 6d, lower panel).

As shown in Figure 6e both cisplatin and paclitaxel-induced activation of caspase-3 is decreased in stable A549 cells silenced for p53 (Figure 6e). However, at difference of paclitaxel, the cisplatin-induced levels of p53 were unaffected by miR-34c-5p (Figure 6f) thus suggesting that resistance of cisplatin might involve the way to induce p53 in these cells.

Taken together these data indicate that transfecting miR-34c-5p in the presence of a proapoptotic insult, as paclitaxel, may protect cells from apoptosis likely by interfering with the levels of p53 likely by tuning the levels of c-myc and suggest that miR-34c-5p-induced p53 attenuation depends on MDM2 activity.
Discussion

Signaling initiated by death receptors causes the recruitment and activation of the initiator caspases (caspase-8 and caspase-10) thus triggering the activation of either intrinsic or extrinsic apoptotic pathways that ultimately leads to cell death. However, distinct multiple signaling pathways have been recently shown to be initiated by some of these receptors that rather promote activation of pro-survival proteins such as NF-KB, protein kinase B (PKB)/Akt, and MAP kinases that ultimately lead to a number of non-cytotoxic functions as cell proliferation or inflammation (Guicciardi and Gores, 2009).

In order to dissect miRNAs implicated in the pro-apoptotic pathway we engineered a NSCLC derived cell line to induce caspase-8 dependent cell death. By using a functional selection we identified miRNAs whose expression may protect cells to undergo apoptosis. Eleven different miRNAs have been selected that if expressed, either alone or in combination, confer resistance to caspase-8 dimerization. Among these miRNAs, miR-34c revealed to be of particular interest. Indeed, miR-34c belongs to a conserved miRNA family consisting of three members: miR-34a, generated from a transcriptional unit on the human chromosome 1p36; miR-34b and miR-34c, which are generated by processing of a bicistronic transcript from chromosome 11q23. Both transcripts have been shown to be under the direct positive control of p53 (Chang et al., 2007; He et al., 2007a; Raver-Shapira et al., 2007; Tarasov et al., 2007; Bommer et al., 2007; Corney et al., 2007) and Elk1 (Christoffersen et al., 2010). Several converging evidence demonstrated that miR-34 members mediate p53 action to negatively regulate cell cycle thus acting as “bona fide” tumor suppressor genes (Hermeking, 2010; Liu et al., 2011; Li et al., 2009; He et al., 2007b). Though exceptions exist (Dacic et al., 2010; Lee et al., 2011), miR-34a/b/c have been consistently found poorly expressed in several tumor and tumor derived cell lines (He et al., 2007a; Corney et al., 2010:).

In apparent contradiction with its tumor suppressive potential, here we demonstrate that forced expression of miR-34c may confer resistance to caspase-8-induced apoptosis thus allowing to the selected cell clones to survive and proliferate even in the presence of lethal doses of the caspase-8 dimerizer AP20187. Indeed, in A549-FK cells, the drug induces the activation of the mitochondrial pathway through the activation of several proapoptotic members of the Bcl2 family, including BID, Bmf, and Bax, that is clearly impaired by ectopic miR-34c. Resistance is unlikely due to the occurrence of new clonal critical mutations in the
apoptotic pathway, indeed, 1): in two analysed cell clones, CI-K and CI-T, caspase-8 sensitivity is restored by antagonizing miR-34c-5p action with a specific antimiR; 2) apoptosis is readily activated by treating the same cell clones with an unrelated proapoptotic drug; and 3) transfecting miR-34c-5p mimics protects the A549-FK cells by AP20187-induced cell death.

Furthermore, our data clearly show that protection is not restricted to the non-physiological pathway induced by the artificial caspase-8 dimerizer that we used for selection, in fact activation of the mitochondrial pathway by paclitaxel was as well specifically impaired by miR-34c. Paclitaxel is a lead anticancer compound that prevents cell progression through mitosis (Allan et al., 2007; Shiff et al., 1979) causing cells to arrest and undergo apoptosis through activation of BID and caspase-9. However, even if our data indicate that miR-34c acts by interfering with the complete activation of the mitochondrial pathway, two observations show that its protective action is only unveiled under specific conditions. First, transfection of miR-34c doesn’t protect cells from other pro-apoptotic compounds, as TRAIL, or cisplatin; second, transfecting miR-34c has no protective effect on the more sensitive H460 NSCLC cell line, raising the question of which are the mechanisms that determine miR-34c to play opposite roles on cell survival or death. Yet, in the absence of definitive evidence on the critical players involved, it seems plausible that the action of miR-34c may depend on the balance of specific intracellular mediators of apoptosis. On the other hand, the protective action of miR-34c is not completely unexpected. Indeed, it has been recently shown that the cognate miRNA, miR-34a, may confer resistance to bortezomib-induced apoptosis by downregulating p53 and that survival and p53 downregulation depend on the expression levels of c-myc (Sotillo et al., 2011). Indeed, c-myc is a key regulator of cell proliferation since its induction sustains cell proliferation and transformation. However, in concert with p53, elevated levels of c-myc may sensitize tumor cells to proapoptotic stimuli and promote cell death (Maclean et al., 2003; Cannell et al., 2010; Murphy et al., 2008; Soucek and Evan, 2010; Allen et al., 2011).

In an attempt to understand which are the critical targets of miR-34c we assumed that, given the protocol of functional selection adopted, some of these targets should be common to multiple miRNAs selected and found in combination with miR-34c (see Legend to Fig. 4 and Text for details). Based on this assumption, we identified among the genes with predicted consensus sites for miR-34c, the proapoptotic protein Bmf, a common target with predicted consensus binding sites for all the miRNAs analysed. Indeed, attenuation of
Bmf expression, even poorly, protects cells from caspase-8-induced apoptosis and thus is a promising target candidate of miR-34c, likely by directly targeting one of the predicted consensus sites in its 3’ UTR at position 2283. Further, according with recent reports that indicate c-myc as target of miR-34a/c (Cannell and Buschell, 2010; Christoffersen et al., 2010; Kress et al., 2011) here we show that c-myc is downregulated by miR-34c-5p and that its downregulation is by its own sufficient to mediate much of the protective effects of miR-34c, thus proving that in A549 cells regulation of c-myc is critical to mediate survival. On the other hand, in good agreement with previous reports (Sotillo et al., 2011) our results indicate that the effects of miR-34c involve, besides c-myc, the MDM2 and p53 loop. Indeed, miR-34c attenuates the paclitaxel-dependent induction of p53 by a mechanism that is unlikely determined by a decrease in the extent of p53 acetylation since the levels of the deacetylase SIRT-1 (a target of miR-34c) are as well decreased. Our results rather show that inhibiting MDM2 induces p53 expression, but induction becomes insensitive to the intracellular levels of miR-34c thus indicating that MDM2 activity could be required for attenuation. Therefore, a plausible explanation of the fact that miR-34c is unable to attenuates the levels of cisplatin-induced p53, while the silencing of p53 confers resistance to both cisplatin and paclitaxel, may likely rely on the genotoxic action of cisplatin that, at difference of paclitaxel, causes MDM2 inhibition. A primary way by which c-myc regulate MDM2 activity implicates the regulation of the MDM2 regulatory protein, p14/Arf, that is however deleted in A549 cells (Lu et al., 2002) thus making elusive in these cells the link between c-myc and MDM2 for protection by miR-34c. On the other hand, it has been shown that active p53 directly induces miR-34c expression that in turn indirectly increases p53 activity thus establishing a positive feedback loop leading to cell cycle arrest (Corney et al., 2007; Yamakuchi et al., 2008; Yamakuchi et al., 2009; Hermeking, 2010). How this loop is attenuated is still unknown. In light of our results, it seems reasonable to hypothesize that increased levels of miR-34c may contribute to switch off the loop and thus interfering with paclitaxel-induced cell cycle arrest.

These results can be relevant both for the understanding of molecular mechanisms that governs the interplay between miR-34c, p53 and c-myc to control cell proliferation and apoptosis, and for the development of targeted specific new therapeutic agents.
Materials and Methods

Cell Cultures

Human NSCLC A549 and H460 (American Type Culture Collection) were grown in RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Corp. Saint Louis, MO, USA), 2mM L-glutamine and 100 U/ml penicillin–streptomycin. A549 cells carry a deletion in the ARF locus (Lu et al., 2002). A549-FK, clone Cl-K and clone Cl-T were cultured in the same medium supplemented with 5 µg/ml puromycin, while A549 stably silenced for p53 were cultured in medium supplemented with 1 µg/ml puromycin.

Immunoprecipitation and immunoblotting

Total cell lysates were prepared in JS buffer (50 mM Hepes ph 7.5, 150 mM NaCl, 1%Glycerol, 1%Triton X-10, 1.5 mM MgCl₂, 5 mM EGTA, 1 mM Na3VO4 and protease and phosphatase inhibitors) and then boiled in SDS/β-mercaptoethanol sample buffer. 40 or 30 µg samples were loaded into 15% or 12% polyacrylamide gels and the proteins were separated by electrophoresis and then blotted onto PVDF membranes (Millipore, Billerica, MA, USA) by electrophoretic transfer. The membranes were then blocked with 5% dried milk in TBS containing 0,1% Tween 20 and incubated at 4°C overnight with the following primary antibodies: anti-caspase 3, anti-caspase 9, anti-BID, anti-Bcl-Xl, anti-Bmf, anti-Acetyl-p53 (Lys382), anti-cyclin D1 (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-procaspase-3 and anti-procaspase-9 (Abcam plc, Cambridge, UK), anti-c-myc (9E10), anti-p53 (DO-1), anti-Chk1 (G-4), anti-cyclin B1 (H-433), anti-SIRT1 (H-300) (Santa Cruz, CA, USA), anti-α-tubulin (Sigma-Aldrich Corp. Saint Louis, MO, USA) and anti-MDM2 (mAB 2A10) (Calbiochem).

For Bax activation analysis total cell lysates were, instead, prepared in 1% Chaps buffer (5mM MgCl₂, 137 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Chaps, 20 mM Tris-HCl ph 7.5 and protease inhibitors. 500 µg proteins were immunoprecipitated with anti-Bax antibody (6A7; BD Pharmingen) at 4°C for 2 h. Immunoprecipitates were captured by a Protein A/G PLUS-Agarose (Santa Cruz, CA, USA) in lysis buffer at 4°C overnight. Immunoprecipitates were then recovered by centrifugation and washed three times in 1%
Chaps buffer. Immunoprecipitates and total extracts (40 µg) were separated on 12% polyacrylamide gel. After SDS-PAGE, proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and then blocked with 5% dried milk in PBS-0.1% Tween 20. Membranes were then incubated with primary anti-Bax (N20) (Santa Cruz, CA, USA) and peroxidase (HRP)-conjugated secondary antibodies in 10% dried milk in PBS containing 0.1% Tween 20 and detected with ECL Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ).

Cell proliferation and cell death analysis

Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO₂ incubator.

AP20187 (ARIAD Pharmaceuticals, Inc., Cambridge, MA, USA) was used for 3 h, 6 h and 24 h at 10 nM. Cell viability was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega BioSciences Inc. San Luis Obispo, CA, USA), according to the manufacturer’s protocol. Metabolically active cells were detected by adding 20 µl of MTT to each well. After 20 min of incubation, the plates were analysed in a Absorbance Microplate Reader (ELx800 BioTek Instruments, Inc.). Apoptosis was assessed using propidium iodide assay followed by flow cytometric analysis and caspase-3 and -9 fluorimetric assays. For propidium iodide assay, 24 h after transfection, cells were seeded in triplicate in 96-well plates at 3.6×10³ cells per well and grown overnight at 37°C in a 5% CO₂ incubator. Then cells were treated with 10 nM AP20187 (ARIAD Pharmaceuticals, Inc., Cambridge, MA, USA) for 6 h or with 100 nM Paclitaxel (Sigma-Aldrich Corp. Saint Louis, MO, USA) for 24 h. The next day analysis of DNA content by propidium iodide (Sigma-Aldrich Corp. Saint Louis, MO, USA) incorporation was performed in permeabilized cells by flow cytometry.

For caspase-3 and -9 fluorimetric assays, 50 µg of total cell lysates were incubated for 1 h at 37°C with DEVD-AFC or LEHD-AFC substrate respectively, according to the manufacturer’s protocol (BioVision Inc. Mountain View, California). After incubation samples have been read in a fluorimeter equipped with 400 nm excitation filter and 505 nm emission filter.

For cell cycle analysis cells transfected with 70 nM of miRNA precursor hsa-miR-34c-5p or a scrambled molecule were recovered 48h and 72h post-transfection, fixed in 70% ethanol, stained with 50 µg/ml
propidium iodide (Sigma-Aldrich Corp. Saint Louis, MO, USA) and analysed by FACS (Fluorescence-activated cell sorter).

Cell transfections

The day before transfection cells were seeded in 10% FBS medium without antibiotics. All transfections were performed in serum-free Opti-MEM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. To alter miRNA level cells were transfected with 70 nM of miRNA precursor hsa-miR-34c-5p, hsa-miR-34c-3p, hsa-miR-124, hsa-miR-124*, hsa-miR-362-5p, hsa-miR-362-3p and Negative Control #1 (Ambion) or 50 nM of LNA anti-miR-34c-5p, anti-miR-34c-3p and anti-miR negative control inhibitor (Exiqon). To alter c-myc and Bmf level cells were transfected with 60 nM siRNA c-myc (Sigma-Aldrich Corp. Saint Louis, MO, USA) and 120 nM siRNA Bmf (Santa Cruz, CA, USA).

For p53 and Bmf stable gene silencing, A549 cells were transfected in 100 mm dishes with 10 μg of shRNAp53, shRNA Bmf or shRNAcntrl (Open Biosystems). For stable clone selection RPMI medium supplemented with 1 μg/ml puromycin was used.

Luciferase assays

For target validation the two Bmf 3’ UTR sequences were PCR amplified using the following primers: BMF S1 Fw: 5’-CTAGAAGGATTTCCTGGCCAGCTCCT-3’, BMF S1 Rw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S2 Fw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S2 Rw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S3 Fw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S3 Rw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S4 Fw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’; BMF S4 Rw: 5’-TCTAGAAGGATTTCCTGGCCAGCTCCT-3’ and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega BioSciences Inc. San Luis Obispo, CA, USA). We deleted a sequence in S2 and S3 miRNA-binding site by using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) using the
following primers: BMF S2 mut Fw 5’-CTGGGGCAGGTGGAAGCTGCACTGTC-3’  BMF S2 mut Rw 5’-
GACCCCGTCCACCTTCGACGTGACAG-3’; BMF S3 mut Fw: 5’-GGTTCTTACTTTCTAGCTGCTGCTCCACCTCCAGCA-3’; S3 mut Rw: 5’-TGCTGGAGGTCAGGCTAGAA
GTAAGAACC-3’.

The megakaryoblastic cell line, MEG-01, as widely used efficient recipient was use for luciferase assays. Cells were cotransfected with 1,2 \( \mu \)g of each generated plasmid and 400 ng of a Renilla luciferase expression construct pRL-TK (Promega BioSciences Inc. San Luis Obispo, CA, USA) with Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post-transfection and assayed with Dual Luciferase Assay (Promega BioSciences Inc. San Luis Obispo, CA, USA) according to the manufacturer’s instructions. Three independent experiments were performed in triplicate.

For p53 promoter activity analysis cells were transfected with Lipofectamine 2000 (Invitrogen) with 500 ng of pLuc-MDM2 expression vector (kindly provided by S. Soddu), 900 ng of miRNA precursor hsa-miR-34c-5p or Negative Control #1 (Ambion), together with 40 ng of pCMV-Renilla in 6 well plates. Luciferase and Renilla activity were assessed with Luciferase assay system and Renilla Luciferase assay system (Promega BioSciences Inc. San Luis Obispo, CA, USA) 48 h after the transfection. The Luciferase activity was normalized for the Renilla activity. All the experiments were performed three times in triplicate and the mean±s.d. was reported.

**qt-RT-PCR analysis**

Reverse transcription reaction was done starting from 1 µg total RNA and using miScript Reverse Transcription Kit (QIAGEN, Milan Italy), according to the manufacturer’s protocol. The expression of mature hsa-miR-34c-5p, has-miR-34c-3p, hsa-miR-362-5p, hsa-miR-362-3p, hsa-miR-124, hsa-miR-124* and of U6 RNA, as housekeeping gene, was assayed using the miScript SYBR Green PCR Kit (QIAGEN) and Real-time PCR was done in triplicate for each case. MiRNA expression was measured using Ct (threshold cycle). The \( \Delta \Delta \text{Ct} \) method for relative quantization of gene expression was used to determine miRNA expression levels. The \( \Delta \text{Ct} \) was calculated by subtracting the Ct of U6 RNA from the Ct of the miRNA of interest. The \( \Delta \Delta \text{Ct} \) was calculated by subtracting the \( \Delta \text{Ct} \) of the reference sample (A549-FK cells
not transduced with miRNAs) from the ΔCt of each sample. Fold change was generated using the equation 2-ΔΔCt.

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References


Legend to Figures

**Figure 1** Cell clones expressing miR-34c-5p are protected from AP20187 induced apoptosis.

(a) MTT assay for resistance to AP20187 of cell clones that express miR-34c. A549-FK (Cntrl) cells and individual cell clones were plated in triplicate in 96-well plates and treated with 10 nM AP20187 for 3 h. Values are expressed as the per cent of the untreated control; (b) parental A549-FK (lanes 1-3) or Cl-K (transduced with miR-34c, miR-362 and miR-124-1) or Cl-T (transduced with miR-34c) were treated for the times indicated with AP20187 (10 nM) and levels of caspase-3 and caspase-9 cleaved products were analysed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. (c) The same as in (b) except that levels of BID cleaved products were analysed. (d) A549-FK, Cl-K or Cl-T were treated for 3 h with AP20187 (10 nM) or left untreated and cell lysates analysed by immunoblot with anti Bax N-20 antibody. Lysates were either first immunoprecipitated with anti Bax6A7 antibody, specific for the active conformation of Bax (upper panel) or directly used for immunoblot analysis (lower panel). Intensity of bands was measured by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition. Values were each normalized for the corresponding values in the direct blot and are expressed as the per cent of the intensity measured in the second lane (labeled with asterisk). (e) Parental A549-FK cells or Cl-K or Cl-T were treated for 24 h with cisplatin (50µg/ml) and levels of caspase-3 cleaved products were analysed by immunoblot. Filters were hybridized with anti α-tubulin to confirm equal loading. The blots (in b,c,d,e) are representative of at least four independent experiments. (f) A549-FK or Cl-T cells were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either anti-miR-34c-5p inhibitor sequence or a corresponding scrambled sequence was transfected and cell lysates analysed for caspase-3 (upper panel) and caspase-9 (lower panel) activity as measured by hydrolysis of Ac-DEVD-AFC or Ac-LEHD-AFC respectively. The value of miR-34c-5p in Cl-T+antimiR-34c-5p, quantified by qt-RT-PCR and expressed as $2^{-ΔΔct}$, is 0.09±0.01 compared to Cl-T+scrambled.

**Figure 2** miR-34c-5p interferes with AP20187 induction of apoptosis.

(a) A549-FK cells (Cntrl) were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic sequence or a corresponding scrambled
sequence was transfected. The per cent of cells in the pre-G1 fraction was determined by FACS analysis as a measure of apoptotic cells. (b, c) A549-FK cells were treated for 3 h with AP20187 (10 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic or a corresponding scrambled sequence was transfected and caspase-9 (b) and caspase-3 activity (c) analysed as in Figure 1f. (d) A549-FK cells (Cntrl) cells were treated with AP20187 (10 nM) as indicated. Where indicated two different scrambled sequences, miR-34c-5p, miR-34c-3p mimic sequences or both miRNAs together were transfected and cell viability was determined by MTT assay as per cent of corresponding untreated cells (see legend to Figure 1a). The values of miR-34c-5p in A549-FK cells transfected with the mimic, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, are: 112.87±5.56 (a), 135.02±7.09 (b,c); 130.21±7.25 in A549-FK+miR-34c-5p and 101.26±6.31 in cotransfected cells (d). The values of miR-34c-3p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, are 137.55±5.30 in A549-FK+miR-34c-3p and 138.03±9.28 in cotransfected cells (d). For all samples levels are compared to the scrambled controls.

**Figure 3** miR-34c-5p protects from paclitaxel-induced apoptosis.

(a) A549 cells were transfected with either miR-34c-5p mimic sequence (left panel) or miR-34c-3p (right panel) and treated for 24 h with paclitaxel (100 nM). Levels of caspase-3 and caspase-9 cleaved products were analysed by immunoblot. Filters were hybridized with anti-α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments. (b) A549 cells (Cntrl) were treated for 24 h with paclitaxel (100 nM) (white columns) or left untreated (black columns). Where indicated either miR-34c-5p mimic sequence or a corresponding scrambled sequence was transfected. The per cent of cells in the pre-G1 fraction was determined by FACS analysis as a measure of apoptotic cells. (c) A549 cells were transfected with LNA anti-miR-34c-5p, anti-miR-34c-3p or anti-miR negative control inhibitor. Levels of caspase-3 and caspase-9 cleaved products were analysed by immunoblot. Filters were hybridized with anti-α-tubulin to confirm equal loading. Values below the blot indicate signal levels of the 17kDa fragment of caspase-3 relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 1. Each blot is representative of at least four independent experiments. (d,e) A549 cells transfected with either miR-34c-5p mimic sequence or a corresponding scrambled sequence were treated with TRAIL (d) or cisplatin (e)
for times and concentrations indicated. Levels of caspase-3 and caspase-9 cleaved products were analysed by immunoblot. Filters were hybridized with anti-α-tubulin to confirm equal loading. Each blot is representative of at least four independent experiments. The values of miR-34c-5p, quantified by qRT-PCR and expressed as $2^{-\Delta\Delta C_t}$, are: 110.92±5.43 (a left panel); 96.37±5.02 (b) 0.19±0.02 (c); 133.72±4.43 (d); 129.77±6.07 (e). The values of miR-34c-3p are: 141.27±7.52 (a right panel) and 0.32±0.03 (e). For all samples levels are compared to the scrambled controls.

**Figure 4** Bmf as target of miR-34c-5p.

(a) Bmf 3’UTR contains two predicted miR-34c-5p binding sites. In the figure is shown the alignment of the two seed regions (S1 and S2) of miR-34c-5p with Bmf 3’UTR. The sites of target mutagenesis (deletion) are indicated by red dashes and red XXX. (b) A549 or A549-FK cells were transfected with either miR-34c-5p or control scrambled sequence and cell lysates analysed for the expression of Bmf. Values below the blot indicate signal levels relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 1. (c) A549-FK, Cl-K and Cl-T cell lysates were analysed for the expression of Bmf. Values below the blot indicate signal levels relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 1. In the insert are reported the miRNAs transduced in the Cl-K and Cl-T and their relative (as compared to A549-FK control cells) expression levels. (d) Cl-T was transfected with LNA anti-miR-34c-5p, anti-miR-34c-3p or anti-miR negative control inhibitor and levels of Bmf were analysed compared with that of A549-FK control cells. Filters were hybridized with anti-α-tubulin to confirm equal loading. The blots are representative of at least four independent experiments. (e) pGL3-Bmf luciferase constructs, containing a wild type (left side of the histogram) or mutated (right side of the histogram) Bmf 3’UTRs, are transfected into MEG01 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results. The predicted recognition site of BMF at position 737-765 (S1) is less conserved (only bosTau2 genome) than that at position 2283-2311 (S2) that is conserved in bosTau2 and rat (rm4). The predicted free energies were calculated and are: -15.1 kJ for BMF S1 and -24.2 kJ for BMF S2, (f) A549 cells were transfected with miR-124, miR-124*, miR-362-5p, miR-362-3p or a control scrambled sequence and Bmf protein levels were
analysed at indicated times by immunoblot. Filters were hybridized with anti-α-tubulin to confirm equal loading. The blots are representative of at least three independent experiments. (g) Bmf 3′UTR contains predicted binding sites for miR-362-5p and miR-362-3p. In the figure is shown the alignment of the two seed regions of miR-362-5p (S3) and miR-362-3p (S4) with Bmf 3′UTR. The sites of target mutagenesis (deletion) are indicated by red dashes and red XXX. pGL3-Bmf luciferase constructs, containing wild type (either for miR-362-5p and miR-362-3p) or mutated (for miR-362-5p) Bmf 3′ UTRs, are transfected into MEG01 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results.

Diana Lab: miTG score for Bmf (miR-34c, 5.72; miR-124, 10.89; miR-362-5p, 3; miR-362-3p, 2.11; miR-345, 5.0; miR-346, 2).

The values of miR-34c-5p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, are: 133.53±4.96 (b left panel); 148.84±6.58 (b right panel); 0.20±0.02 (d); 184.05±8.46, 153.27±6.33, 142.26±7.07, 154.27±5.93, 112.37±4.13 (in Bmf S1, Bmf S2, Bmf S1+S2, Bmf S2 mut and Bmf S1+S2 mut respectively, e). The value of miR-34c-3p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, is 0.34±0.02 (d). The values of miR-362-5p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, are 227.98±9.54 in Bmf S3 and 198.33±5.26 in Bmf S3 mut (g). The value of miR-362-3p in Bmf S4 is 239.85±13.98 (g). For all samples levels are as compared to the scrambled controls.

**Figure 5.** *c-myc mediates miR-34c-5p effects.*

(a) Bmf stable silenced A549 cells (shRNA Bmf) were transfected either with miR-34c-5p mimic or with a scrambled control molecule. After paclitaxel treatment, as indicated, levels of caspase-3 cleaved products were analysed by immunoblot and compared to control cells (shRNAcntrl) transfected with scrambled. Reported folds were calculated as reported in Figure 3c. (b) A549-FK cells transfected with the control scrambled sequence, the miR-34c-5p mimic or a c-myc siRNA were treated with 10 nM AP20187 and levels of caspase-3 and c-myc were analysed by immunoblot as indicated. (c) A549 cells were transfected with a c-myc siRNA or a control siRNA and treated with paclitaxel, as indicated, and and the levels of caspase-3 cleaved products and c-myc were analysed by immunoblot. Values below the blot (in b and c) indicate signal
levels of c-myc relative to control cells (lane 1, labeled with asterisk). Quantization was done as in Figure 1.

(d) A549 cells were transfected with a c-myc siRNA alone or together with miR-34c-5p. In all samples the amount of transfected molecules was kept constant using corresponding scrambled controls as indicated. After treatment with paclitaxel levels of caspase-3 cleaved products, c-myc and Bmf were analysed by immunoblot. (e) A549 cells were transfected with miR-34c-3p mimic or with a scrambled sequence and levels of Bmf and c-myc were analysed by immunoblot 72h post-transfection. The target prediction algorithms (Diana-microT) predicts Bmf but not c-myc as target of miR-34c-3p. (f,g) A549 cells were transfected with miR-34c-5p mimic or with a scrambled sequence and 48h or 72h post-transfection cells were recovered, stained with 50 µg/ml propidium iodide and analysed by FACS (f), or cell lysates were extracted for cyclin B1, cyclin D1 and Chk1 analysis by immunoblot (g). (h) A549 cells transfected with miR-34c-5p mimic or with a scrambled sequence were treated with paclitaxel as indicated and cyclin B1, cyclin D1 and Chk1 levels were evaluated by immunoblot analysis. Filters in a,b,c,d,e,g and h were hybridized with anti-α-tubulin to confirm equal loading. The blots are representative of at least three independent experiments.

The values of miR-34c-5p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, are: 115,62±5,56 (a); 103,98±5,13 (b) 127,89±6,43 (d); The value of miR-34c-3p, quantified by qt-RT-PCR and expressed as $2^{-\Delta\Delta ct}$, is 109,73±4,92 (e). For all samples levels are as compared to the scrambled controls.

**Figure 6.** miR-34c-5p attenuates p53 activity.

(a) A549 cells were treated with paclitaxel 100 nM for 24h. Cell lysates of untransfected cells (Cntrl) and of cells transfected with a control scrambled sequence or with miR-34c-5p mimic were analysed with anti-Acetyl–p53 and anti-p53 antibodies. (b) A549 cells were transfected with a reporter construct containing the promoter region of MDM2 gene fused to luciferase. Luciferase activity was measured after treatment with paclitaxel of cells transfected with the reporter gene together with miR-34c-5p mimic or with a scrambled sequence as control. The reporter assays were performed three times with essentially identical results. (c) A549 cells transfected with either scrambled or miR-34c-5p mimic were treated with paclitaxel, as indicated, and were analysed for MDM2 and SIRT-1 protein expression. (d) A549 cells were transfected with a control scrambled sequence and with miR-34c-5p mimic (upper panel) or with an LNA anti-miR negative control.
inhibitor and an anti-miR-34c-5p inhibitor (lower panel). After treatment with 10 µM Nutlin-3 for 24 h p53 protein levels were analysed by immunoblot compared to untrasfected cells (Cntrl). Fold decrease of miR-34c-5p (lower panel), quantified by qRT-PCR, was 0.5 (untreated), 0.33 (10 µM Nutlin-3) and 0.88 (20 µM Nutlin-3) for samples in the presence of antimiR-34c-5p as compared to the respective controls. (e) p53 stable silenced A549 cells (shRNAp53) or mock stable trasfected cells (shRNAcntrl) were treated with paclitaxel or cisplatin as indicated and levels of caspase-3 and caspase-9 cleaved products were analysed by immunoblot. (f) A549 cells were transfected with either miR-34c-5p mimic or with a scrambled sequence and treated for 24 h with cisplatin (50 µg/ml). Levels of p53 were analysed by immunoblot. Filters (in a,c,d,e and f) were hybridized with anti-α-tubulin to confirm equal loading. Each blot is representative of at least three independent experiments.

The values of miR-34c-5p, quantified by qRT-PCR and expressed as $2^{-\Delta\Delta ct}$, are: 135.11±7.12 (a); 118.55±4.44 (b) 105.95±5.92 (c); 139.88±6.81 (f). For all samples levels are as compared to the scrambled controls.
Fig. 1
Fig. 2
Fig. 3
**Fig. 4**

**a**

BMF S1

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**b**

A549 A549-FK

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Fold

| 1.00* | 0.40 |
| 1.00* | 0.40 |

a-tubulin

18 kDa

52 kDa

**c**

A549-FK

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a-tubulin

18 kDa

52 kDa

**d**

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Fold

| 18 kDa |
| 52 kDa |

antimiR-34c-3p

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Fold

| 18 kDa |
| 52 kDa |

**e**

% luciferase activity

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**f**

Bmf

Cntrl miR-124 miR-124* miR-362-5p miR-362-3p

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**Fig. 4**
Fig. 5
**a**

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**b**

- Untreated
- Paclitaxel 100 nM 24h

**c**

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