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INCREASING SENSITIVITY TO DRUGS IN LEUKEMIAS BY MODULATION OF MICRORNA EXPRESSION

AUMENTO DA SENSIBILIDADE A FÁRMACOS EM LEUCEMIAS ATRAVÉS DA MODULAÇÃO DA EXPRESSÃO DE MICRORNAS

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas - Biologia Celular e Molecular, apresentada à Faculdade de Farmácia da Universidade do Porto

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Legend for the figures present on the cover (from left to right):

Figure 1. K562 cell visualized under electron microscopy

Figure 2. Representation of a flow cytometry diagram

Figure 3. Representation of miRNA biogenesis

Figure 4. Representation of a DLS diagram

Figure 5. Proteins expression observed by Western blot

FACULDADE DE FARMÁCIA DA UNIVERSIDADE DO PORTO

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AUTHOR'S DECLARATION

Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro", is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

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ABSTRACT

miRNAs are small, single stranded, non-coding RNAs that regulate gene expression at a post-transcriptional level. They are known to be involved in several biological processes and diseases such as cancer. Indeed, their abnormal expression alters the regulation of various cancer-related genes.

The role of miRNAs in leukemia has been thoroughly studied but the function of specific miRNAs in the pathogenesis of the disease is not fully understood. The work performed in this thesis aimed at further understanding the role of some miRNAs in leukemia. The specific aims were: i) further understand the role of miR-21 in chronic myeloid leukemia (CML) cell lines; ii) investigate if miR-21 is present in various extracellular vesicles from CML cells and iii) study the role of miR-128 in an acute myeloid leukemia (AML) cell line.

The overexpression of miR-21 has been observed in many cancers, including leukemia. The involvement of this miRNA in cancer related processes, such as chemoresistance, has been shown but its correlation with autophagy had never been addressed. In this thesis it is shown that by downregulating mR-21 expression with antimiRs, in K562 (CML) cells, there was a decrease in cellular viability and a decrease in cellular proliferation. miR-21 downregulation also caused increased programmed cell death and a decrease in the expression levels of Bcl-2 protein, although PARP cleavage was not observed. Thus, the possible involvement of autophagy was analysed. It was verified that miR-21 downregulation increased the expression of the autophagy related proteins Beclin-1, Vps34 and LC3-II. Accordingly, an increase in autophagic vacuoles was seen, both by monodansylcadaverine and acridine orange staining as well as by transmission electron microscopy. In addition, downregulation of miR-21 expression increased the sensitivity of both K562 and KYO-1 (CML) cells to etoposide and doxorubicin, which was reverted by pre-treating cells with an autophagy inhibitor, 3-MA. Therefore, these results described for the first time autophagy induction via miR-21 downregulation and its involvement in drug sensitivity.

Extracellular vesicles (EVs) have recently emerged as important intermediates of intercellular communication. These EVs, shed by donor cells, may contain proteins and both coding and non-coding RNAs, which can be transferred to recipient cells. In particular, it has become evident that EVs may carry miRNAs that are responsible for drug resistance, from resistant to sensitive cells. However, it is not known if miR-21 is

transported in EVs “shed” by CML cells, and moreover if different types of EVs carry different levels of miR-21. Indeed, there are various types of EVs, such as exosomes and microvesicles, but their isolation is still a technical problem. In this thesis, different EVs isolation protocols from K562 cells were employed, providing different EVs populations. This study showed that miR-21 was found in all extracted EVs and that its levels were not dependent on the type (size) of EVs isolated, suggesting that miR-21 is not specifically “packaged” into a particular type of EVs.

Another miRNA known to be involved in leukemia is miR-128. This miRNA has been described, together with other miRNAs, to allow the discrimination between acute myeloid leukemia (AML) and acute lymphoblastic leukemia. Moreover, miR-128 is included in miRNA signatures which allowed the characterization of a particular subtype of AML as well as being associated with worse clinical outcome in a subgroup of patients with high-risk molecular features of AML. However, no functional studies have been performed regarding miR-128 and leukemia. In this thesis, miR-128 overexpression was performed with miR-mimics in an AML cell line (HL-60). This overexpression resulted in decreased cellular viability and increased sensitization to chemotherapeutic agents, etoposide and doxorubicin. miR-128 overexpression also increased programmed cell death but did not affect cell cycle, apoptosis or autophagy. In addition, increased DNA damage was observed upon miR-128 overexpression, which was confirmed by an increase in the comet’s tail intensity in the comet assay, an increase in the number of DNA repair foci stained with either γ-H2AX or 53BP1, as well as an increase in the levels of these proteins. These results showed for the first time an association of miR-128 with DNA damage in the leukemia context.

Overall, the work presented in this thesis provided more insight into the role of miRNAs and drug response in leukemia, contributing to the overall knowledge in this field.

Keywords: miRNAs; CML; AML; drug sensitivity; extracellular vesicles

RESUMO

miRNAs são pequenos RNAs não codificantes, de cadeia simples, que regulam a expressão génica a um nível pós-transcricional. É reconhecido o seu envolvimento em vários processos biológicos bem como em doenças como por exemplo o cancro. De facto, a sua expressão anormal altera a regulação de vários genes relacionados com o cancro.

O papel dos miRNAs na leucemia foi já bastante estudado mas a função específica de alguns miRNAs na patogénese da doença ainda não é completamente compreendida. O trabalho apresentado nesta tese teve como objectivo compreender melhor o papel de alguns miRNAs na leucemia. Os objectivos específicos foram: i) compreender o papel do miR-21 em linhas celulares de leucemia mielóide crónica (LMC); ii) investigar se o miR-21 está presente em várias vesículas extracelulares de células de LMC e iii) estudar o papel do miR-128 numa linha celular de leucemia mielóide aguda (LMA).

A sobre-expressão do miR-21 foi já descrita em vários cancros incluindo na leucemia. O envolvimento deste miRNA em processos relacionados com o cancro, tal como a resistência a fármacos antineoplásicos, foi já demonstrado mas a sua correlação com autofagia nunca foi investigada. Nesta tese, demonstra-se que por diminuição da expressão do miR-21, com antimiRs, na linha celular K562 (de LMC), ocorreu uma diminuição da viabilidade celular assim como uma diminuição da proliferação celular. A diminuição de expressão do miR-21 também causou um aumento da morte celular programada e uma diminuição dos níveis de expressão da proteína Bcl-2, embora não se tenha observado clivagem da proteín PARP. Assim, o possível envolvimento de autofagia foi analisado. Verificou-se que a diminuição de expressão de miR-21 aumentou a expressão de proteínas relacionadas com a autofagia: Beclina-1, Vps-34 e LC3-II. Em concordância, foi também observado um aumento de vacúolos autofágicos, por marcação com monodansilcadaverina ou com laranja da acridina, bem como por microscopia electrónica de transmissão. Para além disso, a diminuição de expressão do miR-21 aumentou a sensibilidade das linhas celulares K562 e KYO-1 (de LMC) ao etoposídeo e doxorrubicina, sensibilidade esta que foi revertida com pré-tratamento das células com um inibidor de autofagia, 3-MA. Assim, estes resultados descrevem, pela primeira vez, a indução de autofagia através da diminuição da expressão do miR-21, bem como o envolvimento dessa autofagia na sensibilidade a fármacos.

As vesículas extracelulares (VEs) apareceram recentemente como importantes mediadores da comunicação intercelular. Estas VEs, libertadas por célula dadoras, podem conter proteínas e RNAs codificantes e não-codificantes, que podem ser transferifos para

células receptoras. Em particular, tornou-se evidente que as VEs podem transportar miRNAs que são responsáveis pela resistência a fármacos, transferidos de células resistentes para células sensíveis. No entanto, não se sabe se o miR-21 é transportado em EVs libertadas por células de LMC, e além disso, se diferentes tipos de EVs transportam diferentes níveis de miR-21. De facto, existem vários tipos de EVs, como exossomas e microvesículas, mas o seu isolamento ainda se debate com problemas técnicos. Nesta tese, diferentes protocolos de isolamento EVs a partir de células K562 foram usados, originando diferentes populações de EVs. Este estudo mostrou que o miR-21 foi encontrado em todos as VEs extraídas e que os seus níveis não são dependemtes do tipo (tamanho) das VEs isoladas, sugerindo que o miR-21 não é 'empacotado' de forma específica num determinado tipo de VEs.

Outro miRNA que está envolvido em leucemia é o miR-128. Este miRNA foi descrito, juntamente com outros miRNAs, como permitindo a discriminação entre LMA e leucemia linfoblástica aguda. Para além disso, o miR-128 está também incluído em assinaturas de miRNAs que permitem a caracterização de sub-tipos de LMA, bem como associado com pior evolução clínica num subgrupo de doentes com características moleculares de LMA de alto risco. Contudo, não existem estudos funcionais realizados com miR-128 em leucemia. Nesta tese, a sobre-expressão de miR-128 foi conseguida com mímicos de miRNAs numa linha celular de LMA (HL-60). Esta sobre-expressão resultou numa diminuição da viabilidade celular e aumento da sensibilidade a dois agentes antineoplásicos: etoposídeo e doxorrubicina. A sobre-expressão de miR-128 também aumentou os níveis de morte celular programada sem, no entanto, afetar o ciclo celular, apoptose ou autofagia. Para além disso, observou-se um aumento nos danos do DNA após sobre-expressão do miR-128, que foi confirmado por um aumento na intensidade da cauda dos cometas no 'ensaio de cometas', por um aumento do número de focos de reparação de DNA, marcados com H2AX ou 53BP1 e por um aumento dos níveis de expressão destas mesmas proteínas. Estes resultados mostram, pela primeira vez, uma associação do miR-128 com danos no DNA, em leucemia.

De um modo geral, o trabalho apresentado nesta tese proporcionou mais conhecimento sobre o papel dos miRNAs na resposta a fármacos citotóxicos na leucemia, contribuindo para o conhecimento global neste domínio.

Palavras-chave: miRNAs; LMC; LMA; sensibilidade a fármacos; vesículas

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ABBREVIATIONS LIST

5-FU	5-Fluorouracil
ABL	Abelson murine leukemia
ABT-263	Navitoclax (first-in-class BCL2 family inhibitor)
ADAM17	A Disintegrin and Metalloprotease-17
AG1478	EGFR inhibitor
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APAF-1	Apoptotic peptidase activating factor 1
APL	Acute promyelocytic leukemia
Ara-C	Cytarabine
ASO	Antisense oligonucleotides
ATM	Ataxia telangiectasia mutated
ATO	Arsenic trioxide
ATR	ATM-Rad3-related
ATRA	All-trans retinoic acid
AZD6244	Small molecule inhibitor of the MEK (MAP/ERK kinase)
BAALC	Brain and Acute Leukaemia, Cytoplasmic
BCR	Breakpoint cluster region
BIM	BCL2-like 11
BM	Bone marrow
Bmf	Bcl-2-modifying factor
BNP2	BCL2/adenovirus E1B 19 kDa protein-interacting protein 2
CBF	Core binding factor
CCNG1	Cyclin G1
CDDP	Cisplatin
CDS2	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2
CEBPA	CCAAT/enhancer-binding protein alpha
CML	Chronic myeloid leukemia
CN	Cytogenetically normal
COX-2	Cyclooxygenase-2
CPT	Camptothecin
CREB	Cyclic AMP-responsive element binding protein
CSCs	cancer stem cells
CTX	Cyclophosphamide
CYP	Cytochrome P450

DDR	DNA damage response
DEX	Dexamethasone
DGCR8	DiGeorge syndrome critical region gene 8
DHFR	Dihydrofolate reductase
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNR	Daunorubicin
DOX	Doxorubicin
DR4	TRAIL Death Receptor-4
DTX	Docetaxel
EAG1	Ether-à-go-go
EGFR	Epithelial growth factor receptor
EMT	Epithelial to mesenchymal transition
EPR	Epirubicin
ERRFI-1	ErbB receptor inhibitor-1
Evs	Extracellular vesicles
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridization
FLT3-ITD	Fms-like tyrosine kinase 3 - internal tandem duplication
GSTP1	Glutathione S-transferase P1
HB-EGF	Heparin-binding EGF-like growth factor
HCC	Hepatocellular carcinoma
HCPT	Hydroxycamptothecin
HDAC	Histone deacetylase
HIPK2	Homeodomain-interacting protein kinase-2
HMEC	Human mammary epithelial cells
hMSH2	Human DNA MutS homolog 2
HNSCC	Head and neck: squamous cell carcinoma
HSPC159	Galectin-related protein
HSPG2	Heparin Sulfate Proteoglycan 2
IGF-IR	Insulin-like growth factor-I receptor
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1
L-OHP	Oxaliplatin
MAGE-A	Melanoma Antigen Family A
MPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau

MARCKS	Myristoylated alanine-rich protein kinase c substrate
MDM4	Murine double minute 4
MDR	Multidrug resistance
MIF	Macrophage inhibitory factor
MEPE	Matrix extracellular phosphoglycoprotein
miR, miRNA	MicroRNA
MLL	Mixed-lineage leukemia
MMC	Mitomycin C
MMR	Mismatch repair
MN1	Meningioma 1
mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated protein kinase
MTDH	Metadherin
MTX	Methotrexate
MX	Mitoxantrone
MYBL2	v-Myb myeloblastosis viral oncogene homolog-like2
NPM1	Nucleophosmin
NSCLC	Non-small cell lung cancer
OSCC	Oral squamous cell carcinoma
PDCD4	Programmed cell death 4
P-gp	P-glycoprotein
PHLPP2	PH Domain And Leucine Rich Repeat Protein Phosphatase 2
PKCε	Protein kinase C epsilon
PLX4720	Specific inhibitor of B-RAF^{V600E}
PTPN12	Protein tyrosine phosphatase, nonreceptor type 12
PTX	Paclitaxel
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RRM2	Ribonucleotide Reductase M2
RT-PCR	Reverse transcription-polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SNP	Single nucleotide polymorphism
SRC	Sarcoma viral oncogene homolog
SFPQ	Splicing factor proline and glutamate- rich
SIRT1	Silent mating type information regulation 2 homolog 1
SLC4A4	Na/bicarbonate cotransporter 1
SMARCC1	SWI/SNF Related, Matrix Associated, actin dependent regulator of chromatin, subfamily C, member 1

SOCS3	Suppressor of cytokine signaling 3
STS	Staurosporine
TAM	Tamoxifen
TDX	Tomudex
TfR1	Transferrin receptor 1
TGF	Transforming growth factor
TKD	Tyrosine kinase domain
TMZ	Temozolomide
TOP2B	DNA topoisomerase 2-beta
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TWF1	Actin-binding protein twinfilin 1
TYMS	Thymidylate synthase
U0126	Selective inhibitor of MEK1/2 kinase
UTR	Untranslated region
VCR	Vincristine
VM-26	Teniposide
VP-16	Etoposide
WT1	Wilms tumor 1
ZEB	zinc finger E-box–binding homeobox

CHAPTER I.

INTRODUCTION

PART I.

LITERATURE REVIEW

1. LEUKEMIA

1.1 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a genetically pleomorphic disease characterized by a clonal disorder of hematopoietic stem cells that disturb normal mechanisms of self-renewal, proliferation and differentiation. Therefore, there is a subsequent accumulation of cells at different stages of inadequate maturation as well as reduction of healthy hematopoietic cells (reviewed in Dohner and Gaidzik 2011; Estey and Dohner 2006; Schlenk et al. 2008; reviewed in Garzon et al. 2008b). AML is defined by a rapid disease tempo which progresses over weeks to months and is classified as a biologically heterogeneous disease with distinct clinical presentations in different morphological and cytogenetic subtypes.

AML has traditionally been classified based on morphologic characteristics, immunohistochemistry and immunophenotyping according to the French–American–British (FAB) classification. More recently, cytogenetic and molecular diagnostic analyses have been added as a complement for a better classification of AML (reviewed in Vardiman et al. 2009). The new classification of hematological malignancies published by the World Health Organization in 2008 (Swerdlow et al. 2008) defines 108 new diagnostic entities in hematopathology, including 50 new or provisional leukemia entries (reviewed in Betz and Hess 2010). AML diagnosis starts with morphologic examination to confirm the presence of at least 20% blasts in bone marrow or blood and its characterization is followed by cytogenetical analysis. Between 50 and 60% of AMLs present cytogenetical alterations with around 45% having an abnormal karyotype with a recurrent chromosomal alteration, and about 15% presenting 3 or more cytogenetic alterations (complex karyotype) (reviewed in Mrozek et al. 2004). Prognosis and genetics of AML are tightly linked. Risk stratification based on cytogenetics divides patients into three main groups, those with favorable, intermediate, and unfavorable cytogenetics (Table 1). Favorable prognosis subtypes include acute promyelocytic leukemia (associated with the translocation t(15;17)) and the core binding factor (CBF) leukemias (that include the translocations t(8;21)(q22;q22) and t(16;16)(p13.1;q22) and the inversion inv(16)(p13.1;q22)). An intermediate prognosis group includes the translocation t(9;11)(p22;q23) and trisomy of chromosome 8. A third group with adverse prognosis encompasses complex karyotypes, translocations t(3;3)(q21;q26.2) or t(6;9)(q23;q34), inversion inv(3)(q21;q26.2), deletion of chromosomes 5 or 7 or abnormalities of 17p as well as abnormalities of 11q23 (reviewed in Brown et al. 2012).

Table 1. Cytogenetic and molecular-genetic risk stratification in AML

Risk profile	Risk stratification	
	Cytogenetic	Molecular genetic abnormalities that further stratify CN-AML
Favorable	acute promyelocytic leukemia t(15;17)	
	t(8;21)(q22;q22)	NPM1 mutation (without FLT3-ITD mutation)
	t(16;16)(p13.1;q22)	biallelic CEBPA mutation
	inv(16)(p13.1q22)	
Intermediate	normal karyotype	
	t(9;11)(p22;q23)	
	trisomy 8	
Unfavorable	complex karyotype	FLT3-ITD mutation
	t(3;3)(q21;q26.2)	
	t(6;9)(p23;q34)	
	inv(3)(q21q26.2)	
	-5 or del(5q)	
	-7	
	abnormalities of 17p	
	abnormalities of 11q23	
	monosomal karyotype	

Adapted from (Dohner et al. 2010; Brown et al. 2012)

Monosomal karyotypes (two or more autosomal monosomies or one autosomal monosomy with at least one other structural abnormality) are a particular bad prognosis group (Breems et al. 2008). Besides the cytogenetic alterations, there are many patients that do not present any detectable chromosomal alteration – cytogenetically normal (CN) – but who harbor mutations or altered expression of specific genes and they represent about 40-50% of AML patients being the largest subset of AML (reviewed in Mrozek and Bloomfield 2006). Mutations in *NPM1*, *CEBPA* and *FLT3*-ITD have allowed stratifying patients in terms of prognosis (Table 1). For example, *NPM1* or biallelic *CEBPA* mutations confer a favorable prognosis (similar to the favorable group defined by karyotype) and patients harboring *FLT3*-ITD mutations have a worse prognosis (Schlenk et al. 2008). There are other molecular alterations that can be found in AML including mutations in *c-KIT*, *IDH1*, *IDH2*, *WT1*, *RUNX1*, *TET2* and *DNMT3A* but they still do not present a prognostic value (Patel et al. 2012).

Nowadays, more than 90% of AML patients can be categorized on the basis of either cytogenetic (chromosomal banding technique and fluorescence in situ hybridization - FISH) or molecular genetic characteristics (reviewed in Seca et al. 2010).

1.2 CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the neoplastic transformation of hematopoietic stem cells (reviewed in Ren 2005). It is normally divided in two distinct phases: i) a benign, chronic phase, which median duration is 3-4 years, that is characterized by the expansion and accumulation of functionally normal myeloid precursors and mature cells that leave the bone marrow; ii) an accelerated blastic phase where CML progenitor cells lose their capacity to differentiate, leading to a rapid expansion of myeloid or lymphoid blast cells in peripheral blood or bone marrow (reviewed in O'Hare et al. 2012; reviewed in Ren 2005).
The majority of CML cases (95%) is characterized, molecular and cytogenetically, by the occurrence of an aberrant chromosome, named Philadelphia (Ph) chromosome (Figure 1).

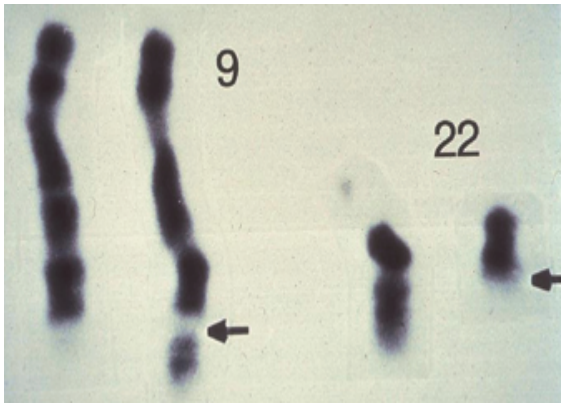

Figure 1. Philadelphia chromosome - banded chromosomes 9 and 22 show the result of the reciprocal translocation of 22q to the lower arm of chromosome 9 and 9q to the lower arm of chromosome 22 (arrows indicate breakpoint regions). Adapted from http://www.nature.com/scitable/content/the-philadelphia-chromosome-25070: accessed in 26Oct2013)

This chromosome is the consequence of a reciprocal translocation between the Abelson murine leukemia (ABL) gene on chromosome 9 and the breakpoint cluster region (BCR) gene on chromosome 22, resulting in the fusion gene *BCR-ABL* (reviewed in Jabbour and Kantarjian 2012). Depending on the place of break in the *BCR* gene there could be different chimeric proteins with variable size and molecular weight (reviewed in Faderl et al. 1999). These different proteins are related with phenotypic differences of the disease (reviewed in Kurzrock et al. 2003). In fact, BCR-ABL protein expression is responsible for the phenotypic abnormalities observed in the chronic phase of CML and is both crucial and sufficient for the malignant transformation of CML (Salesse and Verfaillie 2002; reviewed in Daley et al. 1990). BCR-ABL chimeric protein has constitutively tyrosine-kinase activity (Ben-Neriah et al. 1986) which, considering its cytoplasmic localization

(Wetzler et al. 1993), allows access to cellular substrates that promote growth and replication through the activation of pathways such as RAS, JUN kinase, MYC and STAT (Sawyers et al. 1992; Raitano et al. 1995; Mandanas et al. 1993; Ilaria and Van Etten 1996; Carlesso et al. 1996). The consequence of this downstream activation is cell proliferation independent of growth factors associated with an increase in apoptosis resistance (reviewed in Shet et al. 2002; reviewed in Calabretta and Perrotti 2004). BCR-ABL expression has been shown to exert dose-dependent effects on growth factor dependence, clonogenicity and migration (Barnes et al. 2005; Melo and Barnes 2007) as well as disease progression (Gaiger et al. 1995; Elmaagacli et al. 2000).

1.3 RESPONSE TO THERAPY

Leukemias are characterized by clonal expansion of bone marrow hematopoietic cells with increased blasts and aggressive clinical course.

Hematologic malignancies are, most often, currently treated with non-targeted, highly cytotoxic drugs, radiation and/or bone marrow transplantation.

There are few small molecule inhibitors and specific monoclonal antibodies that have been shown to significantly improve the patient clinical situation. Among these are small molecule inhibitors of BCR-ABL kinase activity for the treatment of BCR-ABL positive leukemias (Hughes et al. 2010; Druker et al. 2001) (Figure 2) and all-trans retinoic acid (ATRA) for the treatment of acute promyelocytic leukemia (APL) that express the fusion protein PML-RARα (reviewed in Sanz et al. 2009).

However, even with the identification of the oncogenic driving force, the results achieved with these therapies are regularly insufficient to completely eradicate tumor cells and eventually the malignancy relapses.

BCR-ABL tyrosine kinase inhibitors, led by imatinib, have completely changed the treatment of CML being able to sustain the disease in its chronic phase. Nevertheless, most patients will require lifetime treatment, as leukemic stem cells will persevere (Corbin et al. 2011; Hurtz et al. 2011). Moreover, advanced BCR-ABL positive leukemias show mild responses to BCR-ABL inhibitors with relapse occurring even if therapy is maintained (Foa et al. 2011). There is, however, a small subset of patients who reach complete molecular response (CMR) after treatment with imatinib and are able to maintain this CMR even after stopping treatment, suggesting that imatinib can ‘cure’ a small amount of CML patients (Mahon et al. 2010).

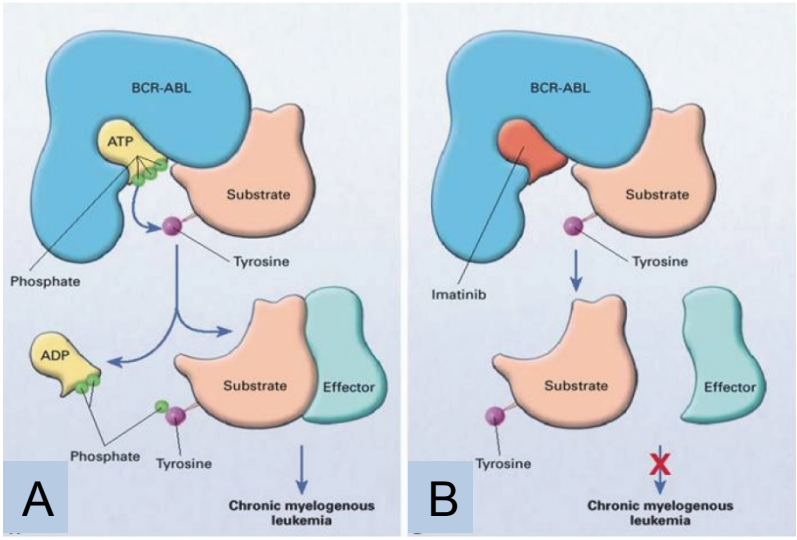

Figure 2. Mechanism of action of the BCR-ABL tyrosine kinase and its inhibition by imatinib - When imatinib is in the kinase pocket (Panel B), the action of BCR-ABL is inhibited, by preventing phosphorylation of its substrates. Adapted from (Savage and Antman 2002)

Contrary to CML, AML does not have a targeted therapy that treats most of the patients and several therapy methods have been employed in the treatment of this leukemia, such as radiotherapy, chemotherapy, immunotherapy, and bone marrow transplant. Although bone marrow transplant is considered an important therapeutic weapon to achieve complete remission, there are a lot of clinical restrictions to this procedure as well as to finding suitable donors. Chemotherapy using daunorubicin and cytarabine has been, for over three decades, the standard treatment for AML (reviewed in Burnett 2012). The rates of remission with standard induction chemotherapy, in patients with AML, vary between 50% and 85% (Farag et al. 2005). However, the majority of patients will relapse and die of the disease within 2 years of accomplishing a remission. Remission rates and overall survival are dependent on several characteristics such as age of patient, cytogenetic alterations, other molecular changes or comorbid illness (reviewed in Shipley and Butera 2009).

Age and cytogenetics are closely related in AML. Adverse cytogenetic alterations increase with increasing age as the percentage of favorable cytogenetics drops from 17% in patients aged younger than 56 years to 4% in those aged older than 75 years. Moreover, the percentage of patients with unfavorable cytogenetics increases from 35% in those younger than 56 years to 51% in patients older than 75 years (Farag et al. 2005; Cancer et al. 2006). Both CML and AML patients, despite the better overall survival that new treatments have provided, relapse due to drug resistance which can be classified as intrinsic or extrinsic (reviewed in Stegmeier et al. 2010; reviewed in Fojo 2007).

Intrinsic resistance is associated with an innate property of the cancer cells that prevents an optimal response to therapy, while extrinsic (or acquired) resistance refers to a characteristic gained (or selected for) during therapy and leads to the loss of clinical response. The mechanisms of intrinsic resistance encompass those that mediate cell quiescence (Kumari et al. 2012; Graham et al. 2002), downregulation of pro-apoptotic molecules (San Jose-Eneriz et al. 2009a), overexpression of anti-apoptotic molecules (Konopleva et al. 2006; Kohl et al. 2007) or promotion of autophagy (Bellodi et al. 2009). Extrinsic resistance mechanisms have been associated with mutations or amplification of the gene that encodes for the protein that is targeted by the selected therapy, amplification of other genes that lead to the triggering of alternative or redundant pathways in the cell and increased expression of cellular energy-dependent drug transporters (efflux pumps) (le Coutre et al. 2000; Thomas et al. 2004; Aceves-Luquero et al. 2009). Mutations in the tyrosine kinase domain (TKD) of BCR-ABL are a good example of acquired resistance as they have been shown to thwart drug binding (Gorre et al. 2001; Sherbenou et al. 2010). Similar mechanisms of acquired resistance to FLT3 inhibitors involve mutations in the TKD of FLT3 and have been described in AML (Moore et al. 2012; Bagrintseva et al. 2004). The most common cause of acquired drug resistance is the cellular expression of drug efflux pumps, a superfamily of transport proteins called ATP-binding cassette, which transfer anticancer drugs from the inside to the outside of the cell. Alterations in the expression of these drug efflux pumps have been shown to occur both in AML and CML (reviewed in Mahon et al. 2008; Mahadevan and List 2004). Since efflux pumps are not substrate specific, cells overexpressing these pumps, such as the P-glycoprotein (P-gp), are multidrug-resistant, defining a phenotype known as multidrug resistance (MDR) (reviewed in Gottesman et al. 2002).

Intra-tumoral genetic heterogeneity, with multiple clones within the same tumor showing different kinds of aggressiveness (Gerlinger et al. 2012) or sub-clones of the same tumor displaying amplification of three different receptor tyrosine kinases in a mutually exclusive fashion (Snuderl et al. 2011), is another feature, likely present in almost all tumor types, that may represent a major obstacle for targeted therapeutic strategies being responsible for cancer drug resistance (reviewed in Gerlinger and Swanton 2010).

2. MICRORNAS

A broad variety of RNAs expressed in the genome have assorted functions, other than being only the "messengers" from DNA to protein expression. These RNAs, named non-coding RNAs (ncRNAs), include an increasing amount of transcripts previously assumed to be "trash" but that are now starting to be classified and thoroughly studied. Among these ncRNAs, microRNAs (miRNAs or miRs) have excelled. miRNAs were first described in 1993, in two papers published in Cell, where lin-4, the first miRNA, was identified in a genetic screen for defects controlling the developmental timing of C. elegans, and it was shown to target lin-14 mRNA (Lee et al. 1993; Wightman et al. 1993). By then, genetic screens used to end up with protein-coding genes as these were the most obvious and functional targets, but the ncRNA lin-4 showed intriguing characteristics. It was a small ~22-nt RNA that repressed the expression of the lin-14 gene by multiple imperfect pairing in the 3'UTR of lin-14 messenger RNA (mRNA). However, it took 8 years for 'microRNA era' to develop with dozens of miRNAs described in C. elegans, Drosophila and humans (Lee and Ambros 2001; Lau et al. 2001; Lagos-Quintana et al. 2001). Nowadays the miRNA database (http://www.mirbase.org/) already has over 24000 entries for a variety of species and over 2500 mature sequences for humans.

miRNAs are a class of single-stranded ncRNAs that range from 19 to 25 nucleotides in length and have significant roles in gene regulation (reviewed in Bartel 2004). In humans, miRNAs represent 1-4% of the genome and are spread through its entire length besides the Y chromosome with about half of the miRNAs situated in intergenic regions while the other half can be found within coding genes (Rodriguez et al. 2004; Saini et al. 2008).Taking into consideration their location in the genome, miRNAs can be classified in four different categories: i) intronic miRNAs in non-coding transcripts, such as the miR-15a~16-1 cluster found in the intron of a well-defined ncRNA gene, DLEU2 (Calin et al. 2002); ii) exonic miRNAs in non-coding transcripts, such as miR-155 which was found in the ncRNA gene, BIC (Tam 2001); iii) intronic miRNAs in protein-coding transcripts, such as the miR-25~93~106b cluster, which is embedded in the intron of the DNA replication licensing factor MCM7 transcript (Kim and Kim 2007); iv) exonic miRNAs in protein-coding transcripts, like miR-985 which is found in the last exon of CACNG8 mRNA (Kim and Kim 2007). With about 40% of all miR loci found in intronic regions of protein-coding transcripts it would be expectable that those miRNAs shared common promoters and were expressed together with their host genes (Baskerville and Bartel 2005; Wang et al. 2009). However, there are some studies reporting that co-expression of miRNAs and host genes is rare or happens only in a cell/tissue specific context (Biasiolo et al. 2011; He et al. 2012).

2.1 MIRNAS BIOGENESIS

The miRNAs have a standard biogenesis that comprises three major phases: 1) transcription; 2) maturation and 3) assembly (reviewed in Winter et al. 2009) (Figure 3). This canonical biogenesis of miRNAs starts in the nucleus with the transcription of long dsRNA molecules, known as primary-microRNAs (pri-miR), by RNA polymerase II.

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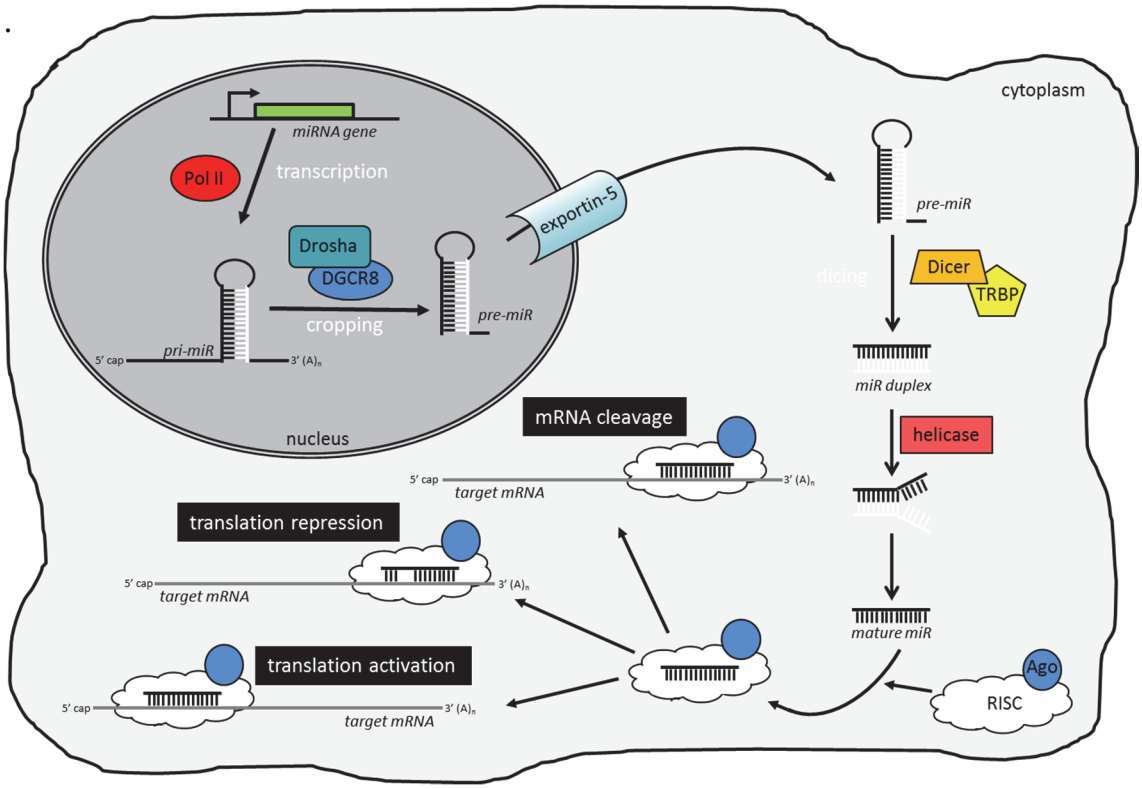

Figure 3. The miRNA biogenesis process.

These pri-miRs are characterized by a stem-loop structure and have some common features with mRNAs such as 5'cap and 3'poly adenylation (Cai et al. 2004). While still in the nucleus, pri-miR is cropped to precursor microRNA (pre-miR), a 70-100 nucleotides hairpin structure, by an RNase III enzyme, Drosha, and its interacting partner DGCR8 (Lee et al. 2003; Denli et al. 2004). Some miRNAs, namely miRtrons, bypass the Drosha-mediated cleavage with miRs being generated as by-products of splicing events (Ruby et al. 2007). The next processing step occurs in the cytoplasm, following the transport of pre-miR from the nucleus to the cytoplasm by exportin-5 (Lund et al. 2004). In the cytoplasm, pre-miR is thereafter converted to a miRNA duplex by another RNase III enzyme – Dicer (Hutvagner et al. 2001) and its partner TRBP, an essential RNA binding protein (Chendrimada et al. 2005). Following Dicer/TRBP processing, the double stranded miRNA is separated by an RNA helicase (Salzman et al. 2007) and while the mature

strand (~22nt) is loaded together with Argonaute (Ago) protein into the RNA-induced silencing complex (RISC), the passenger strand is degraded (Gregory et al. 2005). The mature strand is thought to be the one whose 5' end is thermodynamically more unstable, thus becoming more easily unwounded by the helicase (Schwarz et al. 2003; Khvorova et al. 2003). Association of miRNA loaded RISC with mRNAs through partial or full complementarity to its 3'-UTR region leads to post-transcriptional suppression of the target mRNA expression: if there is full complementarity between miRNA and mRNA, mRNA cleavage occurs, while partial complementarity causes translational repression (reviewed in Filipowicz et al. 2008; reviewed in Valencia-Sanchez et al. 2006; reviewed in Liu 2008). mRNAs have been shown to accumulate and/or be degraded in P-bodies (cellular structures that are enriched in mRNA-catabolizing enzymes and translational repressors) (Parker and Sheth 2007; reviewed in Liu et al. 2005).

2.2 REGULATION OF GENE EXPRESSION BY MIRNAS

The pairing of miRNA with its target mRNA occurs mainly through the binding of the miRNA to the mRNA 3'-UTR. Nevertheless, binding to the coding region or to the 5'UTR may also occur (Orom et al. 2008). One mRNA may have numerous binding sites for the same miRNA or enclose binding sites for different miRNAs (Brodersen and Voinnet 2009; reviewed in John et al. 2004). This promotes the opportunity for various miRNAs binding the same mRNA and thereby regulating cooperatively the expression of a single mRNA, singling the complex system that underlies miRNA regulation of mRNA expression (reviewed in Berezikov 2011; reviewed in Winter et al. 2009; reviewed in He and Hannon 2004). Moreover, some miRNAs regulate specific individual targets while others regulate the expression of several genes simultaneously.

In addition to their canonical function, as inhibitors of mRNA expression, miRNAs can exert a non-canonical function as positive regulators of gene expression (Place et al. 2008; Dong et al. 2011; Vasudevan et al. 2007), by binding to protein-coding exons (reviewed in Rigoutsos 2009) or promoters (Orom et al. 2008; Piriyapongsa et al. 2012).

Recognition of a target mRNA by a miRNA is mainly done by the first 2-7 nucleotides which are situated in the 5'end of the mature miRNA, known as the "seed" sequence (Lewis et al. 2003). Besides the perfect match between the "seed" sequence of a miRNA and its target mRNA, other features of site context may boost the efficacy of miRNA-mRNA interaction (Shin et al. 2010; Grimson et al. 2007). The complexity of the interaction between miRNAs and their target mRNAs may be better understood with the help of bioinformatics. Indeed, there are currently a great number of bioinformatic tools such as TargetScan, miRanda, PICTAR, Diana-microT, miRWalk which have been

developed to predict the target sites of miRNAs *in silico* (Dweep et al. 2011; Min and Yoon 2010). Their algorithms are mainly based on base pairing interactions between miRNAs and their target mRNAs. Still, their output data varies, making biological validation unavoidable and essential to understand their targets and functional roles in the cell. To confirm if a predicted target is indeed a real target, an assay involving a luciferase reporter is used as the gold standard reporter assay. This assay consists in verifying if the wild-type 3'-UTR of a predicted target is repressed by an overexpressed miRNA and if the inclusion of a point mutation in the target sequences of the 3'-UTR abrogates this repression; if those two situations happen, then the studied mRNA is considered a target of that specific miRNA.

As miRNA-mediated regulation controls the expression of a wide amount of different mRNAs involved in multiple key cellular processes (Fu et al. 2012; Lima et al. 2011; Li and He 2012; Bueno and Malumbres 2011; Bueno et al. 2008b), their biogenesis and relative abundance must also be regulated in a tight manner, especially in what concerns their transcription, processing and cellular localization levels (reviewed in Slezak-Prochazka et al. 2010; Davis-Dusenbery and Hata 2010; reviewed in Melo et al. 2010). Transcription factors, enhancers and chromatin modifications may alter miRNA expression levels, either positively or negatively (Ozsolak et al. 2008; Wang et al. 2010a). Recently, miRNAs have been shown to be included into extracellular vesicles therefore targeting neighboring cells (Yang et al. 2011; Umezu et al. 2012), as well as to enter the blood stream therefore reaching distant cells (Ogawa et al. 2010; Zhang et al. 2010).

Ultimately, by being expressed in the entire organism and interfering in most of the cellular mechanisms, miRNAs 'control' the cellular phenotype in processes such as development (reviewed in Amiel et al. 2012) and differentiation (reviewed in Hinton et al. 2012). Moreover, their aberrant expression is often associated with a wide variety of illnesses broadening from diabetes (reviewed in Natarajan et al. 2012), to cardiovascular disease (reviewed in van Rooij and Olson 2012) or to various types of cancers (reviewed in Liu 2012; reviewed in Singh et al. 2012; reviewed in Schetter et al. 2012; reviewed in Seca et al. 2010).

2.3 MIRNAS AND CANCER

miRNAs were first linked with cancer in 2002, when miR-15a and miR-16-1 were associated with chronic lymphocytic leukemia (Calin et al. 2002). This discovery was the trigger for the investigation of the chromosomal location of miRNAs as well as for the study of the profile of miRNAs expression in various human cancers. Interestingly, miRNA genes were shown to be frequently located at fragile sites, as well as in minimal

regions of loss of heterozygosity, minimal regions of amplification or common breakpoint regions (Calin et al. 2004). Expression profiles of miRNAs showed an altered expression of miRNAs in tumors when compared to normal tissue (Calin and Croce 2006; reviewed in Lu et al. 2005; Volinia et al. 2006) with miRNAs being able to accurately classify different tumour types as well as poorly differentiated tumours and trace the tissue of origin of cancers of unknown primary origin (Lu et al. 2005; Rosenfeld et al. 2008).

Besides the relevant information that aberrant expression profiles of miRNAs provides, understanding the mechanisms and biological alterations that underlie such deregulations is also very important. Transcriptional deregulations, epigenetic alterations, mutations, DNA copy number abnormalities and defects in the miRNA biogenesis machinery have been shown to contribute to miRNA deregulation in human cancer (reviewed in Deng et al. 2008; reviewed in Iorio and Croce 2012). This deregulation, depending on their target mRNAs, prompts miRNAs to act as tumor suppressors (named "tumor suppressor miRNAs") or oncogenes (named "oncomirs") (reviewed in Esquela-Kerscher and Slack 2006). Therefore, miRNAs whose expression is decreased in cancer, and normally target oncogenes, are "tumor suppressor miRNAs". On the other hand, miRNAs whose expression is increased in cancer and confers them an oncogenic role, usually targeting tumor suppressor genes, are "oncomirs" (reviewed in Zhang et al. 2007). However, this classification needs to be used with caution, as the same miRNA can function as an oncomir or a tumor suppressor miRNA depending on the cellular context/tissue (reviewed in Chen et al. 2012a).

Abnormal miRNA expression can be regarded as a common feature of human cancers and therefore the involvement of miRNAs in cancer can be described by the role they play in the "hallmarks of cancer": t sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (reviewed in Hanahan and Weinberg 2011; reviewed in Ruan et al. 2009).

2.4 MIRNAS AND POTENTIAL APPLICATIONS IN THE ONCOLOGY CLINIC

Since cancer is one of the leading causes of death worldwide (reviewed in Jemal et al. 2011) there is a need for better diagnostic tools as well as biomarkers for early detection. This is relevant since the stage of the tumor at the time of diagnosis is related to survival and prognosis (with early detection normally being associated with better prognosis).

MiRNAs may have potential useful clinical applications, since they: i) are resistant to ribonuclease degradation; ii) can be accessible from small biopsies, frozen samples, formalin-fixed paraffin-embedded tissues and circulating blood,; iii) may be quantified with highly sensitive measurements (Xi et al. 2007).

The fact that miRNAs are deregulated in cancer makes their expression pattern useful and ideal to be used as cancer biomarkers. In fact, miR-21 and miR-205 have been used for early detection of pancreatic ductal adenocarcinoma cancer, as their overexpression preceded phenotypic changes in the pancreas ducts (du Rieu et al. 2010). miRNAs have also been shown to be able to identify the tissue of origin of a tumor (Lu et al. 2005) as well as to separate different cancer types (Volinia et al. 2006) or discriminate different subtypes of breast cancer (Blenkiron et al. 2007). However, and despite their promising value, miRNA expression profiles of tumours are not yet used in the clinic.

Another focus of current research is the study of circulating miRNAs in serum, plasma, peripheral blood mononuclear cells and other body fluids (reviewed in Cortez et al. 2011). The presence of miRNAs in body fluids may represent a gold mine of noninvasive biomarkers in cancer, as their circulating levels mimic the pattern of expression observed in the tumor (Lawrie et al. 2008). For example, let-7 expression could classify lung cancer samples from patients who underwent potentially curative resection into two major groups, with reduced let-7 expression associated with significantly shorter survival after resection (Takamizawa et al. 2004). In a study of non-small-cell lung cancer, a set of 11 serum miRNAs was differentially expressed between patients with longer or shorter survival, with four (miR-486, miR-30d, miR-1, and miR-499) associated with decreased overall survival of patients (Hu et al. 2010b). Moreover, in a study of acute leukemias, there was a decrease of miR-92a in the plasma samples of all patients compared with controls (Tanaka et al. 2009).

The ability of miRNAs to predict the response to a particular therapy is also of great value to clinicians. Overexpression of miR-21 was associated with shorter overall survival of pancreatic cancer patients treated with gemcitabine (Giovannetti et al. 2010) while downregulation of miR-26, in hepatocellular carcinoma patients, led to a good response to interferon-α treatment, resulting in improved survival (Ji et al. 2009).

Deregulation of miRNA expression is a common feature in cancer and their biological role has been established. Therefore, the usage of molecules that directly interfere with the amount of miRNAs in the cell, such as miR-mimics (that replace the loss of expression of a tumor suppressor miRNA) or antimiRs, antagomiRs or miR sponges (that decrease the endogenous highly expressed oncomir) would be of great interest as therapeutic tools. Drugs that modulate the expression of miRNAs by targeting their transcription and processing might also be of great value (Gumireddy et al. 2008; Melo et al. 2011).

One example came from studies of downregulation of miR-122, an abundant liver-specific miRNA, after injection of antagomiRs (oligonucleotides chemically modified with 2´-O-methyl and phosphorothioate) in the tail vein of mice, which was long lasting and efficient. This was the first successful report of miRNA targeting *in vivo* (Krutzfeldt et al. 2005). Indeed, since then, antimiR-122 has been developed for clinical use (hepatitis C) having so far reached phase II clinical trials (ClinicalTrials.gov Identifier: NCT01200420).

The miRNA sponges are constructs that competitively inhibit endogenous miRNAs by presenting various binding sites for these miRNAs, thus decreasing their quantity (Ebert et al. 2007). miRNA sponges have been applied in functional studies, for example, to study miR-31 in metastasis (Valastyan et al. 2009). One advantage is that a unique miRNA sponge can 'capture' an entire miRNA family (reviewed in Ebert and Sharp 2010).

3. miRNAs AND AML

3.1 miRNA SIGNATURES IN AML

Different studies have been published concerning miRNA profiling in different cancer types (reviewed in Bhatti et al. 2009; reviewed in Dillhoff et al. 2009), amongst which AML profiling has also been documented (Isken et al. 2008; Garzon et al. 2008b; Dixon-McIver et al. 2008; Jongen-Lavrencic et al. 2008; Diaz-Beya et al. 2013). The first miRNA expression profile in AML (Mi et al. 2007) evaluated if miRNA signatures would discriminate between AML and acute lymphoblastic leukemia (ALL). Samples from 11 ALL patients, 47 AML patients (both with similar chromosomal translocations), as well as from 7 cell lines of each malignancy were evaluated. 27 miRNAs were found differently expressed between ALL and AML. Of those, miR-128a, miR-128b, let-7b and miR-223 had significantly altered expression between ALL and AML. Using any two of these four miRNAs signatures was sufficient to accurately (97–99%) discriminate ALL from AML (Mi et al. 2007). A more recent study has addressed the same question: to find miRNAs differentially expressed between AML and ALL. Analysis of 23 miRNAs in 53 AML and 32 ALL bone marrow samples showed that 16 miRNAs were differentially expressed between AML and ALL. Eight had already been reported (Mi et al. 2007) and the other eight were newly identified. Correlating miRNA expression signatures with outcome of patients showed that the expression signatures of a group of miRNAs were associated with overall survival of patients. Of them, three (miR-146a, miR-181a/c, and miR-221) and five (miR-25, miR-26a, miR-29b, miR-146a, and miR-196b) miRNAs were significantly associated with overall survival of ALL and AML, respectively. Particularly, the expression signature of miR-146a was shown to be significantly inversely correlated with overall survival of both ALL and AML patients (Wang et al. 2010b).

Comparison of 50 AML bone marrow samples with 7 normal bone marrow samples as well as 5 CD34+ blood samples was performed, by analysing 154 miRNAs. Four miRNAs were found differently expressed in AML (Isken et al. 2008). From those, miR-23b was downregulated and miR-34a, miR-221 and miR-222 were upregulated in AML samples when compared to controls. Three other miRNAs (miR-26a, miR-26b and miR-29b) were differentially expressed between the groups, with an intermediate expression level in AML, being highly expressed in normal bone marrow samples and having low expression in CD34+ cells, appearing to be related with differentiation. Indeed, these miRNAs were shown to be upregulated upon ATRA induced differentiation of HL-60 cells (Isken et al. 2008).

Another study comparing microRNA levels by microarray between newly diagnosed AML patients and CD34+ bone marrow cells from 10 normal donors identified 26 downregulated miRNAs in AML (Garzon et al. 2008b). The results from 7 of the downregulated miRNAs (miR-93, miR-106b, miR-125a, miR-126, miR-130a, miR-135 and miR-146) were validated by quantitative RT-PCR. The results from the microarray analysis were further confirmed, in six more AML samples and four CD34+ blood samples, for all the miRNAs except miR-135. Of these miRNAs, miR-93, miR-125a, miR-126, miR-130a and miR-146 were not only downregulated in AML samples, when compared with CD34+ cells, but also downregulated in healthy precursors and mature peripheral blood myeloid cells. These results indicate that the downregulation of these miRNAs is probably not directly related to disease but rather to differentiation (Garzon et al. 2008b). In a study performed in 100 AML patients (analysing 157 miRNAs), representative of the range of karyotypes known in AML (Dixon-McIver et al. 2008), 17 upregulated and 16 downregulated miRNAs were found in AML when compared to bone marrow controls, with some of the upregulated miRNAs having been previously described as hematopoietic tissue-specific: miR-142-5p, miR-155 and miR-181 (Chen et al. 2004; Georgantas et al. 2007; Debernardi et al. 2007; reviewed in Seca et al. 2010). Additional miRNAs found upregulated in AML were miR-221 and miR-222, which was in agreement with other publications (Isken et al. 2008). Amongst the miRNAs found downregulated were some previously described as having anti-oncogenic potential in other cancers – miR-26a, miR-34c and miR-199a (Gaur et al. 2007; Corney et al. 2007; reviewed in Seca et al. 2010).

Some of the studies reporting miRNA profiles took into account the diverse cytogenetic alterations present in AML patients. A study with 215 genetically defined de novo AML patients and four samples of CD34+ cells from healthy donors analysed 260 miRNAs. This study allowed to cluster together AMLs with t(8;21), t(15;17), inv(16), NPM1 or CEBPA mutations with an unsupervised clustering approach. By using a supervised analysis, miRNA signatures that could characterize cytogenetic abnormal AMLs – t(15;17), t(8;21) and inv(16) – were identified, as well as signatures that distinguished NPM1 mutations, CEBPA mutations or FLT3 internal tandem duplication (FLT3-ITD). This study showed that although miRNAs were able to distinguish different genetic AMLs, the mRNA expression profiling was more accurate in predicting the same AMLs and it required a smaller number of probes. Nonetheless, a very small number of miRNAs were studied when compared with the number of mRNAs analysed (260 miRNAs compared with 54675 mRNAs), indicating the potential of profiling miRNAs for the molecular diagnosis of AML (Jongen-Lavrencic et al. 2008; reviewed in Seca et al. 2010).

Two studies have concentrated on AMLs harbouring the translocation t(8;21) or inv(16). In one of the studies, upregulation of miR-126 and miR-126* was exclusive of these cytogenetic alterations (Li et al. 2008). In the other study, downregulation of miR-133a and upregulation of miR-146a characterized t(8;21) AML, whilst upregulation of miR-99a, miR-100 and miR-224 defined inv(16) AMLs (Dixon-McIver et al. 2008; reviewed in Seca et al. 2010) (Table 2).

Patients bearing the translocation t(15;17) were characterized by different miRNA expression alterations, some of them described by several independent reports. For example, upregulation of miR-127, miR-323, miR-368 and miR-382 was described in two of three different studies (Dixon-McIver et al. 2008; Li et al. 2008; Jongen-Lavrencic et al. 2008). Curiously, miR-134, miR-154, miR-154*, miR-299, miR-370 and miR-376a, as well as the above-mentioned miRNAs, are located in chromosome 14q and were all described as being upregulated in translocation t(15;17) AMLs (Jongen-Lavrencic et al. 2008; Dixon-McIver et al. 2008; reviewed in Seca et al. 2010) (Table 2).

The MLL-rearrangement AMLs have also been characterized by miRNA profiling and different groups have come to various conclusions. In one study, the absence of expression of miR-10a, miR-331 and miR-340 was reported to define this cytogenetic alteration in AML (Dixon-McIver et al. 2008), whilst another study found an altered expression of 24 miRNAs in MLL rearrangements, seven of which belonging to the polycistronic miRNA cluster, mir-17-92 (Li et al. 2008). Another miRNA signature for MLL-rearrangement AMLs was identified, having 8 upregulated and 14 downregulated miRNAs (Garzon et al. 2008b) (Table 2). Amongst the downregulated miRNAs were some tumour suppressor miRNAs previously known to target critical oncogenes: miR-34b [targets CDK4 and CCNE2 (He et al. 2007)], miR-15a [targets BCL-2 (Cimmino et al. 2005)], let-7 family [targets RAS (Johnson et al. 2005)], miR-29 family [targets MCL-1 (Mott et al. 2007)] and miR-196 [targets HOX-A7, HOX-A8, HOX-D8 and HOX-B8 (Yekta et al. 2004; reviewed in Seca et al. 2010)].

The miRNA profile of 7 AML patients harbouring the translocation t(8;16)(p11;p13), compared with 113 other types of AML, revealed a distinctive signature of 94 miRNAs, most of which were found to be downregulated. A bioinformatic analysis showed that 29 of the miRNAs found to be downregulated might be regulated by methylation. This was confirmed for 27 of those miRNAs, by treating the t(8;16) AML samples with 5-aza-20-deoxycytidine (5-AZA-dC) and trichostatin A, since they were re-expressed after such treatment. Cross-correlation of mRNA and miRNA expression identified RET as a potential target of several miRNAs. In fact, miR-15a, miR-27b, miR-128, miR-195 and miR-218 were further confirmed, by Renilla-luciferase assay and flow cytometry after transfection with pre-miRs, to directly target RET (Diaz-Beya et al. 2013).

Analysis of miRNAs expression profile of 17 AML samples with complex karyotype and with known TP53 status (altered - loss and/or mutation of TP53 - or unaltered) identified 22 differentially expressed miRNAs, with miR-34a and miR-100 as the most significantly down- and upregulated miRNAs, respectively. Moreover, a gene expression profile enriched for genes belonging to p53-associated pathways (and implicated in cell cycle progression or apoptosis) was found in correlation with miR-34a expression. Clinically, low miR-34a expression and TP53 alterations were predictors of chemotherapy resistance and worse outcome (Rucker et al. 2013).

miRNA signatures for specific types of AMLs bearing characteristic mutations have also been described. In 85 *de novo* AML samples (55 with NPM1 alteration and 30 with wild-type NPM1) a distinctive pattern of miRNAs had altered expression between the two groups, with 36 upregulated and 21 downregulated miRNAs in NPM1 mutated samples (Garzon et al. 2008a) (Table 2). Amongst the upregulated miRNAs were miR-10a and miR-10b which were able to differentiate the two groups, as was also shown by others (Jongen-Lavrencic et al. 2008; Becker et al. 2010b; reviewed in Seca et al. 2010) (Table 2).

A microarray analysis of 203 miRNAs in 21 AML samples with NPM1 mutations and 5 BM samples from normal stem cell healthy donors was performed, to detect differential expression patterns. Fourteen miRNAs were identified as having distinct expression levels between leukemic and non-leukemic samples; in addition, NPM1 mutant AML samples presented a markedly overexpression of miR-10a (Ovcharenko et al. 2011) (Table 2). In addition, *in vitro* miR-10a overexpression induced differential gene expression, with murine double minute 4 (MDM4) standing as a candidate target for miR-10a. MDM4 was further validated as a direct target of miR-10a and an inverse association between miR-10a and MDM4 expression in NPM1 mutated AML samples was shown, implying that miR-10a expression may influence biological properties in AML by interfering with the p53 machinery, partly regulated by MDM4 (Ovcharenko et al. 2011).

A locked nucleic acid (LNA) based miRNA microarray platform was used to profile bone marrow samples of patients with cytogenetically normal AML (CN-AML) and five miRNAs emerged (miR-10a, let-7b and let-7c found to be overexpressed and miR-130a and miR-335 found to be underexpressed) as capable of dichotomising AML patients according to their NPM1 mutational status (Bryant et al. 2012) (Table 2).

Another study analysed miRNA and gene expression profiles of 43 cytogenetically normal AML patients (23 with NPM1 mutations and 20 wildtype). Out of 177 putative miRNA-target mRNA interactions that were identified, 77 novel candidates with known or potential involvement in leukemogenesis were validated, such as IRF2-miR-20a, KIT-miR-20a and MN1-miR-15a. Furthermore, it was shown that overexpression of tumor

suppressor miRNAs, such as miR-29a and miR-30c, which is observed in NPM1 mutated AML, may contribute to sensitivity to cytarabine (Russ et al. 2011).

A study focusing on BAALC (Brain and Acute Leukaemia, Cytoplasmic) using miRNA microarrays, reported that from over 50 patients – 24 with high and 26 with low expression of BAALC – no differences were found in miRNA profiles. However, miRNAs predicted (by *in silico* analysis) to target BAALC mRNA were further analysed and miR-148a presented a strong inverse correlation with BAALC expression (Langer et al. 2008). Additionally, AMLs harbouring CEBPA mutations revealed a characteristic miRNA signature, with 2 downregulated and 15 upregulated miRNAs (Marcucci et al. 2008a) (Table 2). Of the latter, 8 belonged to the miR-181 family, known to be involved in erythroid and lymphoid differentiation (Chen et al. 2004; Marcucci et al. 2008a; Choong et al. 2007; reviewed in Seca et al. 2010). Microarray analysis of miRNA expression in the AML cell line Kasumi-1 conditionally expressing CEBPA showed that 18 miRNAs presented altered expression, when compared to the parental cell line. Among those was miR-29b, which was shown to be downregulated in AML patients with impaired CEBPA function or loss of chromosome 7q. As CEBPA selectively regulates miR-29b, this study provided a rationale for miR-29b suppression in AML patients with loss of chromosome 7q or CEBPA deficiency (Eyholzer et al. 2010b).

Another study profiled a miRNA signature for meningioma 1 (MN1) high-expression AMLs. This study identified five upregulated miRNAs (miR-126, miR-126*, miR-129-5p, miR-130b and miR-424) and five downregulated miRNAs (miR-16, miR-19a, miR-20a, miR-100 and miR-196a) (Langer et al. 2009) (Table 2).

Additionally, when analysing AMLs bearing FLT3-ITD, three miRNAs – miR10a, miR-10b and miR-155 – were found to be upregulated in FLT3-ITD when compared with FLT3-wt patients (Garzon et al. 2008b) (Table 2). A study including 64 CN AMLs with adverse molecular characteristics – FLT3-ITD, wildtype NPM1 or both, found 8 miRNAs associated with event-free survival (Marcucci et al. 2008b), none of them overlapping with previously published results (Garzon et al. 2008b; reviewed in Seca et al. 2010). Another study showed that overexpression of miR-124, miR-128-1, miR-194, miR-219-5p, miR-220a and miR-320 was positively associated with the risk of an event (failure to achieve complete remission, relapse or death) whilst miR-181a and miR-181b overexpression was negatively associated with that risk. These authors also investigated gene-expression signatures to identify genes that could be regulated by the depicted miRNAs. Some predicted targets of the miR-181 family had an inverse correlation with these miRNAs' expression (TLR4, CARD8, CASP1 and IL1B), suggesting that miR-181 family downregulation can be linked to more aggressive AMLs (Marcucci et al. 2008b). Another study was able to directly correlate miR-181a expression with a better prognosis in CN

AML, independently of other variables including CEBPA mutations (Schwind et al. 2010; reviewed in Seca et al. 2010). More recently, it was shown that increased expression of miR-181a and miR-181b is also significantly associated with favourable overall survival in cytogenetically abnormal AML (CA-AML) patients (Li et al. 2012). Moreover, when analysing 183 CA-AML patients, upregulation of a gene signature composed of 4 potential miR-181 targets (including HOXA7, HOXA9, HOXA11, and PBX3) associated with downregulation of miR-181 family members, was shown to be an independent predictor of adverse overall survival. Furthermore, *in vitro* and *in vivo* studies have shown that ectopic expression of miR-181b promoted apoptosis and inhibited viability and proliferation of leukemic cells, delaying leukemogenesis; in addition, these effects could be reversed by forced expression of PBX3. These results showed that upregulation of the above mentioned homeobox genes resulted from the downregulation of miR-181 family members and probably contribute to the poor prognosis of patients with nonfavorable CA-AML (Li et al. 2012).

Overexpression of miR-191 and miR-199 was correlated with shorter overall survival and shorter event-free survival in a group of intermediate and poor prognosis karyotypes (Marcucci et al. 2008b). Cytogenetic prognostic risk could also be determined by the expression of two other miRNAs: miR-9 and let-7b, whose expression was found to be low in good prognosis groups and high in intermediate or adverse AMLs (Dixon-McIver et al. 2008; reviewed in Seca et al. 2010).

Mutations in the Wilms tumor 1 gene (WT1) occur in approximately 10% of adults with CN-AML, and conflicting results have been reported that they confer a worse outcome (Virappane et al. 2008; Summers et al. 2007) or have no prognostic impact (Gaidzik et al. 2009; Damm et al. 2010). The synonymous single nucleotide polymorphism (SNP) rs16754 of this gene has also been reported to be associated with outcome but results have not been concordant between studies. An association between the homozygous A allele and significantly shorter relapse-free survival and overall survival has been found in some studies (Damm et al. 2010) while other studies have found no association with such outcomes (Hollink et al. 2010; Ho et al. 2011). Therefore, gene and miRNA expression profiles of 433 AML patients were analysed for associations with rs16754 polymorphism; however, no distinct expression patterns were found to be associated with any rs16754 genotype (Becker et al. 2011).

Table 2. Highlighted upregulated and downregulated miRNAs in AML according to 'genetic alteration'.

Genetic alteration	Highlighted upregulated miRNAs	Highlighted downregulated miRNAs	References
t(8;21)	miR-126, miR-126*		(Li et al. 2008)
	miR-146a	miR-133a	(Dixon-McIver et al. 2008)
inv(16)	miR-126, miR-126*		(Li et al. 2008)
	miR-99a, miR-100, miR-224		(Dixon-McIver et al. 2008)
t(15;17)	miR-127, miR-134, miR-323, miR-376a, miR-382		(Jongen-Lavrencic et al. 2008)
	miR-127, miR-154, miR-154*, miR-299, miR-323, miR-368, miR-370		(Dixon-McIver et al. 2008)
	miR-368, miR-382		(Li et al. 2008)
MLL rearrangements		miR-10a, miR-331, miR-340	(Dixon-McIver et al. 2008)
	miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-92		(Li et al. 2008)
		let-7, miR-15a, miR-29a, miR-29b, miR-29c, miR-34b, miR-196a	(Garzon et al. 2008b)
NPM1 mutation	miR-10a, miR-10b		(Jongen-Lavrencic et al. 2008)
	miR-10a, miR-10b		(Garzon et al. 2008a)
	miR-10a, miR-10b		(Becker et al. 2010b)
	miR-10a		(Ovcharenko et al. 2011)
	miR-10a, let-7b, let-7c	miR-130a, miR-335	(Bryant et al. 2012)
CEBPA mutation	miR-181a, miR-181a*, miR-181b, miR-181c, miR-181d		(Marcucci et al. 2008a)
high MN1	miR-126, miR-126*, miR-129-5p, miR-130b, miR-424	miR-16, miR-19a, miR-20a, miR-100, miR-196a	(Langer et al. 2009)
FLT3-ITD	miR-10a, miR-10b, miR-155		(Garzon et al. 2008b)

Adapted from (Seca et al. 2010)

Recently, a study focusing on leukemias of ambiguous lineage (which represent a heterogeneous category of acute leukemias which cannot be classified as either AML or ALL) analysed their miRNA expression profiles. The study analysed sixteen cases of acute leukemia of ambiguous lineage and 17 cases of clinically defined acute leukemias: AML (n

= 7), B-ALL (n = 6) and T-ALL (n = 4) were analysed. Leukemias of ambiguous lineage did not segregate as a separate entity but exhibited miRNA expression profiles similar to AML, B-ALL or T-ALL. Using only 5 of the most lineage discriminative miRNAs (Wang et al. 2010b; Mi et al. 2007) was enough to define acute leukemia of ambiguous lineage as either AML or ALL (de Leeuw et al. 2013).

MiRNA microarray analysis of 15 chemoresistant and 18 chemosensitive AML bone marrows identified 3 significantly upregulated miRNAs: miR-363, miR-532-5p and miR-342-3p in chemoresistant samples (Mosakhani et al. 2013b).

In order to identify miRNAs that are significant prognostic determinants, independent from other known prognostic factors, a miRNA analysis was performed in a discovery cohort (n = 167) and a validation cohort (n = 409) of a heterogeneous AML population. miR-212 was shown to be an independent prognostic factor, significantly associated with prolonged overall survival and event-free and relapse-free survival in the discovery cohort. These results were subsequently confirmed in the independent validation cohort, with the prognostic significance of high expression of miR-212 not correlating with any (cyto)genetic subtypes of AML (Sun et al. 2013).

3.2 THE ROLE OF MIRNAS IN AML

3.2.1 MIRNAS IN HEMATOPOIETIC DIFFERENTIATION

As mentioned above, AML is a malignance associated with lack of differentiation of hematopoietic cells. This cellular process is therefore a candidate pathway for deregulated miRNAs to interfere with, leading to AML progression. Indeed, some miRNAs have been shown to play a role in hematopoietic differentiation and have been linked with AML progression in specific genetic backgrounds. AML1/ETO is a fusion protein characteristic of translocation t(8;21) in AMLs. AML1 normal expression was shown to downregulate miR-24 transcription but patients bearing the translocation t(8;21) overexpressed miR-24, which inhibited a mitogen-activated protein kinase (MAPK) phosphatase (MKP-7), thus leading to the activation of downstream partners (Zaidi et al. 2009). miR-24 was also shown to block myeloid differentiation and to accelerate cellular proliferation (Zaidi et al. 2009; reviewed in Seca et al. 2010).

The miR-193a has been shown to be downregulated in t(8;21) AMLs. AML1/ETO has been shown to act as a transcriptional repressor of AML1 target genes triggering the heterochromatic silencing of miR-193a. Suppression of miR-193a is associated with increased oncogenic activity of the fusion protein AML1-ETO, because miR-193a directly represses the expression of multiple target genes such as AML1-ETO, DNMT3a, HDAC3,

CCND1 and MDM2. Demethylation, RNAi inhibition of AML1/ETO or ectopic expression of miR-193a induced G1 arrest, apoptosis and restored leukemic cell differentiation. A feedback circuitry involving miR-193a and AML1/ETO/DNMTs/HDACs seems to be involved in AML differentiation. Moreover, *in vivo* experiments showed that ectopic expression of miR-193a reduced tumor size (Li et al. 2013a).

Another miRNA that has been correlated with AML t(8;21) carriers is miR-223 (Fazi et al. 2007). Patients' primary leukemia blasts showed a low level of miR-223, a previously known regulator of myelopoiesis (Fazi et al. 2005). This miRNA expression is decreased by the interaction of the AML1/ETO fusion protein with the miRNA promoter region. AML1/ETO has the capacity to oligomerise with histone deacetylase (HDAC) and DNA methyltransferases (DNMTs) bringing them to the AML1-binding site on the pre-miR-223 gene, hence silencing miR-223. Demethylating treatment, RNAi against AML1/ETO or ectopic expression of miR-223 restored cell differentiation, showing the importance of this miRNA in the normal myelopoiesis. Interestingly, another study focusing on miR-223 and AML showed no association between the expression of this miRNA and hypermethylation of its promoter region; furthermore, this study showed that miR-223 suppression in AML was rather due to impaired miR-223 upstream factors mediated by the conserved CEBP/PU.1 responsive element in front of the pri-miR-223 transcript start site (Eyholzer et al. 2010a). The known role of miR-223 in myelopoiesis has been supported by a recent study that correlates CEBPA and E2F1 transcription factors levels with miR-223 expression. CEBPA was shown to upregulate miR-223 expression, which in turn directly targets E2F1 3'-UTR, inhibiting its translation. Further studies elucidated that E2F1 is also a repressor of miR-223 by binding to its promoter region, producing a negative feedback loop. It was also shown that overexpression of miR-223 blocked cell proliferation allowing cells to differentiate, thus confirming its role in myelopoiesis (Pulikkan et al. 2010; reviewed in Seca et al. 2010).

miR-125 has been associated with the t(2;11) cytogenetic translocation (Bousquet et al. 2008). Indeed, AML patients with t(2;11)(p21;q23) translocation showed a big increase in miR-125b expression; additionally, *in vitro* experiments showed that miR-125b was able to both hamper primary human CD34+ cell differentiation and inhibit myelo-monocytic differentiation in HL-60 and NB4 leukemic cell lines. Accordingly, upregulation of miR-125b may represent a new mechanism of myeloid cell transformation in patients carrying the t(2;11) translocation (Bousquet et al. 2008; reviewed in Seca et al. 2010). In another study, a bone marrow transplantation model was used to analyse the role of miR-125b in leukemogenesis. Transduction of miR-125b resulted in expansion of hematopoietic cells although no leukemic transformation was observed as in other studies (Bousquet et al.

2010). miR-125b was shown to accelerate myeloid tumours induced by a C-terminal mutant of CEBPA (Enomoto et al. 2012).

Another miRNA shown to be specifically overexpressed in AML patients with MLL rearrangement is miR-196b (Popovic et al. 2009). The overexpression of miR-196b in bone marrow progenitor cells seems to increase proliferation and survival capacity, as well as to partially block differentiation, indicating that miR-196b plays a role in the development of MLL leukemias (Popovic et al. 2009; reviewed in Seca et al. 2010). More recently, miR-196b has been shown to directly target the ERG transcription factor. In addition, during *in vitro* differentiation of CD34+ cells the miR-196b expression decreased with time, indicating a role for miR-196b in early hematopoiesis (Coskun et al. 2011).

HIF-1α has been shown to induce differentiation of AML cells (Liu et al. 2006; Song et al. 2008). miR-17 and miR-20a were shown to be repressed by HIF-1α, through its downregulation of c-Myc expression. Ectopic expression of miR-17 or miR-20a partially rescued the growth inhibition and G1 arrest and attenuated HIF-1a-triggered differentiation, indicating that there is a tight loop regulation between these factors. This rescue was achieved through miR-17 and miR-20a direct interaction with p21 and STAT3, reducing their expression. Both p21 and STAT3 have been shown to be involved in the Jak-STAT signalling pathways, reported as closely related to cell differentiation (Weber-Nordt et al. 1998). Moreover, *in vivo* experiments showed that miR-20a contributes to HIF-1α-induced differentiation of leukemic cells (He et al. 2013).

The analysis of 48 BMs from AML patients, compared with BM samples from healthy donors, revealed a higher expression of miR-100 in AML samples. miR-100 overexpression was associated with an arrest of human granulocyte and monocyte differentiation and increased cell survival. It was shown to directly target RBSP3, a phosphatase shown to be is involved in myeloid cell differentiation and cell proliferation (Kashuba et al. 2009), via the dephosphorylation of RB and the release of E2F1, thus inducing progression through the G1/S transition and promoting S-phase entry (Zheng et al. 2012).

The let-7c was shown to be downregulated in PML/RARα-positive AML blasts when compared to normal promyelocytes (Careccia et al. 2009). Ectopic expression of let-7c promoted granulocytic differentiation of AML cell lines and primary blasts. PBX2, a protein whose aberrant expression enhances HoxA9-dependent leukemogenesis, was identified as a novel let-7c direct target, showing its involvement in the AML phenotype (Pelosi et al. 2013).

Two other miRNAs shown to be downregulated in AML patients were miR-29a and miR-142-3p (Wang et al. 2012a). Their expression was increased during ATRA and PMA induced myeloid differentiation in leukemia cell lines and CD34+ hematopoietic

stem/progenitor cells. Both miRNAs directly inhibited cyclin T2, preventing the release of hypophosphorylated retinoblastoma and resulting in the induction of monocytic differentiation. In addition, miR-29a was shown to directly target cyclin-dependent kinase 6, and miR-142-3p to target TGF-β–activated kinase 1/MAP3K7 binding protein 2, which are involved in the regulation of both monocytic and granulocytic differentiation. Using lentivirus-mediated gene transfer, enforced expression of either miR-29a or miR-142-3p in BM CD34+ cells derived from 3 AML patients led to a decrease in their target protein levels and partially overcame the differentiation arrest in AML blasts (Wang et al. 2012b). Interestingly and opposingly, in another study miR-29a was shown to be highly expressed in both human and mouse hematopoietic stem cells (HSC). In this study, miR-29a chimeric mice showed an abnormal acquisition of self-renewal capability by myeloid progenitors and development of a myeloproliferative disorder that progressed to AML. It also promoted progenitor proliferation by accelerating G1 to S/G2 cell cycle transitions thus suggesting that miR-29a participated in the initiation of AML by converting myeloid progenitors into self-renewing, stem-like cells (Han et al. 2010).

AML cell lines exposed to vitamin D3 acquire features of normal monocytes and are arrested in the G1 phase of the cell cycle, due to the upregulation of $p27^{Kip1}$ and $p21^{Cip1}$. miR-181a was shown to directly target $p27^{Kip1}$, attenuating the effect of vitamin D3 on the expression of monocytic differentiation markers and reducing the G1 block, suggesting that expression of miR-181a may contribute to the malignant phenotype of AML cells (Mishra et al. 2009). Vitamin D3-mediated monocytic differentiation of AML cells induced miR-26a expression, while its expression was shown to be downregulated in AML blasts (Salvatori et al. 2011). E2F7 was identified as a novel direct target of miR-26a and it was shown that E2F7 promotes cell cycle progression and inhibits monocytic differentiation of AML cells through binding to the cyclin-dependent kinase inhibitor p21CIP1/WAF1 promoter, repressing its expression. Thus, miR-26a regulation of E2F7 expression has a role in monocytic differentiation and leukemogenesis (Salvatori et al. 2012).

Analysis of miR-370 expression in BM samples from 48 *de novo* AML patients showed a significantly reduction of this miRNA expression, when compared to healthy controls, while miR-370 expression in patients in complete remission showed an increase in this miRNA expression, close to the expression seen in the controls. miR-370 expression was shown to be upregulated after treatment with 5-aza-20-deoxycytidine, a DNA demethylating agent, providing a possible mechanism for its downregulation. Additionally, FoxM1, a well-established oncogenic factor promoting cell cycle progression (Nakamura et al. 2010), was shown to be a direct target of miR-370. Its ectopic expression, in HL60 and K562 cells, led to cell growth arrest and senescence (Zhang et al. 2012).

High BAALC expression level is associated with poor outcome in CN-AML patients. miR-3151 was discovered in intron 1 of BAALC (Stark et al. 2010; Schotte et al. 2011) and its expression was associated with shorter disease-free and overall survival. Patients exhibiting high expression of both miR-3151 and BAALC had worse outcome than patients expressing low levels of either gene or both genes, with both markers contributing independently to poor outcome in CN-AML patients (Eisfeld et al. 2012).

In a study performed in HL-60 cells, it was shown that transferrin receptor 1 (TfR-1), involved in increased proliferation and decreased differentiation of leukaemic cells (Chitambar et al. 1983), is a target of miR-320 (Schaar et al. 2009). This miRNA emerges as a potential therapeutic target for TfR-1 overexpressing malignancies such as breast carcinoma, colorectal cancer or AML (Schaar et al. 2009; Brookes et al. 2006; Kwok and Richardson 2002; Yang et al. 2001).

Functional loss of CEBPA, a key transcription factor involved in the regulation of cell proliferation and differentiation in the hematopoietic system, can result in a differentiation block in granulopoiesis and contribute to leukaemic transformation (Hackanson et al. 2008). DNA methylation of CEBPA upstream promoter region has been correlated with t(15;17) and inv(16) AMLs. The use of demethylating agents, *in vitro*, led to an increase of CEBPA mRNA but, surprisingly, to a decrease of CEBPA protein expression. In addition, it was shown that miR-124a targets CEBPA mRNA, both by luciferase reporter assay (using a vector containing the 3'-UTR of CEBPA mRNA) and by analysis of CEBPA protein levels following transfection of K562 cells with miR-124a. Indeed this miRNA, often silenced by epigenetic mechanisms in AML, becomes activated after demethylation treatment and targets the 3'-UTR of CEBPA mRNA (Hackanson et al. 2008). This might explain why some patients respond well to epigenetic therapy whilst others do not (Hackanson et al. 2008; reviewed in Seca et al. 2010).

3.2.2 MIRNAS AND EPIGENETIC REGULATION

DNA methylation is a recognised regulatory molecular mechanism that leads to gene transcriptional silencing (Kim et al. 2009) and plays a role in AML development (Plass et al. 2008). The role of miRNAs in the epigenetic field is being unveiled (Kasinski and Slack 2011) and miR-29b has been shown to regulate DNA methyltransferases (DNMTs) in the context of leukaemia (Garzon et al. 2009b). This miRNA targets DNMT3A and DNMT3B directly and DNMT1 indirectly by interfering with Sp1, a DNMT1 transcription factor. Overexpression of miR-29b, in leukaemic cell lines, led to global DNA hypomethylation and re-expression of the tumour suppressor genes ESR1 and cyclin-dependent kinase inhibitor 2B (p15INK4b) (Garzon et al. 2009b; reviewed in Seca et al. 2010).

3.2.3 miRNAs AND APOPTOSIS

It has also been shown that miR-29b expression reduced cell growth and induced apoptosis in cell lines and primary AML samples. Furthermore reduced tumour growth was found in a human leukaemia xenograft model in mice, following injection of miR-29b into the tumours (Garzon et al. 2009a). Moreover, Mcl-1 was shown to be a miR-29b target and the analysis of 45 primary AML samples revealed an inverse correlation between miR-29b and Mcl-1 expression (Garzon et al. 2009a; reviewed in Seca et al. 2010)).

Cyclic AMP-responsive element binding protein (CREB) is occasionally overexpressed in AML (Pigazzi et al. 2007; Shankar et al. 2005). Myeloid cell lines, known to overexpress CREB, presented significantly lower levels of miR-34b. Furthermore, the exogenous expression of miR-34b directly targeted CREB mRNA, diminishing CREB protein levels and altering CREB target gene expression including several proteins known to regulate apoptosis: BCL-2, cyclin A1, cyclin B1, cyclin D, NF-KB, JAK1 and STAT3 (Pigazzi et al. 2009; reviewed in Seca et al. 2010). Downregulation of miR-34b in cell lines and AML patients was shown to be, in part, due to promoter methylation (Pigazzi et al. 2013). Moreover, an inverse correlation between miR-34b and CREB expression was observed in 78 pediatric AML patients, supporting the *in vitro* experimental results (Pigazzi et al. 2009).

miR-181a was shown to be downregulated in a HL-60 drug resistant cell line, when compared with its parental sensitive one. Bcl-2 was shown to be a target of miR-181a, which overexpression activated the apoptosis pathway by cytochrome C release and caspase-9/caspase-3 activation leading to sensitization of cells to cytarabine treatment (Bai et al. 2012). Another member of the miR-181 family, miR-181b, was shown to be overexpressed in AML patients. It directly targets MLK2, a member of the MAPK pathway important for p53-dependent apoptosis (Brown and Benchimol 2006), inducing cell survival through blockage of apoptosis (Chen et al. 2010).

Mutations and/or overexpression of c-Kit proto-oncogene frequently occur in subsets of AML. c-Kit is targeted by miR-193a/b, inhibiting its translation and targeting it for degradation. Their expression is inversely correlated in bone marrow samples and restoration of miR-193a/b expression in AML cells resulted in increased apoptosis and inhibited cell growth (Gao et al. 2011a; Gao et al. 2011b).

Inhibiting miR-32 expression was shown to increase apoptosis and sensitize AML cells to cytarabine. As miR-32 directly targets Bim, a known proapoptotic factor, its downregulation allows Bim increased expression and activity (Gocek et al. 2011).

In HL-60 cells, the inhibition of miR-21 using antisense oligonucleotides, contributed to the sensitization of the leukemic cells to cytarabine through an increase in apoptosis (Li et al. 2010b).

3.2.4 miRNAs AND MULTIDRUG RESISTANCE

In a study using the HL-60 AML cell line, Zhao and colleagues reported that miR-138 could be related with multidrug resistance of leukaemic cells. This miRNA was found in lower amounts in a HL-60 resistant cell line when compared with its parental cell line. Additionally, transfection of miR-138 into the HL-60 resistant cells was able to reverse P-glycoprotein-mediated resistance (by regulation of MDR1 transcription) and to induce apoptosis via regulation of the expression of Bcl-2 and Bax proteins (Zhao et al. 2010; reviewed in Seca et al. 2010).

3.3 CAUTION NEEDED IN THE ANALYSIS OF FUNCTIONAL STUDIES

Publications on miRNA expression profiles have not always been providing concordant results. This observation could be explained by the different AML samples used, with different cytogenetic and molecular characteristics, and the different platforms used to generate miRNA expression profiles. Normalisation of miRNA expression to other small RNAs (snRNA or tRNAs) other than miRNAs themselves may provide more reliable results. One of the above-mentioned studies has made a normalisation of the results for miR-223 expression which was shown to be uniformly expressed across the set of samples (Debernardi et al. 2007). However, miR-223 was shown to be differently expressed amongst AML samples in other studies (Fazi et al. 2007; Pulikkan et al. 2010). The method of RNA extraction and analysis of RNA quality could also interfere with the specific miRNA quantities detected (Becker et al. 2010a; Ach et al. 2008).

Despite these drawbacks, miRNA profiling can add extra knowledge towards better treatment of AML. Along with copy number alteration studies (Kim et al. 2012; Walter et al. 2009), miRNA expression profiles and functional studies of individual miRNAs aberrantly expressed in AMLs can introduce new insights into the perception of leukemogenesis, possibly improving diagnosis and identifying better prognostic markers as well as contributing to more tailored patient treatment.

Additionally, miRNAs have been proposed to be promising serum biomarkers for the detection of cancer (Mitchell et al. 2008; Van Roosbroeck et al. 2013; Redova et al. 2013). In fact, various recent studies have revealed that some miRNAs may serve as potential biomarkers in AML. miR-92a expression was found reduced in the plasma of acute

leukemia patients. In addition, the ratio of miR-92a/miR-638 (a miRNA shown to be equally expressed in all the samples, regardless of age, sex or leukemia classification) in plasma was valuable to distinguish healthy donors from acute leukemia patients (Tanaka et al. 2009). The expression of miR-150 and miR-342 in plasma was also shown to be associated with AML (Fayyad-Kazan et al. 2013). Another study found 6 miRNAs (miR-10a-5p, miR-93-5p, miR-129-5p, miR-155-5p, miR-181b-5p and miR-320d) that had significantly different expression levels in AML serum samples compared with control serum samples (Zhi et al. 2013).

4. miRNAs AND CML

CML is associated with the upregulation of miR-17-92 which is directly activated by c-MYC (O'Donnell et al. 2005). miR-17-92 cluster has also been shown to be induced by members of the E2F family of transcription factors (Woods et al. 2007). In addition, it is known that miR-17-5p and miR-20a, two miRNAs members of the miR-17-92 cluster, directly target and downregulate the expression of E2F1 in a negative feedback loop of transcriptional regulation (O'Donnell et al. 2005). This feedback loop counteracts the proliferative and apoptotic roles of E2F transcription factors allowing the fine tuning of this mechanism (Sylvestre et al. 2007; Woods et al. 2007; O'Donnell et al. 2005). Additionally, miR-17-92 cluster was found to be overexpressed in CML $CD34^+$ cells from patients in chronic phase but not in blast crisis, when compared with normal $CD34^+$ cells (Venturini et al. 2007). K562 CML cells treated with imatinib or with siRNAs for BCR-ABL or c-MYC presented downregulation of miR-17-92, showing that the BCR-ABL-MYC pathway controls the expression of these miRNAs (Venturini et al. 2007).

Imatinib resistant K562 cells, which overexpress c-MYC, were shown to have downregulation of miR-144/451. Restoration of miR-144/451 sensitized imatinib resistant cells to apoptosis, providing evidence for the role of Myc-miR144/451 pathway in CML drug resistance (Liu et al. 2012). Some studies have shown that there is a reciprocal regulatory loop between BCR-ABL and miR-451 (Lopotova et al. 2011; Scholl et al. 2012). Additionally, miR-196b, a miRNA whose expression was found decreased in CML patients, was also shown to directly target BCR-ABL decreasing cell proliferation and retarding the cell cycle (Liu et al. 2013b).

CGH (comparative genomic hybridization) analysis identified a tumor suppressor miRNA, miR-203, whose expression is decreased in hematopoietic malignancies, by both genetic (due to its location in a fragile chromosomal region) and epigenetic (due to hypermethylation) mechanisms. miR-203 controls the expression of ABL, and also the expression of BCR-ABL fusion protein. In addition, its reintroduction into the CML cell lines K562 and KCL-22 resulted in decreased expression of ABL and BCR-ABL fusion protein levels, inhibiting tumor cell proliferation in an ABL-dependent manner (Bueno et al. 2008a). In a recent study, overexpression of miR-203 in cells bearing BCR-ABL T315I mutation (known to confer resistance to tyrosine kinase inhibitors) inhibited cell growth and colony formation ability and increased sensitivity to imatinib, therefore antagonizing the main mechanism of resistance (Li et al. 2013c).

miR-30a was also shown to directly target BCR-ABL and is often found underexpressed in bone marrow from CML patients. In K562 CML cells, overexpression of miR-30a reduced

ABL and BCR-ABL protein expression, decreasing proliferation and arresting cell cycle progression between G1 and S (Liu et al. 2013a). In addition, overexpression of miR-30a was shown to decrease Beclin-1 and ATG5 expression (and subsequently autophagy), enhancing imatinib-induced apoptosis (Yu et al. 2012).

miR-138 has also been shown to repress BCR-ABL expression by targeting the coding region of the ABL gene as well as targeting the 3'-UTR of CCND3. In K562 and Ku812 CML cell lines, the tumor suppressor activity of miR-138 was demonstrated by the induction of cell cycle arrest at G0/G1, inhibition of cell proliferation and colony formation and the enhancement of imatinib-induced apoptosis. The upregulation of miR-138 expression upon imatinib treatment is associated with the enhancement of GATA1 activity, which binds to the miR-138 promoter (Xu et al. 2012).

Loss of miR-328 occurs in blast crisis of CML in a BCR-ABL dependent manner. Its re-expression restored differentiation and hampered survival of leukemic blasts by targeting the mRNA encoding the survival factor PIM1 and through inhibition of the translational regulator poly(rC)-binding protein hnRNP E2, inhibiting its interaction with hematopoietic transcription factor CEBPA (Eiring et al. 2010).

In K562 CML cells, miR-181a was shown to directly target RalA, a downstream molecule of BCR-ABL fusion protein in the Ras signaling pathway. miR-181a overexpression led to a decrease in cell growth and induced G2-phase arrest and apoptosis partially through targeting RalA (Fei et al. 2012). Other members of the miR-181 family have been shown to be deregulated in CML. In a model of Lyn-mediated imatinib-resistant CML, miR-181b and miR-181d were shown to be downregulated and miR-181b was shown to directly target Mcl-1 3´-UTR (Zimmerman et al. 2010). A recent study designed to find miRNAs involved in imatinib response, in 9 CML patients including 4 imatinib-resistant and 5 imatinib-responder patients, found miR-181c as the only differentially expressed miRNA (Mosakhani et al. 2013a).

In K562 CML cells CCN3, a negative growth regulator, was shown to be regulated by miR-130a/b, which are BCR-ABL dependent miRNAs. Downregulation of CCN3 expression confers growth advantage and survival means for CML cells (Suresh et al. 2011). By analysis of miRNAs expression in CML cell lines and patients comparing to non-CML cell lines and healthy donors, miR-31, miR-155 and miR-564 were found downregulated in CML with this downregulation being dependent on BCR-ABL activity. The decreased expression of these miRNAs led to the upregulation of several putative targets, namely CBL, E2F3, cyclin D1, K-ras and Akt2 which are involved in distinct signaling pathways such as MAPK, ErbB, mTOR and VEGF related with cell survival and uncontrolled cell proliferation (Rokah et al. 2012). In CML patients the expression of miR-223 is inversely correlated with BCR-ABL suggesting a regulation by the tyrosine kinase over the

expression of this miRNA. PTBP2 and MEF2C, were validated as miR-223 targets, and their expression is associated with cell survival and proliferation which can eventually help in disease progression (Agatheeswaran et al. 2013).

Although the BCR-ABL oncoprotein is a pivotal factor in CML pathogenesis there are some miRNAs that present abnormal expression, independently of BCR-ABL activity. Analysis of miRNA expression in blood samples from newly diagnosed CML patients before and within the first two weeks of imatinib therapy identified an increase in the expression of miR-150 and miR-146a, and a reduction in the expression of miR-142-3p and miR-199b-5p. The aberrant expression levels of these miRNAs tended to normal levels after the two weeks of imatinib therapy (Flamant et al. 2010). Another study, comparing miRNA expression profiles of K562 imatinib sensitive and imatinib resistant cells, identified miR-212 as being downregulated in resistant cells; the authors correlated miR-212 expression with the efflux pump ABCG2 and further confirmed the direct targeting of ABCG2 by miR-212 (Turrini et al. 2012).

Microarray analysis identified differential expression profiles of 49 miRNAs in CML patients at diagnosis, in hematological relapse, therapy failure, blast crisis and major molecular response. Among those miRNAs, miR-150 was shown to be downregulated in patients at diagnosis, in the majority of patients with hematological relapse and in blast crisis (Machova Polakova et al. 2011). It was also shown that miR-150 inversely correlates with the expression of MYB (Machova Polakova et al. 2011), which has been shown to be a target of miR-150, and shown to be required for BCR-ABL dependent leukemogenesis (Lidonnici et al. 2008). Another analysis of miRNA expression comparing mononuclear and $CD34^+$ cells from patients with CML with healthy controls revealed that miR-10a, miR-150 and miR-151 were downregulated, while miR-96 was upregulated in CML cells.

miR-10a expression was shown to be inversely correlated with upstream stimulatory factor 2 (USF2). The USF2 overexpression increased cell growth, while downregulation of miR-10a was shown not to be dependent on BCR-ABL activity and contributed to the increased cell growth of CML cells (Agirre et al. 2008).

In K562 CML cells, miR-29 was shown to target and downregulate the expression of RNase-L, inhibiting proliferation *in vitro* and tumor growth in a xenograft model (Lee et al. 2013). miR-29b was shown to target and decrease the expression of ABL and the enforced expression of his miRNA, in K562 cells, inhibited cell growth and colony formation ability and inducing apoptosis (Li et al. 2013b).

Other studies with K562 cells showed that miR-21 directly targets PDCD4 and that downregulation of miR-21 using antimiR-21 suppressed cell migration, promoted cell apoptosis, and inhibited cell growth (Hu et al. 2010a) with a synergistic effect with arsenic trioxide having been found (Li et al. 2010a; Gu et al. 2011).

miR-326 was recently shown to target Smo, a member of the Hedgehog signaling pathway, resulting in decreased cell proliferation and elevated rate of apoptosis in CML $CD34^+$ cells. As Hedgehog signaling has been proven as a functional pathway for leukemic stem cell, loss of this pathway impairs the development of BCR-ABL-induced CML and depletes CML stem cells (Babashah et al. 2013).

In spite of imatinib's outstanding success in treating CML patients, there are around 20-25% patients that have primary resistance to imatinib and show signs of persistent disease. A miRNA expression signature associated with imatinib resistance was identified by analysis of BM mononuclear cells from patients, with Ph^+ CML, that had complete cytogenetic response at 12 months compared with primary resistant patients. A group of 19 miRNAs that may predict clinical resistance to imatinib was identified, with 18 of them being downregulated (miR-7, miR-23a, miR-26a, miR-29a, miR-29c, miR-30b, miR-30c, miR-100, miR-126#, miR-134, miR-141, miR-183, miR-196b, miR-199a, miR-224, miR-326, miR-422b and miR-520a) and only one upregulated (miR-191). Among these miRNAs, miR-29c, miR-183 and miR-199a have been predicted to target drug transporters, ABCB6, ABCA1 and ABCC5 respectively. As second generation tyrosine kinase inhibitors are effective in patients with imatinib resistance, the identification of predictors of resistance to imatinib is important to distinguish the patients that should not receive imatinib as therapy for CML (San Jose-Eneriz et al. 2009b).

5. miRNAs IN CANCER DRUG RESISTANCE AND DRUG SENSITIVITY

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Abstract
miRNAs are important regulators of distinct biological processes and are tightly related with several diseases, including cancer. In the last years, miRNAs have been shown to play an important role in drug resistance/sensitivity to anticancer drugs. This chapter first addresses the phenomenon of drug resistance in cancer cells. It then describes the role of miRNAs in drug resistance or drug sensitivity, including a review of the role of miRNAs and their validated targets in cancer drug resistance. Furthermore, it focuses on miRNAs that target cellular proteins related to drug resistance such as p53 or proteins involved in cellular processes such as apoptosis, autophagy, DNA damage response, epithelial to mesenchymal transition and cancer stem cell phenotype, drug efflux or drug metabolism. In addition, the non-genetic acquisition of drug resistance through the transfer of miRNAs from drug resistant to drug sensitive cells via microvesicles or exosomes is also discussed. Finally, the known miRNA polymorphisms associated with drug resistance are addressed.

1. Cancer drug resistance

Chemotherapy is an important treatment modality for many types of cancer and it is the main treatment for advanced and surgically resectable cancers. Targeted therapies have been more recently developed with the emergence of small molecules which target specific kinases or receptors in the cell that are known to be deregulated in cancer. Despite the benefits in improving overall survival and quality of life for patients, the therapeutic success of both conventional chemotherapy and targeted therapeutics is often limited due to drug resistance [reviewed in (Fojo, 2007; Broxterman *et al.*, 2009; Stegmeier *et al.*, 2010)].

Cancer drug resistance may be intrinsic, based on genetic characteristics of tumor cells that are insensitive to therapeutic agents even before treatment - intrinsic resistance - or may appear *de novo*, after treatment with a chemotherapeutic agent - acquired resistance. This resistance enables the tumor cells to escape to the cytotoxic/cytostatic effect of anticancer drugs and thus allows the tumor to persist and eventually progress.

Cancer drug resistance has been thoroughly studied in cell lines, animal models and cancer patients, in order to elucidate the cellular and molecular mechanisms involved. These mechanisms may comprise genetic alterations, such as a particular gene amplification, translocations and mutations, or epigenetic modifications that alter the function of pivotal genes [reviewed in (Fojo, 2007; Wilting and Dannenberg, 2012)]. In any of the cases, the mechanisms are generally divided into two main groups: those that disturb the delivery and maintenance of the drugs in the cells and those arising from molecular alterations that affect drug sensitivity [reviewed in (Gottesman *et al.*, 2002)].

Cancer drug resistance has been shown to be very hard to tackle, mostly due to two factors: i) the intra-tumour heterogeneity [(due to expansion of multiple clones, some being more aggressive than others (Gerlinger *et al.*, 2012)] and ii) the tumour dynamics [(with genetic instability and accumulations of mutations throughout tumour development (Sequist *et al.*, 2011)]. In addition, an evolutive adaptation of cancer cells to drugs has been proposed as a mechanism that promotes drug resistance [reviewed in (Gerlinger and Swanton, 2010)]. The most common cause for acquisition of cancer drug resistance is the expression of energy-dependent transporters (or drug efflux pumps), that detect some anticancer drugs and carry out the efflux of such drugs from the cancer cells. Since the drug efflux transporters are not specific for a designated substrate, cells that overexpress drug efflux pumps such as P-glycoprotein (P-gp) are resistant to various drugs, a phenotype known as multidrug resistance (MDR) [reviewed in (Gottesman *et al.*, 2002)]. However, several other mechanisms of drug resistance are known to occur, such as insensitivity to drug-induced apoptosis, enhanced drug metabolism, increased DNA

repair or mutations in drug targets [reviewed in (Gottesman *et al.*, 2002; Fojo, 2007; Borst, 2012)]. Table 1.1 summarizes the most common mechanisms of drug resistance.

Table 1.1 Examples of cancer drug resistance mechanisms.

Mechanism	Example
Overexpression of efflux pumps	overexpression of P-gp conferring resistance (several drugs) [reviewed in (Fletcher *et al.*, 2010)]
Increase drug detoxification	increased activity of the enzyme CYP3A4 conferring resistance to docetaxel [reviewed in (Akhdar *et al.*, 2012)]
Deregulation of apoptosis mechanisms	overexpression of anti-apoptotic proteins, e.g. Bcl-2 (several drugs) [reviewed in (Rebucci and Michiels, 2013)]
Point mutations in genes coding for drug targets	T315I mutation in BCR-ABL confers resistance to imatinib [reviewed in (Garraway and Janne, 2012)]
Overexpression of oncogenes causing dysfunction in signaling molecules and kinases	HER2 (ERBB2) amplification (several drugs) [reviewed in (Garraway and Janne, 2012)]
Activation of alternative mechanisms	MET overexpression with sustained activation of PI3K/Akt signaling confers resistance to TKIs [reviewed in (Kosaka *et al.*, 2011)]
Tumor microenvironment	hypoxia-induced resistance (several drugs) [reviewed in (Rebucci and Michiels, 2013)]
Epigenetic alterations	distinct chromatin state mediated by the histone demethylase KDM5A confers resistance to erlotinib [reviewed in (Garraway and Janne, 2012)]
miRNAs	miR-21 overexpression (several drugs) [reviewed in (Chen *et al.*, 2012c)]

In this work, we will review the current knowledge on the role of miRNAs in drug resistance.

2. Anticancer drugs may affect cellular miRNA expression

miRNA expression has been shown to be altered by anticancer drugs. For example, a panel of 60 human cancer cell lines (NCI-60) which has been used to screen more than 100.000 compounds for their anticancer potential, has been profiled for mRNA and protein expression, mutational status, DNA copy number, chromosomal alterations (Boyd and Pauli, 1995) and miRNA expression (Blower *et al.*, 2007; Gaur *et al.*, 2007b). These studies have shown a correlation between the expression pattern of some miRNAs and the growth inhibitory pattern of certain drugs, which may indicate a role for such miRNAs in drug response (Blower *et al.*, 2007).

Other studies have also shown that anticancer drugs affect miRNAs expression (Rossi *et al.*, 2007; Flamant *et al.*, 2010; Gmeiner *et al.*, 2010). Nevertheless, the results cannot be analysed straightforward as the observed alterations are due to specific characteristics of the experimental design such as drug concentration, time of exposure to the drug or the

type of cells in which the anticancer drugs were tested. Therefore, the precise function of each miRNA might not be easy to assess and still needs extensive work.

3. MicroRNAs involved in cancer drug response: drug sensitivity and drug resistance

The fact that various cancer drugs affect cellular miRNAs expression indicates that miRNAs may be important regulators of mechanisms involved in treatment outcome. Nevertheless, it is not known if those miRNAs have a function in drug response or in drug resistance, or both. In order to confirm this, functional studies, either by overexpressing or silencing the expression of such miRNAs in cell lines have been performed (Table 1.2).

Table 1.2 Role of some miRNAs and validated targets in cancer drug resistance.

miRNA	Manipulation of expression carried out (up or downregulation)	Obtained result	Validated direct target
miR-1	↑	sensitizes lung cancer cells to DOX (Nasser *et al.*, 2008)	MET, FOXP1
miR-7	↑	sensitizes breast cancer cells to CDDP (Pogribny *et al.*, 2010)	ABCC1
	↑	sensitizes head and neck cancer cells to erlotinib (Kalinowski *et al.*, 2012)	EGFR
miR-10a*	↓	inhibition sensitizes glioblastoma cells to TMZ (Ujifuku *et al.*, 2010)	unknown
miR-10b	↑	confers resistance to 5-FU in colon cancer cells (Nishida *et al.*, 2012)	BIM
miR-15a	↑↓	modulates TAM resistance in breast cancer cells (Cittelly *et al.*, 2010a)	BCL2
miR-15b	↑↓	modulates CDDP resistance in tongue cancer cells (Sun *et al.*, 2012)	BMI1
	↑↓	modulates CDDP, DOX, VP-16 and VCR resistance in gastric cancer cells (Xia *et al.*, 2008)	BCL2
miR-16	↑↓	modulates CDDP, DOX, VP-16 and VCR resistance in gastric cancer cells (Xia *et al.*, 2008)	BCL2
	↑↓	modulates TAM resistance in breast cancer cells (Cittelly *et al.*, 2010a)	BCL2
miR-17	↑↓	modulates AZD6244 resistance in lung cancer cells (Dai *et al.*, 2011)	unknown
miR-17- 92	↑↓	confers resistance to DOX and topotecan and modulates VP-16 resistance in mantle cell lymphoma cells (Rao *et al.*, 2012)	BIM, PTEN, PHLPP2
miR-19	↓	inhibition sensitizes breast cancer cells to PTX, VP-16 and MX (Liang *et al.*, 2011)	PTEN
miR-19a	↑↓	modulates 5-FU, CDDP and DOX resistance in gastric cancer cells (Wang *et al.*, 2013a)	PTEN
miR-19b	↑↓	modulates 5-FU, CDDP and DOX resistance in	PTEN

		gastric cancer cells (Wang *et al.*, 2013a)	
miR-20a	↑↓	modulates 5-FU, DOX and VM-26 resistance in colon cancer cells (Chai *et al.*, 2011)	BNIP2
miR-21	↑↓	modulates DTX resistance in prostate cancer cells (Shi *et al.*, 2010)	PDCD4
	↑	sensitizes tongue cancer cells to CDDP (Yu *et al.*, 2010)	unknown
	↑↓	modulates DNR resistance in leukemia cancer cells (Bai *et al.*, 2011)	PTEN
	↑↓	modulates CDDP resistance in neuroblastoma cells (Chen *et al.*, 2012d)	PTEN
	↑↓	modulates gemcitabine resistance in pancreatic cancer cells (Dong *et al.*, 2011)	BCL2
	↑↓	modulates Trastuzumab resistance in breast cancer cells (Gong *et al.*, 2011)	PTEN
	↓	inhibition sensitizes prostate cancer cells to STS (Li *et al.*, 2009a)	MARCKS
	↓	inhibition sensitizes leukemia cells to ATO (Li *et al.*, 2010a)	PDCD4
	↑↓	modulates CDDP and DTX resistance in lung cancer cells (Liu *et al.*, 2013d)	PTEN
	↑↓	modulates DOX resistance in bladder cancer cells (Tao *et al.*, 2011)	PTEN
	↑↓	modulates 5-FU resistance in HCC cells (Tomimaru *et al.*, 2010)	PDCD4, PTEN
	↑	confers resistance to 5-FU in colon cancer cells (Valeri *et al.*, 2010)	hMSH2
	↑↓	modulates DOX resistance in breast cancer cells (Wang *et al.*, 2011c)	PTEN
	↑↓	modulates DEX and DOX resistance in multiple myeloma cells (Wang *et al.*, 2011a)	RHOB
	↓	inhibition sensitizes lung cancer cells to gefitinib (Garofalo *et al.*, 2012b)	unknown
	↓	inhibition sensitizes glioblastoma cells to VM-26 (Li *et al.*, 2009b)	LRRFIP1
	↑	confers resistance to gemcitabine in pancreatic cancer cells (Giovannetti *et al.*, 2010)	PTEN
	↑↓	modulates gemcitabine resistance in cholangiocarcinoma cells (Meng *et al.*, 2006)	PTEN
	↓	inhibition sensitizes CLL cells to fludarabine (Ferracin *et al.*, 2010)	unknown
	↓	inhibition sensitizes lung cancer cells to AG1478 (Seike *et al.*, 2009)	unknown
miR-22	↑	sensitizes p53-mutated colon cancer cells to PTX (Li *et al.*, 2011b)	unknown
miR-23a	↓	inhibition sensitizes tongue cancer cells to CDDP (Yu *et al.*, 2010)	TOP2B
miR-24	↑	sensitizes breast and lung cancer cells to TRAIL (Xie *et al.*, 2013)	XIAP

	↑	confers resistance to MTX in colon cancer cells (Mishra *et al.*, 2007b)	DHRF
	↑	modulates bleomycin and CDDP resistance in CML and HCC cells (Lal *et al.*, 2009)	H2AX
miR-25	↑	modulates TRAIL resistance in cholangiocarcinoma cells (Razumilava *et al.*, 2012)	DR4
miR-27a	↓	inhibition sensitizes ovarian cancer cells to vinblastine (Zhu *et al.*, 2008)	unknown
	↑	sensitizes leukemia cells to DOX (Feng *et al.*, 2011)	ABCB1
	↑↓	modulates PTX resistance in ovarian cancer cells (Li *et al.*, 2010c)	HIPK2
	↓	inhibition sensitizes gastric cancer cells to 5-FU, CDDP, DOX and VCR (Zhao *et al.*, 2011a)	CCND1
mR-29c	↑	sensitizes nasopharyngeal carcinoma cells to CDDP (Zhang *et al.*, 2013)	BCL2, MCL1
miR-30a	↑↓	modulates dasatinib, imatinib and nilotinib resistance in CML cells (Yu *et al.*, 2012b)	BECN1
	↑	sensitizes cervical and gastric cancer cells to CDDP and breast cancer cells to PTX (Zou *et al.*, 2012a)	BECN1
	↑	sensitizes breast cancer cells to PTX (Bockhorn *et al.*, 2013)	unknown
miR-30a*	↑	sensitizes breast cancer cells to DOX and PTX (Bockhorn *et al.*, 2013)	unknown
miR-30b	↑↓	modulates gefitinib resistance in lung cancer cells (Garofalo *et al.*, 2012b)	BIM
miR-30c	↑↓	modulates gefitinib resistance in lung cancer cells (Garofalo *et al.*, 2012b)	BIM
	↑	sensitizes breast cancer cells to DOX and PTX (Bockhorn *et al.*, 2013)	TWF1
miR-31	↑	sensitizes ovarian cancer cells to PTX (Mitamura *et al.*, 2013)	MET
	↑↓	modulates DTX resistance in prostate cancer cells and sensitizes prostate cancer cells to CDDP (Bhatnagar *et al.*, 2010)	E2F6
	↑	sensitizes breast cancer cells to DOX and STS (Korner *et al.*, 2013)	PKCε
miR-34a	↑↓	modulates DTX resistance in breast cancer cells (Kastl *et al.*, 2012)	BCL2
	↑↓	sensitizes colon cancer cells to 5-FU (Akao *et al.*, 2011)	SIRT1
	↑	sensitizes prostate cancer cells to DNR, PTX and VP-16 (Kojima *et al.*, 2010)	SIRT1
	↑↓	modulates DOX resistance in breast cancer cells (Li *et al.*, 2012b)	NOTCH1
	↑	sensitizes Ewing's sarcoma cells to DOX and VCR (Nakatani *et al.*, 2012)	unknown
	↑↓	modulates CDDP resistance in bladder cancer cells (Vinall *et al.*, 2012)	CDK6, SIRT1
	↑	sensitizes medulloblastoma cells to CDDP and MMC (Weeraratne *et al.*, 2011)	MAGE-A

	↑	sensitizes prostate cancer cells to CPT (Fujita *et al.*, 2008)	SIRT1
	↑	sensitizes gastric cancer cells to CDDP, DOX, DTX and gemcitabine (Ji *et al.*, 2008)	BCL2
	↑	sensitizes retinoblastoma cells to topotecan (Dalgard *et al.*, 2009)	unknown
	↑↓	modulates DOX resistance in prostate cancer cells (Rokhlin *et al.*, 2008)	unknown
miR-34c	↑↓	modulates DOX resistance in prostate cancer cells (Rokhlin *et al.*, 2008)	unknown
miR-34c-5p	↑↓	modulates PTX resistance in lung cancer cells (Catuogno *et al.*, 2013)	BMF
	↑	sensitizes gastric cancer cells to PTX (Wu *et al.*, 2013b)	MAPT
miR-93	↑↓	modulates CDDP resistance in ovarian cancer cells (Fu *et al.*, 2012b))	PTEN
	↑↓	modulates TGFβ resistance in gastric cancer cells (Petrocca *et al.*, 2008)	E2F1
miR-96	↑	sensitizes osteosarcoma, cervical and ovarian cancer cells to CDDP (Wang *et al.*, 2012d)	RAD51, REV1
miR-98	↑	sensitizes lung cancer and cells to CDDP (Xiang *et al.*, 2013a)	HMGA2
	↑	confers resistance to CDDP and DOX in HNSCC cells (Hebert *et al.*, 2007)	HMGA2
miR-100	↑	sensitizes lung cancer cells to DTX (Feng *et al.*, 2012a)	PLK1
	↑	sensitizes ovarian cancer cells to everolimus (Nagaraja *et al.*, 2010)	mTOR
miR-101	↑	sensitizes breast cancer cells to TAM (Frankel *et al.*, 2011)	ATG4D, RAB5A, STMN1
	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
	↑	sensitizes HCC cells to curcumin, DOX and VP-16 (Su *et al.*, 2009)	MCL1
miR-103	↑	sensitizes lung cancer cells to gefitinib (Garofalo *et al.*, 2012b)	PKCε
miR-106a	↓	inhibition sensitizes ovarian cancer cells to PTX (Huh *et al.*, 2013)	BCL10, CASP7
miR-106b	↑↓	modulates TGFβ resistance in gastric cancer cells (Petrocca *et al.*, 2008)	E2F1
	↑	sensitizes HMEC to DOX (Ivanovska *et al.*, 2008)	CDKN1A
miR-122	↑	sensitizes HCC cells to DOX and VCR (Xu *et al.*, 2011b)	unknown
	↑	sensitizes HCC cells to DOX (Fornari *et al.*, 2009)	CCNG1
miR-125a/b	↑	sensitizes colon cancer cells to PTX (Chen *et al.*, 2013a)	unknown
miR-125b	↑↓	modulates PTX resistance in breast cancer cells (Zhou *et al.*, 2010)	BAK1

	↑↓	modulates CDDP resistance in ovarian cancer cells (Kong *et al.*, 2011)	BAK1
	↑↓	modulates gemcitabine and PTX resistance in breast cancer cells (Liu *et al.*, 2013c)	BAK1
	↑↓	modulates 5-FU resistance in breast cancer cells (Wang *et al.*, 2012b)	E2F3
	↑	confers resistance to DOX in leukemia cells (Zhang *et al.*, 2011a)	BAK1
miR-125b-2	↓	inhibition confers resistance to TMZ in glioblastoma cells (Shi *et al.*, 2012a)	unknown
miR-126	↑↓	modulates DOX and VCR resistance in lung cancer cells (Zhu *et al.*, 2012c)	VEGFA
	↑	Sensitizes lung cancer cells to gefitinib (Zhong *et al.*, 2010)	unknown
miR-128	↑	sensitizes breast cancer cells to DOX (Zhu *et al.*, 2011)	BMI1, ABCC5
miR-128b	↑	sensitizes ALL cells to DEX (Kotani *et al.*, 2009)	AF4, MLL
miR-128-2	↑	confers resistance to 5-FU, CDDP and DOX in lung cancer cells (Donzelli *et al.*, 2012)	E2F5
miR-129	↑	sensitizes colon cancer cells to 5-FU (Karaayvaz *et al.*, 2013)	BCL2
miR-130a	↑	sensitizes NSCLC cells to TRAIL (Acunzo *et al.*, 2012)	MET
	↑↓	modulates CDDP resistance in HCC cells (Xu *et al.*, 2012d)	RUNX3
	↑↓	modulates CDDP resistance in ovarian cancer cells (Yang *et al.*, 2012c)	unknown
miR-130b	↑↓	modulates CDDP and PTX resistance in ovarian cancer cells (Yang *et al.*, 2012b)	CSF1
miR-134	↑↓	modulates CDDP, DOX and VP-16 resistance in SCLC cells (Guo *et al.*, 2010)	ABCC1
miR-135	↑↓	modulates PTX resistance in lung cancer cells (Holleman *et al.*, 2011)	APC
	↑↓	modulates CDDP resistance in lung cancer cells (Zhou *et al.*, 2013)	MCL1
miR-138	↑	Sensitizes leukemia cells to 5-FU, CDDP, DOX and VCR (Zhao *et al.*, 2010)	ABCB1
miR-140	↑↓	modulates 5-FU resistance and confers resistance to MTX in colon cancer cells (Song *et al.*, 2009a)	HDAC4
miR-141	↑	confers resistance to CDDP in esophageal cancer cells (Imanaka *et al.*, 2011)	YAP1
	↑↓	modulates CDDP resistance in ovarian cancer cells (van Jaarsveld *et al.*, 2012)	KEAP1
miR-143	↑	Sensitizes gastric cancer cells to 5-FU (Borralho *et al.*, 2009)	ERK5
	↑	Sensitizes colon cancer cells to DTX (Xu *et al.*, 2011a)	KRAS
	↑	Sensitizes colon cancer cells to L-OHP (Qian *et al.*, 2013)	IGF-IR

miR-144	↑	Sensitizes CML cells to imatinib (Liu *et al.*, 2012b)	unknown
miR-145	↑	Sensitizes cervical cancer cells to MMC (Shi *et al.*, 2012b)	MUC1, MYC
	↑	Sensitizes glioblastoma cells to CDDP and TMZ (Yang *et al.*, 2012f)	OCT4, SOX2
	↑	Sensitizes lung cancer cells to gefitinib (Zhong *et al.*, 2010)	unknown
miR148a	↑	Sensitizes prostate cancer cells to PTX (Fujita *et al.*, 2010)	MSK1
	↑	Sensitizes esophageal cancer cells to 5-FU and CDDP (Hummel *et al.*, 2011)	unknown
miR-148b	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-152	↑	sensitizes ovarian cancer cells to CDDP (Xiang *et al.*, 2013b)	DNMT1
miR-153	↑	Sensitizes CML cells to ATO (Liu *et al.*, 2012a)	unknown
	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-155	↓	Inhibition sensitizes B-cell lymphoma cells to rituximab (Kim *et al.*, 2012a)	unknown
	↑↓	modulates CDDP resistance in colon cancer cells (Pu *et al.*, 2012)	unknown
	↓	inhibition sensitized lung cancer cells to CDDP (Zang *et al.*, 2012)	unknown
	↑↓	modulates DOX, PTX and VP-16 resistance in breast cancer cells (Kong *et al.*, 2010)	FOXO3A
miR-181a	↑	Sensitizes AML cells to Ara-C (Bai *et al.*, 2012)	BCL2
	↑↓	modulates DNR resistance in CML cells (Li *et al.*, 2012a)	BCL2
	↓	inhibition sensitizes B-cell lymphomas to MX and SAHA (Lwin *et al.*, 2010)	BIM
	↑	sensitizes lung cancer cells to CDDP, L-OHP and carboplatin (Galluzzi *et al.*, 2010)	unknown
miR-181b	↑	Sensitizes gastric and lung cancer cells to 5-FU, CDDP, DOX, VCR and VP-16 (Zhu *et al.*, 2010)	BCL2
miR-182	↑↓	modulates DEX resistance in acute T-cell leukemia cells (Yang *et al.*, 2012a)	unknown
miR-185	↑	sensitizes ovarian cancer cells to CDDP (Xiang *et al.*, 2013b)	DNMT1
miR-186*	↑↓	modulates curcumin resistance in lung cancer cells (Zhang *et al.*, 2010b)	unknown
miR-192	↑	confers resistance to 5-FU in colon cancer cells (Boni *et al.*, 2010)	TYMS
miR-193a	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-193a-3p	↑↓	modulates 5-FU resistance in HCC cells (Ma *et al.*, 2012)	E2F1, SRSF2
miR-193b*	↑	confers resistance to carboplatin in ovarian cancer cells (Ziliak *et al.*, 2012)	unknown

miR-195	↑↓	modulates DOX resistance in colon cancer cells (Qu *et al.*, 2013)	BCL-w
	↓	inhibition sensitizes glioblastoma cells to TMZ (Ujifuku *et al.*, 2010)	unknown
	↑↓	modulates 5-FU resistance in HCC cells (Yang *et al.*, 2012e)	BCL-w
miR-199a	↑↓	sensitizes ovarian cancer cells to CDDP, DOX and PTX (Cheng *et al.*, 2012)	$CD44^+$
	↓	inhibition sensitizes cervical cancer cells to CDDP (Lee *et al.*, 2008)	unknown
miR-199a-3p	↑	sensitizes HCC cells to DOX (Fornari *et al.*, 2010)	mTOR
miR-199a-5p	↑	sensitizes HCC cells to CDDP (Xu *et al.*, 2012e)	ATG7
miR-200b	↑	sensitizes pancreatic cancer cells to gemcitabine (Li *et al.*, 2009c)	unknown
	↑↓	modulates CDDP resistance in tongue cancer cells (Sun *et al.*, 2012)	BMI1
	↑	sensitizes lung cancer cells to DTX (Feng *et al.*, 2012b)	E2F3
	↑↓	modulates CDDP, DOX, VCR and VP-16 resistance in gastric and lung cancer cells (Zhu *et al.*, 2012a)	BCL2, XIAP
	↑↓	modulates gemcitabine resistance in cholangiocarcinoma cells (Meng *et al.*, 2006)	PTPN12
	↑	sensitizes breast cancer cells DOX (Tryndyak *et al.*, 2010)	unknown
miR-200c	↑	sensitizes gastric cancer cells to 5-FU, CDDP, DOX and PTX (Chen *et al.*, 2010c)	unknown
	↑	sensitizes lung cancer cells to CDDP and cetuximab (Ceppi *et al.*, 2010)	unknown
	↑	sensitizes breast cancer cells to epirubicin (Chen *et al.*, 2012b)	ABCB1
	↑	sensitizes ovarian cancer cells to PTX (Cittelly *et al.*, 2012)	unknown
	↓	inhibition sensitizes esophageal cancer cells to CDDP (Hamano *et al.*, 2011)	unknown
	↑↓	modulates DOX resistance in breast cancer cells (Kopp *et al.*, 2012)	unknown
	↑	sensitizes melanoma cells to CDDP, PLX4720 and U0126 (Liu *et al.*, 2012d)	unknown
	↑↓	modulates CDDP, DOX, VCR and VP-16 resistance in gastric and lung cancer cells (Zhu *et al.*, 2012a)	BCL2, XIAP
	↑	sensitizes breast cancer cells to DOX (Tryndyak *et al.*, 2010)	unknown
	↑	sensitizes bladder cancer cells to erlotinib and gefitinib (Adam *et al.*, 2009)	ERRFI-1
miR-203	↑	sensitizes p53-mutated colon cancer cells to PTX (Li *et al.*, 2011a)	AKT2
	↓	inhibition sensitizes breast cancer cells to CDDP (Ru *et al.*, 2011)	SOCS3
	↑	sensitizes lung cancer cells to gefitinib (Garofalo *et*	SRC

		al., 2012b)	
miR-205	↑↓	modulates DTX resistance in prostate cancer cells and sensitizes prostate cancer cells to CDDP (Bhatnagar *et al.*, 2010)	BCL-w
	↑	sensitizes breast cancer cells to gefitinib and lapatinib (Iorio *et al.*, 2009)	HER3
miR-212	↑↓	modulates cetuximab resistance in HNSCC cells (Hatakeyama *et al.*, 2010)	HB-EGF
	↑	sensitizes lung cancer cells to TRAIL (Incoronato *et al.*, 2010)	PED/PEA-15
miR-214	↓	inhibition sensitizes tongue cancer cells to CDDP (Yu *et al.*, 2010)	unknown
	↑↓	modulates CDDP resistance in ovarian cancer cells (Yang *et al.*, 2008a)	PTEN
	↓	inhibition confers resistance to gefitinib (Wang *et al.*, 2012e)	PTEN
	↑	sensitizes cervical cancer cells to CDDP (Wang *et al.*, 2013b)	BCL-w
miR-215	↑↓	modulates MTX resistance in colon cancer cells and confers resistance to TDX in osteosarcoma and colon car cells (Song *et al.*, 2010)	DHFR, TYMS
miR-216a	↑	confers resistance to sorafenib in HCC cells (Xia *et al.*, 2013b)	PTEN, SMAD7
miR-217	↑	confers resistance to sorafenib in HCC cells (Xia *et al.*, 2013b)	PTEN, SMAD7
miR-221	↑↓	modulates TRAIL resistance in NSCLC cells (Garofalo *et al.*, 2008; Garofalo *et al.*, 2009)	CDKN1B/p27^{Kip1}, PTEN, TIMP3
	↑	confers resistance to TAM in breast cancer cells (Miller *et al.*, 2008)	CDKN1B/p27^{Kip1}
	↑	confers resistance to fulvestrant in breast cancer cells (Rao *et al.*, 2011)	CDKN1B/p27^{Kip1}, ERα
	↑↓	modulates gefitinib resistance in lung cancer cells (Garofalo *et al.*, 2012b)	APAF1
	↑↓	modulates TAM resistance in breast cancer cells (Zhao *et al.*, 2008)	ERα
miR-222	↑↓	modulates TRAIL resistance in NSCLC cells (Garofalo *et al.*, 2008; Garofalo *et al.*, 2009)	CDKN1B/p27^{Kip1}, PTEN, TIMP3
	↑	confers resistance to TAM in breast cancer cells (Miller *et al.*, 2008)	CDKN1B/p27^{Kip1}
	↑	sensitizes colon cancer cells to L-OHP and VCR (Xu *et al.*, 2012c)	ADAM17
	↑	confers resistance to fulvestrant in breast cancer cells (Rao *et al.*, 2011)	CDKN1B/p27^{Kip1}, ERα
	↑↓	modulates TAM resistance in breast cancer cells (Zhao *et al.*, 2008)	ERα
	↑↓	modulates gefitinib resistance in lung cancer cells (Garofalo *et al.*, 2012b)	APAF1
	↓	inhibition sensitizes CLL cells to fludarabine	unknown

		(Ferracin *et al.*, 2010)	
miR-224	↓	confers resistance to MTX in colon cancer cells (Mencia *et al.*, 2011)	CDS2, SLC4A4, HSPC159
miR-296	↓	inhibition sensitizes esophageal cancer cells to 5-FU, CDDP, DOX and VCR (Hong *et al.*, 2010)	ABCB1
miR-296-3p	↑↓	modulates imatinib, TMZ and VP-16 resistance in glioblastoma cells (Bai *et al.*, 2013)	EAG1
miR-297	↑↓	modulates DOX, L-OHP and VCR resistance in colon cancer cells (Xu *et al.*, 2012b)	ABCC2
miR-298	↑↓	modulates DOX resistance in breast cancer cells (Bao *et al.*, 2012)	ABCB1
miR-301	↓	inhibition sensitizes breast cancer cells to TAM (Shi *et al.*, 2011)	BBC3, COL2A1, FOXF2, PTEN
miR-302	↓	inhibition sensitizes HNSCC cells to CDDP (Bourguignon *et al.*, 2012)	unknown
miR-320c	↑↓	modulates gemcitabine resistance in pancreatic cancer cells (Iwagami *et al.*, 2013)	SMARCC1
miR-326	↑	sensitizes breast cancer cells to DOX and VP-16 (Liang *et al.*, 2010)	ABCC1
miR-328	↑↓	modulates 5-FU and HCPT resistance in colon cancer cells (Xu *et al.*, 2012f)	ABCG2, MMP16
	↑	sensitizes breast cancer cells to MX (Pan *et al.*, 2009b)	ABCG2
miR-331-5p	↑	sensitizes leukemia cells to DOX (Feng *et al.*, 2011)	ABCB1
miR-337-3p	↑↓	modulates PTX resistance and sensitizes NSCLC cells to DTX (Du *et al.*, 2012)	RAP1A, STAT3
miR-342	↑↓	modulates TAM resistance in breast cancer cells (Cittelly *et al.*, 2010b)	BMP7, GEMIN4
miR-345	↑	sensitizes breast cancer cells to CDDP (Pogribny *et al.*, 2010)	ABCC1
miR-375	↑↓	modulates TAM resistance in breast cancer cells (Ward *et al.*, 2013)	MTDH
	↑	confers resistance to PTX in cervical cancer cells (Shen *et al.*, 2013)	unknown
miR-376	↑↓	modulates CDDP resistance in ovarian cancer cells (Ye *et al.*, 2011)	ALK7
miR-379	↑↓	modulates CDDP, DOX and VP-16 resistance in SCLC cells (Guo *et al.*, 2010)	unknown
miR-421	↑↓	modulates CDDP resistance in nasopharyngeal carcinoma cells (Chen *et al.*, 2013b)	FOXO4
miR-429	↑↓	modulates CDDP, DOX, VCR and VP-16 resistance in gastric and lung cancer cells (Zhu *et al.*, 2012a)	BCL2, XIAP
miR-451	↑↓	Modulates DOX resistance in breast cancer (Kovalchuk *et al.*, 2008)	ABCB1
	↑	Sensitizes CML cells to imatinib (Liu *et al.*, 2012b)	unknown
	↑↓	modulates TAM resistance and sensitizes breast cancer cells to raloxifene and fulvestrant (Bergamaschi and Katzenellenbogen, 2012)	14-3-3ζ

	↓	inhibition sensitizes ovarian cancer cells to vinblastine (Zhu *et al.*, 2008)	unknown
	↑	sensitizes lung cancer cells to CDDP (Bian *et al.*, 2011)	unknown
	↑	sensitizes colon cancer stem cells to irinotecan (Bitarte *et al.*, 2011)	MIF
	↑	Sensitizes glioblastoma cells to imatinib (Gal *et al.*, 2008)	unknown
miR-455-3p	↓	inhibition sensitizes glioblastoma cells to TMZ (Ujifuku *et al.*, 2010)	unknown
miR-494	↑↓	Modulates TRAIL resistance in lung cancer cells (Romano *et al.*, 2012)	BIM
miR-495	↑↓	modulates CDDP, DOX and VP-16 resistance in SCLC cells (Guo *et al.*, 2010)	unknown
miR-497	↑↓	Modulates CDDP, DOX, VCR and VP-16 resistance in gastric and lung cancer cells (Zhu *et al.*, 2012b)	BCL2
miR-504	↑	Confers resistance to VP-16 in osteosarcoma and lung cancer cells (Hu *et al.*, 2010c)	P53
miR-505	↑	Sensitizes breast cancer cells to DOX (Yamamoto *et al.*, 2011)	unknown
miR-512-3p	↑	Sensitizes HCC cells to PTX (Chen *et al.*, 2010a)	c-FLIP
miR-513a-3p	↑	Sensitizes lung cancer cells to CDDP (Zhang *et al.*, 2012b)	GSTP1
miR-519c	↓	Confers resistance to MX in breast and colon cancer cells (To *et al.*, 2009)	ABCG2
miR-520h	↓	Confers resistance to MX in breast and colon cancer cells (To *et al.*, 2009)	ABCG2
miR-558a-5p	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-582-5p	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-591	↑	sensitizes ovarian cancer cells to PTX (Huh *et al.*, 2013)	ZEB1
miR-605	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	unknown
mR-630	↑	confers resistance to CDDP and carboplatin in lung cancer cells (Galluzzi *et al.*, 2010)	unknown
miR-661	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-663	↑↓	Modulates CTX, DTX and DOX resistance in breast cancer cells (Hu *et al.*, 2013)	HSPG2
miR-744*	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	unknown
miR-876-3p	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-886-3p	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1
miR-892b	↑	sensitizes colon cancer cells to ABT-263 (Lam *et al.*, 2010)	MCL1

miR-1915	↑↓	Modulates DOX, L-OHP, MMC and VCR resistance in colon cancer cells (Xu *et al.*, 2013)	BCL2
let-7	↑	Sensitizes pancreatic cancer cells to gemcitabine (Bhutia *et al.*, 2013)	RRM2
let-7a	↑	Sensitizes NSCLC cells to gefitinib (Zhong *et al.*, 2010)	unknown
	↑	Sensitizes breast cancer cells to PTX (Lv *et al.*, 2012)	unknown
	↑↓	Modulates DOX, interferon-γ and PTX resistance in HCC cells (Tsang and Kwok, 2008)	CASP3
let-7b	↑	Sensitizes breast cancer cells to TAM (Zhao *et al.*, 2011b)	ER-α36
let-7c	↑	Sensitizes HCC cells to sorafenib (Shimizu *et al.*, 2010)	BCL-xL
let-7d	↑↓	Inhibition confers resistance to 5-FU, modulates CDDP resistance and sensitizes OSCC cells to PTX (Chang *et al.*, 2011)	unknown
let-7g	↑	Sensitizes ovarian cancer cells to PTX and vinblastine (Boyerinas *et al.*, 2012)	IMP-1
let-7i	↑↓	Modulates CDDP resistance in ovarian cancer cells (Yang *et al.*, 2008b)	unknown
	↑	Sensitizes ovarian cancer cells to PTX (Liu *et al.*, 2012c)	unknown
let-7i	↑	Sensitizes breast cancer cells to TAM (Zhao *et al.*, 2011b)	ER-α36

As can be seen by Table 2, a great number of miRNAs have been shown to modulate drug response. Some of them have been described more frequently than others, granting them more recognition in drug resistance and thus will be described here in more detail.

3.1 miR-21

miR-21 is overexpressed in almost all types of tumors (Volinia *et al.*, 2006) and it is generally accepted as being an "oncomiR". Its expression is often associated with drug resistance, firstly described when downregulation of miR-21 sensitized cholangiocarcinoma cell lines to gemcitabine (Meng *et al.*, 2006). miR-21 has been shown to regulate the translation of several mRNAs such as PTEN, Bcl-2 or PDCD4 (programmed cell death 4), all of which play important roles in cellular mechanisms such as cellular growth, apoptosis, invasion and cell cycle. The miR-21 involvement in drug resistance has been demonstrated in several cancer cell types such as breast (Wang *et al.*, 2011c), colon (Valeri *et al.*, 2010), gastric (Xiang *et al.*, 2013a), prostate (Li *et al.*, 2009a), among others, and it has also been shown to modulate resistance to several drugs, from the conventional chemotherapies such as 5-fluorouracil (Tomimaru *et al.*, 2010), cisplatin (Chen *et al.*, 2012d) or doxorubicin (Tao *et al.*, 2011), to targeted drugs such as AG1478 (Seike *et al.*,

2009), gefitinib (Garofalo *et al.*, 2012b) or trastuzumab (Gong *et al.*, 2011). Although miR-21 expression is most often associated with cancer drug resistance, its expression can modulate the sensitivity of cytotoxic drugs in opposite directions depending on the compound class, indicating that different mechanisms determine toxic and protective effects (Blower *et al.*, 2008). In fact, miR-21 ectopic expression has been associated with chemosensitivity to cisplatin in tongue squamous cell carcinoma cell lines (Yu *et al.*, 2010). Another study has shown a non-canonical mechanism of mRNA regulation by miR-21. Indeed, it has been demonstrated that miR-21 binds the 3'UTR region of Bcl-2 mRNA causing an increase in the expression of Bcl-2 protein and consequent gemcitabine resistance in pancreatic cancer cells (Dong *et al.*, 2011). miR-21 has also been shown to be involved in resistance to doxorubicin and etoposide by induction of autophagy and inhibition of miR-21 expression increased the sensitivity of leukemia cells to the above mentioned drugs (Seca *et al.*, 2013).

3.2 miR-34a

The family of miR-34, which includes miR-34a, miR-34b and miR-34c, is transcriptionally activated by p53 (Gaur *et al.*, 2007b). This prompts the evaluation of miR-34 expression in cancer cells since p53 is a pivotal tumor-suppressor protein often deregulated in cancer. In fact, miR-34a has been shown to be downregulated in many p53 deficient cell lines and this downregulation was associated with drug resistance (Fujita *et al.*, 2008; Ji *et al.*, 2008; Kojima *et al.*, 2010). Furthermore, ectopic expression of miR-34a was shown to sensitize prostate cancer cell to camptothecin (Fujita *et al.*, 2008), bladder cancer cells to cisplatin (Vinall *et al.*, 2012) or breast cancer cells to doxorubicin (Li *et al.*, 2012b). miR-34a has been shown to target several proteins known to be regulated by p53 (Wong *et al.*, 2011). SIRT1 (a protein deacetylase which is involved in apoptosis), Bcl-2 (an anti-apoptotic protein), cyclin D1 and Cdk6 (involved in cell cycle control) have all been shown to be direct or indirect targets of miR-34a (Fujita *et al.*, 2008; Ji *et al.*, 2008; Vinall *et al.*, 2012). The fact that miR-34a inhibits the expression of these proteins underlies its effect in sensitizing different cancer cell types to various cancer drugs. However, a different study has shown that miR-34a targeted Bcl-2 mRNA, thus decreasing its expression, but that its ectopic expression was associated with increased resistance to docetaxel in breast cancer cells through an unidentified mechanism (Kastl *et al.*, 2012).

Two recent papers have described two other targets for miR-34a. Notch1 signaling may contribute to cancer chemoresistance (Gu *et al.*, 2010) and miR-34a overexpression sensitized breast cancer cells to doxorubicin by targeting Notch1 (Li *et al.*, 2012b). MAGE-A, which is aberrantly expressed in many cancers (Kasuga *et al.*, 2008), was also shown to

be a target of miR-34a; in addition, miR-34a overexpression sensitized medulloblastoma cells to both cisplatin and mitomycin C (Weeraratne *et al.*, 2011).

3.3 miR-221/222

miR-221/222 are encoded in the X chromosome and share the same seed sequence between them (Garofalo *et al.*, 2012a). They are commonly regarded as "oncomiRs" as they target several tumor suppressor proteins such as Apaf-1, $p27^{Kip1}$, PTEN or TIMP3 (Garofalo *et al.*, 2012a). In fact, it has been shown that by targeting these mRNAs, miR-221/222 could increase resistance to TRAIL and gefitinib in non-small cell lung cancer (NSCLC) cells (Garofalo *et al.*, 2008; Garofalo *et al.*, 2009; Garofalo *et al.*, 2012b). miR-221/222 has also been shown to target and downregulate ERα expression, thus being partially responsible for resistance to tamoxifen (Zhao *et al.*, 2008). Therefore, downregulation of miR-221/222 sensitized breast cancer cells to tamoxifen-induced cell growth arrest and apoptosis (Zhao *et al.*, 2008). Another study has shown that $p27^{Kip1}$ is also decreased by miR-221/222 in tamoxifen resistant cells (Miller *et al.*, 2008). Other studies showed that ectopic expression of miR-221/222 conferred resistance to fulvestrant in breast cancer cells through the deregulation of multiple oncogenic signaling pathways that involved β-catenin activation and repression of TGF-β (Rao *et al.*, 2011). Interestingly, miR-222 ectopic expression has been also shown to sensitize HCT-8 and HCT116 resistant gastric cancer cells to oxaliplatin. miR-222 overexpression decreased ADAM-17 expression, thus decreasing EGFR activation and TGF-α shedding, which in turn reduces P-gp and MRP1 efflux pump expression (Xu *et al.*, 2012c).

3.4 miR-451

miR-451 is involved in erythropoiesis, being essential for maintenance and/or late-stage maturation of committed erythroid precursors (Dore *et al.*, 2008). Its association with cancer drug resistance was first described in breast cancer cells resistant to doxorubicin which presented a clear upregulation of P-gp protein, a known drug efflux transporter. Ectopic expression of miR-451 targeted MDR1 mRNA and thus sensitized these cells to doxorubicin (Kovalchuk *et al.*, 2008). miR-451 has been also shown to sensitize both chronic myeloid leukemia (CML) and glioblastoma cells to imatinib, although without an identified target (Gal *et al.*, 2008; Liu *et al.*, 2012b). Moreover, miR-451 has been shown to be involved in the inhibition of colon cancer stem cells (CSCs) (Bitarte *et al.*, 2011). By binding the migration inhibitory factor (MIF) mRNA and hampering its translation, as well as indirectly controlling COX-2 expression, miR-451 regulates the Wnt signaling activity [which defines colon CSCs (Vermeulen *et al.*, 2010)] sensitizing colon CSCs to irinotecan (Bitarte *et al.*, 2011). Another study described 14-3-3ζ [factor that binds and

stabilizes key proteins involved in signal transduction and cell cycle regulation (Zannis-Hadjopoulos *et al.*, 2008)] as a direct target of miR-451 that is involved in tamoxifen resistance in breast cancer cells (Bergamaschi and Katzenellenbogen, 2012). Additionally, it has been shown that miR-451 was downregulated in NSCLC tissue, compared to non-cancerous adjacent tissue and that ectopic expression of miR-451 sensitized lung cancer cells to cisplatin (Bian *et al.*, 2011).

4. Regulation of drug response pathways by microRNAs

4.1 Regulation of apoptosis

miRNAs have been shown to target one or simultaneously various mRNAs involved in the core apoptotic signaling, acting both as anti-apoptotic and pro-apoptotic molecules [reviewed in (Lima *et al.*, 2011)]. Moreover, various miRNAs which target apoptosis related proteins, particularly of the intrinsic apoptotic pathway, have already been described to be associated with drug response. The anti-apoptotic Bcl-2 is targeted by several miRNAs and this has been shown to modulate the response of different cancer cellular models to various drugs. For example, miR-15a, -15b, -16, -34a, -129, -181b, -200bc/429cluster, -497 and -1915, all of which downregulate Bcl-2 expression, have been related to the decrease of resistance to tamoxifen in breast cancer cells (Cittelly *et al.*, 2010a) or to other drugs such cisplatin, doxorubicin, etoposide, docetaxel, vincristine, gemcitabine and 5-fluorouracil in colon, gastric, breast or lung cancer cells (Ji *et al.*, 2008; Xia *et al.*, 2008; Zhu *et al.*, 2010; Kastl *et al.*, 2012; Zhu *et al.*, 2012a; Zhu *et al.*, 2012b; Karaayvaz *et al.*, 2013) (Xu *et al.*, 2013). In addition, miR-181a downregulation and consequent upregulation of Bcl-2 was shown to confer resistance to cytarabine in acute myeloid leukemia cells (Bai *et al.*, 2012) while its over-expression sensitized CML cells to daunorubicin by targeting Bcl-2 (Li *et al.*, 2012a). Moreover, miR-21 which has been shown to directly bind to the 3'UTR of Bcl-2 mRNA, increasing its expression, caused gemcitabine resistance in pancreatic cancer cells (Dong *et al.*, 2011).

Another anti-apoptotic Bcl-2 family member is Mcl-1, which has been found to be directly targeted by miR-101, -148b, -153, -193a, -558a-5p, -582-5p, -661, -876-3p, -886-3p and -892b in a study aiming at identifying miRNAs modulators of colon cancer cells sensitivity to ABT-263 (Lam *et al.*, 2010). Another study has also shown the ability of miR-101, found down-regulated in hepatocellular carcinoma (HCC), to sensitize these tumor cells to chemotherapeutic drug–induced apoptosis (Su *et al.*, 2009). Moreover, and at least in part through the modulation of apoptosis via targeting Mcl-1, miR-135 a/b have been suggested to play a role in the development of cisplatin resistance in lung cancer cells (Zhou *et al.*, 2013).

In addition, the downregulation of Bcl-w (antiapoptotic Bcl-2 family protein) by miR-195, -205 and -214 has been described to modulate resistance to cisplatin, doxorubicin, docetaxel or 5-fluorouracil in cervical, colon, hepatocellular or prostate cancer cells (Bhatnagar *et al.*, 2010; Yang *et al.*, 2012e; Qu *et al.*, 2013; Wang *et al.*, 2013b). Moreover, inhibition of Bcl-xL (anti-apoptotic Bcl-2 family member) by let7c has been found to sensitize HCC cells to apoptosis induced by sorafenib (Shimizu *et al.*, 2010).

On the other hand, the pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) is targeted by miR-125b. This has been shown to increase resistance in breast cancer cells to paclitaxel and gemcitabine (Zhou *et al.*, 2010; Liu *et al.*, 2013c), of ovarian cancer cells to cisplatin (Kong *et al.*, 2011) and of leukemia cells to doxorubicin (Zhang *et al.*, 2011a).

Likewise, the regulation of the BH3-only protein, Bim, by miRNAs also plays a role in drug response. Indeed, the blockage of Bim expression by miR-17 in lung cancer cells caused resistance to AZD6244 (a small molecule inhibitor of the MEK pathway) (Dai *et al.*, 2011). Moreover, miR-17~92 cluster, which targets Bim and PTEN, has been shown to confer resistance to doxorubicin and to etoposide in mantle cell lymphoma (Rao *et al.*, 2012). In addition, miR-30b and -30c were shown to modulate the resistance of lung cancer cells to gefitinib (Garofalo *et al.*, 2012b) while miR-494 induced TRAIL resistance in NSCLC, all through a downregulation of Bim (Romano *et al.*, 2012). Moreover, miR-10b expression (which also downregulates Bim) was described as a potential indicator of chemosensitivity to 5-fluorouracil-based chemotherapy regimens in colorectal cancer (Nishida *et al.*, 2012).

Other apoptotic proteins are known to be regulated by miRNAs, altering drug response of cancer cells. For example, by targeting caspase-3, let-7a suppressed HCC death induced by doxorubicin, IFN-γ and paclitaxel (Tsang and Kwok, 2008). Also, the expression of miR-106a, which targets caspase-7 (and also BCL10) has been shown to be associated with paclitaxel resistance in ovarian cancer cells (Huh *et al.*, 2013). Furthermore, by downregulating the apoptotic peptidase activating factor-1 (APAF-1), miR-221 and -222 increased lung cancer cells resistance to gefitinib (Garofalo *et al.*, 2012b).

Finally, the most potent member of the inhibitor of apoptosis family, XIAP, is targeted by miR-200bc/429 cluster (all of which, interestingly, also target Bcl-2) and this seems to be associated with increased sensitivity of gastric and lung cancer cells to cisplatin, doxorubicin, vincristine and etoposide (Zhu *et al.*, 2012a).

4.2 Regulation of p53 levels

The p53 pathway is one of the major players in the regulation of cellular response to drugs. P53, considered the guardian of the genome, may initiate DNA repair, cell-cycle arrest, senescence and, importantly, apoptosis [reviewed in (Vazquez *et al.*, 2008)]. Several miRs have been described to alter p53 expression levels.

In particular, miR-25, -30d and -504 have been shown to directly target p53. Their overexpression reduces p53 protein levels impairing p53 functions, such as p53-mediated transcriptional activation, apoptosis, and cell-cycle arrest as well as conferring resistance to etoposide induced cell death (Hu *et al.*, 2010c; Kumar *et al.*, 2011). In addition miR-375 has also been shown to target and downregulate p53 expression, affecting the response to ionizing radiation and etoposide treatment. In fact, the expression of miR-375 desensitized AGS cells to ionizing radiation and etoposide, abrogating the cell cycle arrest and apoptosis after DNA damage (Liu *et al.*, 2013).

There are other miRNAs such as miR-122 and miR-630 that have been shown to affect p53 stability and activity, leading to alterations in drug sensitivity (Fornari *et al.*, 2009; Galluzzi *et al.*, 2010). Besides the miRNAs that directly or indirectly regulate p53 expression, there are also miRNAs that are regulated by p53 and are involved in drug response, such as miR-34 (Bommer *et al.*, 2007) and miR-519 (Fornari *et al.*, 2012).

4.3 Regulation of autophagy

The role of autophagy in drug response is complex and not fully understood but several studies have shown that autophagy is involved in chemoresistance.

miR-30a is one of the miRNA which is frequently found associated with autophagy. It targets Beclin-1, resulting in decreased autophagic activity. Through the inhibition of Beclin-1 expression, miR-30a regulated autophagy induced by rapamycin in breast and lung cancer cells (Zhu *et al.*, 2009). In addition, in a CML cell line and in CML stem/progenitor cells, overexpression of miR-30a decreased Beclin-1 and ATG5 expression (and subsequently autophagy), enhancing imatinib-induced apoptosis (Yu *et al.*, 2012a; Yu *et al.*, 2012b). Moreover, in a study using cell lines and animal models, cisplatin or taxol treatment were found to increase autophagy but to decrease the levels of miR-30a. Importantly, the inhibition of Beclin-1-mediated tumor cell autophagy which was possible following the increase in miR-30a levels, significantly promoted cisplatin-induced tumor cell apoptosis (Zou *et al.*, 2012b).

miR-101 was identified as potent inhibitor of autophagic flux in MCF-7 breast cancer cells. This miRNA is described to directly target *STMN1, RAB5A* and *ATG4D*, blocking autophagy, and to increase the cytotoxicity of 4-hydroxytamoxifen in breast cancer cells (Frankel *et al.*, 2011).

miR-199a-5p was found to be significantly reduced in HCC patients treated with cisplatin-based chemotherapy and in human HCC cell lines following cisplatin treatment. This cisplatin-induced downregulation of miR-199a-5p resulted in drug resistance. In addition, the decrease in miR-199a-5p has resulted in enhanced autophagy activation by targeting ATG7 (Xu *et al.*, 2012e).

Finally, through microRNA profiling, miR-375 was found to be upregulated in DOX-induced senescent K562 (CML) cells. This upregulated expression was found to be associated with decreased expression of 14-3-3ζ and SP1 genes and with initiation of autophagy (Yang *et al.*, 2012d).

4.4 Regulation of DNA damage response

The mechanisms involved in DNA damage response (DDR) are mediated through several proteins including those acting as DNA damage sensors (such as H2AX, BRCA1), as signal transducers [such as ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related)] and as effectors [including DNA repair, cell-cycle checkpoint (G1/S, intra-S and G2/M) and apoptosis mechanisms] [reviewed in (Green and Lin, 2012)]. Several reports have already described miRNAs which regulate DDR and DNA repair (Lal *et al.*, 2009; Pothof *et al.*, 2009; Bhatnagar *et al.*, 2010; Hu *et al.*, 2010a).

miR-24 was found to downregulate H2AX, suppressing DNA repair in terminally differentiated blood cells. miR-24 upregulation, in post-replicative cells, reduces H2AX and renders cells vulnerable to DNA damage (Lal *et al.*, 2009). Another study has shown that miR-24 overexpression was associated with decreased expression of H2AX and decreased DDR upon etoposide treatment in highly differentiated $CD8^+$ T cells (Brunner *et al.*, 2012). Overexpressing miR-24 in breast cancer cells increased sensitive to cisplatin through direct targeting H2AX (Srivastava *et al.*, 2011). In addition, in an osteosarcoma cell line, miR-138 was also found to enhance cellular sensitivity to cisplatin and camptothecin by directly targeting H2AX gene (Wang *et al.*, 2011b). In another study with cisplatin-resistant human NSCLC cells (A549/DDP), it was shown that the upregulation of miR-138 increased the sensitivity of these cells to cisplatin. In that same study, the excision repair cross-complementation group 1 (ERCC1) was also found to be negatively regulated by miR-138, with its levels being inversely correlated with the levels of miR-138 in A549/DDP cells (Tao *et al.*, 2011).

miR-182 has been described to target BRCA1. In breast cancer cells, miR-182 was shown to mediate BRCA1 downregulation, affecting DNA repair (Moskwa *et al.*, 2011).

ATM was shown to be directly targeted by miR-18a, with their expression being inversely correlated in rectal tumor tissues. The repair of DNA damage induced by etoposide was inhibited by miR-18a, leading to increased apoptosis and accumulation of DNA damage (Wu *et al.*, 2013a). miR-181a/b was also shown to directly target ATM and to sensitize breast cancer cells to PARP inhibitors (Bisso *et al.*, 2013).

The mismatch repair (MMR) system is involved in DNA damage recognition and repair. miR-21 was shown to target the core MMR complex, human mutS homolog 2 (hMSH2) and 6 (hMSH6). Overexpression of this miRNA, in colorectal cancer cells led to a

significantly reduced sensitivity to 5-fluorouracil (Valeri *et al.*, 2010). DNA repair protein RAD51 and the trans-lesion synthesis DNA polymerase REV1 are transcriptionally regulated by miR-96, which directly targets the 3'UTR of REV1 and the coding region of RAD51. Overexpression of miR-96 increases sensitivity to PARP inhibitor AZD2281 *in vitro* and to cisplatin both *in vitro* and *in vivo* (Wang *et al.*, 2012d). Matrix extracellular phosphoglycoprotein (MEPE) is a cofactor of CHK1 that protects cells from DNA damage-induced killing (Zhang *et al.*, 2010c). miR-376a was shown to target MEPE, reducing G2 arrest and sensitizing cervical and liver cancer cells to camptothecin and etoposide (Sheng *et al.*, 2013).

It has been shown that c-Myc overexpression causes DNA damage (Dominguez-Sola *et al.*, 2007). miR-34c has been shown to target and repress c-Myc upon etoposide induced DNA damage, inhibiting DNA synthesis as well as blocking cells in S-phase, thus preventing replication of damaged DNA (Cannell *et al.*, 2010).

4.5. Regulation of cancer stem-like cells and epithelial to mesenchymal transition

CSCs are thought to have a major role in tumor proliferation, invasion or metastasis. Epithelial to mesenchymal transition (EMT) is a process that encompasses a transition from a cobblestone phenotype to a fibroblastic phenotype, together with acquisition of increased cell motility and invasion [reviewed in (Thiery *et al.*, 2009)]. These cellular alterations are accompanied by molecular adjustments including the decreased expression of E-cadherin and β-catenin and increased expression of vimentin, fibronectin or smooth-muscle actin [reviewed in (Thiery and Sleeman, 2006)]. Current data suggests that the attainment of the EMT phenotype and the appearance of CSCs share biological alterations and cooperate in the development of cancer metastasis, recurrence, and chemoresistance (Mani *et al.*, 2008; Hollier *et al.*, 2009).

Analysis of miRNAs that regulate stem cell function and their association with the response to chemotherapy in esophageal cancers pointed to miR-200c, a miRNA known to regulate stem cell function, as being involved in chemoresistance to cisplatin through directly interaction with PPP2R1B, a protein that inhibits tumor invasiveness and is involved in Akt pathway (Hamano *et al.*, 2011). In addition, miR-145 was shown to directly target the 3'UTR of Oct4 and Sox2, two know stem cell markers. Overexpression of miR-145 in glioblastoma (GBM) CSCs reduced the expression of these factors as well as of other stemness genes, including Nanog, c-Myc, and of the oncogene Bmi-1. It also reduced MDR1 and ABCG2 proteins, sensitizing these cells to both cisplatin and temozolomide. All these results demonstrate that miR-145 is a key hub in the stemness network of GBM CSCs (Yang *et al.*, 2012f).

miR-125a/b has been shown to directly target and decrease both Mcl-1 and ALDH1A3, a cancer stem cell marker (Douville *et al.*, 2009). ALDH1-positive cells from the HT29 colon cancer cell line were resistant to paclitaxel and this resistance could be overcome by overexpression of miR-125a/b (Chen *et al.*, 2013a). Intriguingly, miR-125b overexpression was shown to increase CSC population and to confer resistance to taxol and gemcitabine in human breast epithelial cells by targeting Bak1 (Liu *et al.*, 2013c). In another study, docetaxel-resistant pancreatic cells presented various stem cell markers as well as EMT phenotype. Transfection of either miR-200c or miR-205 could revert the phenotype and sensitize these cells through directly targeting ZEB1 and ZEB2 (zinc finger E-box–binding homeobox 1 and 2) (Puhr *et al.*, 2012). ZEB1 was also shown to be directly targeted by miR-591. Moreover, overexpression of miR-591 decreased cell proliferation and migration and sensitized ovarian cancer resistant cells to paclitaxel (Huh *et al.*, 2013). Overexpression of miR-216a/217 was shown to directly target PTEN and SMAD7 and thus activate both TGF-β and PI3K/Akt pathways. This overexpression induced EMT and increased the stem-like cell population conferring resistance to sorafenib in HCC cells (Xia *et al.*, 2013a).

E-cadherin, known to be involved in EMT, is directly targeted by miR-23a. In NSCLC cell lines, overexpression of miR-23a led to increased resistance to gefitinib and increased EMT phenotype (Cao *et al.*, 2012). Bmi1 is another protein reported to be involved in EMT and stem cell self-renewal (Song *et al.*, 2009b; Yang *et al.*, 2010). miR-15b and -200b have been shown to directly target Bmi1, revert EMT phenotype, suppress motility and migration and sensitize tongue squamous cell carcinoma resistant cells to cisplatin (Sun *et al.*, 2012). Other studies have shown the involvement of miR-200 family in the regulation of EMT and drug resistance. In bladder cancer cells, overexpression of miR-200b and -200c inhibited cell migration and sensitized cells to cetuximab by directly binding ZEB1, ZEB2 and ERRFI-1 (Adam *et al.*, 2009). In another study, re-expression of miR-200b in gemcitabine resistant pancreatic cancer cells reverted the EMT phenotype and sensitized cells to gemcitabine (Li *et al.*, 2009c). Additionally, miR-200c was shown to directly target other mesenchymal genes such as class III β-tubulin (TUBB3). Ectopic expression of miR-200c sensitized endometrial Hec50 cancer cells to paclitaxel and vincristine but not to doxorubicin or cisplatin, indicating that targeting TUBB3 is a major mechanism through which miR-200c restores sensitivity to microtubule-binding chemotherapeutic agents (Cochrane *et al.*, 2009).

In tamoxifen resistant cells, re-expression of miR-375 sensitizes cells to tamoxifen, inhibiting invasion and reverting EMT-like properties. miR-375 was shown to directly target metadherin (MTDH), increasing E-cadherin and ZO-1 expression and decreasing

the mesenchymal markers ZEB1 and SNAI2, indicating that the involvement of miR-375 in EMT could, at least in part, be via targeting MTDH (Ward *et al.*, 2013).

4.6 Regulation of drug efflux

By regulating genes involved in drug efflux, several miRNAs have been shown to play a role in drug resistance. In particular, several of these miRNAs are directly or indirectly related with MDR1/P-gp expression, one of the main contributors to the MDR phenotype [reviewed in (Lopes-Rodrigues *et al.*, 2013)].

Various miRNAs have been described to directly target the 3'-UTR of the MDR1 mRNA. An inverse correlation between the expression of such miRNAs and MDR1 mRNA and P-gp is observed. Indeed, overexpression of miR-451, -331-5p or -27a, which target MDR1/P-gp, has increased sensitivity of breast cancer and leukemia cells to doxorubicin (Kovalchuk *et al.*, 2008; Feng *et al.*, 2011). On the other hand, the down-regulation of miR-298 in breast cancer cells increased P-gp expression and resistance to doxorubicin. Importantly, miR-298 expression has been proposed as potential predictor of doxorubicin chemoresistance in human breast cancer (Bao *et al.*, 2012).

Other miRNAs indirectly regulate MDR1/P-gp expression, by targeting mRNAs involved in MDR1 gene activation. The expression of such miRNAs has also been associated with drug response. Indeed, increased miR-21 caused a decrease in the levels of the tumor suppressor protein PDCD4, which was found to be associated with increased translation of several transcripts, including MDR1. miR-21 downregulation enhanced multidrug sensitivity in MCF-7 cells (Bourguignon *et al.*, 2009). Moreover, the downregulation of miR-130a in cisplatin resistant SKOV3 ovarian cancer cells caused an indirect inhibition of MDR1 mRNA and of P-gp expression and reverted resistance to cisplatin (Yang *et al.*, 2012c). In addition, let-7 affected acquired resistance of ovarian cancer cells to taxanes by targeting IMP-1, resulting in MDR1 mRNA destabilization (Boyerinas *et al.*, 2012).

Interestingly, in ovarian and cervical cancer cells, miR-27a and -451 (previously referred as direct MDR1/P-gp regulators) also seem to be activators of P-gp expression (Zhu *et al.*, 2008). Indeed, antagomirs of miR-27a or -451 decreased MDR1 mRNA and P-gp expression, increasing sensitivity to drugs which are P-gp substrates (Zhu *et al.*, 2008).

In addition to the above referred miRNAs, regulating P-gp expression at a post-transcriptional level, some miRNAs have been proposed to regulate MDR1 expression, at a transcriptional level, by interfering with its promoter region, and others through unknown mechanisms [reviewed in (Toscano-Garibay and Aquino-Jarquin, 2012) and (Lopes-Rodrigues *et al.*, 2013)]. miR-138 upregulation in promyelocytic leukemia cells reverted the MDR phenotype by downregulating P-gp (Zhao *et al.*, 2010). In addition, downregulation of miR-27a decreased P-gp expression, possibly reverting drug resistance

in esophageal squamous cells and gastric cancer cells (Zhang *et al.*, 2010a; Dong *et al.*, 2011). miR-200c was down-regulated in breast cancer patients which were non-responders to neoadjuvant chemotherapy (compared to responders) and in doxorubicin-resistant human breast cancer cells MCF-7/ADR (compared to parental MCF-7 cells). miR-200c restoration in MCF-7 cells reduced MDR1 mRNA and P-gp and increased sensitivity to epirubicin (Chen *et al.*, 2012b). The overexpression of miR-122, a liver specific miRNA frequently downregulated in HCC, could modulate the sensitivity of HCC cells to drugs through the downregulation of MDR related genes including MDR1 and MRP (Xu *et al.*, 2011b).

In addition to MDR1/P-gp, the regulation of other efflux proteins by miRNAs has also been associated with drug response/resistance. For example, miR-181a, -328, -519c and -520h target BCRP/ABCG2. This has been shown to modulate colon and breast cancer cells sensitivity to mitoxantrone, 5-fluorouracil or hydroxycamptothecin (Pan *et al.*, 2009b; To *et al.*, 2009; Xu *et al.*, 2012f; Jiao *et al.*, 2013).

miR-326, which was downregulated in etoposide resistant MCF-7 cells (MCF-7/VP) and in a panel of advanced breast cancer tissues, negatively correlated with MRP1/ABCC1 levels. miR-326 overexpression in MCF-7/VP cells sensitized cells to etoposide and doxorubicin (Liang *et al.*, 2010).

Finally, miR-297 targets MRP2/ABCC2 and was down-regulated in a panel of human colorectal carcinoma tissues, as well as in the oxaliplatin resistant cell line HCT116/L-OHP (comparing with its parental cells). The ectopic expression of miR-297 in MDR colorectal carcinoma cells sensitized cells to anticancer drugs *in vitro* and *in vivo* (Xu *et al.*, 2012b).

4.7 Regulation of drug metabolism

Drugs are metabolized mainly by enzymes of the cytochrome P450 (CYP) family, which may be post-transcriptionally regulated by miRNAs. CYP members are overexpressed in a wide range of cancers, metabolizing cancer drugs and probably conferring drug resistance (Murray *et al.*, 1997). The first miRNA found to be associated with a CYP member was miR-27b, which was shown to directly target CYP1B1 in breast cancer cells (Tsuchiya *et al.*, 2006). miR-27b has also been shown to directly bind and regulate CYP3A4 expression (Pan *et al.*, 2009a). Other CYP members have since been found to be targets of miRNAs. CYP24 is targeted by miR-125b (Komagata *et al.*, 2009), CYP2E1 by miR-378 (Mohri *et al.*, 2010), CYP1A1 by miR-892a (Choi *et al.*, 2012) and CYP2J2 by let-7b (Chen *et al.*, 2012a). Besides directly targeting CYP proteins, miRNAs have been shown to target transcription factors that regulate the expression of a variety of drug-metabolizing enzymes, such as Pregnane X receptor (PXR). For example, miR-148a directly targets PXR affecting the expression of CYP3A4 (Takagi *et al.*, 2008).

5. Non-genetic acquisition of microRNAs via microvesicles and exosomes

A possible association between the number of exosomes and cancer drug resistance has been described [reviewed in (Camussi *et al.*, 2010; Muralidharan-Chari *et al.*, 2010)]. In fact, chemoresistant cancer cells were shown to release more microvesicles than chemosensitive cancer cells (Safaei *et al.*, 2005). In addition, it is known that miRNAs may be included in exosomes and transfer drug resistance to other (Jaiswal *et al.*, 2012a). A recent study has shown that microvesicles from P-gp overexpressing drug resistant cells can selectively package some miRNAs into microvesicles, which upon release on the recipient drug sensitive cells can confer MDR by "retemplating" these cells, reflecting the donor resistant phenotype (Jaiswal *et al.*, 2012a; Jaiswal *et al.*, 2012b).

Interestingly, the transfer of miR-21-mediated chemoresistance in pancreatic adenocarcinoma cells has very recently been shown to occur via cell-to-cell contact involving the Systemic RNA Interference-defective-1 Transmembrane Family Member 1 (SIDT1) (Elhassan *et al.*, 2012).

6. MicroRNAs polymorphisms associated with drug resistance

miRSNPs or miRs-polymorphisms have been described as a novel class of functional polymorphisms, usually present at (or near) a miRNA binding site of functional genes and in genes involved in miRNA biogenesis. These polymorphisms may affect gene expression by interfering with a miRNA function. Therefore, this can lead to drug resistance/sensitivity depending on the function of the targeted mRNA that presents the polymorphism [reviewed in (Mishra *et al.*, 2008; Zhang and Dolan, 2010)].

In particular, a miR-200b/200c/429-binding site polymorphism in the 3´-UTR region of AP-2alpha gene (a transcription factor which regulates several genes involved in cell proliferation and apoptosis) was found to associate with resistance to cisplatin in endometrial cancer cells (Wu *et al.*, 2011).

miR-24 expression targeting the common drug target DHFR (dihydrofolate reductase) has been shown to regulate cellular proliferation. Indeed, miR-24 SNP has been shown to interfere with miR-24 function, to increase DHFR mRNA and protein expression and resistance to methotrexate resistance (Mishra *et al.*, 2007a; Mishra *et al.*, 2008; Mishra *et al.*, 2009; Zhang and Dolan, 2010).

Moreover, for the let-7 family of miRNAs, which binds to and regulates the expression of KRAS genes, a polymorphism in the let-7 complementary-binding site (lcs6) of the 3'-UTR of KRAS gene has been described. The presence of this polymorphism has been shown to increase KRAS expression *in vitro* (Chin *et al.*, 2008). Interestingly, let-7 lcs6 polymorphism was shown to predict response in wild-type KRAS patients with metastatic

colorectal cancer treated with cetuximab monotherapy, and may therefore be considered as a predictive marker of cetuximab efficacy in this context (Zhang *et al.*, 2011b).

miRNA polymorphic variants have also been hypothesized as useful predictors of clinical outcome in metastatic colorectal cancer patients treated with the combination of 5-fluorouracil and irinotecan (Boni *et al.*, 2011). In addition, studies in cervical cancer have shown that, in the absence of information on human papillomavirus infection, the tumor necrosis factor-alpha-induced protein 8 (TNFAIP8)-rs11064 SNP may affect the affinity of miR-22 binding to the 3'-UTR of TNFAIP8, regulating its expression. This may contribute not only for the risk of developing cervical cancer but also, the increased in TNFAIP8 protein expression may help predict platinum resistance and clinical outcomes in cervical cancer patients (Shi *et al.*, 2013).

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PART II.

OBJECTIVES

The role of miRNAs in cancer is well documented. In fact, a wide range of miRNA signatures have been documented in different leukemias and the particular function of some miRNAs in both CML and AML has also been addressed. Nonetheless, the function of miRNAs in leukemia drug resistance and drug response in not fully understood.

Thus, the aims of the present work were:

1. Further understand the role of miR-21 in CML cell lines.

The role of miR-21 in CML is not fully understood. To address this issue, the expression of miR-21 was downregulated with antimiRs in two CML cell lines (K562 and KYO-1). Cellular viability along with cellular processes such as cell proliferation, cell cycle, programmed cell death and autophagy were assessed. Further investigation of an associated autophagy mechanism was carried out. Expression of autophagy-related proteins (such as Vps34, Beclin-1 and LC3) was analysed by Western blotting, together with the presence of autophagosomes by fluorescence microscopy. Cellular ultra-structure was analysed by electron microscopy in order to confirm the presence of autophagic structures. Furthermore, the effect of the etoposide and doxorubicin on both cell lines was also analysed, in order to verify if downregulation of miR-21 increased cellular response to these cytotoxic drugs.

2. Investigate if miR-21 is present in various extracellular vesicles from CML cells.

The discovery that different types of extracellular vesicles (EVs) are secreted from cells, and that they can carry cellular content (such as proteins, mRNAs or miRNAs) which can then be incorporated into neighboring cells, has recently increased the scientific interest in these cellular structures. In particular, it has become evident that EVs may carry miRNAs that are responsible for drug resistance, from resistant to sensitive cells. However, it is not known if miR-21 is transported in EVs “shed” from CML cells, and moreover if different types of EVs carry different levels of miR-21. To address this issue, EVs from K562 (CML) cells were extracted using four different protocols, in order to isolate different types of EVs. Furthermore, miR-21 expression levels were analysed in those different types of EVs.

3. Study the role of miR-128 in an AML cell line.

miR-128 was shown to be associated with AML in several miRNA profiles but none of them exploited the functional mechanism that could explain this association. In order to address this question, overexpression of miR-128 was carried out in an AML cell line (HL60). Following miR-128 overexpression, cellular viability and sensitivity to doxorubicin and etoposide was assessed. Several cellular processes, such as cell cycle, cell proliferation, apoptosis, autophagy and DNA damage were studied. Moreover, miR-128 expression was analysed in AML bone marrow samples in order to confirm the levels of this miRNA in these patient samples when compared to controls.

CHAPTER II.

RESULTS AND DISCUSSION

The experimental results obtained in the scope of this thesis were organized in the form of research articles presented in this chapter. Thus, Aim 1 was addressed in Article 1, Aim 2 was addressed in article 2 and Aim 3 was addressed in Article 3.

ARTICLE 1. TARGETING MIR-21 INDUCES AUTOPHAGY AND CHEMOSENSITIVITY OF LEUKEMIA CELLS

Hugo Seca, Raquel T. Lima, Vanessa Lopes-Rodrigues, José E. Guimarães, Gabriela M. Almeida and M. Helena Vasconcelos

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Targeting miR-21 Induces Autophagy and Chemosensitivity of Leukemia Cells

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Abstract: Overexpression of oncomiR-21 has been observed in most cancer types, such as leukemia. This miR has been implicated in a number of cellular processes, including chemoresistance, possibly by directly modulating the expression of several apoptotic related proteins. It was recently shown to directly target Bcl-2 mRNA and upregulate Bcl-2 protein expression. Nevertheless, the possible effect of miR-21 in autophagy has never been addressed. This study investigates the effects of targeting miR-21 with antimiRs on chronic myeloid leukemia cellular autophagy and on associated drug sensitivity. We observed that miR-21 downregulation decreased cellular viability and proliferation, although no changes to the normal cell cycle profile were observed. miR-21 downregulation also caused increased programmed cell death and a decrease in the expression levels of Bcl-2 protein, although PARP cleavage was not affected, indicating that apoptosis was not the relevant mechanism underlying the observed results. Treatment with antimiR-21 caused an increase in the autophagy related proteins Beclin-1, Vps34 and LC3-II. Accordingly, autophagic vacuoles were visualized both by monodansylcadaverine (MDC) and acridine orange (AO) staining and also by transmission electron microscopy (TEM). Additionally, miR-21 downregulation increased K562 and KYO-1 cellular sensitivity to etoposide or doxorubicin. This chemosensitivity was reverted by pre-treating cells with 3-MA, an autophagy inhibitor. Finally, serum starvation (an autophagy inducer) also increased sensitivity to these drugs, confirming that autophagy sensitized these cells to the effect of these drugs. To the best of our knowledge, this is the first description of autophagy induction via miR-21 targeting and its involvement in drug sensitivity.

Keywords: AntimiRs, autophagy, cancer, chemoresistance, miR-21.

1. INTRODUCTION

Autophagy is understood as an evolutionary conserved survival process, by interfering with cellular metabolism. Nonetheless, following excessive autophagy mediated via Beclin-1, the cells may undergo an autophagic-mediated cell death [1, 2].

Autophagy has been shown to be either related with chemoresistance [3] or chemosensitivity [4], which may depend on the cell model used or the drug tested [5, 6].

microRNAs (miRNAs or miRs) have been described as playing a role in several malignancies, as well as being associated with disease pathogenesis and response to therapy [7, 8]. miRs are short non-coding RNAs that regulate gene expression in a post-transcriptional manner *i.e.* by binding to the target-mRNAs and causing their degradation or inhibition of translation [9]. miRs have been shown to regulate several cellular processes (*e.g.* apoptosis, differentiation, migration or proliferation [10-12]), and also to be implicated in tumor cell resistance/sensitivity to chemotherapy [13]. miR-21 is overexpressed in almost all types of solid tumors [14] as well as in hematological malignancies [15]. Evidence for miR-21 oncogenic potential is stated in different studies performed in numerous cell lines [16, 17]. In addition, miR-21 has been shown to be involved in chemoresistance in a variety of cancer cells [18-20].

Drug resistance associated with miR-21 involves decreased chemotherapy-induced apoptosis of cells overexpressing this miR. In fact, miR-21 has been described to target several genes known to be involved in apoptosis, namely Pdcd4 [21], FasL [22] and PTEN [23], decreasing the expression of those proteins. miR-21 has also been shown to target Bcl-2 through a non-canonical mechanism, as its binding to Bcl-2 mRNA increased Bcl-2 expression [24]. It is known that Bcl-2 inhibits Beclin-1-dependent autophagy [2]. Furthermore, it is also known that autophagic signaling networks in cancer may be regulated by miRs [25]. Indeed, miR-30a, miR-101 and miR-130a have also been previously shown to directly target autophagy related mRNAs [26-28].

Therefore, since miR-21 is associated with cancer chemoresistance and since it upregulates Bcl-2 protein ex-

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pression (known to be involved both in apoptosis and autophagy), we hypothesized that miR-21 might modulate autophagy and sensitivity towards drugs that induce autophagy. To confirm this, we targeted miR-21 expression with antimiRs in two chronic myeloid leukemia (CML) cell lines (K562 and KYO-1) and studied the effect on autophagy and drug sensitivity. To our knowledge, this study is the first description that miR-21 may influence autophagy.

2. MATERIALS AND METHODS

2.1. Cell Lines

K562 and KYO-1 cell lines (both CML) were genotyped and routinely cultured in RPMI 1640 medium with ultraglutamine I (Lonza) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories). They were maintained at 37ºC in a humidified atmosphere containing 5% CO_2.

2.2. Transfection of K562 and KYO-1 Cells with AntimiRNAs

K562 and KYO-1 cells ($5x10^5$ cells/mL) were transfected with the antimiRs [anti-miRNA for miR-21 (antimiR-21) and negative control anti-miRNA - Anti-miR™ Negative Control #1 (antimiR-NC), – both from Life Technologies] using Lipofectamine™ and Plus™ reagents (Life Technologies), according to the manufacturer's instructions. All transfections were carried out in serum free medium conditions for the initial 3 h using 200 nM of antimiRs. FBS was then added to obtain a final concentration of 10%.

2.3. Treatments of K562 and KYO-1 Cells with Doxorubicin, Etoposide, 3-MA (3-Methyladenine) or Serum Starvation

Following the 3 h of transfection (as described above), doxorubicin (30 nM), etoposide (200 nM), 3-MA (1mM) or a combination of these drugs was added to the cells. A control was included with the equivalent volume of vehicle of the drug (DMSO). For serum starvation experiments, cells were seeded in serum-free medium and kept without FBS throughout the 48 h. Cell number and viability were determined 48 h later, with the Trypan Blue exclusion assay.

2.4. Protein Expression and Immunoprecipitation Analysis

Cells were lysed 48 h following transfection and protein expression was analyzed by Western blot, as previously described [29, 30]. The following primary antibodies were used: Bcl-2 (1:100, Dako), Beclin-1 (1:1000), LC3 (1:1000) and Vps34 (1:1000) from Cell Signaling; PARP (1:2000) and Actin (1:2000) from Santa Cruz Biotechnology, Inc.; caspase-3 (1:2000) from Milipore. Secondary antibodies from Santa Cruz Biotechnology, Inc. were used (anti-mouse IgG–HRP, 1:2000; anti-rabbit IgG-HRP, 1:2000; anti-goat IgG-IIRP, 1:2000). Immunoprecipitation was performed 48 h following cells transfection. Briefly, approximately 350 μg of protein extracts were incubated overnight with Beclin-1 antibody (1:100, Cell Signaling) followed by beads incubation (Protein G, Sepharose beads, Amersham) for 50 min. After consecutive washes, samples were centrifuged and supernatants were loaded in an acrylamide gel and analyzed by Western blot using Beclin-1 and Bcl-2 antibodies, as previously described [29, 30].

2.5. microRNA Expression Analysis

Total RNA was extracted from cells 24 h following transfection, using TRI Reagent® (Life Technologies) following the manufacturer's instructions, except for the use of chloroform instead of 1-bromo-3-chloropropane. Quantitative real-time PCR (qRT-PCR) was carried out with the miScript System (Qiagen) as previously described [31]. The primers were: miR-21 (Hs_miR-21_2) and U6 (Hs_RNAU6B_2, used as control) from Qiagen. For each primer, serial dilutions of K562 or KYO-1 cDNA were used for the standard curve determination. A dissociation curve was generated and a single peak obtained. Reactions from three independent experiments were run in duplicate and negative control reactions without RT or template were included.

2.6. Programmed Cell Death, BrdU Proliferation Assay, Monodansylcadaverine and Acridine Orange Staining and Cell Cycle Analysis

All of these assays were carried out 48 h following transfection of the cells with the antimiRs. Programmed cell death was assessed using the "*in situ* cell death detection kit" (Roche), as previously described [31]. For cell proliferation studies, following a pulse of BrdU (10 μM) for 1 h, cells were fixed in 4% paraformaldehyde (PFA) in PBS. Cytopsins were prepared and incubated with 2 M HCl for 20 min. Cells were incubated with mouse anti-BrdU (1:10, Dako) for 1 h and further incubated with anti-mouse IgG-FITC (1:100, Dako). Slides were mounted in Vectashield Mounting Media with DAPI, cells observed in a DM2000 microscope (LEICA) and a semi-quantitative evaluation was performed by counting a minimum of 500 cells per slide. For the analysis of the MDC, cells were incubated for 1 h with freshly prepared MDC (50 μM). Cells were fixed as described above. Cytopsins were prepared and mounted in Vectashield Mounting Media with DAPI. Cells were analyzed using a fluorescence microscope (Axio Imager.Z1 coupled with ApoTome Imaging System microscope, Zeiss). For acridine orange (AO) staining analysis, cells were incubated for 15 min with 1μM of AO, cytospined, mounted in Vectashield Mounting Media with DAPI and visualized immediately using a fluorescence microscope (Axio Imager.Z1 coupled with ApoTome Imaging System microscope, Zeiss). For the cell cycle profile analysis, cells were fixed in ice-cold 70% ethanol and further analyzed by flow cytometry as previously described [32].

2.7. Transmission Electron Microscopy

Cells were fixed 48 h following transfection with 2% glutaraldehyde and 2% PFA in 0.1 M Phosphate buffer (PB) and then washed with 0.1% Millipore-filtered PB tannic acid. Cells were postfixed with 1% PB osmium tetroxide for 1 h and stained with 1% Millipore-filtered uranyl acetate. Samples were dehydrated in increasing concentrations of ethanol and finally in propylene oxide for 30 min. They were then infiltrated and embedded directly in Epon Resin and polymerized in a 70ºC oven for 2 days. Samples were cut in ultrathin (50–100 nm) sections with a Leica Ultracut micro-

tome and each section was stained with 5% uranyl acetate solution and with Reynold's lead citrate solution. Images were examined in a JEM 1400 transmission electron microscope at an accelerating voltage of 80 kV (Jeol). Digital images were obtained using Orius 1100W (Gatan).

2.8. Statistical Analysis

All data was statistically analyzed with the *two-tailed* paired Student's t-test.

3. RESULTS

3.1. miR-21 Downregulation Decreases Cellular Viability of K562 Cells

In order to understand the effect of targeting miR-21 in a CML cell line, K562 cells were transiently transfected with antimiR-21 or a control miR (antimiR-NC). Effective miR-21 downregulation was achieved, as confirmed by qRT–PCR for miR-21, which was analyzed 24 h following transfection. Results confirmed that a clear decrease in miR-21 expression was obtained in K562 cells transfected with antimiR-21 (downregulation of expression of 64.5% that is 35.5% relative expression when compared to cells transfected with antimiR-NC, Fig. **1A**). The effect of miR-21 downregulation on viable cell number was then evaluated. Downregulation of miR-21 expression decreased K562 viable cell number to 81.7% or to 78.1%, 24 h or 48 h following transfection, respectively, when compared to Blank cells (untreated, exponentially growing). This decrease was considered small but statistically significant at both time-points analyzed (Fig. **1B**). On the other hand, transfection of cells with antimiR-NC only caused a very small and non-significant reduction in viable cell number.

3.2. miR-21 Downregulation Decreases Cell Proliferation, Does Not Affect Cell Cycle Profile and Increases Programmed Cell Death

Since antimiR-21 transfection caused a significant decrease in viable cell number, the possible mechanisms underlying this effect were studied. Therefore, the effect of miR-21 downregulation on cellular proliferation, cell cycle profile and on programmed cell death was investigated. Results from the BrdU incorporation assay showed that miR-21 downregulation caused a 10% decrease in the number of

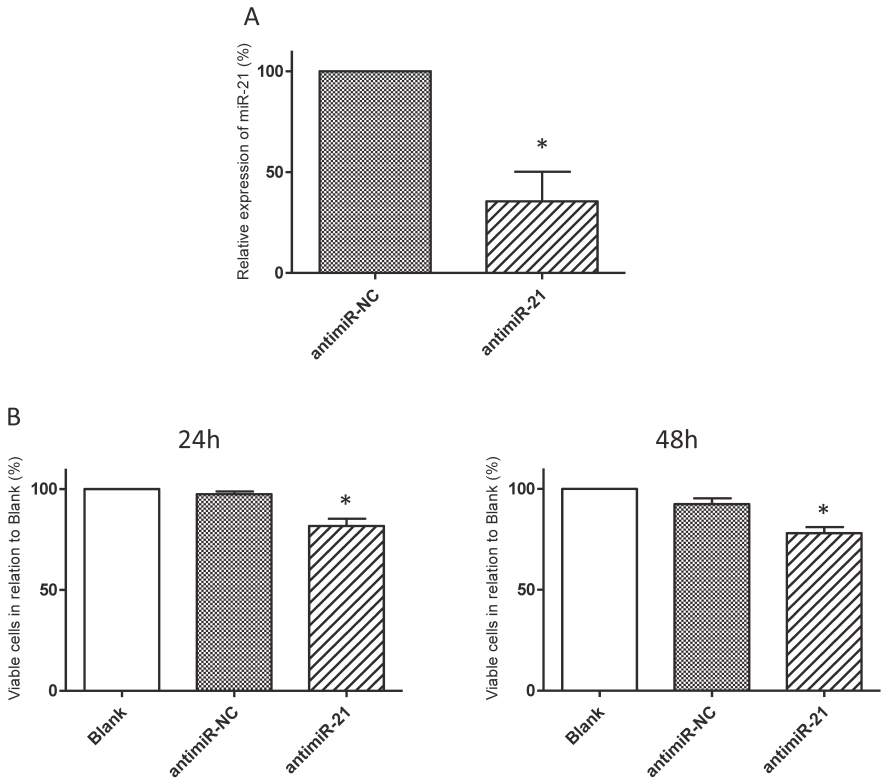

Fig. (1). Targeting miR-21 decreases K562 viable cell number. (**A**) qRT-PCR analysis of miR-21 levels, 24 h after transfection. Results, expressed after normalization of the miR-21 values with the values obtained for U6, were analyzed as % of the control cells (transfected with antimiR-NC) and represent the mean ± SE of three independent experiments, performed in duplicate. (**B**) Viable cell number, 24 h and 48 h after transfection with antimiR-NC or antimiR-21. Results are presented as a % of the Blank cells and are the mean ± SE of four independent experiments. * $p<0.05$ antimiR-NC *vs.* antimiR-21.

proliferating cells (43.9%, absolute value) when compared to cells transfected with antimiR-NC (53.1%, absolute value) ($p<0.01$) (Fig. **2A**). Curiously, miR-21 downregulation did not affect the cell cycle profile (Fig. **2B**). Programmed cell death was analyzed with the TUNEL assay. Results showed that antimiR-21 transfected cells presented a very small, but statistically significant ($p<0.01$), increase in TUNEL labeled cells, when compared to antimiR-NC transfected cells (Fig. **3A**). This increase was from 4.5% in antimiR-NC transfected cells to 6.5% in antimiR-21 transfected cells. Given these results, the expression levels of some apoptotic related proteins, namely Bcl-2, caspase-3 and PARP, were examined by Western Blot (Fig. **3B**). miR-21 downregulation caused a decrease in procaspase-3 expression levels, but did not cause alterations in the expression levels of PARP, a known target of caspase-3, or of cleaved PARP. The levels of Bcl-2 were decreased.

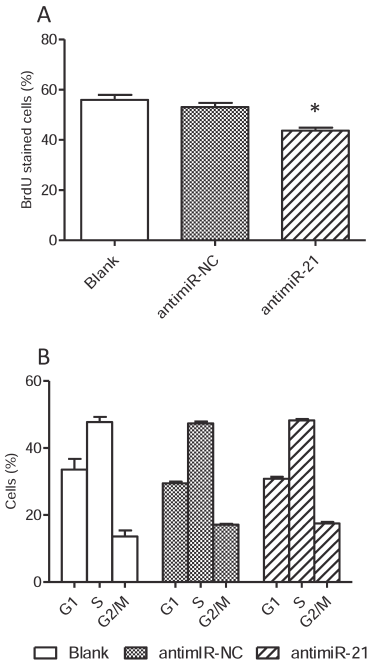

Fig. (2). Effect of targeting miR-21 on K562 cell proliferation and cell cycle profile. (**A**) BrdU incorporation analyzed 48h after transfection. Results are represented as the % of BrdU positive cells and are the mean ± SE of three independent experiments. (**B**) Cell cycle profile analyzed by flow cytometry. Results are represented as the % of cells in each phase of the cell cycle analyzed 48h after transfection and are the mean ± SE of three independent experiments. * $p<0.05$ antimiR-NC *vs.* antimiR-21.

3.3. miR-21 Downregulation Increases Autophagy

Since targeting miR-21 with antimiRs reduced viable cell number, slightly decreased cellular proliferation and marginally increased programmed cell death (although there was no evidence of apoptosis, according to the above mentioned Western blot results) and decreased Bcl-2 levels (Fig. **3B**) and since Bcl-2 binds Beclin-1 and is involved in autophagy regulation [2, 33], the effect of miR-21 downregulation on the association of Bcl-2 with Beclin-1 was analyzed. After performing immunoprecipitation for Beclin-1, it was verified that the amount of Bcl-2 protein that was associated with this protein was clearly decreased (Fig. **4A**). Furthermore, the expression of some autophagy related proteins (Beclin-1, LC3 and Vps34) were examined, by Western Blot. antimiR-21 transfected cells presented a clear increase in the expression of Beclin-1, Vps34 and LC3-II proteins 48 h after transfection (Fig. **4B**), which was indicative of increased autophagy. To further confirm these observations, the presence of autophagosomes was analyzed both with MDC and AO staining, by fluorescence microscopy. The number of MDC-positive as well as AO-positive vacuoles was increased in cells transfected with antimiR-21, when compared with cells transfected with antimiR-NC (Fig. **4C**). Both the increased levels of autophagy related proteins and MDC and AO-positive stained cells indicated that autophagy was being induced. This was confirmed by electron microscopy, which allowed observing the presence of many autophagic structures following targeting miR-21 with antimiRs (Fig. **5**).

3.4. miR-21 Downregulation and Autophagy Induction Increases Sensitivity to Chemotherapy Drugs in K562 Cells

To determine if miR-21 downregulation increased the sensitivity of K562 cells to two conventional chemotherapeutic drugs (etoposide and doxorubicin), we analyzed the effect of miR-21 downregulation on cellular sensitivity to 48 h treatment with these drugs. The antimiR-NC caused a slight reduction in the number of viable cells when compared to the Blank cells, probably due to a non-specific effect. Downregulation of miR-21 (*per se*) decreased the number of viable cells (first group of columns, Fig. **6A**). In addition, downregulation of miR-21 expression sensitized cells to the effects of etoposide or doxorubicin (striped columns, Fig. **6A**). To confirm that autophagy sensitized to the effect of these drugs, cells were serum starved (a known autophagy induction mechanism) and further treated with etoposide and doxorubicin. Serum starvation sensitized the cells to both chemotherapeutic drugs (Fig. **6B**). To further demonstrate that autophagy was indeed responsible for the cellular sensitization to these drugs, cells were treated together with the drugs and 3-MA (an inhibitor of autophagy). Treatment with 3-MA prevented the increased chemosensitivity effect towards both doxorubicin and etoposide, which had been verified upon antimiR-21 transfection (Fig. **6C**).

3.5. Confirmation of Autophagy-Mediated Chemosensitization in Another CML Cell Line (KYO-1)

A clear decrease in miR-21 expression was observed in KYO-1 cells transfected with antimiR-21 (41.9% relative expression, compared to cells transfected with antimiR-NC, Fig. **7A**). This downregulation of miR-21 expression in KYO-1 cells decreased viable cell number to 68.7%, 48 h following transfection when compared to Blank cells (with statistical significance) although antimiR-NC also reduced viable cell number to 86.7% when compared to Blank (Fig. **7B**). This reduction is probably due to transfection

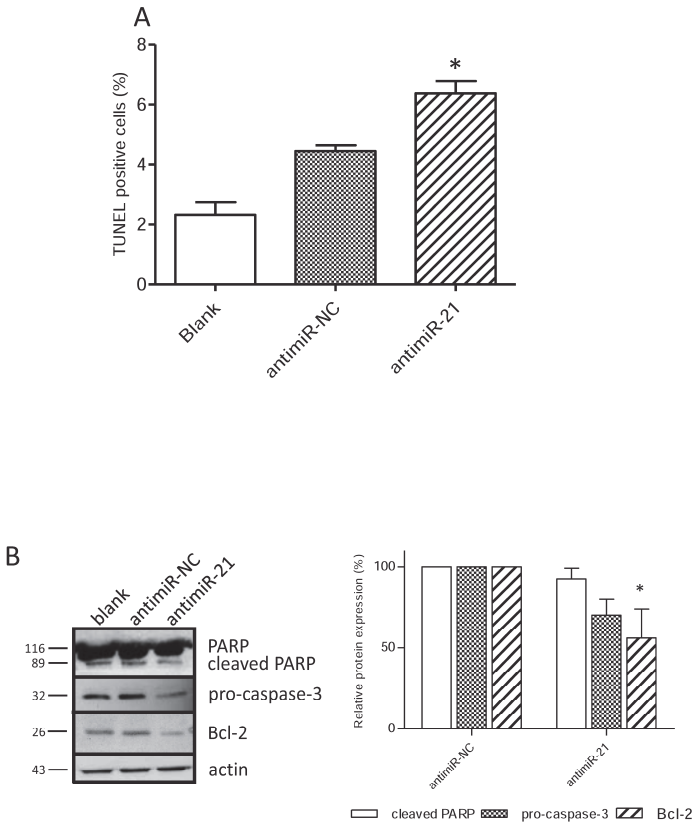

Fig. (3). Effect of targeting miR-21 on K562 cells programmed cell death. (**A**) TUNEL assay. Results are the mean ± SE of three independent experiments. (**B**) Western blot analysis of cell death related proteins. Blots are representative of three independent experiments. Densitometry analysis of the Western blots for cleaved PARP, pro-caspase-3 and Bcl-2. Results are expressed after normalization of the values obtained for cleaved PARP, pro-caspase-3 or Bcl-2 with the values obtained for actin, and also expressed as a % of the values obtained for the antimiR-NC. Each bar represents the mean ± SE from 3 independent experiments. * $p<0.05$ antimiR-NC *vs.* antimiR-21.

cytotoxicity in KYO-1 cell line (first group of columns, Fig. **7B**). Furthermore, downregulation of miR-21 expression sensitized cells to the effects of etoposide or doxorubicin (striped columns, Fig. **7B**). To further confirm that autophagy was responsible for the cellular sensitization, cells were treated together with etoposide or doxorubicin and 3-MA. Treatment with 3-MA prevented the increased chemosensitivity effect towards both drugs, which had been verified after decreasing miR-21 expression (Fig. **7B**).

4. DISCUSSION

miRs have recently emerged as pivotal post-transcriptional regulators of gene expression and may function as 'oncogenes' or 'tumour suppressor genes' by controlling multiple cellular mechanisms [7]. The miR-21 stands out as an important regulator of oncogenic mechanisms such as apoptosis, invasion, metastasis or proliferation [34].

Drug resistance is frequent in cancer and is associated with poor prognosis [35]. miRs control several molecular processes in the cell and play a role in cancer drug resistance [13]. In particular, miR-21 has been associated with drug resistance in several tumor models [18, 20]. A recent publication has shown that miR-21 binds to the 3'-UTR of Bcl-2 mRNA and causes an increase (instead of a decrease) in Bcl-2 expression, by a non-canonical mechanism [24]. On the other hand, inhibition of Bcl-2 expression has increased the efficacy of drug treatment by inducing apoptosis and autophagy (reviewed in [2]). Therefore, we hypothesized that targeting miR-21 with antimiRs would cause an increase in autophagy.

In the present study, antimiRs were used to target and downregulate miR-21 expression in two CML cell lines (K562 and KYO-1) and to further evaluate this effect in autophagy. Similar chemically modified oligonucleotides have been used to target and downregulate other miRs [36] and one antimiR in particular (antimiR-122) has been developed for clinical use (hepatitis C), having so far reached phase II clinical trials [37].

Our results showed that targeting miR-21 *per se* decreased cellular viability, confirming previously reported results [38]. Nevertheless, miR-21 downregulation did not affect the cell cycle profile, contrary to what was

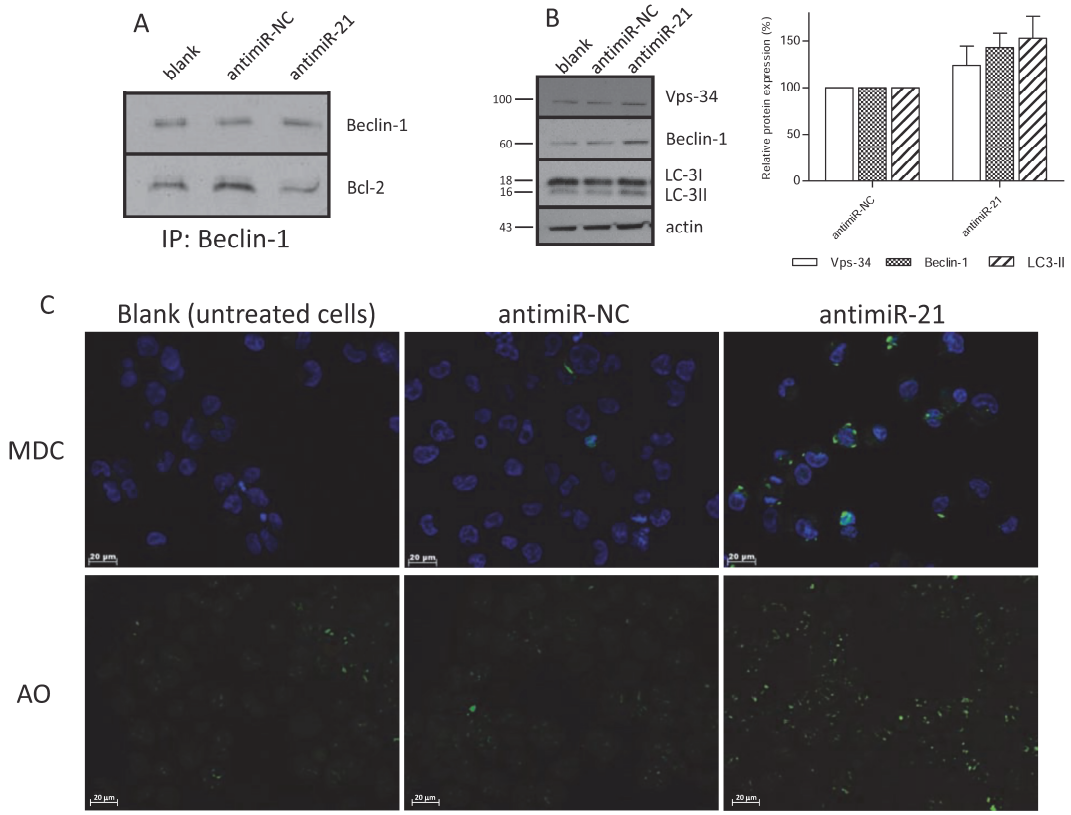

Fig. (4). Effect of targeting miR-21 on cellular autophagy in K562 cells. (**A**) Bcl-2 protein analysis following immunoprecipitation of Beclin-1 (**B**) Western blot analysis of autophagy related proteins. Blots are representative of three independent experiments. Densitometry analysis of the Western blots for Vps34, Beclin-1 and LC3-II. Results are expressed after normalization of the values obtained for Vps34, Beclin-1 or LC3-II with the values obtained for actin, and also expressed as a % of the values obtained for the antimiR-NC. Each bar represents the mean ± SE from 3 independent experiments. Results were not statistically significant. (**C**) Fluorescence Microscopy analysis of MDC stained autophagic vacuoles (Green) and Dapi stained nuclei (Blue) (representative of two independent experiments) and of AO stained autophagic vacuoles (yellow) and cytoplasm (green) (representative of three independent experiments).

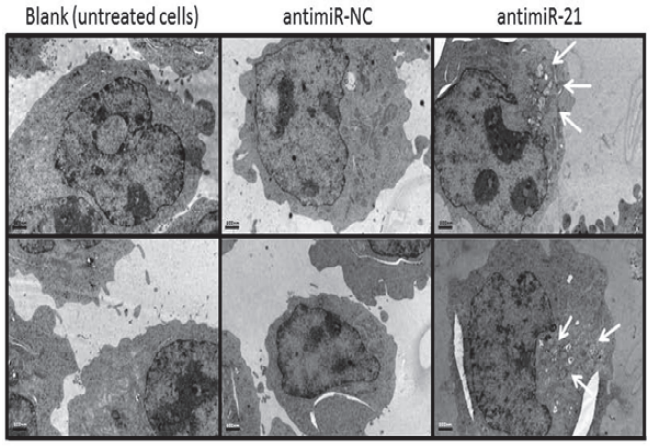

Fig. (5). Effect of targeting miR-21 on cytoplasmic structures of K562 cells analyzed by transmission electron microscopy. Arrows indicate autophagic vacuoles. Results are representative of several cells in two independent experiments.

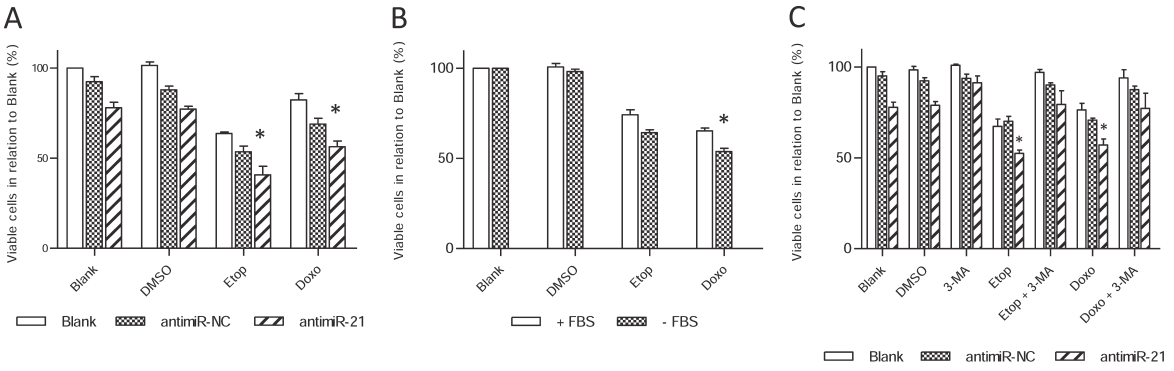

Fig. (6). Effect of targeting miR-21 and of serum starvation on K562 cellular sensitivity to doxorubicin and etoposide. (**A**) antimiR-21 transfected cells have increased sensitivity to etoposide and doxorubicin. Results are represented as a % of Blank cells analyzed 48h after transfection with antimiRs and are the mean ± SE of four independent experiments. * $p<0.05$ antimiR-NC *vs*. antimiR-21. (**B**) Serum deprived cells have increased sensitivity to etoposide and doxorubicin. Results are represented as a % of Blank cells analyzed 48h after serum starvation and are the mean ± SE of three independent experiments. * $p<0.05$ with (+) FBS *vs*. without (-) FBS. (**C**) Treatment with 3-MA (autophagy inhibitor) reverts the chemosensitivity to etoposide and doxorubicin induced by antimiR-21 treatment. Results are represented as a % of Blank cells analyzed 48h after transfection with antimiRs and are the mean ± SE of three independent experiments. * $p<0.05$ antimiR-NC *vs*. antimiR-21.

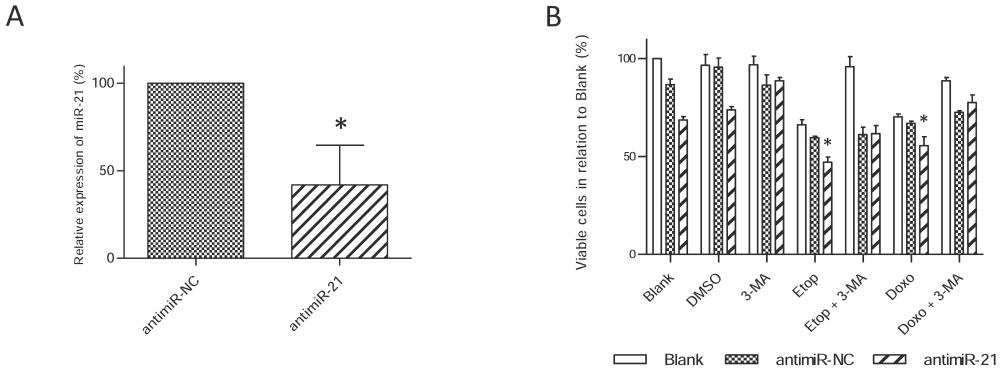

Fig. (7). Effect of targeting miR-21 on KYO-1 cellular sensitivity to doxorubicin and etoposide. (**A**) qRT-PCR analysis of miR-21 levels, 24 h after targeting miR-21 with antimiR-21. Results, expressed after normalization of the miR-21 values with the values obtained for U6, were analyzed as % of the control cells (transfected with antimiR-NC) and represent the mean ± SE of three independent experiments, performed in duplicate. (**B**) AntimiR-21 transfected cells have increased sensitivity to doxorubicin and etoposide and this effect is reverted by treatment with 3-MA (autophagy inhibitor). Results are represented as a % of Blank cells analyzed 48h after transfection with antimiRs and are the mean ± SE of three independent experiments. * $p<0.05$ antimiR-NC *vs*. antimiR-21.

demonstrated in other cell types [39] as well as in the K562 and also in the HL-60 leukemia cell lines [40]. Decreasing miR-21 expression significantly reduced the number of proliferating cells, as had been shown in other studies [41]. It is possible that antimiR-21 slows down the proliferation of cells without changing the cell cycle profile, by slowing down the entire cell cycle.

A small increase in programmed cell death, verified by TUNEL assay, led us to analyze the expression levels of certain apoptotic related proteins such as Bcl-2 and PARP. Bcl-2 expression, an antiapoptotic protein, was decreased, which is in agreement with the previous results that miR-21 increased Bcl-2 expression [24]. However, there was no alteration in cleaved PARP expression, an established apoptosis indicator downstream of caspase activation [42] which was an indication that there was no significant cell death by apoptosis occurring at this time point. Indeed, the TUNEL assay is not specific for apoptosis, which could justify the contradictory results between the TUNEL assay and the cleavage of PARP. In fact, TUNEL positive labeling might occur in non-apoptotic cells [43, 44] and cells presenting markers of autophagy could also be TUNEL positive [45, 46]. Therefore, even though it is possible that some degree of apoptosis might be occurring, these results raised the possibility that autophagy may also be involved in antimiR-21 cellular response.

Indeed, we showed that the amount of Bcl-2 protein associated with Beclin-1 was decreased and the expression of

autophagy related proteins such as Beclin-1, Vps34 and LC3-II was increased, upon miR-21 downregulation with antimiR-21. Beclin-1 is known to form a complex with Vps34, which is essential for the early stages of the autophagosomal formation [47]. Moreover, Bcl-2 has been shown to suppress autophagy by disrupting this complex and forming a Beclin-1/Bcl-2 complex which sequesters Beclin-1, thus avoiding the triggering of autophagosome formation and, consequently, autophagy [48]. Therefore, decreased Bcl-2 protein together with decreased association of Bcl-2 with Beclin-1 and increased expression of the autophagy related proteins Beclin-1, Vps34 and LC3-II supports the concept that downregulation of miR-21 causes autophagy induction. Indeed, both MDC and AO-staining and transmission electron microscopy (TEM) confirmed the presence of autophagic vacuoles when miR-21 was downregulated with antimiR-21. To the best of our knowledge, this is the first description of induction of autophagy by targeting miR-21.

Finally, our results show that targeting miR-21 with antimiRs increased K562 and KYO-1 cellular sensitivity to the cytotoxic drugs doxorubicin and etoposide. We further confirmed that autophagy induction sensitized cells to the effect of these drugs, by subjecting the cells to serum deprivation (which is known to induce autophagy) followed by treatment with the drugs. Moreover, we confirmed that autophagy participated in antimiR-21 induced sensitization to the drugs by showing that 3-MA inhibited this effect. Similar results showing that increased autophagy sensitizes to chemotherapeutic drugs have been demonstrated by others [5, 49] although there are published studies where increased autophagy is responsible for chemoresistance [6, 50]. These contradictory results may be due to the different drugs used and also to the different degree of autophagy induction achieved in the various experiments reported in the published data.

The results here reported might be relevant for targeting miR-21 in order to chemosensitize other tumor cells. We are currently verifying if this is an effect common to other tumor cell types that may have impact in the therapy of cancer.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ARTICLE 2. miR-21 IS PRESENT AT IDENTICAL LEVELS IN VARIOUS EXTRACELLULAR VESICLES ISOLATED FROM K562 CELLS

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miR-21 is present at identical levels in various extracellular vesicles isolated from K562 cells

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Abstract

Within the last years, extracellular vesicles (EVs) have emerged as important mediators of intercellular communication. These EVs, shed by a donor cell, are specifically equipped to mediate this communication since they contain proteins as well as mRNAs and miRNAs, which may be transferred to recipient cells. In particular, it has become evident that EVs may carry miRNAs that are responsible for drug resistance, from resistant to sensitive cells. However, it is not known if miR-21 (involved in drug resistance) is transported in EVs "shed" from chronic myeloid leukemia (CML) cells, and moreover if different types of EVs carry different levels of miR-21.

Among the EVs, exosomes and microvesicles are the most described ones. In spite of the recent interest in the study of EVs, the technical problems associated with the isolation of pure populations of EVs hinder their classification, as well as the determination of their specific content. Therefore, the aim of this work was to extract EVs from K562 (CML) cells using four different protocols, in order to isolate different types of EVs. Furthermore, to analyse miR-21 expression levels in those different types of EVs.

EVs were isolated from K562 cells with different isolation methods. Depending on the method of EVs' isolation, different population size distributions were found. Analysis of miR-21 expression in the EVs isolated with different protocols showed no significant differences in the levels of this miRNA. In conclusion, even though it is important to characterize isolated EVs and to define isolation protocols as a central tool for the study of EVs content, miR-21 was present at identical levels in the various EVs isolated from K562 cells.

Keywords:

Extracellular vesicles (EVs); miRNAs; intercellular communication; isolation protocols

Introduction

Increasing interest is being given to the intercellular communication mediated by the transfer of extracellular vesicles (EVs) (Raposo and Stoorvogel 2013; Lee et al. 2012). Release of vesicles in several specific circumstances had been widely accepted, such as that the release of apoptotic bodies during apoptosis (Nunez et al. 2010). However, it was only recently shown that EVs were also shed from healthy cells, having been found in most bodily fluids (Andaloussi et al. 2013).Indeed, EVs have emerged not only as playing important roles in physiological processes but particularly as being associated with several diseases, including cancer (Muralidharan-Chari et al. 2010; Jorfi and Inal 2013; Azmi et al. 2013).

According to their biogenesis, EVs are often classified into three main classes: exosomes, microvesicles and apoptotic bodies. All of them are cell-derived vesicles, enclosed by a lipid bilayer with sizes ranging from 30 nm to 2000 nm in diameter, depending on their origin. While apoptotic bodies (sized 500-2000 nm) are originated from disintegrating dying cells, microvesicles (sized 50-1000 nm) are generated by budding from the plasma membrane and exosomes (sized 30-120 nm) are derived from the endolysosomal pathway (Andaloussi et al. 2013). However, there is still lack of knowledge of the exact molecular mechanisms which are responsible for their formation. In addition, the isolation of pure populations (consisting of only one type of EVs), from the heterogeneous populations with overlapping size distributions is still technically demanding (with the isolation methods available to date), which hampers not only their proper identification but also the understanding of which molecules are specifically or "packaged" in the different EVs (Guduric-Fuchs et al. 2012; Gyorgy et al. 2011).

Nevertheless, several molecules have been described as being enclosed into EVs, such as lipids, proteins or mRNAs (Andaloussi et al. 2013; Raposo and Stoorvogel 2013). In addition, microRNAs (miRNAs), which are small RNA molecules that regulate gene expression through the binding to target mRNAs, thereby causing their degradation or inhibition of translation (Filipowicz et al. 2008; Valencia-Sanchez et al. 2006), have also been described as being transferred between cells via EVs (Valadi et al. 2007; Umezu et al. 2013). miRNAs are known to regulate several important cellular processes. Alterations in miRNA's expression are associated with several diseases, including cancer (Iorio and

Croce 2012; Farazi et al. 2013). Moreover, miRNAs deregulation has been shown to be implicated in tumor cellular response to chemotherapy (Allen and Weiss 2010; Giovannetti et al. 2012). Importantly, some miRNAs are possibly being selectively packaged into EVs (Chen et al. 2012). This may account for the fact that the "EVs cargo" may not reflect the miRNA profiles of the donor cells (Valadi et al. 2007; Skog et al. 2008; Mittelbrunn et al. 2011) and also for the existence of a cellular selection mechanism through which miRNAs are either retained or transferred between cells (Collino et al. 2010; Ohshima et al. 2010; Pigati et al. 2010).

One miRNA in particular, oncomiR-21 has already been described in EVs (Zhou et al. 2013). This miRNA is found overexpressed in almost all tumors (Volinia et al. 2006) and its involvement in chemoresistance has been addressed in various cancers. OncomiR-21 has been described in microvesicles from glioblastoma (Skog et al. 2008) and its levels were found to be higher in serum microvesicles from glioblastoma patients than in healthy controls (Skog et al. 2008). In addition, exosomal miR-21 expression was found to be up-regulated in serum from patients with esophageal squamous cell cancer when compared to serum from patients who have benign diseases without systemic inflammation. In that study, the presence of miR-21 in exosomes positively correlated with tumor progression and aggressiveness (Tanaka et al. 2013).

The aim of the present study was to extract EVs from K562 (CML) cells using four different protocols, in order to isolate different types of EVs. Furthermore, to analyse miR-21 expression levels in those different types of EVs.

Materials and methods

Cell culture

Chronic myeloid leukemia cell line K562 (ECACC, *European Collection of Cell Cultures*, UK) and its derived cell line K562Dox (a kind gift from Professor J. P. Marie, Paris, France) were cultured in RPMI 1640 with Ultraglutamine I medium (Lonza) with 10% fetal bovine serum (FBS, PAA). Cells were maintained at 37 ºC in a humidified atmosphere containing 5% CO2. Cellular viability and concentration were determined by the Trypan Blue exclusion assay.

EVs isolation by Differential Centrifugation

Confluent cells (8-10x10^5 cells/ml), growing in the previous described medium (35ml) were centrifuged at 500 *g* for 5 min to pellet whole cells. The supernatant was then centrifuged at 15000 *g* for 1 h at 15 ºC to pellet the vesicles. The final pellet was resuspended in HBSS buffer (*Sigma-Aldrich*) and centrifuged at 2000 *g* for 2 min to remove debris. The clear vesicles suspension was further centrifuged at 18000 *g* for 30 min at 15 °C to pellet the vesicles.

EVs isolation with 'ExoQuick-TC' from Systems Biosciences

Confluent cells (9x10^5 cells), growing in the previous described medium were centrifuged at 3000 *g* for 15 min to remove cells and debris. The supernatant was then transferred to a new eppendorf and 150 µl of ExoQuick-TC Exosome precipitation solution were added to this cell-free culture media. The culture media/precipitation solution mixture were then incubated at 4 °C overnight and centrifuged at 1500 *g* for 30 min to pellet the vesicles.

EVs isolation with 'Total Exosome Isolation kit' from Invitrogen

Confluent cells (9x10^5 cells), growing in the previous described medium were centrifuged at 2000 g for 30 min to remove cells and debris. The supernatant was transferred to a new eppendorf and 500 µl of Total Exosome Isolation reagent were added to the cell-free culture media. The culture media/reagent mixture were then incubated at 4 °C overnight and centrifuged at 10.000 g for 1h at 4 °C to pellet the vesicles. The pellet was resuspended in the appropriate buffer.

EVs isolation by Ultracentrifugation

Confluent cells, growing in the previous described medium (10 ml) were centrifuged at 300 *g* for 10 min to pellet cells. Supernatant was then centrifuged at 2000 *g* for 10 min at 4 ºC to pellet debris. Supernatant was further centrifuged at 10.000 *g* for 30 min at 4 ºC to remove larger vesicles and apoptotic bodies. The clear supernatant was finally centrifuged at 100.000 *g* for 70 min at 4 °C to pellet the vesicles. Lastly, the vesicles were washed with PBS 1x and re-centrifuged at 100.000 *g* for 70 min at 4 °C.

Dynamic light scattering (DLS)

Extracellular vesicles were resuspended in PBS. Particle size (nm) and count rates (kilo cycles per second, kcps) were measured using a Nano series Malvern Zetasizer Instrument (Prager Instruments). The measurement duration was adjusted automatically with three measurements per sample.

miRNA expression analysis

Total RNA was extracted from cells or from the isolated vesicles using the miRvana miRNA isolation kit (Ambion) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out with the miScript System (Qiagen) as previously described (Seca et al. 2013) using miR-21 primers form Qiagen (Hs_miR-21_2). Serial dilutions of K562 cDNA were used for standard curve determination to assess PCR reaction efficiency. A dissociation curve was generated and a single peak obtained. Reactions from three independent experiments were run in duplicate or triplicate and negative control reactions without RT or template were included.

Results and Discussion

Comparison of protocols and analysis of the populations of EVs isolated with the different protocolss

Several studies have shown that the term "EVs" may account for different types of "nanosized particles". The technology available to date does still not allow the isolation and characterization of pure populations of EVs, having specific sizes. Therefore, in the present study four different protocols were compared regarding the isolation of EVs from the same cell line (K562): i) the commercially available 'ExoQuick-TC' (from System Biosciences); ii) the 'Total Exosome Isolation kit' (from Life Technologies); iii) a protocol based on differential centrifugation (Jaiswal et al. 2012) and iv) a protocol based on ultracentrifugation (Thery et al. 2006).

Table 1 presents a comparison between the different protocols, showing the advantages and disadvantages of each one. From this comparison, it is possible to see the used kits provided faster extractions of EVs, with lower amounts of cells required and higher amount of EVs recovered. On the other hand, both the centrifugation protocols required considerable longer times, bigger amounts of cells and provided lower amounts of EVs.

The EVs isolated (from K562 cells) with the different protocols were analysed regarding their size distribution using DLS analysis (Figure 1). Some differences in the size profile of the populations of EVs isolated were clearly observed between some of the methods, although with the two commercially available kits similar sizes of EVs were obtained. Indeed, the size profiles of the EVs isolated with the 'ExoQuick-TC' and the 'Total Exosome Isolation kit' presented peaks around 35 nm. Since the results were very similar with the commercially kits, it was decided to the use 'ExoQuick-TC' for further comparisons, which from the two methods had the advantage of being a slightly faster protocol. Therefore, when comparing the 'ExoQuick-TC' with the ultracentrifugation protocol, the results were also similar in terms of size population distribution, although a slender peak of EVs (indicative of more identical vesicles) lower than 100 nm was observed with the ultracentrifugation protocol. On the other hand, differences were clearly observed when comparing these results with EVs isolated using the differential centrifugation protocol. Indeed, EVs isolated with this later method presented two peaks of sizes, a very small one (representing a small percentage of the EVs isolated) around 200 nm and another one (representing the majority of EVs) around 800 nm. Analysing all the

results it was possible to observe that the differential centrifugation protocol allowed the isolation of populations of EVs with bigger sizes than the remaining protocols.

Analysis of the miR-21 levels in the isolated EVs

miR-21 is known to be overexpressed in various types of cancer (Volinia et al. 2006; Seca et al. 2013). Moreover, miR-21 has been found in EVs released from cancer cells (Skog et al. 2008; Tanaka et al. 2013). However these studies were carried out using the 'ExoQuick-TC' or ultracentrifugation protocols only, which, as previously referred, isolate EVs with similar sizes. Therefore, in this study, we decided to analyse and compare the expression levels of miR-21 in EVs with different size ranges, by comparing miR-21 levels in EVs isolated with 'ExoQuick-TC' and differential centrifugation protocols.

Analysis of miR-21 expression levels in the EVs may not be straightforward. One of the major difficulties is the choice of the appropriate internal control which allows the normalization of the experiments. Indeed, although cellular miRNAs expression levels are commonly normalized to the levels of a small nuclear RNA, U6 (Meyer et al. 2010), in the EVs context it is not possible to find proper controls. Examples of controls that have been used include: U6 alone (Ismail et al. 2013; Mocharla et al. 2013), U6 together with U1 (da Silveira et al. 2012), snoRNA U38B and snoRNA U43 (Hunter et al. 2008), or even a pool of the most stable miRNAs (Grange et al. 2011; Collino et al. 2010). However, U6 expression has been shown to be unsuitable for normalization of serum miRNA levels (Benz et al. 2013) and the other mentioned controls may vary between different populations of EVs which makes them unreliable controls. Due to lack of appropriate "house-keeping" RNAs in the EVs, several studies of miRNA's expression analysis have been carried out using the Ct-values, starting with equivalent amounts of total RNA, (Muller et al. 2011; Ogawa et al. 2010; Ng et al. 2013). Taking all this information into account, we have decided to analyse our results using the determined Ct-values (beginning all the experiments with equivalent amounts of total RNA). Results of the levels of miR-21 in the populations of EVs extracted with the two protocols ('ExoQuick-TC' and differential centrifugation) showed the presence of this miRNA in both populations of EVs analysed. Moreover, even though the sizes of the two EV's populations extracted with the different protocols were different, no significantly differences in the amount of miR-21 were observed when comparing the two isolation protocols (Table 2),

These results show that different protocols for EV's isolation provide different EV's populations but that in spite of these differences, the expression of miR-21 does not vary between EVs, implying that its expression is common to different types of EVs.

In conclusion, our study showed that different protocols take different times and require different amounts of cells to initiate the protocol. In addition, different protocols may isolate EVs with different characteristics (sizes). Therefore, when analysing the existing literature it is important to clearly know which protocols were used. Moreover, this study showed that miR-21 levels were not dependent on the type (size) of EVs isolated, suggesting that miR-21 is not specifically "packaged" in a particular type of EVs.

Figure 1

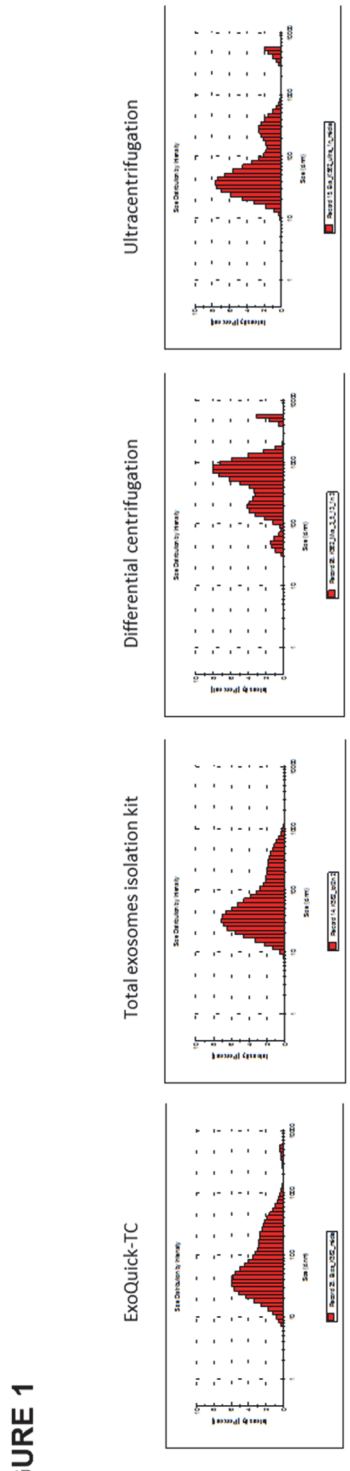

Figure 1 – Size distribution of EVs isolated from K562 analysed by DLS. EVs were isolated from cells using 'ExoQuick-TC' kit', 'Total Exosome Isolation kit', by differential centrifugation or by ultracentrifugation. Results are the mean of 3 independent experiments.

Table 1 – Comparison of various protocols for extracellular vesicles isolation from K562 cells.

	Required Labour Time	Total duration of the extraction	Required sample volume	Required buffer volume	Advantages	Disadvantages
Differential Centrifugation	3h	3h + 2 weeks to grow confluent cells	35 ml	——	. Only requires centrifugations	. Time consuming . Huge sample volume . Low outcome
ExoQuick-TC (Systems Biosciences)	1h	1h + overnight incubation + 48h to grow confluent cells	1 ml	150 µl	. Easy to perform . Small sample volume . High outcome	. Commercial kit cost
Total Exosome Isolation (Invitrogen)	1h40	1h40 + overnight incubation + 48h to grow confluent cells	1 ml	500 µl	. Easy to perform . Small sample volume . High outcome	. Commercial kit cost
Ultra-centrifugation	4h	4h + 1 week to grow confluent cells	10 ml	——	. Only requires centrifugations	. Time consuming . Moderate sample volume . Moderate outcome

*outcome refers to the amount of EVs obtained.

Table 2. miR-21 expression in K562 cells and in EVs extracted from this cell line

	Cell line	EVs	
		'ExoQuick-TC' protocol	Differential centrifugation protocol
miR-21 Ct	19.66 ± 0.12	21.93 ± 0.27	22.51 ± 0.39

Results are mean ± SE of three independent experiments.

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ARTICLE 3. THE ROLE OF miR-128 IN ACUTE MYELOID LEUKEMIA CELLS

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The role of miR-128 in acute myeloid leukemia cells

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Abstract

miR-128 has been associated with cancer, namely with leukemia. In particular, this miR has been described, together with other miRs, to allow the discrimination between AML (acute myeloid leukemia) and ALL (acute lymphoblastic leukemia). In addition, miR-128 is included in miR signatures which not only allow characterizing a particular subtype of AML but that are also associated with worse clinical outcome in a subgroup of patients with high-risk molecular features of AML. Nevertheless, all the published studies are based on data from expression arrays and no functional studies have been performed. Therefore, in order to further understand the role of miR-128 in AML cells and in their response to some chemotherapy, overexpression of miR-128 was achieved with miR-mimics in an AML cell line (HL-60). This resulted in decreased cellular viability and increased sensitization to both etoposide and doxorubicin. Overexpression of miR-128 increased programmed cell death but had no effect on cell cycle profile, apoptosis or autophagy, as no alterations were observed in the protein levels of PARP, pro-caspase-3, Vps34, Beclin-1 or LC3-II. In addition, miR-128 overexpression increased in the levels of DNA damage, as could be concluded by an increase in the comet's tail intensity in the comet assay, an increase in the number of DNA repair foci stained with either γ-H2AX or 53BP1 proteins, and an increase in the levels of these two proteins (observed by Western blot). To the best of our knowledge, this is the first association of miR-128 with DNA damage in a leukemia context.

Introduction

The aberrant expression of microRNAs (miRs) is associated with various diseases, including cancer where they can function as oncogenes or tumor suppressor genes [1-4].

miR-128 has been associated with cancer. This association was first suggested in 2005 when, through miR microarrays analysis, miR-128 expression was found downregulated in glioblastoma tissue and cell lines [5]. Later, in 2007, a study was published which discriminated acute lymphoblastic leukemia (ALL) from acute myeloid leukemia (AML) through the analysis of their miR expression signatures [6]. In this study, miR-128 was one of the four miRs that could accurately distinguish these two types of leukemias being overexpressed in ALL, when compared to AML samples [6].

miR-128 was later found, in a microRNA signature that is associated with clinical outcome, to be associated with a subgroup of patients with high-risk molecular features of AML (those who have FLT3-ITD, wild-type NPM1, or both). miR-128 increased expression was associated with increased risk of incomplete remission, relapse or death [7].

miR-128 (which is a brain-enriched miR) is transcribed from two different gene copies located on chromosomes 2 and 3, named miR-128-1 and miR-128-2, respectively. The resulting two miRs (both miR-128) are identical in their mature forms.

A study by Weiss and collaborators was the first to describe a direct target for miR-128 [8]. In this study, downregulation of EGFR was shown to occur through miR-128 direct interaction. In addition, this work showed that in lung cancer cell lines and also in clinical specimens, mir-128 deletion had a significant correlation with positive patient response and survival to EGFR tyrosine kinase inhibitor treatment with gefitinib [8].

Since then, other targets for this miR have been described. In neuroblastoma cells, miR-128 was shown to target and downregulate the expression of Reelin and DCX, two proteins involved in the migratory potential of neural cells [9]. In addition, the oncogene/stem cell renewal factor, Bmi-1, has also been described as a target of miR-128. In fact, miR-128 was found to be downregulated in gliomas and its overexpression reduced glioma cell proliferation and self-renewal through Bmi-1 targeting [10].

miR-128 was also shown to target E2F3a mRNA, preventing its translation and thus inhibiting the proliferation of glioma cells [11]. Low miR-128 expression may contribute to glioma and glioblastoma increase in proliferation by coordinately upregulating the described transcription regulators (Bmi-1 and E2F3a) as well as ARP5 (another of its described targets known to regulate cell regeneration and proliferation), resulting in uncontrolled proliferation of brain cells [12]. There are other studies that have also depicted Bmi-1 as one of the main targets of miR-128. In one of such studies, by increasing intracellular reactive oxygen species levels via inhibition of Bmi-1, miR-128 inhibited cell growth by promoting senescence in medulloblastoma cancer cells [13]. In pituitary tumor cells, miR-128 was found to be downregulated when compared to normal tissue and confirmed (by luciferase assay) that miR-128 targets Bmi-1, contributing to the involvement of miR-128 in the regulation of tumorigenicity and invasiveness. This regulation involved the binding of Bmi-1 to PTEN promoter, decreasing PTEN expression levels and leading to an increase in AKT activity in the pituitary tumor cells [14].

Ectopic expression of miR-128 in breast tumour-initiating cells (BT-ICs) led to a decrease in cell viability, increase in apoptosis and in DNA damage upon further treatment with Doxorubicin. In this study, miR-128 directly decreased the mRNA translation not only of Bmi-1 but also of ABCC5 (multidrug resistance-associated protein 5 involved in the export of xenobiotics from the cell). The same authors also found that patients with high miR-128

expression have significantly longer overall survival, after being submitted to chemotherapy, than those with low miR-128 expression [15].

Apoptosis related proteins have also been described as being (direct or indirect) targets of this miR. miR-128 was found to bind Bax mRNA, leading to its decreased translation in human embryonic kidney cells and breast cancer cells [16, 17], and to indirectly increase Bcl-2 expression in neuroblastoma cells [18]. miR-128 was also shown to induce apoptosis via PUMA upregulation, by interfering with SIRT1 expression in breast and colon cancer cells [19].

miR-128 has also been suggested to target the TGFβ signaling pathway [20]. Indeed, it was shown to decrease TGFβRI protein expression by binding to the 3'UTR region of its mRNA in breast cancer cells [20]. In gliomas, miR-128 has also been shown to inhibit tumor growth and angiogenesis by directly targeting p70S6K1 (a key downstream target of mTOR pathway) and its signaling molecules (namely HIF-1α and VEGF) [21].

miR-128 was suggested as a tumor suppressor miR that is globally found downregulated in human glioma, exerting a tumor suppressive function by increasing cell cycle arrest and increasing apoptosis. miR-128 also represses glioma-initiating neural stem cells growth, enhancing neuronal differentiation by directly targeting two oncogenic receptor tyrosine kinases - the epithelial growth factor receptor (EGFR) and the platelet-derived growth factor receptor-α (PDGFR-α) [22].

In prostate, miR-128 levels were elevated in benign epithelial cell lines compared with invasive cancer cells and silencing of miR-128 induced invasion in the benign epithelial cells, whereas its overexpression attenuated invasion in cancer cells [23].

miR-128 was also shown to target and downregulate the colony stimulating factor-1 (CSF-1) mRNA and protein expression in ovarian cancer cell lines leading to their decreased cell motility and adhesion [24].

Very recently miR-128 was described to belong to a group of miRs which target the RET proto-oncogene in acute leukemia [25].

Interestingly, treatment with some drugs/natural products has been shown to increase miR-128 levels. Indeed, NVP-LDE-225, a drug in early-stage clinical trials, increased miR-128 expression therefore inhibiting Bmi-1 and thus regulating stemness and cancer stem cell growth [26]. Also in glioma, miR-128 was found to be upregulated upon treatment with the ginsenoside Rh2, a component of the traditional medicinal plant ginseng, mediating a Rh2 anti-proliferative effect [27].

AML is a clonal disorder of hematopoietic stem cells characterized by a rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. The neoplastic myeloblasts are "frozen" at various stages of incomplete maturation, with a loss in the normal hematopoietic function due to alterations in the mechanisms of self-renewal, proliferation and differentiation [28-30].

Several reports have associated miR-128 and leukemia [6, 7, 25, 31, 32].These reports have shown that miR-128 belongs to a set of miRs that can distinguish between AML and ALL (acute lymphoblastic leukemia) [6, 31]. Moreover, that a particular subtype of AML with the translocation t(8;16)(p11;p13) may be characterized by a distinctive miR signature that includes miR-128 [25] or that miR-128 is part of a miR signature that is associated with worse clinical outcome in a subgroup of patients with high-risk molecular features of AML [7]. Nevertheless, all these studies were based on expression arrays and in none of them functional studies have been carried out nor analysed miR-128 expression regarding therapy response, in order to understand the role of miR-128 in leukemia cells. Therefore, to further understand the effect of miR-128 in acute myeloid leukemia, the overexpression of miR-128 in an AML cell line (HL-60) was performed with miR-mimics. In addition, the effects of this overexpression on DNA damage and in drug sensitivity were analysed.

Material and Methods

Human samples

A total of 13 human normal blood and 11 bone marrow AML samples were obtained from Centro Hospitalar São João, Porto, Portugal. The study was approved by the São João Hospital's ethical review board and it was designed according the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants. Samples were separated using Ficoll-Paque PLUS (GE Healthcare). Briefly, samples were mixed with sterile PBS, placed on Ficoll-Paque PLUS and centrifuged for 30 min at 400g. White blood cells were harvested and washed in PBS before freezing at -80ºC. RNA was then extracted and analysed following the protocol mentioned below in "*microRNA-128 expression analysis"* of this section.

Cell line

HL-60 (acute myeloid leukemia) cell line, purchased from DSMZ, was genotyped and maintained in RPMI 1640 medium with ultraglutamine I (Lonza) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories) at 37ºC in a humidified atmosphere containing 5% CO_2 in air. Cellular density and viability were routinely analysed with Trypan Blue exclusion assay.

Transfection of HL-60 cells with pre-miR-128

HL-60 cells ($5x10^5$ cells/mL) were transfected with the following pre-miRs from Life Technologies: pre-miRNA for miR-128 (miR-128) and negative control Pre-miR™ miRNA Precursor Molecules Negative Control #1 (miR-NC). For transfection, Lipofectamine™ and Plus™ reagents (Life Technologies) were used following manufacturer's instructions. All transfections were performed in serum free medium for

the initial 3 h using 50 nM of pre-miRs. FBS was then added to achieve a final concentration of 10%. Cells were further incubated or processed according to the following protocols.

Treatments with Doxorubicin and Etoposide

Following 3 h of transfection (as described above), doxorubicin (30 nM) or etoposide (200 nM) were added to the cells. The equivalent volume of the drug vehicle (DMSO) was added to control cells. Cell number and viability were determined 24 and 48 h later, with the Trypan Blue exclusion assay.

miR-128 expression analysis

Total RNA was extracted from human samples and from HL-60 cells (24 h following transfection of pre-miRs), using TRI Reagent® (Life Technologies) following the manufacturer's instructions, except for the use of chloroform instead of 1-bromo-3-chloropropane as previously described [33]. Quantitative real-time PCR (qPCR) was carried out with the miScript SYBR Green System (Qiagen), as previously described [33] using the following primers from Qiagen: miR-128 (Hs_miR-128_1) and U6 (Hs_RNAU6B_2, as control). For each primer, serial dilutions of HL-60 cDNA were used for the standard curve determination. A dissociation curve was generated and a single peak obtained. Reactions from three independent experiments were run in duplicate and negative control reactions without RT or template were included. PCRs were performed in 7500 Fast Real-Time PCR System (Applied Biosystems) and results analysed with 7500 Software v2.0.6 (Applied Biosystems).

Expression of proteins involved in apoptosis, autophagy and DNA damage – Western blot

Following 24 or 48 h transfection with pre-miRs, cell protein lysates were prepared, quantified and protein expression analysis was performed by Western blot, as previously described [34, 35]. The following primary antibodies were used: Beclin-1 (1:1000), LC3 (1:1000) and Vps34 (1:1000) from Cell Signaling; PARP (1:2000), 53BP1 (1:400), p-Histone H2A.X (Ser 139) (1:200) and Actin (1:2000) from Santa Cruz Biotechnology, Inc., and caspase-3 (1:2000) from Millipore. The secondary antibodies used were: anti-mouse IgG–HRP (1:2000); anti-rabbit IgG-HRP (1:2000); anti-goat IgG-HRP (1:2000), from Santa Cruz Biotechnology, Inc.

Analysis of programmed cell death and apoptosis

Programmed cell death was assessed with TUNEL assay using the "*in situ* cell death detection kit" (Roche), as previously described [36], 24 and 48 h following transfection with pre-miRs. Briefly, cells were fixed in 4% paraformaldehyde (PFA) and cytospins prepared. Cells were permeabilized in ice-cold 0.1% Triton X-100 in 0.1% sodium citrate and incubated with TUNEL reaction mixture (enzyme dilution 1:20). Slides were mounted in Vectashield Mounting Media with DAPI and observed in a DM2000 fluorescence microscope (LEICA). A minimum of 300 cells per slide were counted for a semi-quantitative evaluation of cells undergoing programmed cell death.

Apoptosis was quantified 48 h following transfection with pre-miRs by flow cytometry using the Annexin V-FITC/PI apoptosis Kit (Bender MedSystems) according to the manufacturer's instructions as previously described [37]. Flow cytometry analysis of phosphatidylserine externalization was carried out using an Epics XL-MCL Coulter flow cytometer plotting at least 20,000 events per sample and the FlowJo 7.6.5 software (Tree Star, Inc.).

Analysis of cellular proliferation and cell cycle profile

Cellular proliferation was analysed using the BrdU incorporation assay. Briefly, 47 h following transfection with pre-miRs, cells were subjected to a pulse of BrdU (10 μM) for 1 h and then fixed in 4% PFA in PBS as previously described [38]. Cytopsins were prepared and slides incubated with 2 M HCl for 20 min. Cells were incubated with mouse anti-BrdU (1:10, Dako) for 1 h and further incubated with anti-mouse IgG-FITC (1:100, Dako). Slides were mounted in Vectashield Mounting Media with DAPI and cells observed in a DM2000 microscope (LEICA). A semi-quantitative evaluation was performed by counting a minimum of 500 cells per slide.

For cell cycle profile analysis, cells were harvested and fixed in 70% ethanol and subsequently resuspended in PBS containing 0.1 mg/mL RNase A and 5 μg/mL propidium iodide, prior to analysis. The percentage of cells in the G1, S and G2/M phases of the cell cycle were determined using the FlowJo 7.6.5 software (Tree Star, Inc.) after cell debris and aggregates exclusion [39] and plotting at least 10000 events per sample.

Analysis of DNA damage by the alkaline- Comet assay

Cells were harvested 24 and 48 h of after transfection and frozen (in 10% DMSO in FBS) at -80 ºC. After thawing, cells were washed in PBS, resuspended in 0.6% low melting point agarose and quickly poured onto slides, precoated with 1% agarose in water, with the aid of a coverslip. Slides were kept on ice for 10 min to allow agarose to set. After removing the coverslip cells were lysed with ice-cold lysis buffer (100mM Na_2EDTA, 2.5M NaCl, 10mM Tris-HCl, pH 10.0, 1% Triton X-100) overnight, in the dark, at 4 ºC, followed by two 10 min washes in ice-cold distilled water. Slides were transferred to the electrophoresis tank filled with electrophoresis buffer (10 M NaOH; 200mM Na_2EDTA) and incubated for 20 min to allow DNA unwinding. Samples were subjected to

electrophoresis, for 20 min at 23 V, 300 mA. Slides were then flooded with neutralisation buffer (0.4 M Tris, pH 7.5) for 20 min and then rinsed in ice-cold water for 10 min before being allowed to dry at room temperature overnight. Slides were rehydrated with distilled water for 30 min before being flooded with 2.5 μg/mL of propidium iodide solution and incubated for 20 min at room temperature in the dark. Propidium iodide was rinsed with distilled water for 30 min and slides dried at 37 ºC. Comets were visualized using a LEICA DM2000 fluorescent microscope and analysed using the 'Comet Assay IV v4.3' imaging system (Perceptive Instruments). A minimum of 100 cells per condition were analysed.

Expression of proteins involved in DNA damage – immunofluorescence

To visualize 53BP1 and γ-H2AX protein expression, 24 and 48 h after transfection with pre-miRs, cells were fixed in 4% PFA and, after cytospin, incubated for 10 min in 50 mM of ammonium chloride in PBS. Cells were then permeabilized in ice-cold 0.2% Triton X-100 in PBS for 10 min. After blocking with 2% BSA in PBS for 20 min, slides were incubated with a mouse antibody for phospho-Histone H2A.X (Ser139; 1:400 from Millipore) or a rabbit antibody for 53BP1 (1:200, from Santa Cruz Biotechnology) diluted in 2% BSA in PBS, overnight at 4º C. Cells were then washed with 2% BSA in PBS and incubated with an appropriate secondary antibody (mouse or rabbit IgG conjugated to FITC; 1:100; Dako) for 1 h at room temperature. Slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories) and cells visualized using a fluorescence microscope ZEISS Axio Imager.Z1 coupled with ApoTome Imaging System microscope. Images were exported in TIF format, decompressed with Irfanview (ver. 4.35, Irfan Skiljan) and analysed with ImageJ (version 1.46r, http://rsbweb.nih.gov/ij/index.html), using a module written by Dr. Niklas Schultz at GMT Department, Stockholm University, Sweden [40]. This program evaluates the DAPI and

FITC channels independently, identifies cell nuclei and registers foci parameters within the nuclear area. Foci area, intensity and the number of foci per nucleus were given in the software output file.

Statistical analysis

All data was statistically analysed with the *two-tailed* paired Student's t-test. Data was considered statistically significant when $p<0.05$.

Results

miR-128 overexpression in HL-60 cells decreases cellular viability and increases cellular sensitivity to doxorubicin and etoposide

In order to understand the effect of miR-128 in an AML cell line, HL-60 cells were transiently transfected with miR-128 or with a control miR (miR-NC). Upon 24 h of transfection, a clear overexpression of miR-128 was detected, by qPCR, confirming the success of the transfection. Indeed, an average of 1480 times more miR-128 was observed in the miR-128 transfected cells in relation to cells transfected with miR-NC (Figure 1A).

The effect of miR-128 overexpression on HL-60 cells was then assessed by analysing cell viable number. It was possible to observe that miR-128 overexpression decreased HL-60 viable cell number to 92.0%, 84.3% and 81.0%, at 12 h, 24 h and 48 h after transfection respectively, when comparing to Blank cells (untreated cells in exponential growth). Moreover, this decrease was found to be statistically significant at 24 h and 48 h but not at 12 h after transfection (Figure 1B). In addition, a very small decrease in viable cell number was observed in the miR-NC transfected cells but this decrease was not statistically significant.

To confirm if miR-128 overexpression affected the HL-60 cells response to drugs, cells were treated with two chemotherapeutic drugs (doxorubicin and etoposide) following transfection with miR-128 or miR-NC, and the viable cell number was assessed. As previously observed, miR-128 overexpression was found to decrease the viable cell number *per se* (white columns, Figure 1C). In addition, treatment with the drug vehicle (DMSO) did not affect viable cell number (grey columns, Figure 1C). Interestingly, miR-128 overexpression sensitized HL-60 cells to doxorubicin, since a decrease in viable cell number was observed in cells transfected with miR-128 when compared with cells transfected with miR-NC. This sensitization was statistically significant at 24 h (striped columns, Figure 1C). miR-128 overexpression also sensitized HL-60 cells to etoposide as a decrease in viable cell number was also observed in cells transfected with miR-128 when compared with cells transfected with miR-NC. This sensitization was statistically significant at both 24 h and 48 h (checkered columns, Figure 1C).

miR-128 overexpression does not affect cell cycle, cellular proliferation, apoptosis nor the expression of apoptosis-related and autophagy-related proteins

Since miR-128 overexpression was found to significantly decrease HL-60 viable cell number and to sensitize these cells to the effect of the conventional chemotherapeutic drugs, etoposide and doxorubicin, we investigated some of the possible mechanisms involved. Hence, the effects of miR-128 overexpression on cell cycle profile, cellular proliferation, programmed cell death, apoptosis and autophagy induction were studied.

Cells overexpressing miR-128 showed no alterations in cell cycle profile, analysed by PI staining and flow cytometry, both 24 h and 48 h following transfection (Figure 2A). The levels of BrdU incorporation in cells overexpressing miR-128 were also not altered, in relation to blank or miR-NC transfected cells (data not shown). On the other hand, when

analysing programmed cell death (with TUNEL assay), an increase in the number of TUNEL labeled cells was observed following miR-128 overexpression, both at 24 h and 48 h. This increase was statistically significant at 48 h following transfection ($p<0.05$), when comparing to cells transfected with miR-NC. Indeed, cells transfected with miR-128 presented 8.2% TUNEL labeled cells, whereas cells transfected with miR-NC presented 3.4% of (Figure 2B). Taking these results into account and since TUNEL may not only label apoptotic cells, a specific assay for apoptosis was carried out by analysing Annexin-V/PI staining by flow cytometry. Surprisingly, when analysing the levels of apoptosis using this methodology, no major differences between cells transfected with miR-NC and cells overexpressing miR-128 were observed (Figure 2C). In addition, the expression levels of some apoptotic related proteins, namely procaspase-3 and PARP were also analysed by Western Blot and no significant alterations in their levels were observed between cells transfected with miR-NC and miR-128 (Figure 2D). Therefore, since autophagy has also been described as being involved in cell death, the expression of autophagy related proteins was analysed by Western Blot. Nevertheless, the levels of Vps34, Beclin-1 and LC3-II did not present any alterations between miR-NC and miR-128 transfected cells (Figure 2E).

miR-128 overexpression increases DNA damage

In order to understand what was causing the decrease in viable cell number and increase in TUNEL labeling of miR-128 transfected cells, the effect of miR-128 overexpression in DNA damage induction was analysed by the comet assay and immunofluorescence for γ–H2AX and 53BP1. By analysing the results from the comet assay, it was observed that the percentage of Comet Tail DNA (which is indicative of the % of DNA damage in that cell/comet) was higher in cells transfected with miR-128, than in cells transfected with

miR-NC. This increase was from 3.6% in miR-NC transfected cells to 8.1% in miR-128 transfected cells and was statistically significant ($p<0.05$) (Figure 3A). To further confirm that miR-128 overexpression induced DNA damage, DNA repair foci formation was analysed by immunofluorescence using antibodies for γ–H2AX and 53BP1. Moreover, the levels of both these proteins were analysed by Western blot. Results from the total number of γ–H2AX foci in cells (analysed by immunofluorescence) indicated that miR-128 transfected cells presented a higher number of these foci per cell than cells transfected with miR-NC. This was observed both at 24 h and at 48 h following transfection, with this increase being statistically significant at 24 h for γ–H2AX ($p<0.05$) (Figure 3B). Accordingly, the levels of γ–H2AX were also increased in the Western blot analysis 24 h after miR-128 transfection (Figure 3C). Regarding 53BP1, cells overexpressing miR-128 were also found to present a higher number of foci per cell, when compared with miR-NC transfected cells. This was also observed both at 24 h and 48 h, being statistically significant at 48 h ($p<0.05$) (Figure 3D). This increase was further confirmed by observing an increase in 53BP1 protein expression in miR-128 overexpressing cells (Figure 3E).

miR-128 expression in AML patient samples

Expression of miR-128 was analysed by qPCR in samples from PBMCs of 13 healthy donors and from bone marrow of 11 AML patients. Although the differences were not statistically significant, the expression levels of miR-128 in the AML samples were 1.87 times higher than in healthy donors.

Discussion

Over the last decade, the interest on miRs has grown tremendously with thousands of miRs being identified in various organisms [41]. miRs were shown to be crucial in regulating gene expression, mostly by post-transcriptional regulation, and controlling disease processes. Particularly, miRs have been described as being involved in cancer. Indeed, some miRs may act as 'oncogenes', whereas others act as 'tumour suppressor genes' [42].

Although miR-128 has been described as a brain-enriched miR [43] its expression has been shown to be altered in various types of cancer such as breast [17] or lung cancer [8]. Indeed, miR-128 has been shown to be involved in the regulation of important cell oncogenic processes such as cell proliferation [11], invasion [9] or apoptosis [19]. Moreover, miR-128 has been shown to be altered in leukemia [6, 7, 25, 31]. In this context, to date, there are no functional studies on miR-128 in leukemia cells. The present study adds knowledge to this area.

In addition, drug resistance, which is frequently associated with poor prognosis in cancer [44, 45], has also been shown to be indirectly controlled by the expression of some miRs, as these molecules may regulate several cellular mechanisms that are pivotal for cancer drug resistance [46, 47]. Curiously, the involvement of miR-128 in drug resistance has been described in several studies, although some of them present contradictory results. On the one hand, some reports point out miR-128 expression as being related to sensitization to drugs [15], while others associate miR-128 expression with increased drug resistance [17, 48]. In order to clarify this issue, in the present study the effect of miR-128 on cellular sensitization to two chemotherapeutic drugs (doxorubicin and etoposide) was studied.

Results from this work showed that overexpressing miR-128 *per se* decreased the viable cell number of HL-60 cells. This was in agreement with some of the previously published studies in different cancer cell models [11, 13, 21, 49]. In addition, in this study, miR-128

overexpression was also shown to sensitize cells to the chemotherapeutic drugs - doxorubicin and etoposide. This was in agreement with other study in which miR-128 overexpression sensitized breast tumor cells to doxorubicin [15]. Nevertheless, some other studies have shown the opposite effect in breast and lung cancer cells [17, 48]. In addition to this, the proliferation levels and cell cycle profile of HL-60 cells overexpressing miR-128 showed no alterations, although alterations in both had been shown by others, for brain tumor cells and for human embryonic kidney cells [11, 12, 16]. These contradictory results may be explained by the different genetic background and tissue origin of the cell lines used in this study, compared to others.

In this study, the fact that the percentage of TUNEL positive cells (suggesting programmed cell death) was increased in cells overexpressing miR-128 prompted us to further analyse the cellular levels of apoptosis. Nevertheless, when comparing miR-NC with miR-128 transfected cells, no significant alterations were observed, neither in the Annexin-V/PI staining nor in the expression of the apoptosis related proteins procaspase-3 and PARP (the former, an enzyme known to be involved in the first steps of apoptosis and the latter, a recognized target of apoptosis induction). Nevertheless, the TUNEL assay has previously been described as not being specific for apoptosis, as it may also label non-apoptotic cells [50, 51]. In fact, there are studies showing that cells undergoing autophagy activation can also be labeled by TUNEL [52, 53]. This led us to analyse the expression of autophagy related proteins upon miR-128 overexpression. Nonetheless, also no alteration in autophagic protein levels was observed.

Intrinsic DNA damage may arise from errors taking place during DNA replication, from endogenous reactive oxygen species (ROS) produced from normal metabolic byproducts or from extrinsic genotoxic stresses (including ultraviolet light, ionizing radiation, chemo- and radiotherapeutic agents) [54, 55]. miR-128 has been predicted and shown to target

proteins involved in DNA damage response [56-58] and interestingly, miR-128 has been recently shown to increase intracellular ROS levels [13]. The present study shows that miR-128 overexpressing leukemia cells had increased DNA damage. Indeed, results from the alkaline comet assay (a method that quantifies DNA damage by the increasing content of DNA in the tail of the comets) showed a clear increase in DNA tail intensity in miR-128 overexpressing cells, as was already seen by others in breast tumor cells [15]. Confirmation of DNA damage induction was possible by monitoring γ–H2AX and 53BP1 protein expression, whose foci are evidence of persistent DNA double strand breaks (DSBs) [59]. The observed increase in the number of foci of γ–H2AX and 53BP1 indicated an increase in DNA DSBs, which might justify the increased sensitivity to doxorubicin and etoposide of miR-128 overexpressing cells.

Other miRs associated to DNA damage have been already described to interfere with chemosensitivity. For example, when miR-18a was overexpressed in colorectal cancer cells, those cells were less able to recover from etoposide-induced DNA damage when compared to cells transfected with a control miR, reflecting a compromised DNA DSBs repair mechanism [60]. Indeed, it is known that miR-18a reduces ataxia telangiectasia mutated protein, a central molecule in promoting repair of DNA DSBs [60].

In the context of AML, miR-128 may lead to DNA damage (possibly by affecting the expression of proteins involved in DNA repair, since as mentioned above, miR-128 has been shown to target proteins involved in DNA damage response [56-58]), that accumulates without prompt repair causing increased sensitivity to DNA damaging drugs such as etoposide and doxorubicin.

Results from cell lines led us to analyse the levels of miR-128 in AML samples. A slight increase in the expression of miR-128 was found in AML bone marrow samples, when compared to PBMCs from healthy donors. Even though the differences were not

statistically significant, this suggests that these patients may have an increased DNA damage. However, definitive conclusions cannot be taken from these results, as the sample size was very small. Nevertheless, further studies on the levels of DNA damage from these patients might help explaining the role of miR-128 in leukemia. In addition, it would be very interesting to correlate miR-128 levels in these patients to response to DNA damage agents.

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Figure legends

Figure 1. Increasing miR-128 levels decreases HL-60 viable cell number and increases cellular sensitivity to doxorubicin and etoposide. (A) qRT-PCR analysis of miR-128 levels, 24 h after transfection with miR-128 mimic or control miR (miR-NC). Results, expressed after normalization of the miR-128 values with the values obtained for U6, were analysed as % of the miR-NC transfected cells and represent the mean ± SE of six independent experiments performed in duplicate. (B) Viable cell number analysed 12 h, 24 h and 48 h after transfection with miR-NC or miR-128. Results, presented as % of Blank cells, are the mean ± SE of four independent experiments. (C) Results are expressed as % of viable cell number in relation to Blank analysed 24 h and 48 h after transfection with the miR mimics (miR- 128 or miR-NC) and are the mean ± SE of three independent experiments. * $p<0.05$ miR-NC *vs.* miR-128.

Figure 2. Increasing miR-128 levels on HL-60 cells does not affect cell cycle profile, apoptosis or expression of apoptosis and autophagy-related proteins. (A) Cell cycle profile analysed by flow cytometry. Results are represented as % of cells in each phase of the cell cycle analysed 24 h and 48 h after transfection with the miR mimics (miR-128 or miR-NC) and are the mean ± SE of three independent experiments. (B) TUNEL assay. Results were analysed 24 h and 48 h following transfection with the miR mimics (miR-128 or miR-NC) and are the mean ± SE of three independent experiments. *$p<0.05$ miR-NC *vs.* miR-128. (C) Levels of apoptosis analysed with Annexin V/PI staining by flow cytometry. Images are representative of three independent experiments. Western blot analysis of apoptosis-related proteins (D) and of autophagy-related proteins (E). Images are representative of three independent experiments.

Figure 3. Increasing miR-128 levels increases DNA damage of HL60 cells. Cells were transfected with the miR-mimics (miR-128 or miR-NC) and analysed 24 h or 48 h following transfection. (A) Comet assay results analysed 48 h after transfection. Fluorescence Microscopy images of Comet Tail DNA are shown on the left of this panel. Arrows indicate DNA tails. Images are representative of a minimum of 100 cells (from two independent experiments). Quantification of the % of Tail DNA is shown on the right of this panel. (B) γ-H2AX foci analysed 24 h and 48 h after transfection. The left of the panel shows representative fluorescence microscopy photographs of γ-H2AX foci (from FITC labeling) and Dapi stained nuclei (blue) and are the mean ± SE of three independent experiments. Quantification of these foci is shown on the right of this panel. * $p<0.05$ miR-NC *vs.* miR-128. (C) Western blot analysis of γ-H2AX protein. Blot is representative of three independent experiments (left panel). Densitometry analysis of the Western blot for γ-H2AX (right panel). Results are expressed after normalization of the values obtained for γ-H2AX with the values obtained for actin, and also expressed as a % of the values obtained for blank. Results are the mean ± SE from 3 independent experiments. (D) 53BP1 foci analysed 24 h and 48 h after transfection. The left of the panel shows representative fluorescence microscopy analysis of 53BP1 foci (green from FITC labeling) and Dapi stained nuclei (blue) and are the mean ± SE of three independent experiments. Quantification of these foci is shown on the right of this panel. * $p<0.05$ miR-NC *vs.* miR-128. (E) Western blot analysis of 53BP1 protein. Blot is representative of three independent experiments (left panel). Densitometry analysis of the Western blot for 53BP1 (right panel). Results are expressed after normalization of the values obtained for 53BP1 with the values obtained for actin, and also expressed as a % of the values obtained for blank. Each bar represents the mean ± SE from 3 independent experiments. * $p<0.05$ miR-NC *vs.* miR-128.

Figures

Figure 1

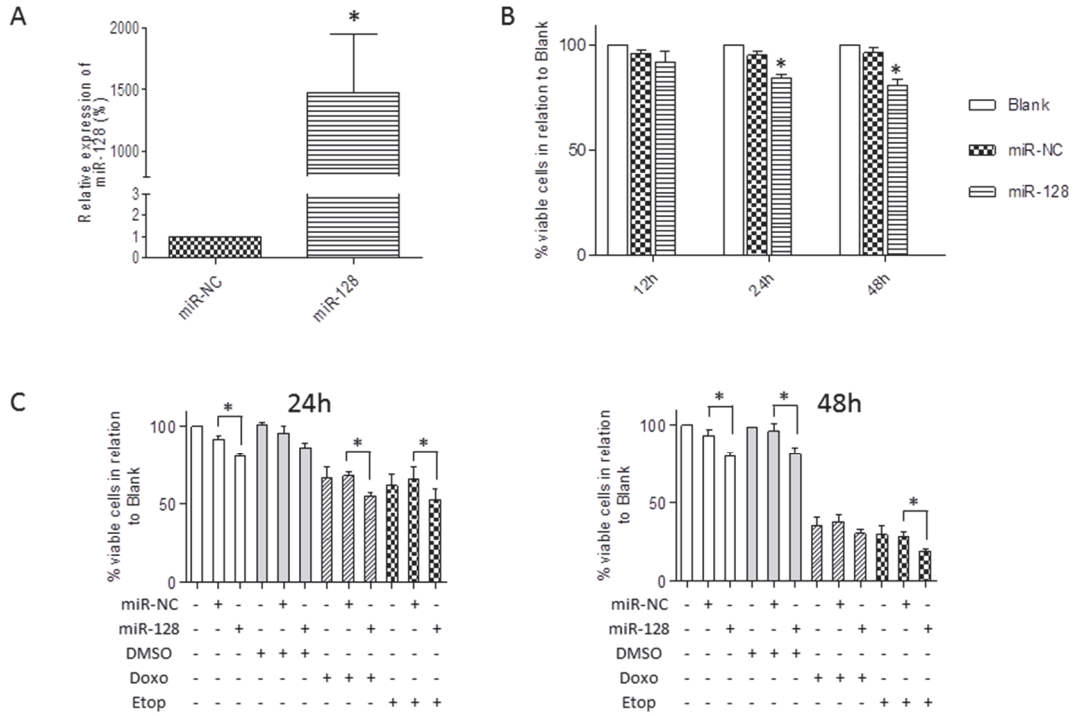


Figure 2

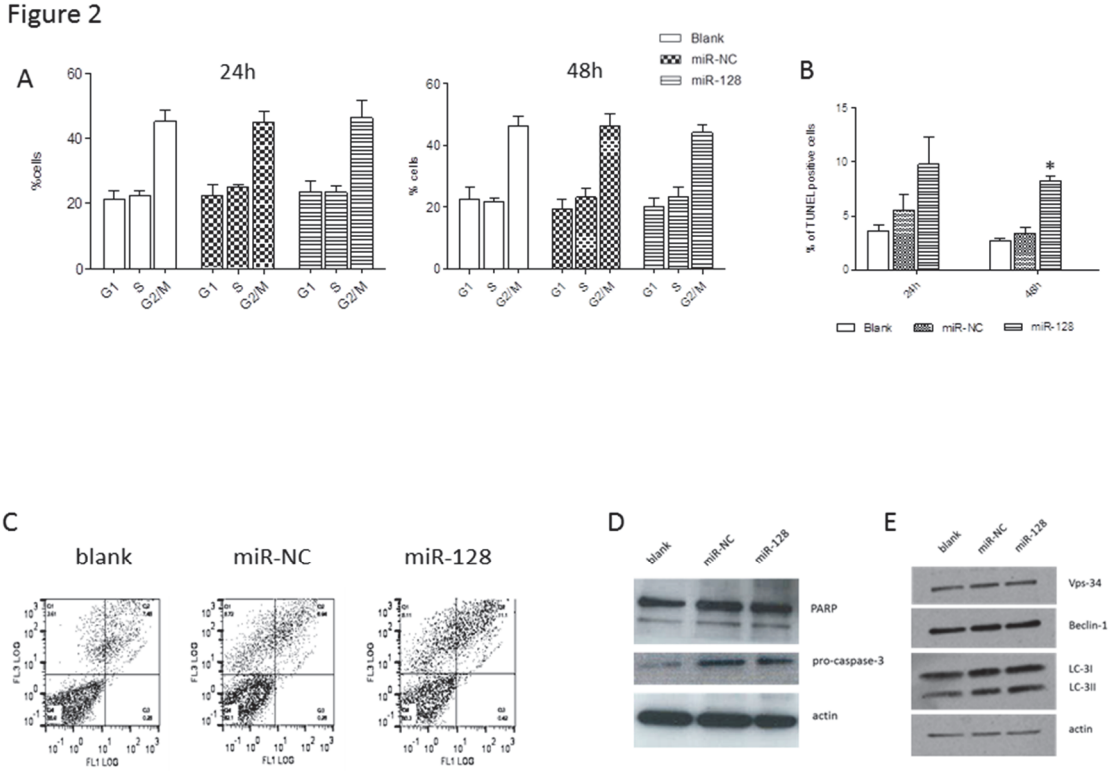


Figure 3

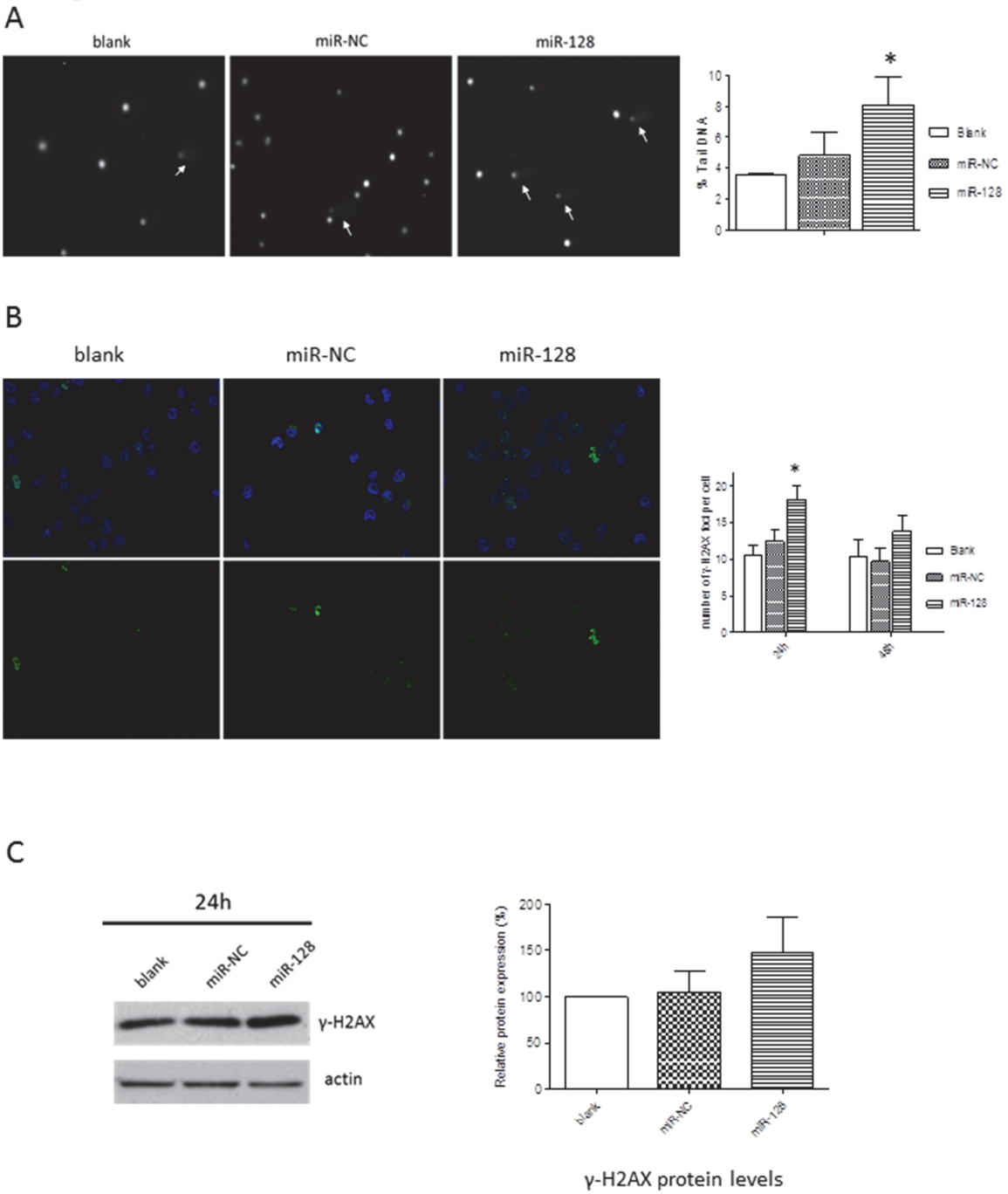
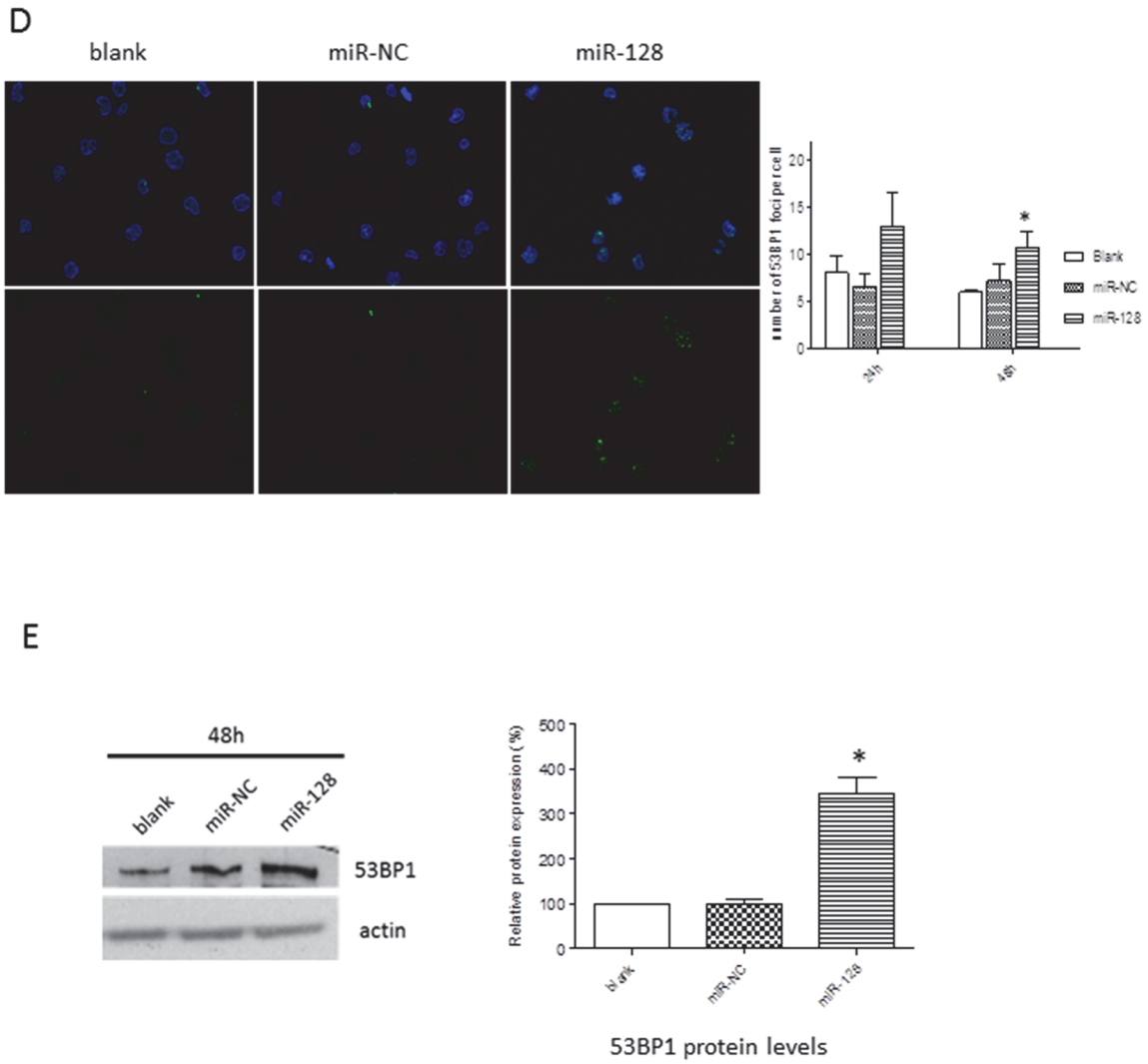

A
blank
miR-NC
miR-128
% Tail DNA
Blank
miR-NC
miR-128
B
blank
miR-NC
miR-128
number of γ-H2AX foci per cell
24h
48h
C
24h
blank
miR-NC
miR-128
γ-H2AX
actin
Relative protein expression (%)
γ-H2AX protein levels

Figure 3


D
blank
miR-NC
miR-128
Number of 53BP1 foci per cell
20
15
10
5
0
*
Blank
miR-NC
miR-128
24h
48h
E
48h
blank
miR-NC
miR-128
53BP1
actin
Relative protein expression (%)
500
400
300
200
100
0
*
blank
miR-NC
miR-128
53BP1 protein levels

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CHAPTER III.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

A new layer of gene expression regulation has been introduced with the discovery of miRNAs. RNAs are no longer bystanders in gene expression and have become 'first class' intervenients, now known to control numerous cellular processes. Since their discovery in human cells (in 1993) miRNAs have been thoroughly studied and their relevance in cancer initiation, development and drug resistance has been well established. In leukemia, miRNAs have also been intensively studied, and various miRNA expression profiles have been performed, to characterize different subtypes of these malignancies as well as to stratify patients in terms of prognosis and response to therapy. Nonetheless, the cellular function of specific miRNAs which have been shown to have altered expression in leukemias has not been fully understood.

Thus, driven by the will to add knowledge to this area, the aim of this work was to understand the cellular functions of two different miRNAs - miR-21 and miR-128 - known to be deregulated in CML and AML, respectively. For such, miRNA overexpression and downregulation approaches were used, and the resulting alteration in cellular mechanisms and response to chemotherapeutic drugs was studied.

miR-21 is well known to be overexpressed in almost all types of cancers (Volinia et al. 2006) including hematological malignancies (Jongen-Lavrencic et al. 2008). Its precursor and mature sequences are highly conserved across many species, suggesting a deeply conserved role for miR-21 in gene regulation (Selcuklu et al. 2009). In fact, its role as an oncomiR has also been well documented (Pan et al. 2010; Selcuklu et al. 2009).

The updated hallmarks of cancer (Hanahan and Weinberg 2011) include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism, and evading immune destruction. Moreover, genome instability and tumor-promoting inflammation are also considered two enabling characteristics of cancer (Hanahan and Weinberg 2011). By suppression of its function, miR-21 has been shown to be implicated in practically all of these mechanisms described by Hanahan and Weinberg (Buscaglia and Li 2011). Although it has a vast number of targets, miR-21 oncogenic function occurs predominantly by interfering with resistance to programmed cell death, particularly to apoptosis (Medina et al. 2010; Hatley et al. 2010).

The findings presented in this thesis show that miR-21 may also participate in autophagy, contributing to chemoresistance of leukemia cells to anti-cancer drugs. Indeed, by decreasing miR-21 expression, we verified that Bcl-2 expression was decreased, in agreement with a previously reported study (Dong et al. 2011) . Besides its anti-apoptotic role, Bcl-2 is also an autophagy inhibitor by binding to Beclin-1. The present work shows that downregulation of Bcl-2 led to a decrease of the amount of this protein that was

bound to Beclin-1, possibly allowing autophagy induction. Thus, this study reports a direct link between miR-21 targeting with antimiRs-21, its consequent decreased expression, and increased cellular autophagy. In another study, using malignant glioma cells, it was shown that decreasing miR-21 expression was associated with radio-sensitivity through enhanced autophagy, although miR-21 downregulation per se did not have any effect in autophagy (Gwak et al. 2012). These differences in the function of miR-21 by itself (without a drug "stimulus") might be related to the different cell types used in the present study and in the one by Gwak et al. (2012).

The present work also shows that autophagy is involved in cellular sensitization to the chemotherapeutic drugs doxorubicin and etoposide. Autophagy is a homeostatic mechanism for intracellular recycling and metabolic regulation and, at the same time, it is also stress responsive, since it is important for the removal of damaged proteins and organelles. In the cancer context, autophagy defects are associated with inflammation which may contribute to cancer formation (Mathew et al. 2007; White et al. 2010). Autophagy induction has been associated both with cancer progression and cancer inhibition, although the specific conditions determining whether autophagy boosts or inhibits cell death are not yet known. On some occasions, autophagy induces death (Ropolo et al. 2012; Gewirtz et al. 2009) and on other occasions, it plays a protective role rather than programming cell death (Ogier-Denis and Codogno 2003; Zou et al. 2012). Moreover, autophagy inhibition has been shown to augment the efficacy of anticancer agents in a variety of tumors (Degenhardt et al. 2006; Carew et al. 2007; Amaravadi et al. 2007). However, autophagy induction has also been associated with increased drug sensitivity as shown by the present work and others (Pardo et al. 2010; Newman et al. 2007).

The conventional view of cell communication is that it is mediated through gradients of soluble ligands which are identified by the cell-associated receptors. However, recent studies demonstrated a novel form of inter-cellular communication, through the shedding of membrane vesicles by donor cells, which can fuse to and be internalized by acceptor cells in the surrounding area, the so called "neighboring cells" (Al-Nedawi et al. 2008; Skog et al. 2008). These extracellular vesicles (EVs) can contain proteins and RNAs, including mRNAs and miRNAs. The importance of the presence of miRNAs in EVs gained additional attention when secreted miRNAs were shown to exert their function in the recipient cells (Kosaka et al. 2010; Cantaluppi et al. 2012). In particular, miR-21 has been shown to be incorporated in EVs (Chen et al. 2012; Chiba et al. 2012).

The term 'EVs' may account for different types of vesicles (from exosomes to microvesicles) and current available technology does not allow isolation of pure

populations of these EVs. Therefore, in this thesis, four different protocols were compared regarding EVs' isolation from K562 (CML) cells. The different isolation methods required different times to be executed and different amounts of cells to initiate the protocol. In addition, the different amounts of EVs recovered gave rise to different size population profiles. In particular, EVs isolated with the 'ExoQuick-TC' and the differential centrifugation protocols were the ones providing the most different population sizes. Nevertheless, even though the types (sizes) of the two EVs' populations extracted were different, no significantly differences in miR-21 levels were observed when comparing these two isolation protocols ('ExoQuick-TC' and differential centrifugation), suggesting that miR-21 is not specifically "packaged" into a particular type of EVs.

Given the fact that EVs are biocompatible, immunologically inert, can be patient-derived if required, have an innate ability to cross major biological barriers including the blood–brain barrier (Alvarez-Erviti et al. 2011) and can carry miRNAs, it is also possible that they could be used as 'drug' vehicles for the delivery of exogenous miRNAs or antimiRs. In fact, some studies have already shown that EVs can mediate the transfer of exogenous nucleic acids (Zhang et al. 2010; Akao et al. 2011; Pan et al. 2012). Furthermore, combinatorial EVs-based therapeutics will possibly be developed in the future.

This work also shows that miR-128 overexpression increases the DNA damage observed in leukemia HL-60 cells, possibly explaining the increased chemosensitivity to doxorubicin and etoposide that these cells (with miR-128 overexpression) presented. However, the chemosensitizing role of miR-128 is not definitively established. Other works have shown the opposite effect for miR-128 overexpression (Donzelli et al. 2012). Even more puzzling is the contradictory effect of miR-128 expression in breast cancer cells when treated with the same drug - doxorubicin (Zhu et al. 2011; Ji et al. 2013), inferring that the genetic background of the cells must play an important role in the outcome.

Once miRNAs are not known to directly cause DNA damage, it is supposed that miR-128 is interfering with the DNA damage response (DDR) signaling network. The DDR is a signal transduction pathway that decides the cell's fate to: i) repair the DNA damage, or ii) undergo apoptosis if there is too much damage. The DDR network has classically been regarded as consisting of a kinase signaling cascade that leads to the inhibition of Cdk-cyclin complexes and a delayed transcriptional response that promotes cell cycle arrest (Boucas et al. 2012). miRNAs act post-transcriptionally and are responsible for a third layer of DDR regulation. They have been shown to target and regulate the expression of several DDR intervenients (Han et al. 2012) and to be involved in (the initiation and

progression of) tumorigenesis and also modulate the sensitivity of cells to DNA damaging agents (Wouters et al. 2011).

DNA damage plays a dual role in cancer. On the one hand it causes mutations and/or chromosomal rearrangements, which may be the trigger for the carcinogenic process, and on the other hand it may be "used as therapeutic approach" since many cytotoxic drug treatments rely on the use of DNA damaging agents, such as radiation, alkylating agents and DNA-crosslinking compounds, to induce cell death in cancer cells. In this context, further studies would be of great interest to clarify possible DDR targets of miR-128 in leukemic cells, which might be responsible for the increased DNA damage and increased sensitivity to chemotherapeutic drugs that was observed in this work. This would be of particular interest as it could be an additional strategy to overcome therapy resistance that is often associated with radio- and chemotherapy.

In this work it was also shown that miR-128 expression in AML bone marrow samples was higher than in peripheral blood mononuclear cells. Although not statistically significant, these results suggest that these patients might have increased DNA damage and thus, might be more susceptible to cytotoxic agents. However, in order to be able to draw conclusions from these results, the sample size needs to be increased. At the same time, collecting post-treatment bone marrow samples from AML patients would also be very valuable, to confirm the hypothesis that these patients have increased susceptibility to cytotoxic agents.

In this thesis, two different strategies have been employed to modulate miRNAs expression: miRNA antagonists and miRNA mimics. These techniques are being developed so that they can be introduced in the clinics as new therapeutic drugs. miRNA antagonists are used to inhibit miRNAs that are overexpressed in human diseases. These antagonists are conceptually similar to other inhibitory approaches, like siRNAs and antisense molecules. During the process of drug development, the miRNA that is being targeted can also be used as a biomarker for pharmacokinetic and pharmacodynamics optimization of the antagonist. miRNA inhibitors are usually single-stranded oligonucleotides, complementary to the targeted miRNA, with chemically modified backbones that allow increased half-life and affinity to the endogenous miRNA and the seizing of these miRNAs in a configuration that is unable to be processed by RISC. Therefore, miRNA inhibition occurs upstream of RISC and is presumably independently of cellular cofactors (Hutvagner et al. 2004; Meister et al. 2004; Orom et al. 2006). In contrast, miRNA mimics are used to restore the expression of miRNAs which show a cellular loss of function. This miRNA replacement allows the delivery of "therapeutic miRNAs" as short double-stranded oligonucleotides (Wiggins et al. 2010) that act

downstream of RISC, thus requiring its enzymatic intervention. In this type of therapeutic approach the miRNAs would themselves be the 'drug', and the rationale for their development as therapeutics is that some of them present decreased expression in particular diseases. Their targets are irrelevant because the mimic carries the same sequence as the endogenous miRNA and is expected to regulate the same genes as in a 'non-disease' situation. the therapeutic activity of miRNA antagonists and miRNA mimics could be monitored through the measurement of endogenous levels of the "targeted miRNA" but phenotypic assays which can capture all miRNA-induced changes to the transcriptome (such as genome wide mRNA expression analysis), would be the preferred method for the monitoring (Bader et al. 2011; Bader et al. 2010).

Most clinical approaches such as the inhibition of some miRNAs (such as miR-10b, miR-21 or miR-451) or increasing the expression of other miRNAs (such as miR-16 or miR-34a) are currently only undergoing pre-clinical studies (Uchino et al. 2013). However, one miRNA antagonist is already undergoing clinical trials. Indeed, the miR-122-specific antagonist, is currently undergoing Phase IIa clinical testing for patients infected with hepatitis C virus (Janssen et al. 2013).

In spite of all these advances in miR-therapeutics, the main challenge for miRNAs ever becoming a 'true' therapy is the successful delivery of these molecules to the target tissue without compromising the integrity of the miRNAs (Davis et al. 2008; Castanotto and Rossi 2009; Whitehead et al. 2009).

In conclusion, this work provided more insight into the role of miRNAs in leukemia, and drug response in leukemia, contributing to the overall knowledge in this field.

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