Apoptomirs: small molecules have gained the license to kill

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Abstract

Apoptosis is a tightly regulated form of cell death and represents an important process during normal development. In the past years, the scientific community has produced remarkable advances in our understanding of cancer biology, realizing that apoptosis and the genes that control it have a profound effect on the malignant phenotype. Recently, a new class of non-coding RNA genes, known as microRNA (miRNA or miR), have been demonstrated to play important roles in diverse biological processes, including development, cell differentiation, proliferation, and apoptosis. This suggests that other oncogenic mechanisms are needed to produce selective pressure to override apoptosis during multistage carcinogenesis. Intriguingly, since most cytotoxic anticancer agents induce apoptosis, it is possible that defects in apoptotic programs may contribute to treatment failure. Several studies strongly suggest a role for microRNAs in modulating sensitive/resistant phenotypes to cytotoxic therapy, calling for further investigation and validation of microRNA functions and targets in order to improve sensitivity to cancer treatments, thus ultimately improving prognosis and survival. Here, we review the current findings about microRNAs focusing on their involvement in the apoptotic process.

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Introduction

MicroRNAs (miRNAs or miRs) are non-protein coding genes thought to regulate the expression of up to 30% of human genes, either inhibiting mRNA translation or inducing its degradation (Lewis *et al.* 2005). Besides a crucial role in cellular differentiation and organism development (Kloosterman & Plasterk 2006), miRs are frequently misregulated in human cancer (Lu *et al.* 2005, Volinia *et al.* 2006) and they can act as either potent oncogenes or tumor suppressor genes (Esquela-Kerscher & Slack 2006).

In humans, miRs are expressed as long precursor RNAs transcribed by RNA polymerase II, which are known as primary miRs (pri-miRs) that are capped at

the 5'-end and have a 3'-poly (A) tail (Lee *et al.* 2002, Cai *et al.* 2004).

Pri-miRs undergo a two-step maturation process to produce functional miRs. The first step occurs in the nucleus and is facilitated by Drosha, a cellular endonuclease, and the double-stranded RNA-binding domain protein DGCR8 (Lee *et al.* 2003, Han *et al.* 2004). Drosha cleaves both strands of the pri-miR in a staggered manner, producing a stem loop precursor molecule ~70 nucleotides in length known as a pre-miR (Lee *et al.* 2003, Kim 2005, Bushati & Cohen 2007). These precursors are subsequently transported to the cytoplasm by an exportin-5-dependent mechanism (Yi *et al.* 2003), where they are cleaved by the DICER enzyme and by a double-stranded RNA-binding domain protein TRBP, resulting in <17–24 nt mature miRs (Ketling *et al.* 2001, Chendrimada *et al.* 2005), which associate with a RNA-induced silencing-like complex (RISC; Bushati & Cohen 2007). The interaction of miR/RISC and its target mRNA results in gene regulation (Hammond *et al.* 2000).

This paper is one of 6 papers that form part of a special Focus Section on microRNAs. The Guest Editors for this section were Professor Alfredo Fusco, Naples, Italy, and Professor Carlo M Croce, Columbus, OH, USA; they were not involved in the handling of this paper, on which they are listed as an author.

The expression patterns, function, and regulation of miRs are largely unknown, but emerging data and the frequent location of miRs at fragile sites, common breakpoints, regions of amplification, or loss of heterozygosity suggest that they may play significant roles in human carcinogenesis. Each miRs has a distinct capability to potentially regulate the expression of dozens of coding genes and thereby modulate several cellular pathways including those involved in proliferation, apoptosis, and stress response (Yanaihara *et al.* 2006).

Apoptosis is an evolutionary conserved process by which organisms remove cells that are superfluous, have outlived their usefulness, or are dangerous for the survival of the organism.

The apoptotic process can be executed intracellularly, which involves the release of a number of factors from mitochondria and can be activated by a diverse set of stressors (intrinsic pathway) or through transmembrane death receptors (DRs), which are activated when they are engaged by their cognate ligands (extrinsic pathway; Fig. 1).

The intrinsic apoptotic pathway hinges on the balance of activity between pro- and anti-apoptotic members of the BCL2 superfamily of proteins, which act to regulate the permeability of the mitochondrial membrane (Coultas & Strasser 2003). The anti-apoptotic BCL2 proteins Bcl-2 and Bcl-xL act to prevent permeabilization of the mitochondrial outer membrane by inhibiting the action of the pro-apoptotic multi-domain BCL2 proteins BAX (a cytosolic protein) and BAK (found in the mitochondrial membrane; Reed 1998). Overexpression of Bcl-2 and Bcl-xL is known to be associated with a number of human malignancies (Igney & Krammer 2002, Bush & Li 2003, Coultas & Strasser 2003). Other pro-apoptotic BCL2 family members, including the BH3-only proteins PUMA and NOXA, act as cytosolic sensors of cell damage or stress (Karst & Li 2007). The intrinsic pathway begins with cell stress triggering the active transcriptional up-regulation of specific members of the pro-apoptotic BCL2 protein family involved in the promotion of apoptosis, such as the BH3-only proteins PUMA and NOXA. These, in turn, activate the multi-domain

Figure 1 Representation of the role of miRs in apoptosis. Pro-apoptotic molecules are depicted in blue, and anti-apoptotic molecules are depicted in fuchsia.

pro-apoptotic proteins BAX or BAK, which move to the mitochondrial membrane and disrupt the function of the anti-apoptotic BCL2 proteins, thereby allowing permeabilization of the mitochondrial membrane (Henry-Mowatt *et al.* 2004). Cytochrome *c* and the pro-apoptotic proteins SMAC/DIABLO are then able to leak from the intermembrane space of the mitochondria into the cytosol (Henry-Mowatt *et al.* 2004). Cytochrome *c* binds the adaptor protein apoptotic protease-activating factor-1, forming a large multi-protein structure known as the apoptosome (Fig. 1). The apoptosome then recruits and activates caspase-9, which, in turn, activates the downstream effector caspases, including caspases-3, -6, and -7, leading to apoptosis. Under normal conditions, caspase activity is held in check by a protein family known as inhibitor of apoptosis proteins (IAPs), of which at least ten have been identified, including XIAP, cIAP1, cIAP2, ILP2, MLIAP, SURVIVIN, and BRUCE (Deveraux & Reed 1999, Lavrik *et al.* 2005). IAPs are characterized by the presence of between one and three specific domains termed baculoviral repeats, which are directly involved in the caspase-inhibitory activity. While not directly involved in apoptotic signaling *per se*, some of these proteins prevent cell death by suppressing endogenous initiator and effector caspase activity. As part of the intrinsic apoptosis pathway, the SMAC/DIABLO protein released from the mitochondria promotes apoptosis by directly interacting with IAPs and disrupting their ability to inactivate the caspase enzymes (Srinivasula *et al.* 2000, Henry-Mowatt *et al.* 2004). Emerging evidence also suggests that IAPs may play a role in modulating cell division (Schimmer 2004). The IAPs survivin and cIAP1 are overexpressed in several malignancies.

The extrinsic pathway begins outside the cell through the activation of specific pro-apoptotic receptors on the cell surface by specific molecules known as pro-apoptotic ligands. These ligands include Apo2L/TRAIL (receptors DR4 and DR5) and CD95L/FasL (receptor CD95/Fas) (Ashkenazi 2002, Debatin & Krammer 2004, Rowinsky 2005, Fulda & Debatin 2006). Unlike the intrinsic pathway, the extrinsic pathway triggers apoptosis independently of the p53 protein (Rieger *et al.* 1998, Ravi *et al.* 2004).

Once activated by extracellular ligand binding, the intracellular domains of these receptors, known as the ‘death domains’, bind to the adaptor protein Fas-associated death domain (FADD), leading to the assembly of the death-inducing signaling complex, or DISC, and recruitment and assembly of initiator caspases-8 and -10 (Boldin *et al.* 1995, Chinnaiyan *et al.* 1995, Kischkel *et al.* 1995, Wang *et al.* 2001).

Caspases-8 and -10 are stimulated and undergo self-processing, releasing active enzyme molecules into the cytosol, where they activate caspases-3, -6, and -7, thereby converging on the intrinsic pathway (Fig. 1).

miRs and apoptosis

The involvement of miRs in apoptosis was first reported in 2003 when miR-14 and *bantam* were shown to regulate cell death in *Drosophila* (Brennecke *et al.* 2003, Xu *et al.* 2003). *Bantam*, originally identified as a gene that causes overgrowth of wing and eye tissue (Hipfner *et al.* 2002), was demonstrated to be a miR and was shown to promote proliferation and inhibit apoptosis by targeting the pro-apoptotic gene *hid1* (Brennecke *et al.* 2003). miR-14, identified in a screen for genes that alter cell death in the *Drosophila* eye, was demonstrated to inhibit apoptosis by regulating the effector caspase *Drice* (Xu *et al.* 2003).

In the following, we will discuss several miRs that are reported to be involved in cell death (summarized in Table 1 and Fig. 1).

miR-1/miR-133

The miR-1 subfamily contains two distinct genes, miR 1-1 and miR 1-2, which are specifically expressed in cardiac and skeletal muscle precursor cells, being direct transcriptional targets of muscle differentiation regulators, including serum response factor MyoD and MEF2 (Zhao *et al.* 2005). Excess miR-1 in the developing heart leads to a decreased pool of proliferating ventricular cardiomyocytes.

miR-1 and miR-133 derive from the same miR polycistron and are transcribed together (Chen *et al.* 2006). Interestingly, they carry out distinct biologic functions: miR-1 promotes myogenesis by targeting histone deacetylase-4, a transcriptional repressor of muscle gene expression, while miR-133 enhances myoblast proliferation by repressing serum response factor.

In addition, miR-1 and miR-133 are involved in regulating cell fate in cardiomyocytes (Xu *et al.* 2007). miR-1 and miR-133 produce opposing effects on apoptosis induced by oxidative stress in H9c2 rat ventricular cell, with miR-1 being pro-apoptotic and miR-133 being anti-apoptotic. Post-transcriptional repression of HSP60 and HSP70 by miR-1 and caspase-9 by miR133 contributes significantly to their opposing actions.

Recently, it has been demonstrated that miR-1 exerts its pro-apoptotic effect by regulating Bcl-2 expression on both mRNA and proteins levels (Tang *et al.* 2009).

Table 1 miRs involved in apoptosis

miR	Chromosome location	Function	Targets relevant to apoptosis
miR-1	20q13	Promotes myogenesis targeting HDAC4	HSP60 HSP70
miR-133	18q11	Enhances myoblast proliferation by repressing SRF	Caspase-9
Let-7/miR-98	Xp11	Development of <i>Caenorhabditis elegans</i>	NF2 Caspase-3
miR-15a	13q14	Regulates pancreatic regeneration	BCL-2
miR-16-1	13q14	Negatively regulates cell cycle progression and cell growth	BCL-2
		Regulates pancreatic regeneration	
miR-21	17q23	Unknown	PTEN TMP1 PDCD4
miR-29	7q32	DNA methylation	Mcl-1
miR-34a	1p36	Regulation of cell cycle	SIRT1 CDK4
miR-34b/c	11q23	Regulation of proliferation and adhesion-independent cell growth	CDK6
miR-106b-25	7q22	Regulates E2F1 involved in TGF β pathway	BIM
miR-17-92	17q31.3		
miR-221	Xp11.3	Regulation of erythropoiesis	p27 ^{Kip1}
miR-222	Xp11.3	Regulation of erythropoiesis	p27 ^{Kip1}

Let-7/miR-98

Let-7 was first identified in *Caenorhabditis elegans* and is one of the founding members of the miR family (Reinhart *et al.* 2000). The let-7 family consists of 11 very closely related genes (Pasquinelli *et al.* 2000). Let-7 is conserved in vertebrates and invertebrates; the expression increases after differentiation and in mature tissue but is barely detectable in the embryonic stage (Lee *et al.* 2005). This is due to the regulation of the processing of the let-7 family of miRs by LIN28, a developmentally regulated RNA-binding protein, which is involved in miR processing and promotes the reprogramming of human somatic cells into induced pluripotent stem cells (Yu *et al.* 2007). LIN28 selectively blocks the processing of pri-let-7 miRs in embryonic stem cells (ES) by specifically binding to conserved nucleotides in the loop region of the let-7 precursor and inhibiting the activity of Drosha and Dicer, thus having a central role in blocking miR-mediated differentiation in ES cells (Rybak *et al.* 2008, Viswanathan *et al.* 2008). These findings also establish a novel role for the miR precursor loop region in the regulated production of mature let-7 (Newman *et al.* 2008). These discoveries have started to reveal the intricate and complex web of regulatory networks through which miRs become part of the integrated mechanism of gene regulation that defines ES cell fate and behavior.

Previous studies suggest that let-7 is a tumor suppressor, as let-7 directly regulates and suppresses the RAS and HMGA2 oncogenes through their 3'-UTR

(Johnson *et al.* 2005, Akao *et al.* 2006, Lee & Dutta 2007). Moreover, a reduced let-7 level was also reported to correlate with elevated RAS expression in lung squamous carcinoma (Johnson *et al.* 2005). Apart from the role as a tumor suppressor, the involvement of let-7 in the cell proliferation pathway in human cells has been reported recently (Johnson *et al.* 2007). Let-7a, one of the let-7 miR members, was found to modulate the interleukin-6-dependent STAT-3 survival signaling in human malignant cholangiocytes by targeting the tumor suppressor gene NF2 (Meng *et al.* 2007a,b). By computer-based sequence analysis, the potential recognition sequence of let-7a was found on the 3'-UTR of caspase-3 but not on the other caspase members. Moreover, down-regulation of caspase-3, but not the other caspases, such as caspase-8 or -9, was observed in cells upon ectopic let-7a expression. By targeting caspase-3, a well-known effector caspase in apoptosis, let-7a was subsequently found to suppress the drug-induced apoptosis in A431 cells and HepG2, cells lines of two different tissue origins. Further confirmation of the role of caspase-3 in let-7a regulation of apoptosis is provided by the observations that the caspase-3 inhibitor attenuated the enhancement effect of let-7a knockdown on doxorubicin-induced apoptosis in HepG2, A431, and A10A cells and that transfection with anti-let-7a inhibitor did not have effect on doxorubicin-induced apoptosis in MCF-7 cells, which do not express the endogenous caspase-3. All these results draw the conclusion that let-7a regulation of apoptosis is mediated by caspase-3 (Tsang & Kwok 2008).

miR-15a and miR-16-1

Using bioinformatics tools, our group found that the miR-15a and miR-16-1 targeted the *BCL2* mRNA sequence (Cimmino *et al.* 2005). Bcl-2 is a central player in the genetic program of eukaryotic cells, favoring survival by inhibiting cell death (Cory & Adams 2002). Overexpression of the BCL2 protein has been reported in many types of human cancers, including leukemia, lymphomas, and carcinomas (Sanchez-Beato *et al.* 2003). In follicular lymphomas and in a fraction of diffuse B-cell lymphomas, the *BCL2* gene expression is deregulated through a translocation mechanism t(14;18)(q32;q21), which places the gene under the control of immunoglobulin heavy chain enhancers (Tsujiimoto *et al.* 1984, 1985). The fact that the *BCL2* gene is overexpressed in 65–70% of B-cell chronic lymphocytic leukemias (CLLs) and that deletions or down-regulation of the miR-15a and miR-16-1 cluster have been reported in the same proportion of CLL samples led us to hypothesize that miR-15a and miR-16-1 could be responsible for the increased levels of Bcl-2 in CLL (Cimmino *et al.* 2005). Indeed, our data showed that miR-15a and miR-16-1 expressions are inversely correlated with Bcl-2 expression in CLL samples and that both miRs negatively regulate Bcl-2 at a post-transcriptional level. Bcl-2 repression by these miRs induced apoptosis in a leukemic cell line model. In MEG-01 cells transfected with the wild-type miR-15/16, we observed an increased rate of apoptosis mediated by cleavage of pro-caspase-9 and poly (ADP-ribose) polymerase, indicating that the reduction in BCL2 protein levels caused by miRs is sufficient to initiate the apoptotic process (Cimmino *et al.* 2005).

Given that Bcl-2 is overexpressed in the majority of CLL malignant lymphocytes and no genetic alteration can explain this aberration, control of Bcl-2 expression by the miR-15/16 cluster can be considered as one of the main molecular mechanisms of this phenomenon. To further shed light on the tumor suppressor action of the miR-15a and miR-16-1 cluster, we analyzed the effects of both miRs on the transcriptome and proteome in CLL cells (Calin *et al.* 2008).

Ectopic expression of miR-15a and miR-16-1 led to the up-regulation of 265 genes and down-regulation of 3307 genes, among them the myeloid cell leukemia sequence 1 (*MCL-1*) anti-apoptotic gene and other predicted targets of miR-15 and miR-16. Interestingly, ~20% of the down-regulated genes had AU-rich elements (AREs), which could further support the finding that ARE-mediated instability is implicated in

the regulation of gene expression by miR-16-1 (Jing *et al.* 2005). Analysis of the proteomic changes after overexpression of the miR-15a and miR-16-1 cluster revealed 27 different proteins that exhibited at least fourfold changes with respect to the control group. Intriguingly, the proteins BCL2 and Wilms tumor 1, a confirmed and predicted target respectively, were among the proteins with prominent changes. Other proteins found are involved in cell growth, the cell cycle, oncogenesis, tumor suppression, and anti-apoptosis (Jing *et al.* 2005).

The tumor suppressor function of miR-15a/16-1 has also been addressed *in vivo*. In nude mice, ectopic expression of miR-15a/16-1 was found to cause dramatic suppression of tumorigenicity of MEG-01 leukemic cells exhibiting a loss of endogenous expression of miR-15a/16-1 (Calin *et al.* 2008). This observation confirms that miR-15a and miR-16-1 genes are tumor suppressors and are highly effective in preventing the growth of leukemic xenografts. The miR-15a/16-1 cluster targeting of BCL2 at the post-transcriptional level implies that miR genes play an important role in regulating apoptosis. Our data show that in cancer cells lacking these miRs, restoration of miR-15a and miR-16-1 triggers apoptosis and suppresses tumorigenicity (Cimmino *et al.* 2005). Therefore, it is possible that perturbation of the expression of oncosuppressor miR genes, such as miR-15a and miR-16-1, may lead to tumorigenesis.

miR-21

miR-21 is the most up-regulated miR across many cancer types (Volinia *et al.* 2006, Krichevsky & Gabriely 2009). Its implication as an anti-apoptotic factor was first reported in human glioblastoma cells where its down-regulation increased apoptotic cell death (Chan *et al.* 2005) and in a mouse model (Si *et al.* 2007). miR-21 has been identified as a potential regulator of the phosphatase and tensin homolog (PTEN) in hepatocellular carcinoma (HCC; Meng *et al.* 2007a,b). *PTEN* is a tumor suppressor gene, which negatively regulates cell proliferation and survival. Impairment of PTEN regulation is thought to play a role in oncogenic transformation (Keniry & Parsons 2008). Inhibition of miR-21 was shown to increase PTEN expression *in vitro*, determining decreased cells proliferation, migration, and invasion. On the other hand, overexpression of miR-21 produced the opposite effect, supporting a role for miR-21 in regulating proliferation and tumorigenesis. The decreased PTEN expression was accompanied by the induction of constitutive phosphorylation of the focal adhesion

kinase (FAK). FAK is a major target of PTEN, which is involved in cell cycle progression, cell survival, and migration (Park *et al.* 2002, Gautam *et al.* 2003). The opposite effect was seen upon inhibition of miR-21 in HCC cells. Furthermore, the regulation of PTEN by miR-21 was shown to increase expression of two matrix metalloproteinase (MMP)-9 and MMP-2, which are involved in invasion (Meng *et al.* 2007a,b) through FAK dephosphorylation in normal hepatocyte cells. Dissimilarly in breast cancer, Frankel *et al.* (2008) showed that overexpression or inhibition of miR-21 caused only subtle changes in PTEN expression, suggesting, as for other miRs, that functional targets of miR-21 may differ in different cell/tissue types.

miR-21 has been demonstrated to regulate the programmed cell death 4 gene (*PDCD4*; Lu *et al.* 2008). *PDCD4* is a tumor suppressor protein that is overexpressed during apoptosis (Lankat-Buttgereit & Göke 2009) and downregulated in human cancers. It has been shown to inhibit promoter-induced neoplastic transformation (Cmarik *et al.* 1999), tumor promotion, and progression (Jansen *et al.* 2005). The regulation of *PDCD4* by miR-21 is mediated by interaction at a miR-21 target site within the *PDCD4* 3'-UTR. This interaction negatively regulates *PDCD4*, influencing invasion and metastatic potentials of neoplastic cell (Asangani *et al.* 2008).

Similarly, a translational regulation of *PDCD4* by miR-21 has been demonstrated in MCF-7 breast cancer cells (Frankel *et al.* 2008), in embryonic kidney cells HEK293T, and in a mouse epidermal cell system (Lu *et al.* 2008). The authors also demonstrated that overexpression of miR-21 increased the colony formation in MCF-7 cells, indicating a role in anchorage-independent transformation in these cells, which supports what had been seen in colorectal cancer (Asangani *et al.* 2008).

Taken together, these data strongly indicate the regulation of *PDCD4* by miR-21, which appears to play a role in invasion and metastasis in both colorectal and breast cancers.

Since miR-21 is overexpressed in colon and breast cancers (Iorio *et al.* 2005, Asangani *et al.* 2008), deregulation of miR-21 may provide a mechanism for cancer initiation and progression in colorectal and breast carcinoma through its regulation of *PDCD4*.

Recently, miR-21 has been reported to target important tumor suppressor genes including the serpin peptidase inhibitor, clade B (ovalbumin), member 5 (*SERPINB5*/maspin; Zhu *et al.* 2008). Members of the human serpin family regulate a diverse array of serine and cysteine proteinases associated with essential biological processes such as fibrinolysis, coagulation,

inflammation, cell mobility, cellular differentiation, and apoptosis. Most serpins are secreted and attain physiological concentrations in the blood and extracellular fluids. However, a subset of the serpin superfamily, the ov-serpins, also resides intracellularly (Silverman *et al.* 2001). Different groups have demonstrated that maspin is involved in sensitizing cells to apoptosis (Tahmatzopoulos *et al.* 2005, McKenzie *et al.* 2008) by targeting AKT.

Hence, the oncogenic potential of miR-21 lies in its ability to regulate multiple cancer-associated pathways, probably via multiple cellular targets, which may partially explain its frequent up-regulation in cancer.

A recent study shows that miR-21 (Singh *et al.* 2008), the levels of which increase following the induction of mouse ES cell differentiation, has potential binding sites in the 3'-UTRs of the mRNAs that encode for Nanog, SOX2, and possibly OCT4. These are key proteins that are involved in ES cell self-renewal (Houbaviy *et al.* 2003). Interestingly, the transcription of miR-21 itself is regulated in ES cells by a transcriptional repressor called the RE1-silencing transcription factor, which directly interacts with *cis* elements upstream of the miR-21 gene (Singh *et al.* 2008).

miR-29

The miR-29 subfamily contains three paralogs: miR-29a, b, and c, which map at the common fragile site FRA7 (Calin *et al.* 2004). miR-29a is constitutively expressed in all phases of the cell cycle, miR-29b is expressed at low levels except in mitotic cells, and miR-29c is not detectable (Hwang *et al.* 2007).

miR-29b, which is down-regulated in lung cancer and in CLL (Calin *et al.* 2005, Yanaihara *et al.* 2006), has been recently reported to target the MCL-1 in cholangiocarcinoma cells (Mott *et al.* 2007). Mcl-1 is an anti-apoptotic member of the BCL2 family of proteins, which binds to pro-apoptotic members Bim and Bid preventing TRAIL-induced apoptosis (Chen *et al.* 2005). The TRAIL-signaling pathway involves the binding of TRAIL ligand to cell surface DR4 and 5 (Degterev *et al.* 2003, Johnstone *et al.* 2008). Upon binding, the receptor trimerizes (Wang & El-Deiry 2003), recruits the adaptor protein FADD, and initiates the caspase cascade, which results in apoptosis (Chinnaiyan *et al.* 1995). The TRAIL-signaling pathway has been shown to induce apoptosis in a number of transformed cell types both *in vitro* and *in vivo* (Schaefer *et al.* 2007). However, we found that some tumor cells display a TRAIL-resistant phenotype

(Garofalo *et al.* 2008). Overexpression of Mcl-1 is common in cancers such as cholangiocarcinoma, where its expression renders cells resistant to TRAIL-mediated apoptosis. Silencing of this protein has been shown to sensitize cholangiocarcinoma cells to TRAIL (Taniai *et al.* 2004). Mott *et al.* (2007) showed that the expression of miR-29 was inversely related to Mcl-1 expression, also demonstrating the ability of miR-29 to sensitize cells to TRAIL-mediated apoptosis.

Enforced expression of miR-29b renders tumor cells more sensitive to apoptosis-inducing activity of TRAIL, suggesting that the miR-29b/MCL-1 connection is functionally important and could be exploited for cancer therapy.

miR-34

The miR-34 family comprises three processed miRs that are encoded by two different genes: miR-34a is encoded by its own transcript on the short arm of chromosome 1 (1p36), whereas miR-34b and miR-34c share a common primary transcript on the long arm of chromosome 11 (11q23) (Hermeking 2009). The members of this miR family share a high similarity, suggesting that they may share the same targets (Chang *et al.* 2007). Different groups, independently, identified miR-34 as a target for p53 (Bommer *et al.* 2007, Chang *et al.* 2007, Corney *et al.* 2007, He *et al.* 2007, Raver-Shapira *et al.* 2007, Tarasov *et al.* 2007). Using a p53-inducible system, they reported that the miR-34 family of miRs is directly regulated by p53, and that miR-34 mediates growth arrest in multiple cell lines via direct 3'-UTR regulation of cell cycle regulatory factors, such as cyclin E2 (CCNE2), cyclin-dependent kinase 4 (CDK4), and the hepatocyte growth factor receptor (c-Met). Ectopic expression of miR-34 also resulted in a reduction of phospho-Rb, supporting the hypothesis that miR-34 regulates CDK4 and CCNE2. Furthermore, miR-34a was shown to directly target E2F3. Ectopic expression of miR-34 caused arrest in the G₁ phase of the cell cycle and induced cellular senescence in primary human fibroblasts (Bommer *et al.* 2007, He *et al.* 2007). In addition, miR-34b/c inhibited proliferation and colony formation in soft agar (Corney *et al.* 2007). Welch *et al.* (2007) reported that miR-34a induces apoptosis when reintroduced into the neuroblastoma cell lines that show decreased expression of miR-34a. Chang *et al.* (2007) showed that miR-34a-induced apoptosis is at least in part dependent on the presence of wild-type p53, indicating that miR-34a may feed back to p53. This observation was confirmed by Yamakuchi *et al.*

(2008) and linked to the targeting of the silent information regulator (*SIRT1*) mRNA by miR-34a. SIRT1 is an NAD-dependent deacetylase, which inhibits several pro-apoptotic proteins in response to oxidative and genotoxic stress (Longo & Kennedy 2006, Brooks & Gu 2009). miR-34a inhibition of SIRT1 increases p53 acetylation on lysine 382 with a subsequent increased transcriptional activity of p53, which determines the induction of p21 and PUMA, which regulate cell cycle and apoptosis respectively. Furthermore, miR-34 leads to apoptosis in cells where the p53 is present (Yamakuchi *et al.* 2008). These results could lead to a possible scenario where the induction of miR-34 genes allows p53 to regulate the expression of a large number of proteins, even after their transcripts have already been synthesized. This type of regulation may be advantageous in situations of cellular stress, as it does not require the translation of additional effector proteins that would presumably take too long to allow time for repair (Hermeking 2009). In addition, targeting of p53-induced mRNAs by miR-34 may contribute to the fine tuning of the p53 response and prevent an uncontrolled, irreversible response to p53 activation (Cohen *et al.* 2006).

miR-106b-25/107-92 clusters

The miR-106b-25 cluster is composed of the highly conserved miR-106b, miR-93, and miR-25, which are overexpressed in different types of cancer, including gastric, prostate, pancreatic neuroendocrine tumors, neuroblastoma, and multiple myeloma (Ambs *et al.* 2008, Petrocca *et al.* 2008, Kan *et al.* 2009, Li *et al.* 2009). These miRs are located in a 515 bp region at chromosome 7q22, in the intron 13 of their host gene *MCM7*, where they are cotranscribed in the context of *MCM7* primary transcript (Petrocca *et al.* 2008). Amplification of this region has been reported in several studies in gastric cancer (Peng *et al.* 2003, Weiss *et al.* 2004, Takada *et al.* 2005), and *MCM7* overexpression is an unfavorable indicator in prostate and endometrial cancers (Li *et al.* 2005, Laitinen *et al.* 2008). This raises the possibility that *MCM7* oncogenic properties could be linked, at least in part, to the hosted miRs. *MCM7* is induced by E2F1, a transcription factor that controls the G₁–S transition activating a variety of genes involved in DNA replication, and belongs to a family of specialized proteins that license chromosomal DNA to undergo replication once, and not more than once, at each cell cycle. It has been proposed that overexpression of *MCM7* may allow rereplication, thereby causing

aneuploidy. However, because *MCM7* mRNA and the intronic miR-106b-25 cluster derive from the same transcript, it is conceivable that MCM7 and miR-106b-25 cooperate in exerting their oncogenic function through different complementary mechanisms. Because *MCM7* mRNA and preliminary miR-106b-25 are cotranscribed, E2F1 regulates the expression of both (Petrocca *et al.* 2008). In fact, E2F1 increases the expression of MCM7 and miR-106b-25 precursors with identical kinetics, whereas E2F1 silencing by RNA interference parallels miR-106b-25 precursor down-regulation. Although the possibility of a miR-106b-25-independent promoter cannot be excluded, there are not known E2F1-binding sites up to –5000 bp from the miR-106b-25 locus, whereas *MCM7* mRNA and miR-106b-25 precursor levels perfectly correlate in primary gastric tumors and normal mucosa. Therefore, at least in the case of E2F1-dependent regulation, MCM7 drives miR-106b-25 cluster expression. It is very reasonable that other MCM7 transcriptional regulators, such as MYC, MYCN, and AIB-1, can also activate this cluster in specific contexts. For example, we observed MYC-dependent regulation of miR-106b-25, whereas MYCN, an oncogene frequently amplified in neuroblastoma, may cause the reported elevation of miR-106b-25 in these tumors. The picture becomes even more complex as mature miR-106b and miR-93 directly regulate E2F1 expression by interacting with highly conserved binding sites on its 3′-untranslated region. The miR-17-92 cluster is a prototypical example of a polycistronic miR gene. In the human genome, the miR-17-92 cluster encodes six miRs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), which are tightly grouped within an 800 bp region of human chromosome 13. Both the sequences of these mature miRs and their organization are highly conserved in all vertebrates. The human miR-17-92 cluster is located in the third intron of a 7 kb primary transcript known as *C13orf25* (Ota *et al.* 2004), and also regulates E2F1 expression through the same sites (Dews *et al.* 2006). This mechanism establishes a negative feedback loop between miR-106b-25/miR-17-92 and E2F1 that may work as a sensor to control E2F1 protein levels, providing cancer cells a way to escape E2F1-induced apoptosis. In fact, as with most oncogenes, overexpression of E2F1 above a critical threshold is perceived as an apoptotic signal. The functional implications of miR-106b-25 overexpression in gastric cancer are tightly linked with the transforming growth factor β (TGF β) tumor suppressor pathway. In fact, these miRs silence two main downstream

effectors of TGF β signaling: the cell cycle inhibitor CDKN1A (p21) and the pro-apoptotic gene *BCL2L1* (*BIM*) (Petrocca *et al.* 2008). Whereas miR-106b and miR-93 suppress p21 expression, which is required for TGF β -induced cell cycle arrest, miR-25 silences BIM expression, which is essential for TGF β -dependent apoptosis. Similarly, miR-17-5p and miR-20a repress p21 expression, whereas miR-92 inhibits BIM expression, suggesting that miR-106b-25 and miR-17-92 cooperate in inactivating the TGF β pathway. It is of note that the physiological role of these miRs emerges exclusively in the context of active TGF β signaling: in fact, their silencing does not visibly alter the proliferation and the survival of gastric cancer cells in the absence of TGF β . Nonetheless, miR-106b-25/miR-17-92 targets occupy critical nodes at the intersection between the TGF β pathway and the MYC network. Whereas the TGF β pathway suppresses proliferation by down-regulating MYC and inducing p21 and other CDK inhibitors, MYC impairs the TGF β pathway mainly by inhibiting p21 transcription. A fine balance between these pathways is crucial to maintain the proper control of cell proliferation and apoptosis. However, in normal cells, MYC is programmed to activate BIM and trigger apoptosis as a safeguard mechanism, if TGF β control should fail (Egle *et al.* 2004). The role of BIM in mediating miR-dependent apoptosis is supported by recent publications showing extensive apoptosis in the pre-B-cell compartment in B-cell-specific Dicer knockout (Koralov *et al.* 2008) and constitutive miR-17-92 knockout mice (Ventura *et al.* 2008) and expansion of the lymphocytic compartment in B-cell-specific miR-17-92 transgenic mice (Xiao *et al.* 2008). In these models, the observed phenotypes were associated with alterations in apoptosis and BIM expression.

That miR-106b-25 and miR-17-92 physiologically control apoptosis is supported by the fact that miR-17-92/miR-106b-25 double knockout mice exhibit a much more severe phenotype, characterized by prenatal lethality and extensive apoptosis in the liver and in other organs, compared with miR-17-92 single knockout mice (Ventura *et al.* 2008).

miRs-221/222

miR-221 and miR-222 genes are clustered on chromosome Xp11.3 (Ciafre *et al.* 2005). They have been reported to be significantly up-regulated in primary glioblastomas, in papillary thyroid carcinoma, and in prostate cancer (Ciafre *et al.* 2005, Galardi *et al.* 2007, Visone *et al.* 2007).

Recently, our group (Garofalo *et al.* 2008) has identified miR-221 and miR-222 as regulators of TRAIL sensitivity in NSCLC. TRAIL-resistant (CALU-1) and TRAIL-sensitive (H460) cell lines were identified; differences in sensitivity to TRAIL were not related to differences in endogenous receptor levels, as receptor levels were demonstrated to be comparable in both cell lines. miR analysis indicated differential expression of seven miRs in TRAIL-resistant cells compared to TRAIL-sensitive H460 cells. Overexpression of two of these miRs, miR-221 and miR-222, in TRAIL-sensitive cells increased resistance to TRAIL-induced cell death by ~40% and reduced activation of caspase-3 and -8. By contrast, inhibition of these miRs in TRAIL-resistant cells resulted in a TRAIL-sensitive phenotype, indicating a role in determining cell sensitivity to TRAIL. Putative targets, the proto-oncogene Kit, and tumor suppressor p27kip1 (Felli *et al.* 2005, Galardi *et al.* 2007), both of which play important roles in the cell cycle and its regulation, were shown to be downregulated in cells exhibiting resistance to TRAIL. *In vitro* inhibition and overexpression of miR-221 and miR-222 modulated expression of both Kit and p27kip1, strengthening evidence that these genes are targets. Silencing of p27kip1 increased cell resistance to TRAIL, implicating a role for this protein in maintaining sensitivity to TRAIL. These data strongly point to a role for miR-221 and miR-222 in determining cellular sensitivity to TRAIL-induced apoptosis in NSCLC, through the regulation of key proteins Kit and p27kip1. Interestingly, a recent study has demonstrated that negative regulation of p27kip1 by miR-221 and miR-222 in breast cancer cells conferred resistance to the chemotherapeutic drug tamoxifen (Miller *et al.* 2008), indicating a common role for these miRs in different cancers.

Conclusion

During the course of cancer development, a normal cell progresses towards malignancy by acquiring defects in the regulatory pathways that control normal cell proliferation and homeostasis. When these mechanisms fail, one or more mutations can become stable and interfere with cell cycle regulation and with repairing processes, thus causing the cell to accumulate more and more mutations. Over the past decades, our efforts were focused on understanding the roles of protein-coding genes in cancer. Recently, it has become quite clear that a novel class of non-coding genes, the miRs, regulate a diverse set of cellular processes from immune function to hematopoietic

lineage commitment, to embryonic development, to cell cycle regulation and apoptosis, supporting the idea that some synergism between several deregulated miRs and their protein-coding counterparts facilitates a favorable environment for cancer formation. The evasion of apoptosis underlies tumorigenesis and represents a major obstacle to successful therapy. Therefore, effort in the characterization of miR targets and function is imperative to improve our understanding of the role of miRs in tumorigenesis and facilitates the design of appropriate therapies targeting this novel group of molecules.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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