MiR-200c Regulates Noxa Expression and Sensitivity to Proteasomal Inhibitors

Mikael Lerner*, Moritz Haneklaus, Masako Harada, Dan Grandér

Department of Oncology-Pathology, Cancer Center Karolinska (CCK), Karolinska Institutet, Stockholm, Sweden

Abstract

The pro-apoptotic p53 target Noxa is a BH3-only protein that antagonizes the function of selected anti-apoptotic Bcl-2 family members. While much is known regarding the transcriptional regulation of Noxa, its posttranscriptional regulation remains relatively unstudied. In this study, we therefore investigated whether Noxa is regulated by microRNAs. Using a screen combining luciferase reporters, bioinformatic target prediction analysis and microRNA expression profiling, we identified miR-200c as a negative regulator of Noxa expression. MiR-200c was shown to repress basal expression of Noxa, as well as Noxa expression induced by various stimuli, including proteasomal inhibition. Luciferase reporter experiments furthermore defined one miR-200c target site in the Noxa 3'UTR that is essential for this direct regulation. In spite of the miR-200c:Noxa interaction, miR-200c overexpression led to increased sensitivity to the clinically used proteasomal inhibitor bortezomib in several cell lines. This apparently contradictory finding was reconciled by the fact that in cells devoid of Noxa expression, miR-200c overexpression had an even more pronounced positive effect on apoptosis induced by proteasomal inhibition. Together, our data define miR-200c as a potentiator of bortezomib-induced cell death. At the same time, we show that miR-200c is a novel negative regulator of the pro-apoptotic Bcl-2 family member Noxa.

Introduction

Death induced by the intrinsic mitochondrial pathway is initiated by perturbation of the mitochondrial membrane, and proceeds via release of cytochrome c and other apoptogenic factors from the intermembrane space of this organelle. This process is tightly regulated by the anti- and pro-apoptotic members of the Bcl-2 family [1]. Cytochrome c release in response to various types of cellular stress is suggested to occur via pores formed by homo and hetero-oligomers of the pro-apoptotic Bcl-2 family members Bak and Bax [2]. The actual ratio of anti- to pro-apoptotic Bcl-2 family members constitutes a sensor and sets the threshold of susceptibility to apoptosis for the cell. That the relative abundance of anti-apoptotic and pro-apoptotic regulators also critically influences tumorigenesis is illustrated by the recurring perturbation of this balance in cancer [3]. Consequently, the expression of Bcl-2 family members is normally tightly regulated at multiple levels including transcriptional activation and proteasomal degradation [1].

In recent years, microRNAs have emerged as important regulators of gene expression. MicroRNAs are 21–23 bp long non-coding RNAs that function mainly through targeting the 3'UTR of specific genes and thereby inhibiting the translation of their encoded protein or degrading the target mRNA [4,5]. With their ability to regulate multiple genes simultaneously, microRNAs have fundamental roles in such diverse processes as proliferation, apoptosis and differentiation. Furthermore, many microRNAs, such as those of the miR-15, let-7, or miR-17 families have been shown to be deregulated in cancer, resulting in the altered expression of target genes important for tumor development [6].

Some Bcl-2 family members have been shown to be regulated by microRNAs, such as Bcl-2, which is regulated by miR-15/16 and miR-148a, [7,8,9] and Mcl-1, which is regulated by miR-29 [10]. However, for many of the Bcl-2 family members, including the pro-apoptotic p53 target gene Noxa, it is unknown whether microRNA regulation takes place. Like other BH3-only proteins, Noxa has the capacity to bind and neutralize pro-survival Bcl-2 family members. However, it has a restricted binding pattern and mainly interacts with Mcl-1 [11]. Among other things, this interaction leads to proteasomal degradation of Mcl-1 [12,13], which in turn has been shown to be a prerequisite for apoptosis in response to for example UV irradiation [14].

Given the ability of Noxa to fine-tune apoptotic signaling in response to various stimuli, and that Noxa protein induction is necessary for cell death to occur following treatment with some cytotoxic cancer drugs [13], we set out to investigate if Noxa is regulated by microRNAs. Any given gene is generally predicted to be regulated by many different microRNAs [15]. One major obstacle in microRNA research is that the numerous bioinformatic tools available for target prediction invariably give a large set of false positive results [16]. Therefore, we made use of a luciferase-based screening method to pick out the most relevant microRNAs that target Noxa. Cloning the 3'UTR of Noxa downstream of a luciferase reporter and introducing this construct into cells allowed us to determine to what degree the reporter activity is repressed in different tissues. This analysis was then complemented...
MiR-200c Regulation of Noxa and Apoptosis

A. Relative luciferase activity

B. miRNA expression levels

C. miRNA targets

D. 

α-GAPDH

α-Noxa
with luciferase experiments using deletion constructs that pinpointed the critical regulatory part(s) of the 3′UTR. Finally, the combined results were then compared with existing microRNA expression profiling data [17] to identify candidate microRNAs(s) that might account for the differential luciferase activity. Using this screening system we identified miR-200c as a new regulator of Noxa. MiR-200c was shown to repress both basal and stress-induced Noxa protein expression. Surprisingly, enforced miR-200c expression at the same time led to increased bortezomib-induced apoptosis. This apparent discrepancy was reconciled by the finding that in cells devoid of Noxa expression, miR-200c caused an even greater increase in apoptosis. These data suggest that miR-200c potentiates apoptosis induced by proteasomal inhibitors but that it concomitantly represses Noxa which leads to an attenuated apoptotic induction.

The data in this study define miR-200c as a novel regulator of Noxa and more generally show that microRNA-induced phenotypes must always be viewed as the complex results of a large number of occurring individual microRNA:mRNA target interactions.

Materials and Methods

Constructs, Inhibitors and Cloning

The miR-200c cluster expression construct was created by nested PCR cloning using the pcDNA™ 3.3 TOPO® TA Cloning Kit (Invitrogen). As first round primers miR200c-F1 (5′-GATTGAGGTTGGTAAATCGG-3′) and miR200c-R1 (5′-AGGCACGTGGTAAATCTG-3′) were used. Nested primers were miR200c-F2 (5′-CTGCGTGTGCTACTCAACTCAG-3′) and miR200c-R2 (5′-GACTGGGTTAATCTGCT-3′) as first round primers and miR200c-F (5′-AGGCACGTGGTAAATCTG-3′) and miR200c-R (5′-CTGCGTGTGCTACTCAACTCAG-3′) as second round primers. The sequence of the cloned insert was verified in its entire length by sequence mutant psiCHK2-Noxa-Mut construct was created using the QuikChange II Site Directed Mutagenesis Kit (Stratagene). The Noxa overexpression plasmid was constructed by amplifying the Noxa coding region from a pool of cell line cDNAs using the KAPA2G Fast PCR mix (KAPABiosystems) with the primers Noxa-F (GGACTGTGTGCTACTCAACTCAG) and Noxa-R (TTTCCATCTAATAGTACTACAGCC). Amplicons were gel-purified and cloned into pcDNA3.3 using the pcDNA™ 3.3 TOPO® TA Cloning Kit (Invitrogen). The wild type sequence of the Noxa open reading frame in the construct was verified by sequencing.

Oligonucleotides and Chemicals

Pre-miR-control (AM17110) and pre-miR-200c (PM11714) were purchased from Ambion. The miR-200c anti-miR™ inhibitor (AM11714) and anti-miR™ Negative Control #1 (AM17010) were obtained from Ambion. Noxa siRNA oligos (5′-GUAUUUAUGACAUUCUG-3′) and control oligos targeting green fluorescent protein (5′-CAAGACUGACCCGAGUC-3′) were purchased from Eurofins MWG and have been described previously [18,19]. Bortezomib (Velcade®) was from Janssen-Cilag. MG132 was obtained from Calbiochem. Doxorubicin was purchased from MEZA AB.

Bioinformatic Sequence Analysis

MiRNAs potentially targeting the 3′UTR of Noxa were predicted using TargetScan (http://www.targetscan.org/) and PicTar (http://pic.tar.mdcb.berlin.de/). In order to qualify as a possible Noxa regulator, a microRNA had to be predicted to have at least one microRNA target site in the Noxa 3′UTR according to at least two out of three prediction algorithms. MicroRNA expression data was obtained from microRNA.org (http://www.microrna.org/).

Cell Lines, Transfections and Treatments

MCF7, HEK293 and U2OS cells, all originally obtained from American Type Culture Collection (ATCC) (http://www.lgsstandards-atcc.org/), were maintained in Dulbecco’s Modified Medium (DMEM/High Modified, Hyclone) containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin. HCT116 cells were grown in McCoy’s 5A medium (with L-glutamine, Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. HCT116 wild type and Dicer1 knockout cells [20] were kindly provided by B Vogelstein and K Kinzler (John Hopkins University and Howard Hughes Medical Institute), siRNA oligos and pre-miRNA oligos were transfected using HiPerFect reagent (Qiagen), according to the manufacturer’s instructions. Transfections of miRNA inhibitors and cotransfections of plasmids and pre-miRNA oligos were performed using Lipofectamine 2000 (Invitrogen). For plasmid transfection experiments, cells were transfected with TransIT®-LT1 Transfection Reagent (Mirus) or GeneJuice® Transfection Reagent (Novagen) according to the manufacturers’ protocol.
A.

Human  TCTTCAGTATTACTT
Chimp   TCTTCAGTATTACTT
Mouse   gCcTCAGcATTAagg
Rabbit gCcTCAGTAGATT
Cow     TCTTCAGTATTACTT
Dog     TCTTCAGTATTACTT
Elephant TtTcCAGTATTACTT
Armadillo TCTTCAGTATTACTT

B.

Noxa wt 3' UTR  5' ...CACAUCCUGUUUUCUUCAGUUAA... 3'
hsa-miR-200c  3' AGGUAGUAAUGGCGGCUCUAUU 5'
Noxa 3' UTR mutant  5' ...CACAUCCUGUUUUCUUCcgcggA... 3'

C.

![Bar chart showing expression levels of Noxa with and without miR-200c](chart)

D.

![Bar chart showing expression levels in different cell lines](chart)
Typically, 1.0 μg plasmid, 100 nM siRNA oligos, 50 nM premiRNA oligos or 200 nM miRNA inhibitors were transfected per 6-well, if not otherwise stated. Generally, cells were transfected, incubated for 24 hours and then treated with the indicated drug for the indicated time period.

Western Blot Analysis and Antibodies

Cells were lysed in NP-40 buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris pH 8.0) containing protease and phophatase inhibitors (Roche) and western blot analysis was performed as described previously [21]. The following primary antibodies were used: mouse monoclonal against Noxa (Calbiochem), rabbit polyclonal against GAPDH (Abcam), rabbit polyclonal against cleaved caspase 3 (Asp175, Cell Signaling) and rabbit polyclonal against cleaved PARP (Asp214, Cell Signaling). As secondary antibodies HRP-conjugated anti-rabbit and anti-mouse antibodies (Cell Signaling) were used.

 Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from indicated cell lines using Trizol reagent (Life Technologies) and reverse transcribed using Superscript II (Invitrogen) with random primers. Real-time PCR for Noxa (primers: Noxa-F 5’-AAGCCGAGCCACATCGCT-3’ and Noxa-R 5’-GAGGAGAGGACTGCC-3’, [22]) was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) in triplicate measurements on a 7900 HT Fast Real-time PCR system (Applied Biosystems). Samples were quantified using three point standard curves for each primer pair. Values were normalized to the relative quantity of GAPDH [primers: GAPDH-F 5’-AGCCGAGGCCACATCGCT-3’ and GAPDH-R 5’-GACACCATCCATCCATCTG-3’] with a similar result was obtained (Figure 1A, lower panel). This raised the possibility that one or several microRNAs that are expressed in MCF7 cells, but not in HEK293 or U2OS cells, regulate Noxa expression. We proceeded to compile the expression of all microRNAs predicted to target Noxa according to the TargetScan, PicTar and miRanda algorithms (Figure 1B). Luciferase reporter experiments were carried out using the Dual-Luciferase Reporter Assay System (Promega) as previously described [23].

Results

Luciferase-based Screening Identifies miR-200c as a Potential Regulator of Noxa

In order to evaluate whether Noxa is regulated by microRNAs, we first analyzed Noxa protein levels in HCT116 cells lacking Dicer1 (HCT116 Dicer1−/−), an RNase III enzyme required for microRNA processing. Noxa protein was increased in HCT116 Dicer1−/− as compared to wild type cells, suggesting that Noxa expression is under the influence of microRNA regulation (Figure S1). In order to identify which microRNAs that regulate Noxa, we first cloned the entire Noxa 3’UTR into the pmiR-REPORT vector downstream of luciferase. This vector was introduced into MCF7, HEK293 and U2OS cells and luciferase activity was measured. As can be seen in Figure 1A, luciferase expression was potently reduced in MCF7 cells while no repression was observed in the other cell lines. In order to exclude the possibility that the difference simply reflected differential usage of the promoter driving luciferase in the different cell lines, the Noxa 3’UTR was also cloned into the psiCHECK2 luciferase vector. Using this construct, a similar result was obtained (Figure 1A, lower panel). This raised the possibility that one or several microRNAs that are expressed in MCF7 cells, but not in HEK293 or U2OS cells, regulate Noxa expression. We proceeded to compile the expression of all microRNAs predicted to target Noxa according to the TargetScan, PicTar and miRanda algorithms (Figure 1B). Luciferase reporter truncation mutants with progressively shorter UTRs were created and introduced into MCF7 cells. Figure 1C shows that luciferase activity was restored already with the longest deletion mutant, indicating that the repressive element is located in the distal 0.5 kb of the Noxa 3’UTR. Of the three candidate microRNAs, only miR-200c has a predicted target site in the 3’UTR of the Noxa protein (Figure 1C). These results strongly suggest that miR-200c regulates the Noxa 3’UTR.

In conclusion, by making use of a luciferase-based screening method together with microRNA expression profiling we could identify miR-200c as a high-probability Noxa-regulating microRNA.

Noxa is a Direct Target of miR-200c

The Noxa 3’UTR contains one miR-200c target site that is evolutionarily conserved down to armadillo Dasypus novemcinctus (Figure 2A). In order to examine whether miR-200c regulates Noxa, we mutated the seed region of this site, which is thought to interfere with microRNA binding (Figure 2B). When cotransfect-
MiR-200c Regulation of Noxa and Apoptosis

A.

|        | 24h | 48h | 72h |
|--------|-----|-----|-----|
| pcDNA3.3 | pcDNA3.3-miR200c-cluster | pcDNA3.3-miR200c-cluster | pcDNA3.3-miR200c-cluster |

α-GAPDH - 40 kDa

α-Noxa - 10 kDa

B.

|        | pre-miR ctrl | pre-miR-200c | pre-miR ctrl | pre-miR-200c |
|--------|--------------|--------------|--------------|--------------|
| HEK293 | α-GAPDH      | α-Noxa       | HEK293       | U2OS         |

C.

![Graph showing protein expression over time](image)

D.

|        | 1 μM MG132 24h |
|--------|---------------|
| pcDNA3.3 | pcDNA3.3-miR200c-cluster |

α-GAPDH - 40 kDa

α-Noxa - 10 kDa

α-Noxa - 10 kDa

Short exp.

Long exp.
miR-200c Downregulates Noxa Expression

We transfected HEK293 cells, which have low endogenous miR-200c expression levels, with a vector encoding the miR-200c cluster and analyzed Noxa protein levels at different timepoints following transfection. As seen in Figure 3A, miR-200c overexpression resulted in a clear downregulation of Noxa expression at all timepoints analyzed. MicroRNA qRT-PCR was used to confirm proper miR-200c processing following plasmid transfection (Figure S2). Since the miR-200c cluster encodes both miR-200c and miR-141, we also transfected a pre-miR-200c oligonucleotide to investigate whether miR-200c expression alone is sufficient to repress Noxa. Expression of the pre-miR-200c oligonucleotide caused a clear downregulation of Noxa in several cancer cell lines (Figure 3B and data not shown). MicroRNAs repress gene expression by promoting RNA degradation and, to a lesser extent, by inhibiting translation [5]. Overexpression of the miR-200c cluster led to a significant downregulation of Noxa mRNA levels as measured by qRT-PCR (Figure 3C). This suggests that miR-200c indeed causes mRNA degradation of Noxa. Under unstimulated conditions, Noxa levels in cells are generally very low, but are known to increase under conditions of cellular stress [13]. Therefore, we assessed whether miR-200c can modulate Noxa levels when Noxa is induced by proteasomal inhibition. HEK293 cells were transfected with the miR-200c cluster or an empty control vector and subsequently treated with the proteasomal inhibitor MG132. As can be seen in Figure 3D, induction of Noxa protein was attenuated in cells with overexpressed miR-200c. Again, overexpression of the pre-miR-200c oligonucleotide resulted in a similar decrease in Noxa protein levels upon proteasomal inhibition (Figure 3D, right panel). This effect was not dependent on cell type as miR-200c-mediated repression of induced Noxa was evident also in HCT116 cells (Figure S3).

Together these results demonstrate that miR-200c can downregulate Noxa RNA and protein under both normal conditions and during cellular stress caused by proteasomal inhibition.

Noxa is Repressed by miR-200c Under Normal Conditions

In order to further examine whether the miR-200c-Noxa interaction takes place when miR-200c is expressed at endogenous levels, we made use of an inhibitor that binds miR-200c and prevents it from binding to its targets. When introduced into MCF7 cells a small, but consistent, increase in Noxa protein levels was observed (Figure 4A and data not shown). A similar picture emerged when cells were treated with MG132, again confirming that the miR-200c-mediated regulation takes place both under normal conditions and when Noxa is induced by exogenous stress (Figure 4B). Taken together, these results suggest that Noxa is regulated by endogenous miR-200c.

miR-200c Potentiates Proteasome Inhibitor-mediated Cell Death

Given the effect of miR-200c on Noxa, we hypothesized that it could modulate cellular sensitivity to apoptosis. We therefore...
A. 

| bortezomib | 10nM | 20nM | 30nM | 50nM |
|------------|------|------|------|------|
| pre-miR ctrl | pre-miR-200c | pre-miR ctrl | pre-miR-200c | pre-miR ctrl | pre-miR-200c | pre-miR ctrl | pre-miR-200c |
| α-GAPDH     |       |      |      |      | - 40 kDa |
| α-cleaved PARP |     |      |      |      | - 90 kDa |
| α-cleaved caspase 3 | |      |      |      | - 25 kDa |
| α-Noxa      |       |      |      |      | - 10 kDa |

B. 

![Bar chart showing % apoptotic cells for pre-miR ctrl and pre-miR-200c](image)

C. 

![Bar chart showing % apoptotic cells for untransfected, pre-miR ctrl + bortezomib, and pre-miR-200c + bortezomib](image)
evaluated the effect of miR-200c on apoptosis induced by the proteasome inhibitor bortezomib. This clinically used drug was chosen since it has been shown that Noxa induction is important for bortezomib-induced cell death [19,24,25]. Treatment of HCT116 cells with clinically relevant doses of bortezomib led to a time- and dose-dependent induction of Noxa protein (Figure S4). As can be seen in Figure 5A, overexpression of miR-200c in HCT116 cells treated with bortezomib led to a downregulation of Noxa at all doses. Surprisingly, at the same time miR-200c overexpression resulted in increased bortezomib-induced apoptosis as assessed by immunoblotting for cleaved caspase 3 and cleaved PARP (Figure 5A). In order to directly test how apoptosis induction is affected by miR-200c overexpression, Annexin V/PI staining was performed on HCT116 left untreated or treated with bortezomib. Again, in both cases miR-200c overexpression led to increased cell death, as compared to a scrambled pre-miR control oligonucleotide (Figure 5B–C). A similar result was obtained in the HEK293 cell line (data not shown). Also, this effect was not restricted to proteasome inhibition, as cells treated with the DNA-damaging drug doxorubicin showed increased apoptosis induction upon miR-200c overexpression as well (data not shown). Since the effects of miR-200c on Noxa and cell death induced by bortezomib apparently contradict one another, we went on to examine the effect of miR-200c on apoptosis in a setting without Noxa expression. Therefore, we knocked down Noxa expression in bortezomib-treated HCT116 cells using siRNA oligos. Knockdown of Noxa led to an expected decrease in both Noxa protein levels and proteasome inhibitor-induced apoptosis as measured by Annexin V/PI staining (Figure 6A). Interestingly, when Noxa was knocked down, miR-200c overexpression had an even more pronounced effect on apoptosis induction. Indeed, in cells transfected with control siRNA oligos, miR-200c overexpression led to a 33% increase in apoptosis, as compared to cells transfected with scrambled pre-miRs. In contrast, in cells with Noxa knocked down the increase in apoptosis was 78% (Figure 6A). To further investigate the relationship between miR-200c, Noxa and bortezomib-induced cell death, we went on to ectopically express a Noxa construct lacking the miR-200c target site (Figure S5). When Noxa was overexpressed in cells left untreated with bortezomib, only a minor effect on apoptosis could be observed (Figure 6B, left panel). However, overexpression of Noxa potentiated the positive effect of miR-200c on bortezomib-induced apoptosis (Figure 6B, 71% versus 97% increase in apoptosis), showing that artificially maintaining high Noxa levels in cells increases the pro-apoptotic effects of miR-200c even further. In summary, these data show that miR-200c sensitizes cells to bortezomib treatment. However, at the same time it represses Noxa, which leads to an attenuated bortezomib response.

Discussion

In this study we identify and validate miR-200c as a regulator of the proapoptotic BH3-only member Noxa. Much is known regarding the transcriptional regulation of Noxa. Several types of cellular stress, such as DNA damage and hypoxia, lead to Noxa induction in both a p53-dependent and independent fashion [13]. However, nothing has so far been reported concerning possible microRNA regulation of Noxa. The identification of miR-200c as a Noxa regulator was facilitated by a methodology that combines a luciferase-based screening with mining of microRNA expression data (Figure 1). This method is broadly applicable to the identification of other microRNA:target interactions.

Obviously, other mechanisms than microRNAs exist that regulate gene expression through the 3' UTR. Several recent studies have demonstrated the importance of for example RNA-binding proteins in posttranscriptional gene regulation [26,27,28]. However, it has also been shown that in many cases there is extensive interplay between microRNAs and RNA-binding proteins [29]. For example, miR-16 is necessary for the regulated turnover of AU-rich element (ARE) containing mRNAs by the ARE-binding protein tristetraprolin [30]. The fact that microRNA-mediated gene repression makes up a substantial part of 3' UTR-mediated regulation was substantiated in a recent report investigating the impact or shortened 3'UTRs on oncogenic transformation. When isoforms of varying 3'UTR length of the IMP-1 oncogene were used in soft-agar colony formation assays, it was demonstrated that the shorter isoforms were more oncogenic than the longer ones. Importantly, this difference in transformation ability was mostly attributed to loss of mRNA targeting, since microRNA target site mutants yielded significantly enhanced transformation from the longer isoforms [31]. One advantage with our method is that one is not restricted to the cell lines used in the current study and it is of course straightforward to change and expand the selection of cell lines to a set that is optimal for a given target gene. Furthermore, as more expression data is emerging, especially given the amounts of information originating from the recently developed mass sequenc-
Figure 6. Noxa attenuates the proapoptotic effect of miR-200c. (A) HCT116 cells were transfected with pre-miR-200c or pre-miR-control oligos together with Noxa or GFP control siRNA oligos for 24 hours. Cells were treated with 20 nM bortezomib for an additional 24 hours and
apoptosis was assessed by Annexin V/PI staining and FACS (upper panel). Cells were also collected and processed for immunoblotting for GAPDH and Noxa to demonstrate efficiency of siRNA knockdown (lower panel). Protein size in kilodaltons (kDa) is shown. (B) HCT116 cells were transfected with the indicated constructs and oligos. Samples were either left untreated (left panel) or treated with 20 nM bortezomib (right panel) and were subsequently analyzed as in (A). Graphs show the mean of percentages of Annexin V-positive cells, including PI-positive and PI-negative, from three independent experiments. Percentage values above graphs show fold increase in apoptosis when comparing indicated samples. doi:10.1371/journal.pone.0036490.g006

expression of, among other genes, E-cadherin and in this way miR-200 microRNAs help to maintain the cell in an epithelial state [33,34,35]. It is known that EMT is intimately linked to cancer development and that metastasizing cells undergo a process that is very similar to EMT. However, cancer cells can also undergo the reverse process, mesenchymal-to-epithelial transition (MET), when colonizing distant sites in the body following extravasation. In light of this it is perhaps not surprising that a complex picture emerges with regard to cancer and miR-200. While many tumor types, such as advanced breast cancer and clear cell carcinoma, show reduced miR-200 levels, some other malignancies instead display overexpressed miR-200 levels [33]. One speculative possibility is that downregulation of miR-200 occurs in some tumors when the cancer cells become invasive and that this is followed by miR-200 upregulation in distant metastases that undergo MET.

While the novel miR-200c target Noxa is dispensable for certain types of cell death, it is crucial for cell death in response to proteasomal inhibition [19,24,25,36,37,38]. The proteasome inhibitor bortezomib (PS-341, Velcade®) has been demonstrated to be clinically beneficial in the treatment of multiple tumor types, including myeloma and mantle cell lymphoma [39,40]. We therefore chose to study its impact in relation to miR-200c. The observed effects of miR-200c on Noxa and cell death induced by bortezomib and other agents might at first appear counterintuitive. Why would miR-200c potentiate apoptosis and repress Noxa at the same time? One possible reason is that is a matter of threshold. MiR-200c keeps Noxa in check to prevent premature or excessive apoptosis to occur. Once Noxa is induced to high enough levels following cellular stress, the interaction between miR-200c and Noxa becomes less relevant and other miR-200c targets play a more important role. Indeed, several targets have been described that could explain the pro-apoptotic effect of miR-200c, such as FAP-1 [41], PLCγ1 [32] and the above-mentioned ZEB1 [42]. In line with this, miR-200c has been described to potentiate apoptosis in response to CD95 signaling and microtubule-targeting agents [41,43]. Also, it is possible that the miR-200c:Noxa interaction plays a more dominant role in other tissues or when Noxa is induced by other stimuli. One has to bear in mind that the phenotypic effect of a given microRNA is dictated by the sum total expression of all its potential targets. Yet another possibility would be that Noxa for some reason has an anti-apoptotic effect in our systems. However, without Noxa expression, the positive effect of miR-200c on apoptosis becomes even more pronounced (Figure 6A), indicating that Noxa indeed potentiates cell death induced by bortezomib. In line with this, ectopic expression of a Noxa construct unresponsive to miR-200c regulation led to potentiation of miR-200c-mediated apoptosis induction (Figure 6B). We thus have a situation where the pro-apoptotic effect of miR-200c is partially counteracted by its repressive effect on Noxa. Interestingly, a similar scenario was described for miR-128. It apparently induces apoptosis in HEK293T cells while at the same time it directly represses the pro-apoptotic Bax protein [44].

In conclusion, we have identified miR-200c as an apoptosis-regulating microRNA that represses Noxa. The data presented have implications for the understanding of apoptosis in general, and Noxa regulation in particular. Furthermore, it can also help explain the mechanism behind bortezomib resistance in different tumors.

Supporting Information

Figure S1  Protein extracts were prepared from HCT116 DICERI wild type and knockout cells and analyzed for Noxa protein levels by immunoblotting. GAPDH was used as a loading control. (TIF)

Figure S2  HEK293 cells were transfected with the miR-200c cluster expression vector or an empty vector control. 48 hours post-transfection, cells were collected and processed for TaqMan qRT-PCR analysis. MiR-200c expression was normalized to that of the small nuclear RNA RNU148 using the comparative Ct method. The expression level in mock-transfected cells is set to 1. (TIF)

Figure S3  HCT116 cells were transfected with pre-miR-200c or pre-miR-control oligos. 24 hours post-transfection, cells were treated with the indicated concentrations of MG132 for an additional 24 hours, and processed for Noxa immunoblotting. GAPDH was used as a loading control. Protein size in kilodaltons (kDa) is also shown. (TIF)

Figure S4  Bortezomib induces a time- and dose-dependent increase in Noxa protein levels. HCT116 cells were treated with increasing concentrations of bortezomib and analyzed for Noxa protein expression (upper panel). HCT116 cells were treated with 20 nM bortezomib, collected at the indicated timepoints and processed for immunoblotting for the indicated proteins (lower panel). While Noxa is induced already after three hours, cleaved PARP and caspase 3 immunoblots demonstrate that apoptosis is not properly executed until after 24 hours of treatment. GAPDH was used as a loading control. Protein size in kilodaltons (kDa) is also shown. (TIF)

Figure S5  HCT116 cells were transfected with empty vector (pcDNA) or with a Noxa overexpression construct (pcDNA-Noxa). Protein extracts were analyzed for Noxa and GAPDH levels by immunoblotting. Protein size in kilodaltons (kDa) is also shown. (TIF)

Acknowledgments

The authors acknowledge Per Johnson for help with the microRNA qRT-PCR data.

Author Contributions

Conceived and designed the experiments: ML M. Haneklaus DG. Performed the experiments: ML M. Haneklaus M. Harada. Analyzed the data: ML M. Haneklaus M. Harada DG. Contributed reagents/materials/analysis tools: ML M. Haneklaus DG. Wrote the paper: ML M. Haneklaus DG. Obtained permission for use of cell line HCT116DICERI-/-: ML.
References

1. Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9: 47–59.
2. Tait SW, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11: 621–632.
3. Yip KW, Reed JC (2008) Bcl-2 family proteins and cancer. Oncogene 27: 6390–6406.
4. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
5. Hunzinger E, Izaurralde E (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 12: 99–110.
6. Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259–269.
7. Cimmino A, Calin GA, Ferracin M, Cimmino MA,ardi A, et al. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102: 15944–15949.
8. Xia L, Zhang D, Du R, Pan Y, Zhao L, et al. (2008) miR-133 and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer 123: 372–379.
9. Zhang H, Li Y, Huang Q, Ren X, Hu H, et al. (2011) MiR-141a promotes apoptosis by targeting Bcl-2 in colorectal cancer. Cell Death Differ.
10. Mott JL, Kobayashi S, Brunk SF, Gores GJ (2007) mir-29 regulates Mcl-1 expression and apoptosis. Oncogene 26: 6133–6140.
11. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JG, et al. (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 17: 393–403.
12. Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, et al. (2007) Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc Natl Acad Sci U S A 104: 6217–6222.
13. Ploner C, Koller R, Villunger A (2008) Noxa: at the tip of the balance between life and death. Oncogene 27 Suppl 1: S84–92.
14. Nijhawan D, Fang M, Traer E, Zhong Q, Guo W, et al. (2005) Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 17: 1475–1486.
15. Watanabe Y, Tomita M, Kanai A (2007) Computational methods for microRNA target prediction. Methods Enzymol 427: 65–86.
16. Bentsch T (2009) Prediction and validation of microRNAs and their targets. FEBS Lett 579: 5904–5910.
17. Landgraf P, Russe D, Shirerid R, Sewer A, Iovino N, et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 132: 1401–1414.
18. Lerner M, Harada M, Loven J, Castro J, Davis Z, et al. (2009) DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle involved in microRNAs miR-15a and miR-16-1. Exp Cell Res 315: 2941–2952.
19. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, Trichet V, Robillard N, et al. (2009) Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. Cancer Res 67: 5418–5424.
20. Cummins JM, He Y, Leary RJ, Pagliarini D, Diaz LA Jr., et al. (2006) The colorectal microRNAome. Proc Natl Acad Sci U S A 103: 3687–3692.
21. Lerner M, Corcoran M, Cepeda D, Nielsen ML, Zubarev R, et al. (2007) The RBCC gene RFP2 (Leu7) encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD. Mol Cell Biol 28: 1670–1682.
22. Lee SM, Kim JH, Cho EJ, Youn HD (2009) A nucleocytoplasmic malate dehydrogenase regulates p35 transcriptional activity in response to metabolic stress. Cell Death Differ 16: 738–748.
23. Thiery J, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, et al. (2002) Mechanisms of Interferon-alpha induced apoptosis in malignant cells. Cell Death Differ 12: 1894–1907.
24. Zhang Q, Huang S, Guo S, Zara D, Moc皇后A, et al. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 120: 623–634.
25. Mayr C, Bartel DP (2009) Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 138: 673–684.
26. Uhlmann S, Zhang JD, Schwager A, Mannsheger H, Rizzarelli S, et al. (2010) miR-200b/c/222 cluster targets PLCGamma1 and differentially regulates proliferation and EGF-driven invasion than miR-200a in breast cancer. Oncogene 29: 4297–4306.
27. Peter ME (2009) Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. Cell Cycle 8: 183–185.
28. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsikkin A, et al. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10: 593–601.
29. Park SM, Gaur AB, Lengyl E, Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22: 894–907.
30. Zhang L, Lopes H, George NM, Liu X, Pang X, et al. (2011) Selective involvement of BH3-only proteins and differential targets of Noxa in diverse apoptotic pathways. Cell Death Differ 18: 864–873.
31. Perez-Galan P, Pous G, Villanor M, Montserrat E, Campo E, et al. (2006) The proapoptotic inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. Blood 107: 257–264.
32. Barou M, Kolihaas SL, Butterworth M, Vogler M, Dinndal D, et al. (2010) Role of NOXA and its ubiquitination in proapoptotic inhibitor-induced apoptosis in chronic lymphocytic leukemia cells. Haematologica 95: 1510–1518.
33. Richardson PG, Mitani C, Schlossman R, Ghobrial I, Hideshima T, et al. (2008) Bortezomib in the front-line treatment of multiple myeloma. Expert Rev Anticancer Ther 8: 1053–1072.
34. Suh KS, Goy A (2008) Bortezomib in mantle cell lymphoma. Future Oncol 4: 149–161.
35. Schickel R, Park SM, Murrmann AE, Peter ME (2010) miR-200c regulates induction of apoptosis through CD95 by targeting FADD. Mol Cell 38: 908–915.
36. Magenta A, Cencioni C, Fasanaro P, Zaccagnini G, Greco S, et al. (2010) miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death Differ 15: 99–107.
37. Cochrane DR, Spechta NS, Howe EN, Nordeen SK, Richer JK (2009) MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. Mol Cancer Ther 8: 1055–1066.
38. Adhikari YK, Saini N (2011) MicroRNA-128 downregulates Bax and induces apoptosis in human embryonic kidney cells. Cell Mol Life Sci 68: 1415–1428.