miR-328 Functions as an RNA Decoy to Modulate hnRNP E2 Regulation of mRNA Translation in Leukemic Blasts

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SUMMARY

MicroRNAs and heterogeneous ribonucleoproteins (hnRNPs) are posttranscriptional gene regulators that bind mRNA in a sequence-specific manner. Here, we report that loss of miR-328 occurs in blast crisis chronic myelogenous leukemia (CML-BC) in a BCR/ABL dose- and kinase-dependent manner through the MAPK-hnRNP E2 pathway. Restoration of miR-328 expression rescues differentiation and impairs survival of leukemic blasts by simultaneously interacting with the translational regulator poly(rC)-binding protein hnRNP E2 and with the mRNA encoding the survival factor PIM1, respectively. The interaction with hnRNP E2 is independent of the microRNA’s seed sequence and it leads to release of CEBPA mRNA from hnRNP E2-mediated translational inhibition. Altogether, these data reveal the dual ability of a microRNA to control cell fate both through base pairing with mRNA targets and through a decoy activity that interferes with the function of regulatory proteins.

INTRODUCTION

Dysfunctional posttranscriptional gene regulation by sequence-specific RNA-binding proteins (RBPs) plays a critical role in the pathogenesis and evolution of human diseases (Carpenter et al., 2006; Glisovic et al., 2008; Keene, 2007), including the blastic phase of chronic myelogenous leukemia (Melo and Barnes, 2007; Perrotti and Neviani, 2007). In this disease stage, increased activity of the BCR/ABL oncoprotein alters processing, export, and/or translation of mRNAs encoding tumor suppressors, oncproteins, and critical regulators of differentiation by aberrantly modulating the expression and function of RBPs with sequence-specific mRNA-binding activity (Eiring et al., 2008; Perrotti and Neviani, 2007). Differentiation arrest in myeloid blasts crisis chronic myelogenous leukemia (CML-BC) depends on the BCR/ABL-MAPK-induced activity of hnRNP E2 (Chang et al., 2007; Perrotti et al., 2002), a poly(rC)-binding protein that controls translation of specific mRNAs (Ostareck-Lederer and Ostareck, 2004). In CML-BC, but not chronic phase (CML-CP) CD34+ bone marrow (BM) progenitors, hnRNP E2 is highly expressed and, upon interaction with the C-rich element in the 5′ untranslated region (UTR) of CEBPA mRNA, suppresses translation (Chang et al., 2007; Perrotti et al., 2002) of this master regulator of myeloid differentiation (Tenen, 2003).

hnRNP E2 RNA-binding activity does not require posttranslational modifications, and its expression is transiently induced by cytokines (e.g., IL-3) in myeloid precursors (Chang et al., 2007). Nonetheless, the early stages of myelopoiesis are characterized by cytokine-dependent proliferation and C/EBPα-mediated differentiation, suggesting that the hnRNP E2:CEBPA interaction might be prevented by a hnRNP E2-interacting protein or RNA, or by another RBP interacting with the CEBPA 5′UTR. Because small noncoding RNAs are regulators of critical cell functions...
including normal and leukemic hematopoiesis (Chen et al., 2004; Garzon et al., 2006), we hypothesized that differentiation arrest of myeloid CML-BC blasts results from impaired expression of a microRNA (miRNA) that, in addition to exerting its gene silencing activity through canonical binding to mRNA regulatory regions (Bartel, 2009; Friedman et al., 2009), also strongly competes with CEBPA mRNA for binding to hnRNP E2. Here, we report the existence of miRNA-dependent posttranslational control of biological processes through both base pairing with complementary mRNAs and sequence-dependent interference with the miRNA-regulatory function of RNA-binding proteins (decoy activity).

RESULTS

BCR/ABL-Dependent Downregulation of miR-328 Expression

Comparative miRNA arrays revealed that a discrete number of miRNAs were more than 3-fold modulated in a BCR/ABL dose- and kinase-dependent manner in cell line models and patient-derived myeloid CML-BC cells versus CML-CPC BM progenitors (not shown). Among the miRNAs downregulated in CML-BC (Garzon et al., 2006), we focused on miR-328 because its mature mature mRNA region (Figure 1A).

Northern blot and qRT-PCR analyses confirmed that miR-328 downregulation is markedly induced by BCR/ABL expression in 32Dcl3 myeloid precursors (32D-BCR/ABL) and primary lineage-negative (Lin−) mouse BM cells (Figure 1B). Reduction of miR-328 expression depends on BCR/ABL kinase activity as imatinib treatment (2 μM; 24 hr) significantly increased miR-328 expression in 32D-BCR/ABL and K562 cells (Figure 1B), as well as in Lin− BM cells from leukemic SCLTα-BCR/ABL mice (Figure 1C), suggesting its possible involvement in blastic transformation.

miR-328 Competes with CEBPA mRNA for Binding to hnRNP E2

To determine whether the C-rich element present in mature miR-328 is a bona fide hnRNP E2-binding site, RNA electrophoretic mobility shift assays (REMSA), UV crosslinking, and RNA immunoprecipitation (RIP) assays were performed using maltose-binding protein (MBP)-tagged hnRNP E2 (MBP-hnRNP E2), 32D-BCR/ABL cytoplasmic cell lysates expressing high levels of hnRNP E2 (Perrotti et al., 2002), and/or lysates of 32Dcl3 and 32D-BCR/ABL cells expressing a Flag-tagged hnRNP E2. REMSA (Figure 2A and Figure S1A available online) and UV crosslinking (Figure 2B) revealed that a 32P-labeled hiRNP E2 oligonucleotide efficiently formed a complex with recombinant, endogenous, and ectopic hnRNP E2 proteins but not with MBP alone or lysates of 32Dcl3 cells. Accordingly, the mobility of hiRNP E2:miR-328 complexes was identical to that formed using the uORF/spacer region of CEBPA mRNA (CEBPA uORF) oligonucleotide (Figures 2A and 2B), and miR-328 binding to hiRNP E2 was four times stronger than that of CEBPA uORF (Figure 2A). By contrast, the BCR/ABL-regulated miR-330, which is not C-rich, neither interacted with hiRNP E2 nor disturbed hiRNP E2:miR-328 complex formation (Figures 2A and 2B). miR-328 and CEBPA uORF binding to endogenous hiRNP E2 was also impaired by imatinib (Figure 2A) that, reportedly, inhibits hiRNP E2 expression in BCR/ABL+ cells (Chang et al., 2007).

To determine whether hiRNP E2:miR-328 interaction requires integrity of the miRNA seed sequence (Figure 2B, underlined nucleotides) that, as known, is essential for canonical miRNA: mRNA target interaction, UV crosslinking assays were performed using 32D-BCR/ABL and 32D-Flag-E2 lysates, as well as a miR-328 oligonucleotide harboring a mutated seed sequence that retains the wild-type C-rich character (miR-328-Mut)
Figure 2. miR-328 Competes with CEBPA mRNA for Binding to hnRNP E2

(A) REMSA shows binding of miR-328, CEBPA uORF, and miR-330 RNA probes to MBP-tagged hnRNP E2 (lanes 2, 4, 6–12), MBP (lanes 1, 3, 5), and cytoplasmic lysates of 32Dcl3 (lanes 14, 19) and untreated or imatinib-treated 32D-BCR/ABL cells (lanes 13, 15–18, 20–22). Cold competitor RNAs were used as indicated (lanes 8–9, 11–12, 17–18, 22).

(B) Top: UV crosslinking shows binding of miR-328 (lanes 1–3 and 10), CEBPA uORF (lanes 4–6), the seed sequence-mutated miR-328 (miR-328-Mut) (lanes 7–9), and miR-330 (lane 11) to hnRNP E2 in 32Dcl3, 32D-BCR/ABL, and 32D-Flag-E2 cell lysates (lanes 1–6). Bottom: Western Blots show levels of hnRNP E2 and GRB2 in the lysates used in UV crosslinking. Sequence of the wild-type and seed sequence-mutated miR-328 with substituted nucleotides indicated by asterisks.

(C) RNA Immunoprecipitation (RIP) assay for miR-328 performed on anti-hnRNP E2 immunoprecipitates (IPs) from lysates of untreated (lane 3), imatinib-treated (lane 5), and pSR-miR-328-transduced (lane 7) 32D-BCR/ABL (6.15 clone) cells. RIP with a nonrelated IgG served as controls (lane 8). IN: input RNA; MWM: molecular weight marker (lane 1); NTC: nontemplate control; -RT: no reverse transcribed PCR reaction. RIP was also observed with ectopically expressed Flag-hnRNP E2 proteins (see Figure S1).

(D) Left: RIP assays for CEBPA mRNA (top) and miR-328 (bottom) performed on anti-hnRNP E2 (lanes 5 and 9) and nonrelated IgG (lanes 3 and 7) IPs from parental (lanes 2–5) and miR-328-expressing (lanes 6–9) 32D-BCR/ABL cells. IN: RNA input; MWM: molecular weight marker (lane 1); NTC: nontemplate PCR control. hnRNP E2 RIP assays for a nonrelated mRNA (i.e., SET) are reported in Figure S1. Inset top right: Northern blot shows levels of ectopic miR-328 in vector- (lanes 1 and 3) and miR-328-transduced (lanes 2 and 4) 32Dcl3 and 32D-BCR/ABL cells. snRNA U6 was analyzed for normalization. Right: Densitometric analyses of the
(Figure 2B). As shown (Figure 2B), miR-328-Mut binds both endogenous and ectopic hnRNP E2 at higher affinity than wild-type miR-328, suggesting that the C-rich character rather than the seed sequence is important for hnRNP E2 interaction. Furthermore, the hnRNP E2:miR-328 interaction was also demonstrated in living cells by RIP assay. In fact, endogenous miR-328 was found associated with endogenous hnRNP E2 in anti-hnRNPs E2 but not anti-IgG immunoprecipitates (IP) from imatinib-treated 32D-BCR/ABL cells (Figure 2C). As expected, no binding was detected in untreated IL-3-cultured 32D-BCR/ABL cells (Figure 2C). Ectopic expression of miR-328 at physiological levels (Figure 2D and Figure S1B) also resulted in complex formation with both ectopic (Figure S1C) and endogenous (Figures 2C and 2D and Figure S1D) hnRNP E2. Note that Flag-hnRNP E2 did not significantly alter total hnRNP E2 levels (Figure S1A) and that miR-328 binding to hnRNP E2 impaired (~80% inhibition) hnRNP E2:CEBPA association (Figures 2D and 2E and Figure S1D). Furthermore, qRT-PCR analyses performed on anti-hnRNP E2 RIPs revealed that 4.26% ± 0.56% (n = 3) of the total miR-328 was associated to hnRNP E2 (not shown). As expected, hnRNP E2 did not interact with either a BCR/ABL-induced mRNA (i.e., SET) or the endogenous C-rich miR-223 (Figure S1D). Accordingly, ectopic miR-181b did neither interact with hnRNP E2 nor significantly alter levels of the hnRNP E2:CEBPA complex (Figure 2E). Thus, miR-328 specifically competes with CEBPA mRNA for binding to hnRNP E2.

hnRNP E2:miR-328 Interaction Does Not Associate with the RISC Loading Complex

To assess whether the hnRNP E2:miR-328 interaction occurs in the presence of major components of the RISC loading complex (Chendrimada et al., 2005), we evaluated whether hnRNP E2 is in complex with Dicer, Ago2, or TRBP2. Anti-hnRNPs E2 and anti-Dicer (Figures 3A and 3B) coimmunoprecipitation (coIP) experiments clearly show that hnRNP E2 did not interact with Dicer, TRBP2, or Ago2. Likewise, hnRNP E2 or Dicer immunodepletion did not alter levels of RISC components (Ago2 and TRBP2) or hnRNP E2, respectively (Figures 3A and 3B). As expected, TRBP2 was readily detectable in anti-Dicer IPs (Figure 3B). Thus, hnRNP E2:miR-328 interaction does not appear to require association with RISC loading components. However, RIP assays revealed miR-328 in complex with both Dicer and Ago2 (Figure 3C), suggesting that miR-328 also functions as a silencer of mRNA targets in a canonical RISC-dependent manner and that, most likely, its binding to hnRNP E2 is not mutually exclusive. Indeed, the presence of readily detectable hnRNP E2:miR-328 complex in RIP assays on Dicer-immunodepleted lysates (Figure 3C, lane 5) suggests that miR-328 might interact with hnRNP E2 in a RISC-independent manner.

miR-328 Rescues C/EBPα-Driven Granulocytic Differentiation of CML-BC CD34+ Progenitors

As loss of miR-328 may influence the phenotype of CML-BC myeloid blasts, we evaluated proliferation, clonogenic potential, levels of CEBPA mRNA and miR-328 associated to hnRNP E2 evaluated by RIP assays (n = 4) performed with parental (light bars) and miR-328-expressing (dark bars) 32D-BCR/ABL cells (mean ± SEM). (E) Left: RIP assays for CEBPA mRNA (top) on anti-hnRNPs E2 IPs from vector (pSuper)- (lane 5), miR-328- (lane 7), and miR-181b-transduced (lane 9) 32D-BCR/ABL cells; RIP assays for miR-181b (middle) and miR-328 (bottom) on anti-hnRNPs E2 IPs from miR-181b-expressing 32D-BCR/ABL cells (lanes 8 and 9). Inset top right: UV crosslinking with miR-328, miR-181b, and miR-330-labeled oligonucleotides and cytoplasmic lysates of 32Dcl3 (lanes 1, 3, and 5) and 32D-BCR/ABL (lanes 2, 4, and 6) cells. Binding of hnRNP E2 to miR-328 is shown in lane 2. Inset bottom left: Graph shows qRT-PCR analysis of miR-181b expression in 32Dcl3 (black), vector- (gray), and pSuper-miR-181b-transduced (white) 32D-BCR/ABL cells (mean ± SEM). Right: Densitometric analysis of the RIP’ed CEBPA mRNA associated to hnRNP E2 (n = 3), expressed as percentage of the input RNA (IN), in vector-, miR-328-, and miR-181b-transduced 32D-BCR/ABL cells (mean ± SEM). Controls for endogenous and ectopic hnRNP E2 protein and miR-328 expression levels and the specificity of the RIP protocol are shown in Figure S1.
Figure 4. miR-328 Rescues Granulocytic Differentiation through Restoration of C/EBPα Expression

(A) miR-328 levels in (left) 32Dcl3 cells undergoing G-CSF-induced differentiation; (middle) Lin−/Sca−/Kit− HSC, CMP/GMP/MEP committed progenitors and mature neutrophil BM subpopulations from wild-type C57BL/6 mice (mean ± SEM); and (right) CD34+ human BM cells undifferentiated (white) and induced to differentiate toward the erythroid (light gray), megakaryocytic (dark gray), granulocytic (red), or monocytic (black) lineages (mean ± SEM).

(B) Wright-Giemsa-stained cytospins of G-CSF-treated (0–7 days) pSuper-, miR-328-, miR-328-Mut-, miR-223-, and/or miR-181b-infected 32Dcl3 and/or 32D-BCR/ABL cells (mean ± SEM). Levels of miR-223 in BCR/ABL+ cell lines and primary cells and effect of ectopic miR-223 on cell proliferation are reported in Figure S2.

(C) % Post-mitotic cells (metamyelocytes, bands, segmented neutrophils)

(D) % Post-mitotic cells

(E) C/EBPα (p42), hnRNP E2 (37 kDa), GRB2

(F) CML-BC-pCDH, CML-BC-miR-223, CML-BC-miR-328

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and G-CSF-driven differentiation in miR-328-transduced GFP\(^+\) BCR/ABL\(^+\) cells, BCR/ABL\(^+\) Lin\(^-\) mouse BM, and/or CML-BC/CDDL cells. As controls, we assessed the effects of ectopic miR-328-Mut, miR-181b, and the myeloid differentiation-related (Chen et al., 2004) miR-223 in BCR/ABL cells. Of note, ectopic miR-328 or miR-223 levels in BCR/ABL cells were similar to those in nontransformed cells (Figure 2D and Figure S2A), thus excluding off-target effects due to overexpression.

Although endogenous miR-328 (Figure 1) and, to a lower extent, miR-223 (Figure S2B) were downregulated in 32D-BCR/ABL and K562 cells, their ectopic expression did not have a significant effect on IL-3-dependent and/or -independent growth (Figure S2B). Likewise, ectopic miR-328 did not accelerate the kinetics of 32Dc3 neutrophil maturation (Figure 4B), consistent with the barely detectable levels of hnRNP E2 in 32Dc3 cells (Perrotti et al., 2002) and the increased expression of endogenous miR-328 in 32Dc3, human CD34\(^+\) (n = 2) and mouse Lin\(^-\)/Sca\(^+\)/Kit\(^+\) (n = 3) BM (NBMT) progenitors undergoing granulocytic differentiation (Figure 4A). Conversely, miR-328 levels were not significantly different in normal CD34\(^+\) BM cells before and after differentiation toward other hematopoietic lineages (Figure 4A). As expected (Fazi et al., 2005), miR-223 enhanced 32Dc3 differentiation (not shown).

In agreement with the potential role of miR-328 as an antagonist of hnRNP E2 differentiation inhibitory activity, forced expression of miR-328 at physiological levels (Figure 2D and Figure S2A) efficiently rescued granulocytic differentiation of newly established (15 days after BCR/ABL infection) 32D-BCR/ABL cells (Figure 4B). In fact, the majority (82.1% \(\pm\) 3.9%) of miR-328-expressing 32D-BCR/ABL cells were postmitotic myelocytes, bands, and segmented neutrophils after 7 days of G-CSF-supplemented culture (Figure 4B). As expected, G-CSF-treated vector- and miR-181b-transduced BCR/ABL\(^+\) cells remained blasts (5.1% \(\pm\) 0.5% and 11.0% \(\pm\) 3.2% postmitotic cells) (Figure 4B). By contrast, miR-328-Mut expression efficiently induced 32D-BCR/ABL differentiation (88.6% \(\pm\) 3.1% postmitotic cells) (Figure 4B).

Although terminal differentiation was also a characteristic of miR-223-overexpressing 32D-BCR/ABL cells, only ectopic miR-328 but not miR-223 expression (Figure 2C) restored G-CSF-driven maturation of GFP\(^+\) CD34\(^+\) BM progenitors from myeloid CML-BC patients (n = 6) (Figure 4C). In fact, both miR-328- and miR-223-Mut-expressing CML-BC/CDDL BM cultures became bands and segmented neutrophils (88.8% \(\pm\) 2.4% and 85.2% \(\pm\) 4.2% postmitotic cells) after 10 days in rh-G-CSF (25 ng/ml). By contrast, morphology of miR-328-expressing CML-BC/CDDL (n = 6) progenitors remained similar to untransduced cells (n = 6), appearing arrested at the myeloblast stage after 7–10 days in G-CSF-containing medium (16.9% \(\pm\) 1.7% postmitotic cells) (Figure 4C) with unchanged levels of the granulocyte/macrophage markers CD11b or CD14 (not shown).

Consistent with the essential role of C/EBP\(_x\) in neutrophil maturation of BCR/ABL\(^+\) blasts (Ferrari-Amorotti et al., 2006; Perrotti et al., 2002; Wagner et al., 2006), C/EBP\(_x\) expression was readily detectable in miR-223- and miR-328-transduced 32D-BCR/ABL myeloid precursors, and in miR-328- but not miR-223-expressing CML-BC/CDDL BM progenitors and miR-181b-expressing 32D-BCR/ABL cells (Figure 4E). This was dependent neither on hnRNP E2 downregulation (Figure 4E) nor on increased CEBPA mRNA levels (Figure 4F and Figure S2C).

In agreement with its ability to bind hnRNP E2 expression (Figure 4E), indicating that the differentiation-promoting effects of miR-328 do not result from the seed sequence-dependent silencing of miR-328 mRNA targets. Furthermore, myeloperoxidase (MPO), a marker of granulocyte/macrophage commitment and a direct transcriptional target of C/EBP\(_x\) (Rosmarin et al., 1989; Wang et al., 2001), was also significantly increased in G-CSF-cultured miR-328- (18.7% \(\pm\) 0.2% [uninfected] versus 53.3% \(\pm\) 7.3% [miR-328]; p < 0.004) but not miR-223- (18.7% \(\pm\) 0.2% [uninfected] versus 22.2% \(\pm\) 3.6% [miR-223]; p = 0.28) expressing CML-BC/CDDL cells (n = 3) (Figure 4D). Finally, the dissimilar response of miR-223-transduced 32D-BCR/ABL versus CML-BC/CDDL progenitors to G-CSF might depend on differences in the mechanism(s) controlling expression of NFI-A (Figure S2D), a miR-223-negative regulator (Fazi et al., 2005).

miR-328 Restores CEBPA mRNA Translation Both In Vitro and In Vivo

As hnRNP E2:mir-328 binding in vitro is more efficient than that of CEBPA uORF (Figure 2), and miR-328 expression antagonizes hnRNP E2:CEBPA interaction (Figures 2D and 2E) most likely by competing for binding to hnRNP E2, it is plausible that miR-328 releases CEBPA from the translation inhibitory effects of hnRNP E2. Thus, we assessed the effect of miR-328 on CEBPA translation in rabbit reticulocyte lysate and in an in vivo mouse model of myeloid CML-BC. In the latter, BCR/ABL\(^+\) cell differentiation is driven solely by ectopic C/EBP\(_x\) expression, which is under the control of its uORF/spacer mRNA element (Chang et al., 2007). As reported (Perrotti et al., 2002), translation of CEBPA mRNA was markedly impaired (~80% inhibition) in vitro translation reactions programmed with a CEBPA construct containing the uORF/spacer intercistronic region (pcDNA3-WT-uORF-C/ EBP\(_x\)) and the recombinant fusion protein MBP-hnRNP E2 (Figure 5A) but not when performed in the absence of exogenous hnRNP E2 (Figure 5A). Addition of 1000-fold excess of mature miR-328 but not miR-330 resulted in an almost 100% increase of newly synthesized \(^{35}\)S-C/EBP\(_x\) protein (CEBPA+hnRNP E2...
versus CEBPA+hnRNP E2+miR-328; p < 0.005) (Figure 5A). Note that addition of miR-328 in the absence of MBP-hnRNP E2 did not significantly affect CEBPA mRNA translation (Figure 5A). Moreover, the large amount of MBP-hnRNP E2 (Figure 5A) might justify the incomplete rescue of CEBPA translation.

To assess whether forced miR-328 expression rescues neutrophilic maturation of differentiation-arrested BCR/ABL blasts through restoration of CEBPA mRNA translation, we used the aberrant 32D-BCR/ABL long-term cultured 6.15 cell clone that exhibits extremely high levels of BCR/ABL and hnRNP E2 but is unable to undergo G-CSF-driven differentiation due to transcriptional suppression of CEBPα expression (Figure 5C). Indeed, 6.15 cells completely rely on translation of ectopic CEBPA mRNA for differentiation. Thus, parental and 6.15 cells expressing a GFP-WT-uORF/spacer-C/EBPα (6.15-WT-uORF), which contains the hnRNP E2 translation inhibitory element, were retrovirally transduced with the pSUPERIOR-retro-puro-miR-328 (6.15-WT-uORF-miR-328) or with the empty vector (6.15-WT-uORF-pSUP). Differentiation assays confirmed that the ability of miR-328 to induce neutrophil differentiation is dependent on and mediated by the presence of CEBPA mRNA, as expression of miR-328 in parental 6.15 cells failed to rescue differentiation, whereas 91.7% ± 6.4% of 6.15-WT-uORF-miR-328 cells were postmitotic after 7 days of culture in G-CSF (Figure 5B). Furthermore, forced miR-328 expression neither decreased hnRNP E2 protein nor increased CEBPA mRNA levels (Figure 5C), suggesting that the restoration of C/EBPα protein expression in 6.15-WT-uORF-miR-328 cells (Figure 5C) results from the ability of miR-328 to interfere with hnRNP E2 translation inhibitory activity. Accordingly, anti-hnRNP E2 RIP assays performed with 6.15-WT-uORF-pSUP and 6.15-WT-uORF-miR-328 lysates revealed that miR-328 expression and, therefore, formation of the hnRNP E2:miR-328 complex (Figure 5D) markedly decreased levels of the hnRNP E2-bound CEBPA mRNA (Figure 5D).

To determine whether miR-328 influences the CML-BC-like disease process induced by transplantation of BCR/ABL-expressing cells, SCID mice (n = 13 per group) were intravenously injected with 6.15-WT-uORF-miR-328 or 6.15-WT-uORF-pSUP cells (5 x 10^5 GFP+puromycin-selected cells/mouse), and engraftment was assessed 1 week later by nested RT-PCR-mediated BCR/ABL detection in peripheral blood (not shown). After 3 weeks, three mice/group were sacrificed for visual and histopathologic examination of hematopoietic organs and for flow cytometric quantification of GFP+/GR1+ differentiated BM cells. Consistent with the almost complete hnRNP E2-dependent translational inhibition of C/EBPα expression in 6.15-WT-uORF cells (Chang et al., 2007), hematoxylin/eosin-stained sections of BM (Figure 5E), spleen, and liver (not shown) from 6.15-WT-uORF-pSUP-injected mice showed splenomegaly and massive infiltration of myeloid blasts with a low degree of differentiation. A few myeloid cells undergoing terminal neutrophil differentiation were occasionally observed in BM from 6.15-WT-uORF-pSUP-injected mice (mean fluorescence intensity [MFI]: 14.98 ± 1.84 GFP+/GR1+ BCR/ABL+ cells). By contrast, spleens from 6.15-WT-uORF-miR-328-injected mice appeared normal in weight or slightly hyperplastic, and histopathologic analysis of BM (Figure 5E), spleen, and liver (not shown) showed marked infiltration by mature neutrophils and myeloid precursors at postmitotic stages of differentiation (MFI: 63.84 ± 5.40 GFP+/GR1+ BCR/ABL+ cells) when compared to age-matched controls (Figure 5E), suggesting that miR-328 also negatively regulates survival pathways in CML-BC although its major effect appears to be on differentiation. In fact, although no significant difference in survival time was noted, the remaining 6.15-WT-uORF-pSUP-injected mice died of a CML-BC-like leukemia, whereas 6.15-WT-uORF-miR-328-injected animals succumbed from an aggressive CML-CP-like myeloproliferative disorder (not shown). Altogether, these in vitro and in vivo results indicate that rescue of granulocytic maturation of differentiation-arrested BCR/ABL+ cells by miR-328 is likely due to its direct binding to hnRNP E2 that, in turn, prevents translational inhibition of CEBPA mRNA.

**A BCR/ABL-MAPK-hnRNP E2 Pathway Suppresses mir-328 Transcription through Inhibition of C/EBPα**

We recently reported that high levels of BCR/ABL expression/kinase activity, as observed in CML-BC (Jamieson et al., 2004; Schultheis et al., 2005), impair C/EBPα expression through the MAPK(ERK1/2)-dependent regulation of hnRNP E2 expression/activity (Chang et al., 2007). To determine whether BCR/ABL uses the same signaling pathway to suppress miR-328 expression in CML-BC, miR-328 levels were evaluated in G-CSF-cultured (24–48 hr) parental and newly established 32D-BCR/ABL cells treated with imatinib (2 μM) or MEK1 inhibitors U0126 (25 μM) and CI-1040 (10 μM) and after overexpression (MSCV-Flag-E2).

**Figure 5. In Vitro and In Vivo Interference of miR-328 with hnRNP E2 Translation Inhibition of C/EBPα Expression**

(A) Levels of newly synthesized 35S-C/EBPα protein in RRL translation reactions programmed with CEBPA mRNA (derived from pcDNA3-WT-uORF-C/EBPα) (black), CEBPA mRNA and mature miR-328 RNA oligonucleotides (dark gray), CEBPA mRNA and recombinant MBP-hnRNP E2 protein either alone (light gray) or in the presence of mature miR-328 (red), or miR-330 (white; negative control) RNA oligonucleotides. Data are expressed as percentage of the mean ± SEM and are representative of three different experiments performed in duplicate. Inset: Western blot shows levels of both endogenous RRL hnRNP E2 and recombinant MBP-hnRNP E2.

(B) Wright-Giemsa-stained cytopsins of G-CSF-treated (0–7 days) 6.15-pSUP, 6.15-miR-328, and 6.15-WT-uORF-miR-328 cells (mean ± SEM).

(C) Left: Levels of hnRNP E2, endogenous and HA-tagged C/EBPα, and GRB2 proteins and miR-328 and snRNA U6 in parental 32Dc13, 6.15-pSUP-transduced, and miR-328-transduced 6.15-WT-uORF cells: right; RT-PCR and qRT-PCR show levels of CEBPA mRNA in 32Dc13, 6.15, 6.15-miR-328, and 6.15-WT-uORF-HA-CEBPA cells either uninfected or infected with pSUP or miR-328 constructs. GAPDH levels were measured for normalization (mean ± SEM).

(D) RIP assays for CEBPA mRNA (top) and miR-328 (bottom) on anti-hnRNP E2 (lanes 5 and 9) and nonrelated IgG (lanes 3 and 7) IPs from 6.15-uORF-pSUP (lanes 2–5) and 6.15-uORF-miR-328 cells (lanes 6–9). IN: input RNA.

(E) Top: H&E-staining of BM shows maturation of BCR/ABL+ cells in mice injected with p-SUPERIOR vector (middle) and miR-328-transduced (right) 6.15-WT-uORF cells. Age-matched mice (left) served as a control. FACS analysis shows mean fluorescence intensity (MFI; mean ± SEM) of differentiated GFP+ GR1+ BCR/ABL+ cells at 3 weeks post-transplant from BM of 3 mice/group. Bottom: Visual analysis and weight of spleens from the same groups of mice (mean ± SEM).
or shRNA-mediated downregulation (pSR-hnRNP E2 shRNA) of hnRNP E2. Inhibition of BCR/ABL or MEK1 kinases strongly enhanced miR-328 expression (Figure 6A), suggesting that BCR/ABL-mediated suppression of miR-328 requires MAPK (ERK) activity. Ectopic hnRNP E2 impaired miR-328 expression in 32Dcl3 cells, with no noticeable effect in 32D-BCR/ABL cells (Perrotti et al., 2002). By contrast, shRNA-mediated downregulation of hnRNP E2 efficiently rescued miR-328 expression (Figure 6B) that already express high levels of hnRNP E2 (Perrotti et al., 2002). By contrast, shRNA-mediated downregulation of hnRNP E2 efficiently rescued miR-328 expression (Figure 6B). Thus, the BCR/ABL-MAPK-induced hnRNP E2 may directly regulate miR-328 nuclear export, processing, and/or stability or indirectly influence miR-328 transcription. Of interest, modulation of hnRNP E2 levels did not alter miR-223 expression in 32D-BCR/ABL cells, whereas hnRNP E2 overexpression inhibited miR-223 in 32Dcl3 cells (Figure S2E), consistent with the notion that hnRNP E2 reduces C/EBPα expression (Perrotti et al., 2002), thereby averting C/EBPα-dependent miR-223 transactivation (Fazi et al., 2005).

Transcription Element Search System-mediated (http://www.cbil.upenn.edu/cgi-bin/tess) sequence analysis revealed four putative C/EBPα-binding sites scattered within 1500 bp upstream of the mouse pre-miR-328 (Figure 6C). Thus, chromatin immunoprecipitation (ChIP) assays were performed using nuclear extracts from GFP-sorted HA-C/EBPα-expressing 32Dcl3 cells (32D-HA-C/EBPα) and four sets of primers, each encompassing one of the potential C/EBPα-binding sites. In vivo physical interaction between HA-C/EBPα and the miR-328 promoter region was detected in ChIP assays performed on anti-HA but not anti-Flag (negative control) immunoprecipitates with primer sets #1 and #3 containing the human/mouse-conserved C/EBPα-binding sites located at nucleotides −1119 to −1112 and −565 to −557, respectively (Figure 6C). Accordingly, ectopic C/EBPα expression markedly induced miR-328 levels in myeloid precursors (Figure 6C), altogether suggesting that a BCR/ABL-MAPK-hnRNP E2 pathway downregulates miR-328 expression through inhibition of C/EBPα, thus impeding enhancement of miR-328 transcription (Figure 6D).

**Figure 6. Pathways Regulating miR-328 Expression**

miR-328 levels in 32Dcl3 and/or 32D-BCR/ABL cells (A) treated with imatinib or the MAPK inhibitors U0126 and CI-1040 or (B) expressing a Flag-hnRNP E2 (left) or a shRNA-targeting hnRNP E2 (right).

(C) Top: Representation of the C/EBPα-binding sites within the miR-328 promoter; bottom: Chromatin immunoprecipitation (ChIP) with anti-HA antibody shows binding of HA-C/EBPα to miR-328 promoter sequences in 32D-HA-C/EBPα cells (left: ChIP blot; middle: densitometric analysis). Anti-Flag immunoprecipitates served as negative controls. Bars indicate the mean ± SEM from three independent experiments; northern and western blots (right) show levels of miR-328 and HA-C/EBPα, respectively, in parental and 32D-HA-C/EBPα cells. U6 snRNA and GRB2 protein levels were used as controls.

(D) Model of the molecular network regulating miR-328 expression in CML-BC and miR-328 decoy activity in BCR/ABL* myeloid cell differentiation by direct interference with hnRNP E2 translation inhibition of C/EBPα expression.

or shRNA-mediated downregulation (pSR-hnRNP E2 shRNA) of hnRNP E2. Inhibition of BCR/ABL or MEK1 kinases strongly enhanced miR-328 expression (Figure 6A), suggesting that BCR/ABL-mediated suppression of miR-328 requires MAPK (ERK) activity. Ectopic hnRNP E2 impaired miR-328 expression in 32Dcl3 cells, with no noticeable effect in 32D-BCR/ABL cells (Perrotti et al., 2002). By contrast, shRNA-mediated downregulation of hnRNP E2 efficiently rescued miR-328 expression (Figure 6B). Thus, the BCR/ABL-MAPK-induced hnRNP E2 may directly regulate miR-328 nuclear export, processing, and/or stability or indirectly influence miR-328 transcription. Of interest, modulation of hnRNP E2 levels did not alter miR-223 expression in 32D-BCR/ABL cells, whereas hnRNP E2 overexpression inhibited miR-223 in 32Dcl3 cells (Figure S2E), consistent with the notion that hnRNP E2 reduces C/EBPα expression (Perrotti et al., 2002), thereby averting C/EBPα-dependent miR-223 transactivation (Fazi et al., 2005).

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**miR-328 Impairs CML-BC<sup>CD34+</sup> Clonogenic Potential and Canonically Suppresses PIM1 Expression**

Ectopic miR-328 expression reduced colony formation in 32D-BCR/ABL, Lin<sup>−</sup> SCL-TCR/BCR/ABL (n = 5), and CML-BC<sup>CD34+</sup> (n = 2) BM cells by 75%, 83%, and 75%, respectively (Figure 7A). The effect of miR-328 on clonogenicity of BCR/ABL<sup>+</sup> cells is independent from hnRNP E2:miR-328 interaction, as shRNA-mediated hnRNP E2 downregulation did not affect BCR/ABL-driven colony formation (Figure 7A). Thus, miRanda, PicTar, and TargetScan bioinformatics algorithms were utilized to identify miR-328 targets regulating CML-BC progenitor cell survival. Among the predicted miR-328 human/mouse mRNA targets,
PIM1 (Figure 7B) kinase is important for survival of BCR/ABL+ cell lines (Nieborowska-Skorska et al., 2002). PIM1 protein was strongly upregulated in a BCR/ABL kinase-dependent manner in cell lines and CD34+ CML-BC (n = 2) versus CML-CP and NBM BM cells (Figure 7C).

Ectopic miR-328 expression in 32D-BCR/ABL, K562, and CML-BC/CD34+ cells decreased PIM1 protein without significantly affecting its mRNA levels (Figure 7D), suggesting that miR-328 might impair mRNA translation upon interaction with the PIM1 3′UTR. To formally demonstrate that miR-328 silences PIM1 expression through its interaction with the miR-328-binding site, we cloned the wild-type (pMX-Flag-WTPIM1-WT3′UTR) and miR-328-binding site-deleted (pMX-Flag-WTPIM1-Δ3′UTR) PIM1 3′UTR (Figure 7E) into a pMX-Flag-WTPIM1 plasmid and transduced these constructs into 32D-BCR/ABL-miR-328 or, as a negative control, 32D-BCR/ABL-miR-223 cells. As expected, ectopic Flag-PIM1 expression was lower in pMX-Flag-WTPIM1-WT3′UTR-transduced 32D-BCR/ABL-miR-328 cells but not in cells transduced with pMX-Flag-WTPIM1-Δ3′UTR or with PIM1 cDNA only (pMX-Flag-WTPIM1) (Figure 7E). Accordingly, Flag-PIM1 expression derived from pMX-Flag-WTPIM1-WT3′UTR was barely detectable in miR-328- but not in miR-223-transduced cells, in which its expression was similar to that of Flag-WTPIM1 and Flag-WTPIM1-Δ3′UTR (Figure 7E), indicating that decreased ectopic PIM1 levels are not due to loss of other mRNA-binding sites within the 196 bp-deleted 3′UTR. Expression of the seed sequence-mutated mir-328 (miR-328-Mut) impaired the ability of miR-328 to canonically silence PIM1 expression through its interaction with the miR-328-binding site (Figure 7F). Thus, miR-328 specifically silences PIM1 expression through interaction with the PIM1 3′UTR. In agreement with the importance of PIM1 for survival of BCR/ABL+ cells, expression of a wild-type PIM1 cDNA lacking the 3′UTR (WT PIM1) but not of a kinase-deficient (KD PIM1) PIM1 cDNA into 32D-BCR/ABL-miR-328 cells (Figure 7G) completely restored IL-3-independent colony formation (Figure 7G), suggesting that miR-328-dependent inhibition of BCR/ABL-driven clonogenic potential results from direct PIM1 downregulation. Note that WT PIM1 alone did not affect 32D-BCR/ABL-pSR-EV clonogenic potential (Figure 7G).

**DISCUSSION**

Altered miRNA expression has been tightly associated with cancer development and progression (Friedman et al., 2009; Garzon et al., 2006). Among the miRNAs differentially expressed in CML, we focused on miR-328 and provided a series of evidence highlighting two important concepts. The first represents a paradigm shift to the notion that miRNAs act primarily as negative posttranscriptional regulators of gene expression and proposes for miRNAs a function termed *decoy activity*. The second identifies miR-328 as a molecular relay, the loss of which is important for the differentiation arrest of progressing CML-BC blasts.

miR-328 Decoy Activity

As miRNAs base pair with mRNA 3′UTRs in a sequence-specific manner (Bartel, 2009), it is conceivable that miRNAs could interfere with the activity of RNA-binding proteins (e.g., hnRNPs), either indirectly by pairing with RBP-binding sites contained in specific mRNAs (George and Tenenbaum, 2006) or directly through binding the RBP itself and impeding RBP:miRNA interaction. Herein we demonstrated that miRNAs can act as direct inhibitors of RBP activity. In fact, miR-328 specifically interacts in a seed sequence-independent manner and, most likely, through its C-rich clusters, with the translational inhibitor poly (rC)-binding protein hnRNP E2. This, in turn, prevents and/or displaces CEBPA mRNA binding to hnRNP E2 and rescues CEBPA mRNA translation both in vitro and in vivo. In support of the notion that miR-328 and, most likely, other miRNAs may act as “decoy” molecules for RBPs, which upon binding could control synthesis, processing, export, stability, and/or translation of specific mRNA subsets, a proteomics-based study in epithelial A431 cells reported that forced miR-328 expression not only decreased levels of different genes but also upregulated a subset of proteins (Wang et al., 2008). Interestingly, 37% of these upregulated proteins have miRNAs with complex 5′UTRs (e.g., uORF or multiple ATGs) containing C-rich elements representing potential hnRNP E2-binding sites. Thus, it is reasonable to speculate that upregulation of some of these proteins might result from interference with hnRNP E2 activity. Furthermore, there is evidence that miRNAs, other components of the RISC complex (Parker and Sheth, 2007) and RBPs (e.g., hnRNP E2) are present in processing bodies (P bodies) (Fujimura et al., 2008), dynamic subcellular structures where mRNAs are complexed with RBPs and/or miRNAs for translational suppression or decay (Parker and Sheth, 2007). In this scenario, miR-328 may compete with CEBPA mRNA for binding to hnRNP E2 that, in turn, releases CEBPA and allows its loading onto polysomes for translation. It is also possible that hnRNP E2 not only prevents C/EBPα-dependent induction of pri-miR-328 transcription but also directly promotes miR-328 decay. Accordingly, an inverse correlation exists between hnRNP E2 and miR-328 expression in CML-BC, and hnRNP E2 more efficiently binds to miR-328 than to CEBPA. However, as hnRNP E2 continuously shuttles between nucleus and cytoplasm as well as in and out of P bodies (Fujimura et al., 2008; Makeyev and Liebhaber, 2002), knowledge of the subcellular location where initial binding of hnRNP E2 to miR-328 or CEBPA occurs remains elusive, although a plausible assumption is the cytoplasm as their association was detected using cytoplasmic extracts.

MicroRNA interaction with sequence-specific RBPs is not unprecedented; however, none of the reported mechanisms match the case of hnRNP E2 and miR-328. For example, hnRNP A1, another RBP upregulated in CML-BC (Perrotti and Neviani, 2007), binds the primary miR-17–92 transcript to allow processing of pre-miR-18a (Guil and Caceres, 2007). Interestingly, expression of the miR-17–92 cluster is upregulated in CML and is important for proliferation and reduced susceptibility to apoptosis of K562 cells (Venturini et al., 2007). However hnRNP E2:miR-328 interaction does not seem to affect miR-328 biogenesis, as no accumulation of primary or precursor miR-328 was detected in BCR/ABL+ cells (not shown). It was also shown that miR-369-3 interacts with the AU-rich region (ARE) of TNF-α mRNA, which recruits the AGO2-FXR1 RBP complex to the ARE element itself and upregulates or represses translation under serum-starved or proliferating conditions, respectively.
Figure 7. miR-328 Impairs Survival through Targeting of PIM1 Kinase mRNA

(A) IL-3-independent or -dependent methylcellulose colony formation (mean ± SEM from triplicates of three independent experiments) of vector- (gray bars) and miR-328-transduced (red bars) 32D-BCR/ABL cells (IL-3-independent), leukemic Lin−/C0SCLtTA-BCR/ABL (n = 5) (IL-3-dependent) cells, CML-BCCD34+ (n = 2) (IL-3 dependent) BM progenitors, and vector- (pSUPER) or hnRNP E2 shRNA (pSUPER-shE2)-infected 32D-BCR/ABL cells (IL-3-independent). Inset: Western blot shows hnRNP E2 levels upon shRNA knockdown.

B

\[ 5' \ldots AT-GGAAGAGGTACAGGGCAAA ... 3' \quad 1893-1915 \text{ hPIM1} \\
\[ 5' \ldots ATGGAAGAGGTACAGGGCAAA ... 3' \quad 1594-1615 \text{ mPIM1} \\
\[ 3' \ldots U-GCUUUCGUCUCUCUCUC ... 5' \quad \text{hsa-miR-328} \]

C

D

E

F

G

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K/E2-dependent CML might favor disease progression by also promoting hnRNP to miR-328 but can be extended to other miRNAs containing
expression of CGG-rich miRNAs (e.g., miR-572 and miR-638). Thus, it is likely that the decoy activity of miRNAs is not limited
activity of the AU-rich element (ARE) RNA-binding proteins AUF1 (hnRNP D) and HuR (Lal et al., 2004), respectively, can
also speculate that the mRNA stabilizing and destabilizing
mRNAs controlling synaptic function (Brown et al., 2001; Darnell et al., 2003), might be influenced by the altered
retardation syndrome and normally regulates translation of
expression of mRNAs encoding cytokine receptors (e.g.,
GM-CSF, TNF-α, and interleukins), oncogenes (e.g., c-Myc and FOS), tumor suppressors (e.g., p53), and cell-cycle regula-
tors (e.g., p21 and cyclin D1) (Hinman and Lou, 2008; Khabar, 2005; Lal et al., 2004). Likewise, the function of the CGG-repeat
binding FMRF RBP, which is the cause of the Fragile X mental retardation syndrome and normally regulates translation of
miRNAs controlling synaptic function (Brown et al., 2001; Darnell et al., 2001; Zaffa et al., 2003), might be influenced by the altered
expression of CGG-rich miRNAs (e.g., miR-572 and miR-638). Thus, it is likely that the decoy activity of miRNAs is not limited
to miR-328 but can be extended to other miRNAs containing
nucleotide sequences resembling the consensus RNA-binding sites for RBPs that are involved in different normal cell functions and in neoplastic as well as non-cancer-related diseases.

**miR-328: A Molecular Relay in CML Disease Progression**

Even though a few miRNAs are aberrantly regulated in CML (Agire et al., 2008; Bueno et al., 2008; Venturini et al., 2007), evidence of their involvement in disease progression is still lacking. The failure of myeloid CML-BC progenitors to undergo maturation depends on increased BCR/ABL activity which, in addition to enhancing survival, proliferation, genomic instability, and self-renewal (Melo and Barnes, 2007), allows the hnRNP E2 inhibitory effect on C/EBPα that, per se, is sufficient to reinstate differentiation of Ph(+) blasts (Ferrari-Amorotti et al., 2006; Perrotti et al., 2002). We demonstrated that loss of miR-328 occurs in CML-BCCD34+ but not CML-CPCCD34+ myeloid progenitors, and that forced miR-328 expression at physiological levels rescues C/EBPα-driven granulocytic maturation and impairs survival of CML-BC blasts. However, the proapoptotic effect of miR-328 does not seem to depend on its decoy activity and, therefore, on C/EBPα-induced differentiation and growth arrest (Keeshan et al., 2003), as shRNA-mediated hnRNP E2 downregulation does not influence BCR/ABL-driven clonogenic potential.

Rather, we showed that impaired colony formation is the consequence of miR-328 canonical activity that targets PIM1 mRNA, thus repressing PIM1 expression and survival-promoting activity (Hoover et al., 2001; Nieborowska-Skorska et al., 2002). Indeed, forced expression of a wild-type, but not a kinase-deficient, PIM1 cDNA lacking the 3′ UTR into miR-328-expressing cells fully rescued BCR/ABL clonogenicity. However, the main in vivo effect of miR-328 seems to be on differentiation rather than survival, as forced miR-328 expression did not delay leukemogenesis but reversed the blast crisis-like phenotype to a disease that resembles a myeloproliferative-like disorder, although the absence of marked splenomegaly may suggest that a portion of miR-328*/BCR/ABL* cells underwent apoptosis. Although miR-223 was also described as a positive regulator of neutrophil maturation in aplastic cells (Fazi et al., 2005), our data in primary leukemic samples and work in miR-223 knockout animals (Johnnidis et al., 2008) argue against a general role for miR-223 as an inducer of myeloid differentiation.

Mechanistically, we showed that BCR/ABL uses the same MAPK(ERK1/2)-hnRNP E2 signaling pathway (see model in

(B) miR-328-binding site (red) within the 3′ UTR of mouse and human PIM1 mRNA.

(C) PIM1 protein levels in 32Dc3, untreated or imatinib-treated 32D-BCR/ABL (wild-type and T315I) and K562 cells (left); in CD34* BM cells from healthy donors (NB, CML-CP, and CML-BC patients (middle); and in the CD34+ , untreated and imatinib-treated CD34+ BM fractions from a CML-BC patient.

(D) Left: Effect of miR-328 expression on PIM1 protein levels in 32D-BCR/ABL, K562, and CML-BCCD34+ BM cells. 32Dc3 cells were used as a negative control; right: PIM1 mRNA expression by qRT-PCR in 32Dc3, vector-transduced, and miR-328-transduced 32D-BCR/ABL. Representative of triplicates from three inde-
dependent experiments (mean ± SEM).

(E) Left: Levels of ectopic Flag-PIM1 proteins from constructs lacking (pMX-Flag-WTPIM1) and harboring the wild-type (pMX-Flag-WTPIM1-T315U) or 196 base pair end-terminal-deleted (pMX-Flag-WTPIM1-Δ3′UTR) PIM1 3′ UTR in miR-223- or miR-328-expressing 32D-BCR/ABL. Northern blot shows levels of miR-223, miR-328, and snRNA U6. Right: Schematic representation of the Flag-PIM1 constructs.

(F) Effect of seed sequence-mutated (miR-328-Mut) on endogenous PIM1 expression in parental 32Dc3 and empty vector (pCDH-), miR-328-, and miR-328-Mut-infected 32D-BCR/ABL cells.

(G) Left: Endogenous and ectopic (wild-type [WT PIM-1] and kinase-deficient [KD PIM-1]) PIM1 protein levels in 32Dc3, pSUPER- and pSR-miR-328-infected 32D-BCR/ABL cells; right: graph shows rescue of IL-3-independent clonogenic activity of miR-328-expressing (white) 32D-BCR/ABL cells to normal levels (black) by ectopic wild-type (red) but not kinase-deficient (yellow) PIM1 construct lacking the 3′ UTR. Effect of PIM1 forced expression on vector-transduced clonogenicity (gray). Bars represent the mean ± SEM of colony numbers from three independent experiments.
Figure 6D) to suppress C/EBPα (Chang et al., 2007) and miR-328 expression. Notably, constitutive MAPK activation by BCR/ABL occurs in CML-BC but not CML-CP (Notari et al., 2006). Moreover, similar to the positive feedback loop described for miR-223 and C/EBPα (Fazi et al., 2005), we showed that C/EBPα also interacts with the miR-328 promoter, thus enhancing its transcription.

In conclusion, the discovery of dual activities for miR-328 that profoundly affect myeloid cell differentiation and survival not only add a new layer to the complexity of mechanisms regulating the phenotype of CML-BC progenitors but, more importantly, highlight the ability of miRNAs to alter mRNA metabolism by acting also as molecular decoys for RNA-binding proteins.

EXPERIMENTAL PROCEDURES

Additional details on all the methods are available online in the Extended Experimental Procedures.

Clonogenic and Viability Analysis

Methylcellulose clonogenic assays were carried out by plating 10^3 32DCl3 and derivative cell lines, 10^4 CML-BC/C0, or 10^4 Lin- SCLTA-BCR/ABL BM progenitors in 0.9% MethoCult (Stem Cell Technologies) in the presence or absence of rIL-3 (100 ng/ml). Colonies (>100 μm) from cell lines and primary cells were scored 7 and 15 days later, respectively. Human and mouse cell lines and primary cell source and culture conditions as well as plasmids and retroviral vectors are reported in the Extended Experimental Procedures.

In Vitro and In Vivo Differentiation Assays

In vitro granulocytic differentiation was induced for 7–10 days with 25 ng/ml rG-CSF. Morphologic differentiation was assessed by Wright/Giemsa staining. For in vivo differentiation, 10-week-old ICR-SCID mice (n = 13 per group) were intravenously injected (6 × 10^5 cells/mouse) with pSUPER.puro or pSUPER-miR-328-transduced 6.15-WT-uroF-CEBPA(GFP+) cells.

RNA Extraction, Northern Blot, and Real-Time PCR

Total RNA was used in northern blot, RT-PCR, and/or qRT-PCR for the analysis of miRNA and mRNA expression. U6 snRNA and GAPDH levels were analyzed for normalization of miRNA and mRNA PCRs, respectively.

REMSA, UV Crosslinking, and RNA Immunoprecipitation

Recombinant MBP-hnRNP E2 (Chang et al., 2007) and 32Dcl3 or 32D-BCR/ABL cytoplasmic extracts were used in REMSA and UV crosslinking as described (Perrotti et al., 2002). RIP and miR-328 RT-PCR were performed as described (Keene et al., 2006; Wang et al., 2008).

In Vitro Translation Assay

In vitro translation assays using the transcription/translation-coupled rabbit reticulocyte lysate system (Promega) were performed with pcDNA3-WT-uORF-C/EBPα or pcDNA3-WT-uORF-C/EBPα in the presence or absence of 1 μg recombinant MBP-hnRNP E2 (Perrotti et al., 2002) either with or without 1000x mature miR-328 or miR-330 oligoribonucleotides.

Western Blotting, Communmunoprecipitation, and ChIP Assays

For western blot, 1 × 10^6 cells were lysed (0°C; 30 min) in 50–100 μl RIPA buffer, clarified, and subjected to SDS-PAGE. For C/EBPα detection, 100 cells were directly lysed in 20 μl Laemmli buffer and denatured prior to SDS-PAGE and transfer to nitrocellulose. For coIP, cells were lysed on ice with immunoprecipitation buffer and 1.0 mg of protein was used in communmunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using an EZ-Chip kit (Millipore).

Statistics

Data were analyzed as follows: (1) two-tailed paired Student’s t test for assays with identical cell lines, untreated and imatinib-treated SCLTA-BCR/ABL cells, RIP assay densitometric and qRT-PCR, and in vitro translation assays; (2) two-tailed independent Student’s t test for clonogenic assays with unpaired miRNA-infected Lin- BM cells; and (3) the Mann-Whitney rank sums test for assays with unpaired CML patient samples. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.cell.2010.01.007.

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**Supplemental Information**

**EXTENDED EXPERIMENTAL PROCEDURES**

**Cell Cultures and Primary Cells**
32Dc3 murine myeloid precursor cells, Ph1(+) erythroleukemia K562 cells, and derivative lines were maintained in IMDM plus 10% FBS and 2 mM L-glutamine ( GibCO). 10% WEHI-conditioned medium was used as the source of mIL-3. 293T cells were maintained in culture in DMEM, 10% FBS and 2 mM L-glutamine. 32D-BCR/ABL, 32D-BCR/ABL(T315I), and miRNA-expressing cells were generated by retroviral infection followed by antibiotics-mediated selection or FACS-mediated sorting of GFP+ cells (Perrotti et al., 2002). Newly established 32D-BCR/ABL cells (which express CEBPA mRNA but not C/EBPα protein) were used in differentiation assays. Murine BM cells from femurs of C57BL/6 or leukemic SCLtTA-BCR/ABL mice (Koschmieder et al., 2005) underwent Lin- magnetic-activated cell sorting (MACS, Miltenyi Biotec) and were grown for 2 days in IMDM medium containing murine IL-3 (2 ng/ml), IL-6 (2 ng/ml), SCF (20 ng/ml), Flt3-ligand (5 ng/ml), and GM-CSF (5 ng/ml) (R&D Systems) prior to infection with MigR1, MigR1-p210BCR/ABL (W. Pear, UPENN, Philadelphia, PA), or miRNA retroviral vectors. All animal studies were performed with approval of The OSU Institutional Animal Care and Use Committee. For patient specimens, frozen mononuclear BM cells from healthy donors were purchased from Cincinnati Children's Hospital, Cincinnati, OH. Patient specimens were obtained from the OSU Leukemia Tissue Bank, Columbus OH; Maisonneuve-Rosemont Hospital, Montreal, Quebec, Canada; City of Hope National Medical Center, Duarte, CA; MD Anderson Cancer Center, Houston TX; and Aarhus University, Denmark. All the performed experiments were approved by The OSU Institutional Review Board. Where indicated, cells were treated with the following kinase inhibitors: 1–2 μM imatinib mesylate (Novartis Oncology), 25 μM U0126 (Promega), or 10 μM CI-1040 (Pfizer).

**In Vitro and In Vivo Differentiation Assays**
In vitro granulocytic differentiation was induced by exposing cells to 25 ng/ml rhG-CSF for 7–10 days. Morphologic differentiation was assessed by Wright/Giemsa staining of cytospins. miR-328 expression in different lineages was extrapolated from miRNA array analysis of hematopoietic precursors obtained by culturing for 14 days human (non-mobilized) CD34+ BM cells (n = 2) in EPO/SCF/IL-3 (erythroid), G-CSF/GM-CSF/SCF/IL-6/IL-3 (granulocytic), TPO/SCF/IL-3 (megakaryocytic) and M-CSF/GM-CSF/IL-6/IL-3/SCF (monocytic). Morphology and lineage-specific antibody staining was used to assess differentiation. Total RNA from lineage-committed cells at different days of culture was hybridized in duplicate to OSU v3.0 miRNA chip. After quantiles normalization, differentially expressed miRNAs were identified by using the univariate t test within the BRB array tools. For in vivo differentiation, 10-week-old ICR-SCID mice (n = 13 per group) were i.v. injected (5 × 105 cells/mouse) with pSUPERior.retro.puro- or pSUP-miR-328-transduced 6.15-WT-uORF-C/EBPA(GFP+) cells. Engraftment was assessed 1 week after cell injection by nested RT-PCR-mediated detection of BCR/ABL transcripts in circulating peripheral blood cells (Eiring et al., 2008). After 3 weeks, mice were sacrificed and BM, spleen, and liver were subjected to visual and histological (H&E staining) analyses and flow-cytometric detection of Gr1+ GFP+ BM cells using PE-conjugated GR1 mAb (PharMingen). Light microscopy was performed on a Zeiss Axioskop 2 Plus microscope equipped with a Plan-Neo 40×/0.75NA objective and a Canon Powershot A70 camera. Images were captured using Canon Remote Capture software and Adobe Photoshop CS.

**Plasmids**
The pSRzMSVtkneo-BCR/ABL, pMSCVpuro-Flag-hnRNP E2, MigRI-HA-CEBPA, pcDNA3-WTuORF-C/EBPα constructs have been described (Perrotti et al., 2002).

**pSUPER-shE2**
The human hnRNP E2 sequence targeting human and mouse mRNAs was subcloned into the pSUPER.retro.neo.GFP vector as previously described (Eiring et al., 2008).

**pSR- and pCDH-miR-223, pSR- and pCDH-miR-328, pCDH-miR-328-Mut, pSUP-miR-328, and pSUP-miR-181b**
Pre-miR-328, pre-miR-223, and pre-miR-181b were PCR amplified from 32Dc3 genomic DNA (see below for primer sequences) and cloned into either the retroviral pSuper.retro.neo.GFP or pSuperior.retro.puro vectors (OligoEngine), or the lentiviral pCDH-CMV-MCS-EF1-copGFP vector (System Biosciences). The pCDH-miR-328-Mut vector was mutated in the seed sequence with the Quik-Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

**pMX-Flag-WTPIM1-WT3’UTR and pMX-Flag-WTPIM1-3’UTR**
The wild-type (658 base pairs) or 3’-deleted (462 base pairs) PIM1 3’UTRs were RT-PCR amplified from 32D-BCR/ABL mRNA (see below for primer sequences) and cloned into the pMX-Flag-WTPIM1 plasmid. pMX-Flag-WTPIM1 and pMX-Flag-KD-PIM1 have been described (Nieborowska-Skorska et al., 2002).
Cloning Primers and Sequences

**pSUPER.retro.neo.GFP**
- miR-223 Forward: `5'-CATAGATCTTCCAGTTGACATCTTCCAGC-3'`
- miR-223 Reverse: `5'-CATAGACCTAAACACGCGTTGCTGTGAGCT-3'`
- miR-328 Forward: `5'-CATAGATCTAAGAGCTCATGGAAACTGTGG-3'`
- miR-328 Reverse: `5'-CATAGATCTTAAACACGCGTTGCTGTGAGCT-3'`
- miR-181b Forward: `5'-CATGGATCCAAAAAGAGAGCTTCATGTTTCATAAGC-3'`
- miR-181b Reverse: `5'-CATGGATCCAAAAAACAGCGTTGCTGTGAGCT-3'`

**pSUPERIOR.retro.puro**
- miR-328 Forward: `5'-CATAGATCTAAGAGCTCATGGAAACTGTGG-3'`
- miR-328 Reverse: `5'-CATGGATCCAAAAAACAGCGTTGCTGTGAGCT-3'`
- miR-328-Mut Primer #1: `5'-GAAAGTATCTACAGCCCCATTCCCGCTCTGCCCTTCCGTCC-3'`
- miR-328-Mut Primer #2: `5'-GGACGGAAGGGCAGAGCGGGAATGGGGCTGTAGATACTTTC-3'`

**pCDH-CMV-MCS-EF1-copGFP**
- miR-223 Forward: `5'-CATGAATTCTCCAGTTGACATCTTCCAGC-3'`
- miR-223 Reverse: `5'-CATGGATCCAAAAAGAGAGCTTCATGTTTCATAAGC-3'`
- miR-328 Forward: `5'-CATGAATTCAAGAGCTCATGGAAACTGTGG-3'`
- miR-328 Reverse: `5'-CATGGATCCAAAAAACAGCGTTGCTGTGAGCT-3'`
- miR-328-Mut Primer #1: `5'-GAAAGTATCTACAGCCCCATTCCCGCTCTGCCCTTCCGTCC-3'`
- miR-328-Mut Primer #2: `5'-GGACGGAAGGGCAGAGCGGGAATGGGGCTGTAGATACTTTC-3'`

**pMX-Flag-WTPIM1**
- WT3 UTR Forward: `5'-CATGCGGCCGCCAGCCTTTCTGCTGCTGTC-3'`
- WT3 UTR Reverse: `5'-CATGCGGCCGCTTGTGCGTTCTGTGTGAGGT-3'`
- Δ3 UTR Forward: `5'-CATGCGGCCGCCAGCCTTTCTGCTGCTGTC-3'`
- Δ3 UTR Reverse: `5'-CATGCGGCCGCCAGCAGAGTTTGAGAAGC-3'`

RNA Extraction, Northern Blot, and Real-Time PCR

Total RNA was used in northern blot, RT-PCR, and/or qRT-PCR for the analysis of miRNA and mRNA expression levels. qRT-PCR for detection of *CEBPA* mRNA levels was performed using the PCR primers indicated below. Total RNA was isolated using Trizol (Invitrogen) and analyzed for miRNA expression by northern blot and/or qRT-PCR. For northern blot, RNA (1–20 μg) was fractionated on a 15% denaturing polyacrylamide-urea gel (Bio-Rad) and subject to hybridization (18 hr; 43°C) with 32P-labeled miR-223, miR-328, or U6 snRNA probes (see below for probe sequences).

Northern Hybridization Probes and Sequences

- miR-328: `5'-ACGGAAGGGCAGAGAGGGCCAG-3'`
- miR-328-Mut: `5'-GGACGGAAGGGCAGAGCGGGAATG-3'`
- snRNA U6: `5'-GCAAGGAGGCGCTTGTGCTGTGAGGT-3'`
- miR-223: `5'-GGGGTATTTGACAAACTGACA-3'`

For qRT-PCR, mature miR-328, miR-223, and miR-181b, as well as U6 snRNA looped primers were used according to the manufacturer's instructions (Applied Biosystems). Where indicated, resulting PCR products were fractionated on a 15% denaturing polyacrylamide-urea gel (Bio-Rad) and subject to hybridization (18 hr; 43°C) with 32P-labeled miR-223, miR-328, miR-328-Mut, or U6 snRNA probes (see below for probe sequences).

REMSA, UV Crosslinking, and RNA Immunoprecipitation

Recombinant MBP-hnRNP E2 (Chang et al., 2007) and 32Dci3 or 32D-BCR/ABL cytoplasmic extracts were used in REMSA and UV crosslinking as described (Perrotti et al., 2002). Briefly, reactions performed with 1 μg MBP-hnRNP E2 or 10 μg cytoplasmic extracts were incubated (30 min, RT) with a 32P-labeled miR-328, miR-328-Mut, miR-330, miR-181b, or *CEBPA* uORF (Perrotti et al., 2002) oligoribonucleotide and resolved in 5% native-PAGE/0.5X TBE for REMSA or in 4%–15% SDS-PAGE for UV crosslinking analysis (see below for RNA oligonucleotide probe sequences). For competition assays, 500- to 2000-fold molar excess of single-stranded oligoribonucleotides was added to the reaction. RNA immunoprecipitation (RIP) was performed as described (Keene et al., 2006). Briefly, 32D-BCR/ABL, 32D-Flag-E2, and 32D-BCR/ABL-Flag-E2, and 6.15-WTuORF cells were transduced with either pSR-miR-328, pSUP-miR-328, pSUP-miR-181b, or the empty vector, lysed (5 min, 0°C) in 100 mM KCl, 5 mM MgCl₂, 10 mM HEPES [pH 7.0], 0.5% NP40, 1 mM DTT, 100 units/ml RNase OUT (Invitrogen), 400 μM vanadyl-ribonucleoside complex and protease inhibitors (Roche), clarified and stored 0°C. Ribonucleoprotein particle-enriched lysates were incubated with either protein G- (anti-Flag or anti-HA), sepharose A+ (anti-Dicer, anti-hnRNP E2, and rIgG), or sepharose A agarose G-coupled beads (anti-Ago2 and rIgG) (4°C; 2 h). Beads were subsequently washed four times with 50 mM TRIS/HCl, pH 7.0, 150 mM NaCl, 1 mM MgCl₂, and 0.05% NP-40, and twice after addition of 1M Urea. IPs were digested with proteinase K (55°C; 30’) and hnRNP E2-associated mRNAs were analyzed by northern blot.
and miRNAs were isolated as described above. RT-PCR for CEBPA was performed with the PCR primers indicated below. RT-PCR for miR-328 was performed as described (Wang et al., 2008).

RNA Oligonucleotides and Sequences

miR-328: 5'-CUGGCCCUUCUCUGCCCUCCGU-3'

miR-328-Mut: 5'-CUUCGGCCUGCCUUCCGU-3'

miR-330: 5'-GCAAAAGCAAGGCCUUCAGAGA-3'

miR-181b: 5'-AACAAUUCAACUGUCUGUCGGGU-3'

CEBPA uORF: 5'-CUGGGCAUGCCCGAGAACUCUAACUCUCCCAUGGAG-3'

Western Blotting and Coimmunoprecipitation

For Western blot, 1 × 10^6 cells were lysed (0°C; 30 min) in 50–100 μl RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris [pH 8.0]) containing 1 mM PMSF, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na3VO4, 50 mM NaF, 10 mM β-glycerol-phosphate, clarified (12,000 × g; 4°C, 30 min), and subjected to SDS-PAGE. For C/EBPα detection, 10^6 cells were directly lysed in 20 μl Laemmli buffer and denatured prior to SDS-PAGE and transfer to nitrocellulose. For coimmunoprecipitation, cells were lysed on ice with immunoprecipitation buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, and 0.1% SDS) supplemented with protease (Complete EDTA free, Roche) and phosphatase (PhosStop, Roche) inhibitors. 1.0 mg of protein in 200 μl of buffer was incubated with 25 μl of sepharose A beads previously coated with 4 μg of antibody (2 hr, 4°C). Half of the immunoprecipitate was then separated by SDS-PAGE and transferred to nitrocellulose. Antibodies used were: rabbit polyclonal anti-hnRNP E2 (Chkheidze et al., 1999; Gamarnik and Andino, 1997; Waggoner and Liebhaber, 2003); anti-PIM1, anti-C/EBPα, anti-NFI-A, anti-Dicer, clone H-212, and anti-TRBP2, clone S-11 (Santa Cruz Biotechnology); anti-phosphotyrosine, clone 4G10 (Upstate); anti-GRB2 (BD Transduction Laboratories); anti-HA.11 (Sigma); and anti-Ago2, clone C34C6 (Cell Signaling). Rabbit IgG (IgG) antibody (Santa Cruz Biotechnology, Inc.) served as an isotype control.

Chromatin Immunoprecipitation

ChIP assays were performed using an EZ-Chip kit (Millipore). Briefly, MigR1-HA-C/EBPα-transduced (Perrotti et al., 2002) 32Dcl3 cells were formaldehyde-crosslinked, lysed and incubated with protein G-coupled anti-HA.11 and anti-Flag (negative control) antibodies for immunoprecipitation (IP) of ectopic HA-C/EBPα. Chromatin was then separated by SDS-PAGE and transferred to nitrocellulose. Antibodies used were: rabbit polyclonal anti-hnRNP E2 (Chkheidze et al., 1999; Gamarnik and Andino, 1997; Waggoner and Liebhaber, 2003); anti-PIM1, anti-C/EBPα, anti-NFI-A, anti-Dicer, clone H-212, and anti-TRBP2, clone S-11 (Santa Cruz Biotechnology); anti-phosphotyrosine, clone 4G10 (Upstate); anti-GRB2 (BD Transduction Laboratories); anti-HA.11 (Covance); anti-Flag, clone M2 (Sigma); and anti-Ago2, clone C34C6 (Cell Signaling). Rabbit IgG (IgG) antibody (Santa Cruz Biotechnology, Inc.) served as an isotype control.

PCR Primers and Sequences

C/EBPα ChIP: miR-328 Primers

CEBP1 Forward: 5’-CCACAGGCTAGAACAAGGATGGAC-3’

CEBP1 Reverse: 5’-CTTTCTCCATCACTAGTACAC-3’

CEBP2 Forward: 5’-AGGTATGCGCTATAGGAGAGA-3’

CEBP2 Reverse: 5’-AAGCTATGTTTGCCTGTATCC-3’

CEBP3 Forward: 5’-GAGTGATGAGAGGGCTCTGG-3’

CEBP3 Reverse: 5’-CTGGTTAACGACTCTCAATCGTC-3’

CEBP4 Forward: 5’-ATAGGTTGAGGGCATTACCTTGTG-3’

CEBP4 Reverse: 5’-CTGAATAAGACCTGGAAGGAGATG-3’

CEBPA RT-PCR Primers

CEBPA Forward: 5’-GCCAGCACGAGACGTATAGA-3’

CEBPA Reverse: 5’-GCCCAGAAACTGCTGTTGAA-3’

SUPPLEMENTAL REFERENCES


Figure S1. miR-328 Competes with CEBPA mRNA for Binding to hnRNP E2, Related to Figure 2

(A) Western blot shows levels of Flag-tagged and endogenous hnRNP E2 in parental and Flag-hnRNP E2-expressing 32D-BCR/ABL cells. GRB2 levels were analyzed for controls.

(B) Northern blot shows levels of miR-328 in 32DcI3 (lane 1), in vector- (lane 2) and in pSR-miR328-transduced (lane 3) 32D-BCR/ABL-Flag-E2 cells. snRNA U6 levels served as a loading control.

(C) RIP assay shows association of mature miR-328 to ectopic hnRNP E2 in anti-Flag RIPs from miR-328-expressing 32D-Flag-E2 and 32D-BCR/ABL-Flag-E2 cells. Anti-HA RIPs were used as negative controls.

(D) RIP assay shows association of miR-328 and CEBPA mRNA, but not miR-223 or SET mRNA, to endogenous hnRNP E2 in anti-hnRNP E2 RIPs from empty vector- and miR-328-expressing 6.15 and 6.15-uORF cells.
Figure S2. Regulation of miR-223 Expression in BCR/ABL+ Cells, Related to Figure 4

(A) Levels of ectopic miR-223 or miR-328 (left) and their effect on proliferation of (right) 32Dcl3, 32D-BCR/ABL, and K562 cells.

(B) Left: Northern blot and qRT-PCR analyses show expression of miR-223 in 32Dcl3, untreated and imatinib-treated BCR/ABL+ cell lines. Right: qRT-PCR analyses show expression of miR-223 in CD34+ bone marrow progenitors from CML-CP (n = 6) and CML-BC (n = 6) patients (mean ± SEM). U6 snRNA was used for normalization. Notably, qRT-PCR revealed no significant changes (p = 0.35) in miR-223 levels in CML-BCCD34+ (n = 6) versus CML-CPCD34+ (n = 6) BM cells (mean ± SEM).

(C) Left: qRT-PCR shows levels of miR-223 and miR-328 in vector (pCDH)-, miR-223-, and miR-328-infected CML-BC progenitors (mean ± SEM). Right: qRT-PCR shows levels of CEBPA in empty vector-, miR-223-, or miR-328-infected 32D-BCR/ABL cells (mean ± SEM).

(D) Left: Western blot shows that NFI-A expression is induced in a BCR/ABL kinase-dependent manner. Middle: Ectopic miR-223 could efficiently reduce NFI-A levels in 32D-BCR/ABL and K562 cells. Right: By contrast, NFI-A was not influenced by ectopic miR-223 expression in CML-BCCD34+ progenitors. While NFI-A was slightly induced in CML-CPCD34+ compared to CD34+ progenitors from normal bone marrow (NBMCD34+), its expression was not different in CML-CPCD34+ versus CML-BCCD34+ progenitors (n = 2), and was inhibited rather than induced by imatinib treatment (n = 3).

(E) Left: Northern blot analysis shows miR-223 levels in parental and Flag-hnRNP E2-expressing 32Dcl3 and 32D-BCR/ABL cells. Right: Effect of shRNA-mediated hnRNP E2 downregulation on miR-223 levels. U6 snRNA and GRB2 protein levels were used as controls.