Down-Regulation of *hsa-miR-10a* in Chronic Myeloid Leukemia CD34+ Cells Increases USF2-Mediated Cell Growth

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Abstract

MicroRNAs (miRNA) are small noncoding, single-stranded RNAs that inhibit gene expression at a posttranscriptional level, whose abnormal expression has been described in different tumors. The aim of our study was to identify miRNAs potentially implicated in chronic myeloid leukemia (CML). We detected an abnormal miRNA expression profile in mononuclear and CD34+ cells from patients with CML compared with healthy controls. Of 157 miRNAs tested, *hsa-miR-10a, hsa-miR-150*, and *hsa-miR-151* were down-regulated, whereas *hsa-miR-96* was up-regulated in CML cells. Down-regulation of *hsa-miR-10a* was not dependent on BCR-ABL1 activity and contributed to the increased cell growth of CML cells. We identified the upstream stimulatory factor 2 (*USF2*) as a potential target of *hsa-miR-10a* and showed that overexpression of *USF2* also increases cell growth. The clinical relevance of these findings was shown in a group of 85 newly diagnosed patients with CML in which expression of *hsa-miR-10a* was down-regulated in 71% of the patients, whereas expression of *USF2* was up-regulated in 60% of the CML patients, with overexpression of *USF2* being significantly associated with decreased expression of *hsa-miR-10a* ($P = 0.004$). Our results indicate that down-regulation of *hsa-miR-10a* may increase USF2 and contribute to the increase in cell proliferation of CML implicating a miRNA in the abnormal behavior of CML. (Mol Cancer Res 2008;6(12):1830–40)

Introduction

MicroRNAs (miRNAs) are noncoding, single-stranded RNAs of 21 to 25 nucleotides recently implicated in the regulation of several biological processes, such as cell cycle, apoptosis, differentiation, and development or tumorigenesis (1, 2). miRNAs inhibit the expression of specific genes either by decreasing mRNA stability or by blocking mRNA translation (2-5). *In silico* studies have estimated and annotated more than 450 human miRNAs[8] (6, 7), many of them evolutionary conserved and localized in cancer-associated fragile genome regions (8). The fact that each miRNA may regulate multiple targets implies that a third of the protein-coding genes in humans may be regulated by miRNAs (9).

The first evidence for the involvement of miRNAs in human cancer was described in chronic lymphocytic leukemia in which deletion of a chromosomal region (13q14) was associated with down-regulation of *miR-15a* in the majority of chronic lymphocytic leukemia patients (68%; ref. 10). It was later shown that *miR-15a* and *miR-16-1* negatively regulate expression of the BCL2 protein at the posttranscriptional level,

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[8] http://microrna.sanger.ac.uk/

inducing apoptosis in leukemic cells (11). Abnormal expression of miRNAs has currently been described in numerous solid tumors, such as lung cancer (12, 13), breast cancer (14), glioblastoma (15), hepatocellular carcinoma (16), endocrine pancreatic tumors (17), papillary thyroid carcinoma (18), testicular germ cell tumors (19), and colorectal cancer (20, 21), but also in hematologic malignancies, such as chronic lymphocytic leukemia (22), lymphomas (23), acute promyelocytic leukemia (24), and more recently acute lymphoblastic leukemia (25).

Chronic myeloid leukemia (CML) represents a remarkably useful model to study new mechanisms of tumorigenesis due to the fact that the hallmark of the disease is the presence of the Philadelphia chromosome that is present in >95% of the patients and is responsible for the disease (26-30). Information about the implication of miRNAs in the pathogenesis of CML is limited to the description of abnormal expression of certain miRNAs (*miR-15a, miR-16, miR-142, miR-155, miR-181, miR-221*, and *let-7a*) in the CML cell line K562 (31, 32) and up-regulation of the polycistronic *miR-17-92* cluster in CML patients (33). Based on the analysis of the miRNA profile expression in patients with newly diagnosed CML, we have determined that *hsa-miR-10a, hsa-miR-150*, and *hsa-miR-151* are down-regulated, whereas *hsa-miR-96* is up-regulated in CML cells. Down-regulation of *hsa-miR-150* and *hsa-miR-151* is mediated by BCR-ABL1 expression, whereas down-regulation of *hsa-miR-10a* is BCR-ABL1 independent. In this study, we show that down-regulation of *hsa-miR-10a* results in increased expression of the upstream stimulatory factor 2 transcription factor (*USF2*), which contributes to the increased cell growth of CML cells.

Results

A miRNA Expression Pattern Discriminates CML Patients from Healthy Donor Samples

We analyzed the expression profile of 157 miRNAs by quantitative real-time PCR (Q-RT-PCR) using Taqman methodology (PE Applied Biosystems) in mononuclear cells (MNC) and $CD34^+$ cells from patients with CML ($n = 6$) at diagnoses and control healthy donors ($n = 6$). For comparison of miRNA expression, data on every sample were normalized using the expression of the housekeeping genes *GUSB* or *U6* as described (34, 35). Relative quantification and supervised analysis comparing bone marrow (BM) MNCs of CML patients identified 53 differentially expressed miRNAs, 23 of them up-regulated and 30 down-regulated in CML patients with respect to healthy donors. Cluster unsupervised analysis based on differentially expressed miRNAs generated a tree that clearly distinguished between both groups (Fig. 1A). To assess a more homogeneous population of cells and because CML is a clonal stem cell disease, we did the same analysis using $CD34^+$ cells from the same samples. Eleven miRNAs were differentially expressed, 10 of them down-regulated and 1 up-regulated in CML patients. Cluster unsupervised analysis based on differentially expressed miRNAs generated a similar tree distinguishing CML samples from normal donors (Fig. 1B).

When a further statistical analysis using Statistical Package for the Social Sciences was done comparing CML and healthy donor MNCs, only 34 of the 53 differentially expressed miRNAs maintained their statistically significant differences ($P < 0.05$; Supplementary Table S1). The same analysis done with $CD34^+$ cells from patients and donors confirmed the differential expression of the 11 miRNAs (Supplementary Table S2). The results of the analysis were the same whether we used *GUSB* or *U6* genes for normalization. We also did a comparison between the miRNA expression profile of CML MNC and $CD34^+$ cells and healthy donor MNC and $CD34^+$ cells, which showed 61 and 45 miRNAs to be differentially expressed in CML patients and normal donors, respectively, between MNC and $CD34^+$ cells (Supplementary Fig. S1).

The analysis of those miRNAs differentially expressed both in BM MNC and $CD34^+$ cells of CML patients in comparison with healthy donors showed that six miRNAs were differentially expressed in both comparisons, five of them down-regulated (*hsa-miR-10a, hsa-miR-125a, hsa-miR-150, hsa-miR-151*, and *hsa-miR-199s*) and one up-regulated (*hsa-miR-96*) in CML patients with respect to healthy donor samples, indicating a higher likelihood of these miRNAs to be involved in CML pathogenesis. To confirm the results, we analyzed the expression of five of these six miRNAs (*hsa-miR-10a, hsa-miR-96, hsa-miR-125a, hsa-miR-150*, and *hsa-miR-151*) in MNC and $CD34^+$ cells from another 16 newly diagnosed CML patients and another 6 healthy donors by Q-RT-PCR using individual Taqman miRNA assays. *hsa-miR-199s* miRNA expression was not analyzed because this miRNA has been eliminated from the miRNA annotation list. The analysis confirmed the statistically significant differences in the expression of four of five miRNAs (*hsa-miR-10a, hsa-miR-96, hsa-miR-150*, and *hsa-miR-151*) between CML and healthy donors (Table 1), suggesting that these miRNAs could be directly implicated in the pathogenesis of CML.

Down-Regulation of hsa-miR-10a *in CML Is Not Mediated by BCR-ABL1 Activity*

As CML is characterized by the presence of the fusion protein BCR-ABL1, which shows an increased tyrosine kinase activity, we reasoned that abnormal expression of *hsa-miR-10a, hsa-miR-150, hsa-miR-151*, and *hsa-miR-96* observed in CML patients could be dependent on BCR-ABL1 or its kinase activity. To test this hypothesis, we analyzed the expression of these miRNAs by Q-RT-PCR in the Mo7e and Mo7e-p210 cell line before and after treatment with imatinib, a specific inhibitor of BCR-ABL1 kinase activity, for 24 hours (36). Whereas Mo7e-p210 cells showed a reduced expression of *hsa-miR-150* and *hsa-miR-151* in comparison with Mo7e cells, treatment with imatinib induced an up-regulation of expression of both miRNAs in Mo7e-p210 cells. These results suggest that BCR-ABL1 kinase activity induces down-regulation of *hsa-miR-150* and *hsa-miR-151* in CML (Supplementary Fig. S2A and B). No expression of *hsa-miR-96* was detected in any of the cell lines before or after treatment with imatinib. Whether a genetic abnormality is responsible for the lack of *hsa-miR-96* expression in the parental Mo7e cell line is currently unknown.

Unlike what we observed in patients with CML in which expression of *hsa-miR-10a* was significantly down-regulated in 71% of the patients, the expression of *hsa-miR-10a* was increased in Mo7e-p210 and treatment with imatinib had no statistically significant effect on miRNA expression

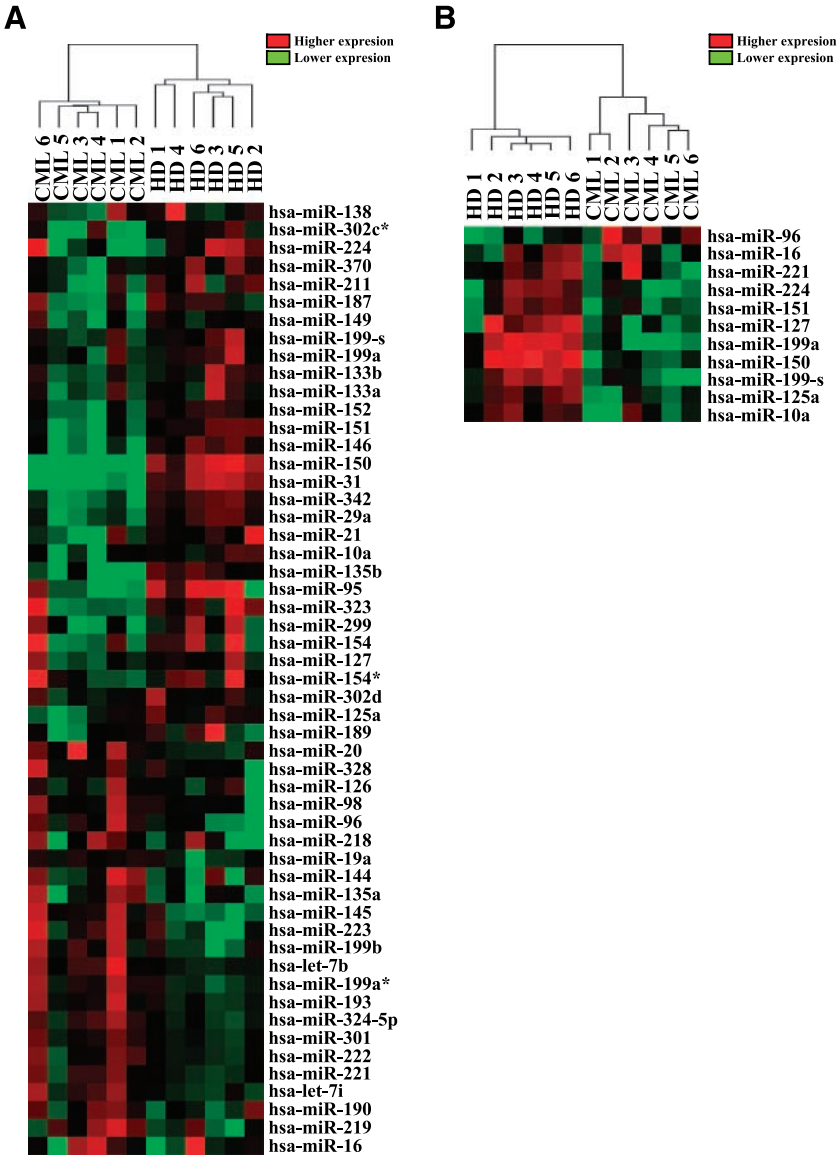

FIGURE 1. Hierarchical cluster analysis of miRNA expression in CML patients and healthy donor samples. Cluster analysis of expression of 157 miRNA using MNC (**A**) or $CD34^+$ cells (**B**) of patients with CML versus healthy donors. The dendrogram is shown on the top of the figure. miRNAs differentially expressed are depicted with the names included.

(Supplementary Fig. S2C). Expression of *hsa-miR-10a* was also increased in TCC-S cells, whereas BV173 and KU812 cell lines showed a reduced *hsa-miR-10a* expression in comparison with Mo7e (Fig. 2A). Expression of *hsa-miR-10a* in CML-derived cell lines was confirmed by Primer extension analysis (Fig. 2B). Quantification of the primer extension gave a similar result than the normalization of *hsa-miR-10a* expression obtained by Q-RT-PCR, ensuring the reliability of both techniques (data not shown). Although *hsa-miR-10a* is down-regulated in CML cells, which express BCR-ABL1, expression of *hsa-miR-10a* in these cells is unlikely to depend on BCR-ABL1 activity based on the following facts: (*a*) Mo7e-p210 does not show a down-regulation of *hsa-miR-10a* in comparison with Mo7e, (*b*) treatment with imatinib does not significantly affect the expression of *hsa-miR-10a*, and (*c*) CML-derived cell lines show different *hsa-miR-10a* levels.

Table 1. miRNAs Differentially Expressed in BM MNC and $CD34^+$ Cells between CML Patients and Healthy Donor

miRNA	miRNA Sequence	Genome Location	*P* value $CD34^+$ CML/HD	*P* value MNCs CML/HD
hsa-miR-10a	uacccuguagauccgaauuugug	17q21.32	0.003	0.04
hsa-miR-96	uuuggcacuagcacauuuuugc	7q32.2	0.004	0.127
hsa-miR-125a	ucccugagacccuuuaaccuguga	19q13.41	0.955	0.568
hsa-miR-150	ucucccaacccuuguaccagug	19q13.33	0.018	0.003
hsa-miR-151	acuagacugaagcuccuugagg	8q24.3	0.004	0.018

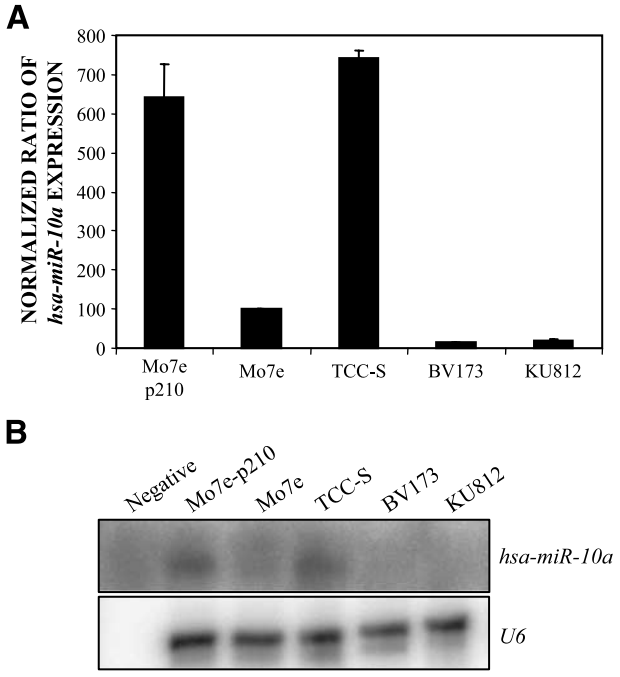

FIGURE 2. Expression analysis of *hsa-miR-10a* in CML-derived cell lines. **A.** *hsa-miR-10a* expression was analyzed in Mo7e, Mo7e-p210, TCC-S, KU812, and BV173 cell lines by Q-RT-PCR. Expression of miRNAs was normalized and expressed as a ratio of Mo7e expression. Columns, mean of three different experiments; bars, SD. **B.** *hsa-miR-10a* expression analysis by primer extension in Mo7e, Mo7e-p210, TCC-S, KU812, and BV173 cell lines. Negative, buffer/oligonucleotide sample. U6 oligonucleotide was used as a loading control.

USF2 *Is a Potential Target of* hsa-miR-10a

As biological significance of miRNA deregulation relies on their gene targets, we analyzed the predicted targets of *hsa-miR-10a*. The analysis was done using four algorithms, mirBase, miRanda, TargetScan, and PicTar, commonly used to predict human miRNA gene targets. Because any of the four approaches generate an unpredictable number of false positives, results were intersected to identify the genes commonly predicted by at least three of the algorithms. Results are shown in Supplementary Table S3.

We next analyzed whether any of the predicted targets of *hsa-miR-10a* were up-regulated in CML $CD34^+$ cells. Thus, we compared the list of putative targets with the list of up-regulated genes found in gene array studies done comparing the gene expression profile of $CD34^+$ cells from CML patients at diagnoses and healthy donor $CD34^+$ cells (37). We found that the expression of transcription factor *USF2* showed an inverse correlation with the expression of *hsa-miR-10a* being up-regulated in CML $CD34^+$ cells (Supplementary Table S4). As predicted by PicTar,[9] there was complementarity between *hsa-miR-10a* and *USF2* 3′-untranslated region (UTR).

To show whether *hsa-miR-10a* could regulate the expression of *USF2*, RKO cells were transfected with the pre-miR-10a or anti-miR-10a molecules and the expression of *hsa-miR-10a, HOXA1*, and *USF2* mRNA was analyzed by Q-RT-PCR. This cell line was used for technical reasons, as the colon carcinoma–derived RKO cell line can be efficiently transfected unlike leukemia cell lines. Expression of *HOXA1* mRNA, a known *hsa-miR-10a* target (38, 39), was used as control. Transfection of RKO cells with Silencer FAM-labeled negative control showed a cell viability of 93% and a transfection efficiency of 88% by fluorescence-activated cell sorting (Supplementary Fig. S3). Transfection of RKO with anti-miR-10a induced a significant reduction of *hsa-miR-10* expression level and an increase in the mRNA levels of *HOXA1* and *USF2* in comparison with cells transfected with the anti-miR scrambled control or negative control (Supplementary Fig. S4). In contrast, a significant increase of *hsa-miR-10* expression level and a decrease of mRNA levels of *HOXA1* and *USF2* were found in RKO cells transfected with pre-miR-10 (Supplementary Fig. S4).

To further show the role of *hsa-miR-10* in CML, nucleofection of the CML-derived KU812 cell line (viability was 78% and transfection efficiency was 96% by fluorescence-activated cell sorting using BLOCK-iT Fluorescent Oligo; Supplementary Fig. S5) was done. KU812 cells were chosen as they show low levels of *hsa-miR-10*, similar to what was observed in CML cells from patients (Fig. 2A). Transfection with anti-miR-10a induced a decrease in the expression of *hsa-miR-10*, which was smaller than the one observed in RKO cells, but a higher increase in the levels of *HOXA1* and *USF2* mRNA. Similarly, KU812 cells nucleofected with pre-miR-10 showed an increase in expression of *hsa-miR-10a* and a decrease in *HOXA1* mRNA and *USF2* mRNA and protein levels (Fig. 3A and B). These data indicate that *hsa-miR-10a* regulates the expression of *USF2* (at least) through decrease in mRNA stability. In addition, we analyzed the expression of other genes presumably not regulated by *hsa-miR-10a*, such as *GAPDH, RPS18*, and *CCND1*, and found them not to be regulated by this miRNA (Supplementary Fig. S6).

To evaluate whether USF2 is a potential target of *hsa-miR-10a*, we did a *Renilla* luciferase reporter assay with a vector containing the putative *USF2* 3′-UTR target site downstream of a *Renilla* reporter gene (*Renilla*-USF2 3′-UTR vector). Predicted base pairing between *hsa-miR-10a* and a putative target site in the 3′-UTR of *USF2* shows perfect complementarity between nucleotides 2 to 8 of *hsa-miR-10a* and the target, resulting in a seed of seven nucleotides (Fig. 4A). *Renilla*-USF2 3′-UTR vector, control vector containing firefly luciferase, and pre-miR-10a or negative precursor-miR control were transfected into RKO cell line. Firefly luciferase activity was evaluated to normalize for transfection efficiency. *Renilla* luciferase activity of RKO cells transfected with pre-miR-10a was significantly lower ($P = 0.001$) than RKO cells transfected with negative precursor-miR control (Fig. 4B). These data indicate that *USF2* is a potential target of *hsa-miR-10a*.

USF2 *Is Up-Regulated in CML Patients*

To confirm our results in a more clinically relevant model, we analyzed the expression of *USF2* in BM MNCs from a group of patients with newly diagnosed CML ($n = 85$) and in healthy donors ($n = 21$). Normalized ratios for *USF2* expression determined in BM specimens, from healthy individuals

[9] http://pictar.bio.nyu.edu

(N_{USF2} ratios) fell between 82% and 100% (mean N_{USF2}: 94 ± 7%). However, CML patient mean N_{USF2} was 225 ± 205% (range, 89-1,057%), significantly higher than ratios found in healthy donors ($P = 0.01$). Moreover, a N_{USF2} value above 125% (determined as the mean from healthy donors plus 3 SD) was chosen to define overexpression of *USF2* in CML samples. Using this cutoff value, up-regulation of *USF2* was found in 60% (51 of 85) of CML patients.

We also analyzed *hsa-miR-10a* expression in the same group of CML patients. Mean expression of *hsa-miR-10a* was 37% (range, 0.05-6,690%) being down-regulated in 60 of 85 (71%) CML patients (down-regulation was considered when expression of *hsa-miR-10a* was lower than the mean − 4 SD) compared with normal BM cells. CML patients with a decreased expression of *hsa-miR-10a* miRNA showed overexpression of *USF2* mRNA, whereas CML patients with normal levels of *hsa-miR-10a* presented normal *USF2* mRNA expression (*hsa-miR-10a* expression was 85 ± 159% in CML patients with up-regulation of *USF2*, and 515 ± 1,210% in patients with normal *USF2* expression; $P = 0.004$). These results lead us to suggest that *hsa-miR-10a* down-regulation may cause increased levels of USF2 also in CML cells from patients.

Down-Regulation of hsa-miR-10a *and Overexpression of USF2 Increase CML Cell Growth*

We wanted to study whether down-regulation of *hsa-miR-10a* or up-regulation of USF2 could lead to increased cell growth of CML cells and, therefore, could be involved in the development or progression of CML. To evaluate this, *hsa-miR-10a* expression levels were altered in KU812 cells by nucleofection with pre-miR-10a or anti-miR-10 and cell growth was evaluated and compared with that of cells transfected with the pre-miR or anti-miR scrambled controls, respectively. Interestingly, an increase in *hsa-miR-10a* expression (Fig. 5A), which was associated with a decrease in USF2 protein expression (Fig. 5B), induced a decrease in cell growth (Fig. 5C), whereas a decrease in *hsa-miR-10a* expression resulted in an increase in cell growth (Fig. 5D).

Finally, to examine whether USF2 expression could participate in *hsa-miR-10a*–mediated regulation of cell growth, small interfering RNAs (siRNA) against USF2, or siRNA Silencer Negative Control-1, were nucleofected in KU812 cells. siRNAs against USF2 efficiently decrease USF2 expression as detected by Q-RT-PCR (Fig. 6A) or by Western blot analysis (Fig. 6B). Down-regulation of USF2 with siRNAs resulted in a decrease in cell growth compared with control cells (Fig. 6C and D). These results indicate that down-regulation of *hsa-miR-10a* and consequently overexpression of *USF2* participate in the abnormal growth of CML cells.

Discussion

Treatment of CML has notably been improved by the development of imatinib mesylate, a tyrosine kinase inhibitor that blocks the kinase activity of BCR-ABL1, defining this drug as the gold standard first-line therapy for CML patients (36, 40, 41). In contrast, it has been recently shown that imatinib mesylate does not inactivate all BCR-ABL1–activated signaling pathways that are essential for CML cell survival

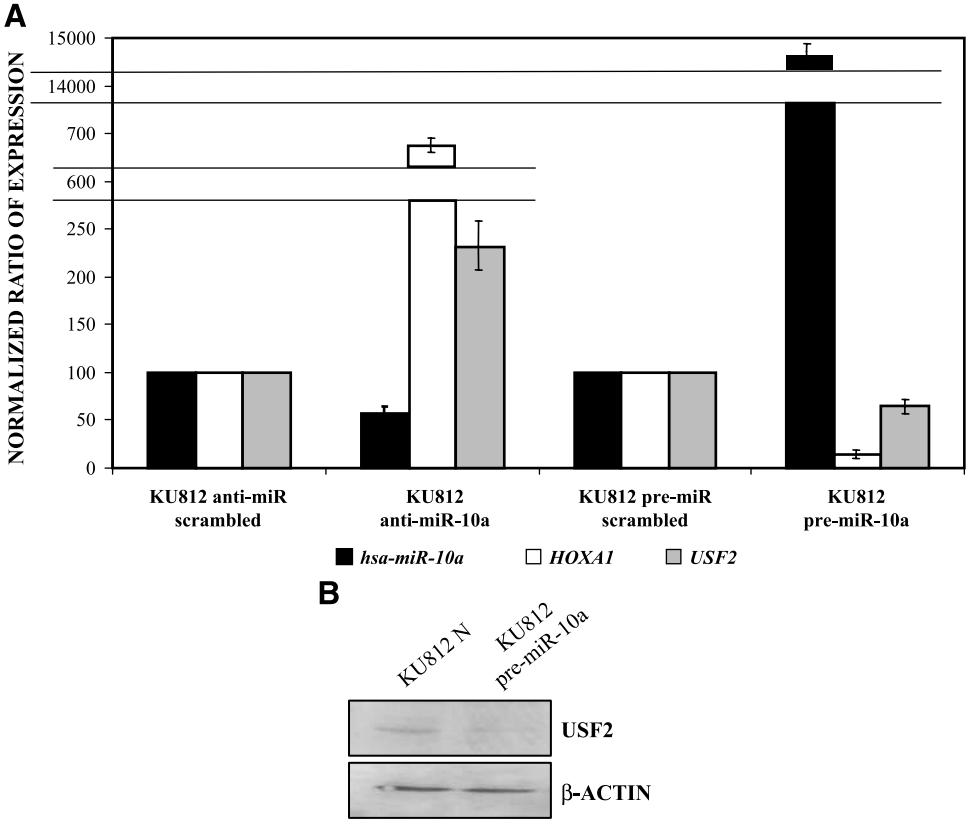

FIGURE 3. Quantitative expression of *hsa-miR-10a, HOXA1*, and *USF2* mRNA levels in KU812 cells. **A.** KU812 cells were transfected by nucleofection with pre-miR-10a, scrambled precursor control, anti-miR-10a, and scrambled anti-miR control as described in Materials and Methods. Cells were harvested 48 h after transfection, and *hsa-miR-10a, HOXA1*, and *USF2* expression was analyzed by Q-RT-PCR. *HOXA1* and *USF2* levels increased in cells nucleofected with anti-miR-10a and decreased in cells nucleofected with pre-miR-10a. Columns, mean of three different experiments; bars, SD. **B.** Western blot analysis of USF2 protein expression 48 h after transfection with the pre-miR-10a or scrambled precursor control. β-Actin was used as a loading control.

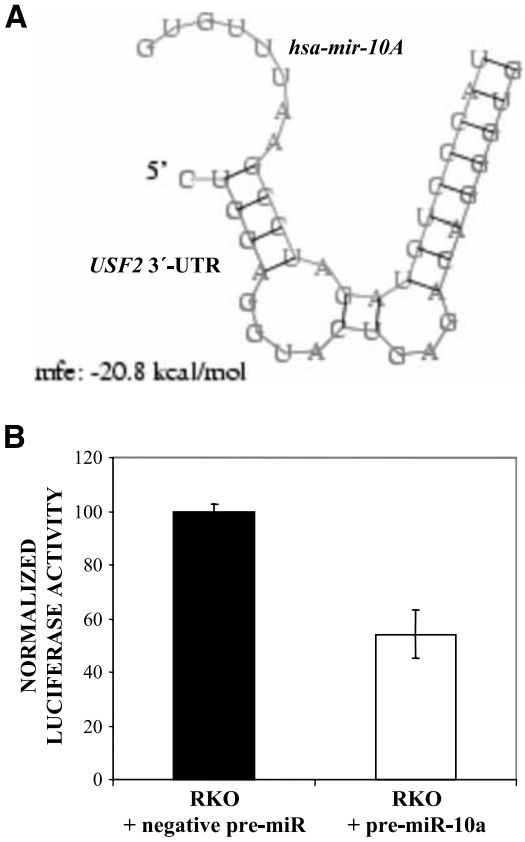

FIGURE 4. *USF2* is a target of *hsa-miR-10a*. **A.** Complementarity between *hsa-miR-10a* and the *USF2* 3′-UTR as predicted by PicTar. **B.** *Renilla* luciferase reporter assay of RKO cell line transfected with *Renilla*-USF2 vector, control vector containing luciferase, and pre-miR-10a or negative precursor-miR control. Columns, mean of three experiments from three independent transfections; bars, SD.

(42). This implies that persistent malignant progenitors can be a potential source of relapse in CML patients and that there is a need to improve our understanding of the biology of CML to provide new targets for therapy. The recent implication of miRNAs in the regulation of important biological processes altered in CML, such as cell cycle, apoptosis, and adhesion (2), establishes these small RNA molecules as potential players in CML pathogenesis.

The result of the current study identifies certain miRNAs (*hsa-miR-10a, hsa-miR-96, hsa-miR-150*, and *hsa-miR-151*) to be abnormally regulated in patients with CML and thus provides support to our hypothesis that miRNAs could be implicated in CML. While this article was in preparation, another study described the up-regulation of the polycistronic *miR-17-92* cluster in $CD34^+$ cells of CML patients (33). Unlike that study, we did not observe an up-regulation of *miR-17-92* in CML $CD34^+$ cells. The different results could be explained on the bases of the different strategies used for normalization. Whereas normalization of miRNA expression was done using the expression of *hsa-miR-16* miRNA in the study from Venturini et al. (33), we used expression of *GUSB* and *U6* genes for normalization as this strategy has been validated in recent studies (34). It is important to point out that the analysis of differentially expressed miRNAs in $CD34^+$ cells of CML patients indicates that *hsa-miR-16* is down-regulated, thus making this miRNA inappropriate for normalization of data. Furthermore, *hsa-miR-16* negatively regulates the antiapoptotic protein BCL2 (11) that has also been implicated in CML (43). The use of a certain miRNA for normalization of the data based on the assumption that expression of the specific miRNAs is constant regardless of the tissue or the disease may not be a reliable way of normalizing the results (10, 12, 21, 44).

As we and others have previously shown, the presence of the p210 BCR-ABL1 fusion oncoprotein regulates directly or indirectly the mRNA and/or the protein expression of several genes that participate in the deregulation of the cell cycle, apoptosis, and adhesion observed in CML cells (45, 46). We decided to examine whether the abnormal expression of *hsa-miR-10a, hsa-miR-96, hsa-miR-150*, and *hsa-miR-151* could be dependent of BCR-ABL1 and its kinase activity. The result showed that *hsa-miR-150* and *hsa-miR-151* decreased as a result of functional expression of BCR-ABL1. Further studies are required to address the molecular mechanism of BCR-ABL1–mediated down-regulation and whether down-regulation of *hsa-miR-150* and *hsa-miR-151* is essential for BCR-ABL1 role in CML.

The expression of *hsa-miR-10a* was independent of BCR-ABL1 activity and this could in fact suggest that abnormal expression of *hsa-miR-10a* is associated with the development of the disease. Interestingly, in a recent study in hematopoietic $CD34^+$ cells, *hsa-miR-10* has been designated as one of the 33 miRNAs implicated in hematopoiesis (hematopoietic-expressed miRNAs; ref. 47). According to this model, expression of these miRNAs would be responsible for controlling differentiation of hematopoietic cells so that once differentiation is activated certain miRNAs are down-regulated. Consistent with Georgantas et al. (47), *hsa-miR-10a* was normally expressed in BM MNC and $CD34^+$ cells of healthy donor samples, but the expression of this miRNA was down-regulated in CML patient samples. Furthermore, reexpression of *hsa-miR-10a* in CML cells decreased cell growth, thus supporting the potential role of *hsa-miR-10a* in CML disease.

Different mechanisms, such as (22) localization of miRNA in a loss of heterozygosity region (8), epigenetic methylation of the CpG islands of miRNAs (48, 49), mutations in miRNA sequences (22), or altered miRNA maturation or stability (50), could be implicated in the regulation of miRNA expression. *hsa-miR-10a* is localized in 17q21.32 genome region, which is not a chromosome fragile site or a commonly deleted chromosome region in CML. Furthermore, a defined CpG island could not be found near the *hsa-miR-10a* chromosome localization, making unlikely that an epigenetic methylation mechanism participates in the regulation of *hsa-miR-10a* in CML. In addition, we did not detect an up-regulation of *hsa-miR-10a* expression after treatment of KU812 cells with the inhibitor of histone deacetylase trichostatin A or the demethylating agent 5-aza-2′deoxycytidine (data not shown), further indicating that *hsa-miR-10a* expression is not regulated by abnormal methylation or histone acetylation.

Down-regulation of *hsa-miR-10a* in CML could not be explained by the presence of gene mutations, as we did not find

any point mutations in the 201-bp genomic region that includes the *hsa-miR-10a* sequence using any of the studied samples of CML $CD34^+$ (data not shown). We also compared the expression of *DROSHA* and *DICER* genes, components of the miRNA maturation machinery, by Q-RT-PCR in 16 BM samples of Philadelphia chromosome–positive, chronic-phase CML patients and 10 healthy donors to determine whether the maturation of miRNA could be responsible for the abnormal expression of *hsa-miR-10a*. No statistically significant differences in the expression of *DROSHA* or *DICER* were found between CML and healthy donors, suggesting that alterations in the processing machinery of miRNAs are not responsible for the decreased expression of *hsa-miR-10a* in CML (data not shown). We also believe that *hsa-miR-10a* should be properly matured in CML cells as we failed to detect accumulation of *hsa-miR-10a* pre-miRNA or pri-miRNA precursors by primer extension (data not shown). Further studies will be required to identify the mechanisms that regulate the *hsa-miR-10a* expression in CML.

In conclusion, our study shows that the expression of the *hsa-miR-10a* miRNA is down-regulated in CML patients and that *hsa-miR-10a* regulates expression of *USF2*. Further, these abnormalities are independent of the BCR-ABL1 activity and are implicated in the abnormal growth of CML cells. Further studies will be necessary to identify other *hsa-miR-10a* targets and to show the exact role of *hsa-miR-10a* and its target genes in the development of CML.

Materials and Methods

Cell Lines and Patient Samples

Human-derived Mo7e, Mo7e transfected with p210 isoform of BCR-ABL1 (Mo7e-p210), and CML-derived cell lines TCC-S, KU812, and BV173 were grown in RPMI 1640 supplemented with 20% fetal bovine serum, 20 mmol/L HEPES buffer, and penicillin-streptomycin (all of them from BioWhittaker) with (Mo7e) or without (Mo7e-p210, TCC-S, KU812, and BV173) 10 μg/μL of recombinant human interleukin-3 (PeproTech) as described (45, 46). In some experiments, Mo7e-p210 cells were treated with 2 μmol/L imatinib for 24 h. Colon carcinoma–derived RKO cell line was grown in MEM (Life Technologies) supplemented with 10% fetal bovine serum and penicillin-streptomycin (BioWhittaker).

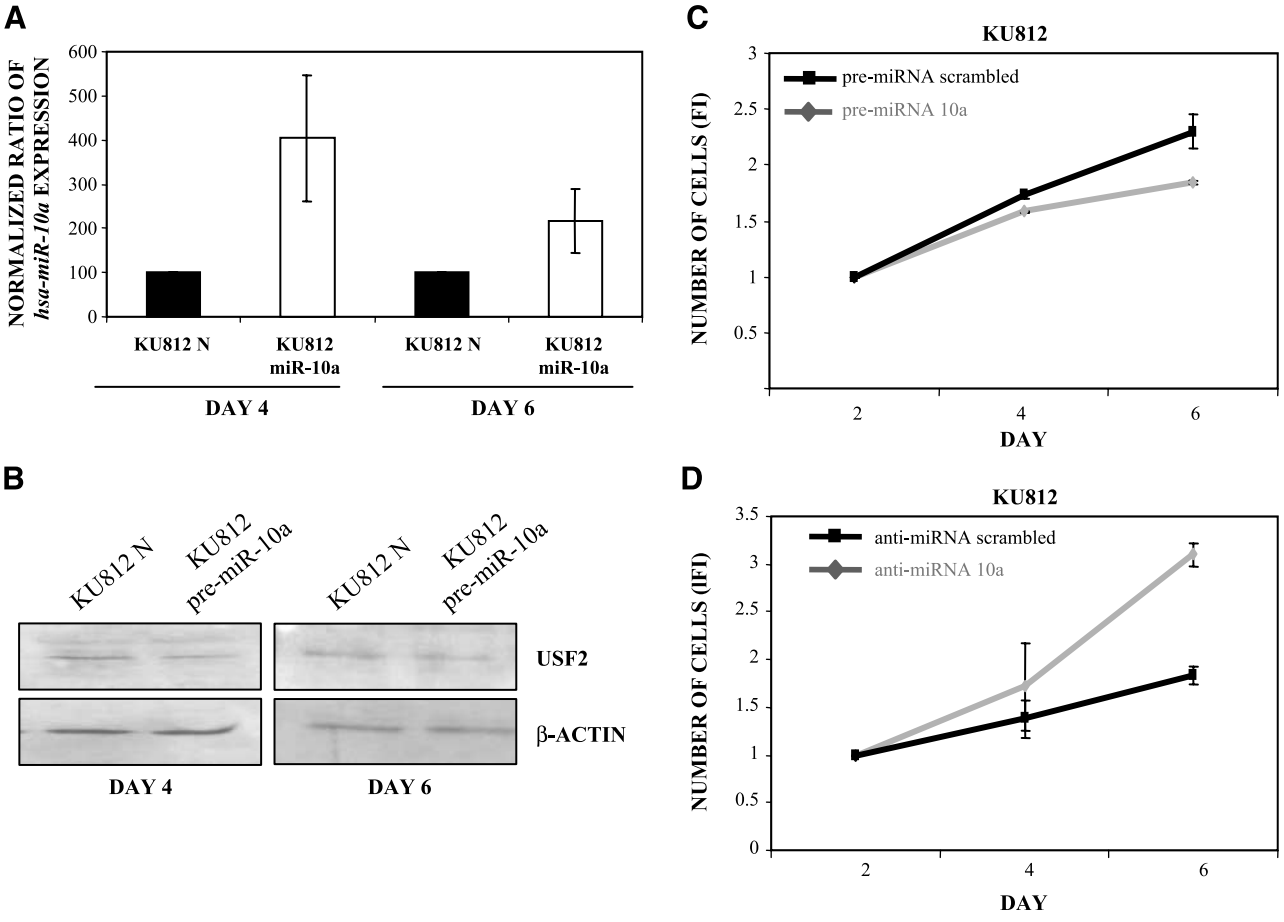

FIGURE 5. *hsa-miR-10a* transfection decreases growth of CML cells. **A.** *hsa-miR-10a* expression analysis by Q-RT-PCR 4 and 6 d after pre-miR-10a nucleofection. **B.** Western blot analysis of USF2 protein 4 and 6 d after pre-miR-10a nucleofection. β-Actin was used as a loading control. **C** and **D.** KU812 cells were transfected by nucleofection with pre-miR-10a molecule and scrambled precursor control (**C**) and anti-miR-10a and anti-scrambled control (**D**). Cell viability and total cell counts were determined at various times by trypan blue exclusion. KU812 N, KU812 scrambled cells; KU812 miR-10a, KU812 transfected with pre-miR-10a molecule. Points, mean of three experiments; bars, SD.

BM cells were obtained from patients with CML at diagnosis and from normal volunteer donors. All CML patients were 100% Philadelphia chromosome positive by conventional cytogenetic analysis. MNCs were isolated by sedimentation on Ficoll-Hypaque gradients. $CD34^+$ cells were isolated using the MACS $CD34^+$ isolation kit (Miltenyi Biotec) and the AutoMACS selection device as described (45). $CD34^+$ purity was always above 90%. All studies were approved by the Investigational Review Boards at the University of Navarra. All the samples were obtained after informed consent of the patients and donors.

RNA Extraction and Reverse Transcription

Total RNA was extracted with Ultraspec (Biotecx) following the manufacturer's instructions. Total RNA (5 ng) was used to synthesize a specific cDNA of each analyzed miRNA according to the Taqman MicroRNA Assay protocol (PE Applied Biosystems). Reverse transcriptase reactions contained 1.5 μL of miRNA-specific stem-loop reverse transcriptase primer and 6 μL of Master Mix composed of 5 ng RNA, 1× reverse transcriptase buffer, 0.25 mmol/L of each deoxynucleotide triphosphate, 3.33 units/μL of MultiScribe reverse transcriptase, and 0.25 unit/μL of RNase inhibitor (PE Applied Biosystems). The 7.5 μL reactions were incubated in a PE Applied Biosystems 2720 Thermo Cycler in a 96-well plate for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C.

Expression Analysis by Q-RT-PCR

Expression of 157 miRNAs was analyzed using specific primers and Taqman probe for each miRNA according to the Taqman MicroRNA Assay protocol. Q-RT-PCR was done in a PE Applied Biosystems 7300 Sequence Detection system using 0.7 μL of reverse transcriptase product of each miRNA in a reaction volume of 10 μL with 1× Taqman Universal PCR master mix and 1 μL of primer and probe mix according to the Taqman MicroRNA Assay protocol (PE Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 10 min. The C_t data were determined using default threshold settings.

Validation of miRNAs was done by Q-RT-PCR using the standard curve method [User Bulletin N°2, part number (PN) 4303859; PE Applied Biosystems]. Individual Taqman miRNA assays for *hsa-miR-10a* (PN: 4373153), *hsa-miR-96* (PN: 4373010), *hsa-miR-125a* (PN: 4373149), *hsa-miR-150* (PN: 4373127), and *hsa-miR-151* (PN: 4373179) were used (PE Applied Biosystems).

Expression of *HOXA1* was analyzed using Assay-on-Demand (Hs 00171793_m1; PE Applied Biosystems). Q-RT-PCR for *USF2, DROSHA*, and *DICER* was done with the LightCycler technology using 1 μL cDNA in 20 μL reaction volume with 0.4 μm/L of each primer [*USF2*, 5′-AAAAATTGATGGAACCAGAACACC-3′ (forward) and 5′-TCCCGTCTTGCTGTTGTC-3′ (reverse); *DROSHA*, 5′-CAAGATGACCCAACTCCCT-3′ (forward) and

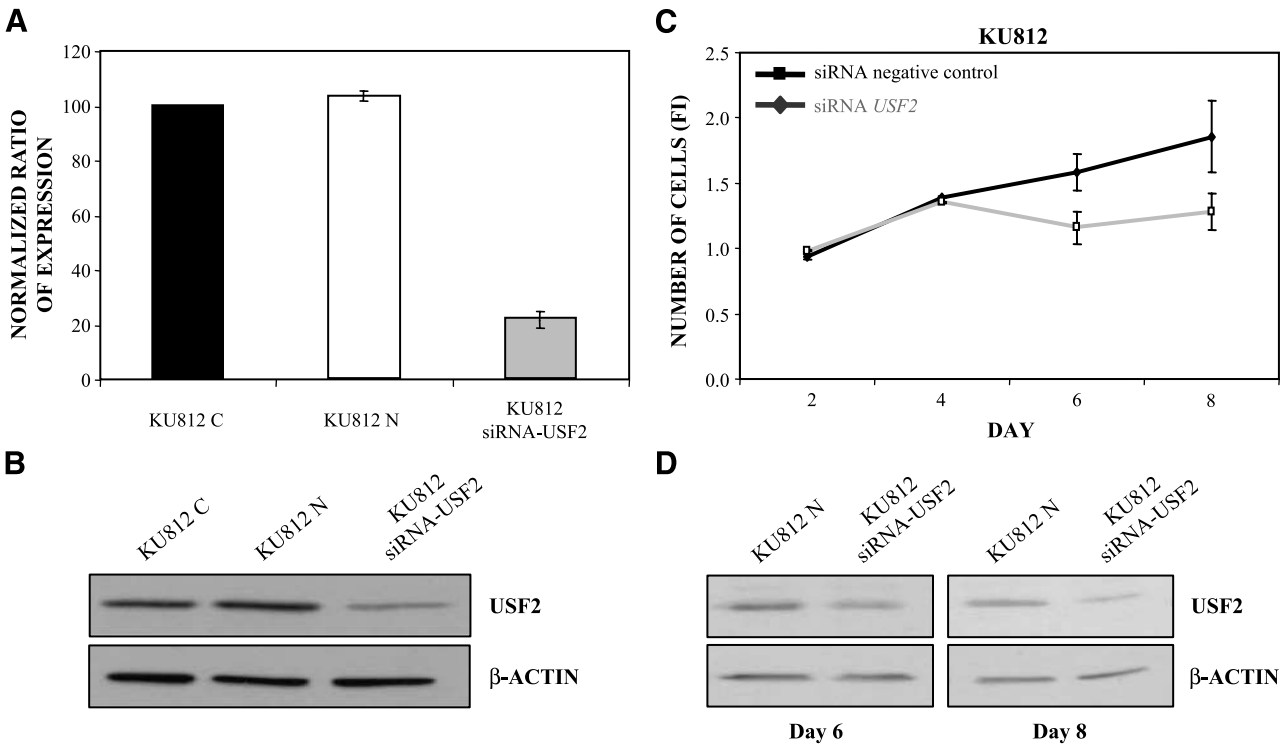

FIGURE 6. Inhibition of *USF2* by siRNA induces a decrease in cell growth. KU812 cells were transfected by nucleofection with *USF2* siRNA and Silencer Negative Control-1. **A.** *USF2* mRNA analysis by Q-RT-PCR. **B.** Western blot analysis of USF2 protein. β-Actin was used as a loading control. **C.** Cell viability and total cell counts of transfected KU812 cells were determined at various times by trypan blue exclusion. **D.** Expression of USF2 protein by Western blot analysis at 6 and 8 d after siUSF2 nucleofection. β-Actin was used as a loading control. KU812 N, KU812 scrambled cells. **A** to **C.** Values represent mean ± SD of three experiments.

5′-TTCTGAACAATGGCAGTCCG-3′ (reverse); and *DICER*, 5′-TCTGCCAATTAACTCACCTC-3′ (forward) and 5′-TAACTATCCCTCAAACACTCTG-3′ (reverse)] and 2 μL of 10× LightCycler FastStart DNA Master SYBR Green I. The final Mg^{2+} concentration in the reaction mixture was adjusted to 3.5 mmol/L. The following program conditions were applied for Q-RT-PCR running: 95°C for 10 min; amplification program, consisting of 45 cycles at 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s; melting program, 1 cycle at 95°C for 0 s, 40°C for 60 s, and 90°C for 0 s; and cooling program, 1 cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, which was 0.2°C/s between 40°C and 90°C. *U6* gene was used as reference gene. To reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes versus their controls/calibrators in relation to the reference gene was used. Calculations were automatically done by the LightCycler software (RealQuant, version 1.0, Roche). The normalized ratios of *USF2* (N_{USF2}), *DROSHA* (N_{DROSHA}), and *DICER* (N_{DICER}) expression were obtained from the following equation and expressed as percentage of the control/calibrator:

$$\text{Normalized ratio (N)} = (E_{\text{target}})^{\Delta \text{Cp target (control}-\text{sample)}} / (E_{\text{ref}})^{\Delta \text{Cp ref (control}-\text{sample)}}.$$

Efficiencies (E) of each gene were calculated from the slopes of crossover points (Cp) versus DNA concentration plot according to the formula $E = 10^{(-1/\text{slope})}$. ΔCp corresponded to the difference between control/calibrator Cp and sample Cp, either for the target or for the reference sequences. The selected controls/calibrators were BM specimens from healthy donors. They were considered as 100% expression.

Primer Extension

hsa-miR-10a expression was analyzed by Primer extension technique as described previously (51) using (5′-TACACAAATTCGGATCTACA-3′) oligonucleotide for *hsa-miR-10a* and (5′-TGCTAATCTTCTCTGTATCGT-3′) oligonucleotide for *U6*. Six picomoles of γ-ATP–labeled *hsa-miR-10a* oligonucleotide were incubated with 3 μg of total RNA and only 0.25 μg of RNA with oligonucleotide *U6*. Samples were loaded onto a 14% polyacrylamide gel and separated by electrophoresis. Gels were dried and exposed to a screen that was developed in a Cyclone phosphorimager (Perkin-Elmer).

Normalization and Data Analysis

Expression of miRNAs was normalized using the expression of *GUSB* or *U6* genes in each sample as previously described (34). Primers for expression analysis of *GUSB* were GUS-R (5′-CCGAGTGAAGATCCCCTTTTTA-3′) for the reverse transcription and primers GUS-F (5′-GAAAATATGTGGTTGGAGAGCTCATT-3′), GUS-R, and probe GUS-P (5′-^{FAM}C-CCAGCACTCTCGTCGGTGACTGTTCA^{TAMRA}-3′) for the Q-RT-PCR. For expression of *U6*, we used Taqman RNU6B assay (PN 4373381; PE Applied Biosystems). Relative quantification of expression of analyzed 157 miRNAs was calculated with the $2^{-\Delta\Delta Ct}$ method (User Bulletin N°2, PN 4303859; PE Applied Biosystems). The data are shown as ΔC_t of the relative quantity of target miRNAs, normalized, and compared with expression in healthy donor samples.

An unsupervised cluster analysis was carried out using Cluster and TreeView software (52). Hierarchical clustering based on the average linkage method with the centered correlation metric was done. To identify genes with statistically significant changes in expression between both groups, we did a supervised analysis using the algorithm significant analysis of microarrays (53). All data were permuted over 100 cycles by using the two-class (unpaired) format.

Finally, miRNA expression was compared between CML and healthy donor samples using Statistical Package for the Social Sciences package, version 13.0 (SPSS). Differences between two independent groups were analyzed by the Student's *t* test or the Mann-Whitney *U* test depending on variable distribution (54). A *P* value of ≤0.05 was considered significant.

Cell Line Transfection

The pre-miR-10a precursor molecule, negative precursor-miR control, anti-miR-10a inhibitor, negative anti-miR control, *USF2* siRNA (AM16708), and Silencer Negative Control-1 were purchased from Ambion. The colon carcinoma–derived RKO cell line was transfected with each of the precursor and inhibitor molecules at final concentration of 50 nmol/L using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's instruction. In a different set of experiments, the CML-derived cell line KU812 was nucleofected using 100 μL of Cell Line Nucleofector Solution V (Amaxa GmbH) with each of the precursor-miR, inhibitor-miR molecules, *USF2* siRNA, and Silencer Negative Control-1 at a final concentration of 50 nmol/L using program T-06 from the Nucleofector device (Amaxa GmbH). After 48 h, cells were harvested to analyze the expression of *hsa-miR-10a, HOXA1*, or *USF2* by Q-RT-PCR. Transfection efficiency was determined by flow cytometry using the Silencer FAM-labeled Negative Control (Ambion) for the RKO cell line and BLOCK-iT Fluorescent Oligo (Invitrogen Life Technologies) for the KU812 cell line. Cell viability and total cell counts were determined at various times by trypan blue exclusion and found to be always above 80%.

hsa-miR-10a *Genomic Sequence Analysis*

DNA from CML cells was extracted using QIAamp DNA Mini kit (Qiagen). PCR for amplification of the 201-bp genomic sequence where *hsa-miR-10a* is located was done in a total volume of 25 μL using 1 μL genomic DNA, 1 unit AmpliTaq Gold DNA Polymerase (PE Applied Biosystems), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, and 20 pmol of each primer (10a-D: 5′-CTGGAAAATTTCTGGGCCAA-3′ and 10a-R: 5′-CCAGACTGTCCTCATTCAGAAAAA-3′). Amplification products were purified using QIAquick PCR Purification kit (Qiagen) and sequenced with ABI PRISM d-Rhodamine Terminator Cycle Sequencing kit in an ABI PRISM 377 DNA Sequencer (PE Applied Biosystems).

Renilla *Luciferase Assay*

Renilla luciferase construct *Renilla*-USF2 3′-UTR was made by ligating oligonucleotides (USF2-3′-UTR-D: 5′-CTAGAGCTGCCTCCTGCTCTCTGGAGGTACTGAGACAGGGTGCTGATGGGAAGGAGGAGC-3′; USF2-3′-UTR-R: 5′-GGCCGCCCTCCTTCCCATCAGCACCCTGTCTCAGTACCTCCAGAGAGCAGGAGGCAGCT-3′) containing the putative target site of *USF2* 3′-UTR into *Xba*I-*Not*I site of the *Renilla* reporter vector (pRL-SV40, Promega). Cells (250,000) of RKO cell line were cotransfected using Lipofectamine (Invitrogen) with 0.5 μg of *Renilla*-USF2 3′-UTR vector, 2 μg of the control vector containing firefly luciferase (pGL3-Promoter, Promega), and 50 mmol/L of pre-miR-10a or negative precursor-miR vector. *Renilla* luciferase activity was measured 48 h after transfection using Dual Luciferase System (Promega) in a Berthold Luminometer (Lumat LB 9507) as previously described (51).

Western Blot Analysis

Proteins extracted from the CML cell line KU812 nucleofected with *USF2* siRNA and Silencer Negative Control-1 were analyzed by Western blot as previously described (55). The membranes, after being blocked, were incubated first with primary antibody for USF2 (diluted 1:1,000 for 2 h; Santa Cruz Biotechnology) or β-actin (1:4,000 for 1 h; Sigma) and then with alkaline phosphatase–conjugated secondary antibodies (1:10,000 for 1 h; Sigma). Bound antibodies were revealed by a chemiluminescent reagent (Tropix) and detected using Hyperfilm enhanced chemiluminescence (Amershan Biosciences). β-Actin was used as a loading control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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