miR-223 Regulates Cell Growth and Targets Proto-Oncogenes in Mycosis Fungoides/Cutaneous T-Cell Lymphoma

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The pathogenesis of the cutaneous T-cell lymphoma (CTCL), mycosis fungoides (MF), is unclear. MicroRNA (miRNA) are small noncoding RNAs that target mRNA leading to reduced mRNA translation. Recently, specific miRNA were shown to be altered in CTCL. We detected significantly reduced expression of miR-223 in early-stage MF skin, and further decreased levels of miR-223 in advanced-stage disease. CTCL peripheral blood mononuclear cells and cell lines also had reduced miR-223 as compared with controls. Elevated expression of miR-223 in these cell lines reduced cell growth and clonogenic potential, whereas inhibition of miR-223 increased cell numbers. Investigations into putative miR-223 targets with oncogenic function, including *E2F1* and *MEF2C*, and the predicted miR-223 target, *TOX*, revealed that all three were targeted by miR-223 in CTCL. E2F1, MEF2C, and TOX proteins were decreased with miR-223 overexpression, whereas miR-223 inhibition led to increased protein levels in CTCL. In addition, we showed that the 3′-UTR of *TOX* mRNA was a genuine target of miR-223. Therefore, reduced levels of miR-223 in MF/CTCL lead to increased expression of E2F1, MEF2C, and TOX, which likely contributes to the development and/or progression of CTCL. Thus, miR-223 and its targets may be useful for the development of new therapeutics for MF/CTCL.

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INTRODUCTION

MicroRNA (miRNA) are small, 18–22 bp, noncoding RNAs that negatively regulate protein translation (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001; Bartel, 2004). They elicit their effects on protein translation through the binding of the 3′ untranslated region (3′-UTR) of mRNA, primarily causing inhibition of translation or mRNA cleavage (Reinhart *et al.*, 2000; Llave *et al.*, 2002; Bartel, 2004). Since the discovery that a reduction in miR-15/16 may be a causal factor in the development of chronic lymphocytic leukemia (Calin *et al.*, 2002), there has been tremendous interest in the role of miRNA in the development and progression of malignancies, as well as in the use of miRNA in diagnostic signatures and cancer biomarkers.

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL) and is increasing in incidence (Weinstock and Horm, 1988). It classically presents as patches and plaques, and progresses to tumors, with eventual blood and visceral involvement. We have few therapeutic options in advanced MF, and the 5-year survival markedly reduces from 94% in stage IA to 48% in stage IIB and to 18% in stage IV (Agar *et al.*, 2010). In addition, little is known about the pathogenesis of MF. Although efforts have been made to identify potential causative infectious agents, genetic mutations, and chronic antigenic stimulants, there is no clear understanding of the etiology. Recently, studies have identified altered miRNA expression in MF and CTCL. Ballabio *et al.* (2010) reported decreased levels of miR-342 and miR-223 in Sézary syndrome, a leukemic form of CTCL. Another group identified a ''diagnostic signature'' of miRNA (miR-155, -203, and -205) in CTCL to help differentiate it from benign dermatoses (Ralfkiaer *et al.*, 2011). It is clear that miRNA are altered in MF/CTCL, but it is unknown what role these alterations have in the oncogenesis or progression of the disease. In a malignancy with increasing incidence, poor 5-year survival, and limited therapeutic options, it is imperative that we make efforts to further understand MF/CTCL.

miR-223 is an intergenic miRNA under the control of its own dedicated promoter (Pulikkan *et al.*, 2010). Interestingly,

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Abbreviations: CTCL, cutaneous T-cell lymphoma; MEF2C, myocyte-enhancing factor 2C; MF, mycosis fungoides; miRNA, microRNA; PBMC, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time PCR; TOX, thymocyte selection–associated high mobility group box; UTR, untranslated region

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it has been shown to be elevated in immature or blastic T-cell malignancies (Mavrakis *et al.*, 2011) but was found to have reduced expression in CTCL, a mature T-cell malignancy (Ballabio *et al.*, 2010; Narducci *et al.*, 2011; Ralfkiaer *et al.*, 2011). However, other groups have not detected a significant alteration in miR-223 expression between CTCL and controls (van Kester *et al.*, 2011; Qin *et al.*, 2012). On the basis of these data, the precise alteration and role that miR-223 has in CTCL has not been firmly established.

miR-223 has been shown to target potential oncogenic transcription factors, *E2F1* (Pulikkan *et al.*, 2010) and the myocyte-enhancing factor 2C (*MEF2C*) (Johnnidis *et al.*, 2008), in myeloid cells. A proposed miR-223 target, the thymocyte selection–associated high mobility group box (*TOX*) that is necessary for the development of $CD4^+$ T-lymphocytes, was recently found to be overexpressed in early-stage MF (Lewis *et al.*, 2005; Wang and El Naqa, 2008; Wang, 2008; Aliahmad *et al.*, 2012; Zhang *et al.*, 2012). Here we show that miR-223 is expressed at decreased levels in MF and CTCL and that miR-223 targets *TOX*, *E2F1*, and *MEF2C* in CTCL. Moreover, increasing miR-223 led to reduced levels of TOX and the oncogenic proteins, E2F1 and MEF2C, with an associated decrease in CTCL growth and clonogenic potential, indicating that miR-223 may be an inhibitor of CTCL development and/or progression.

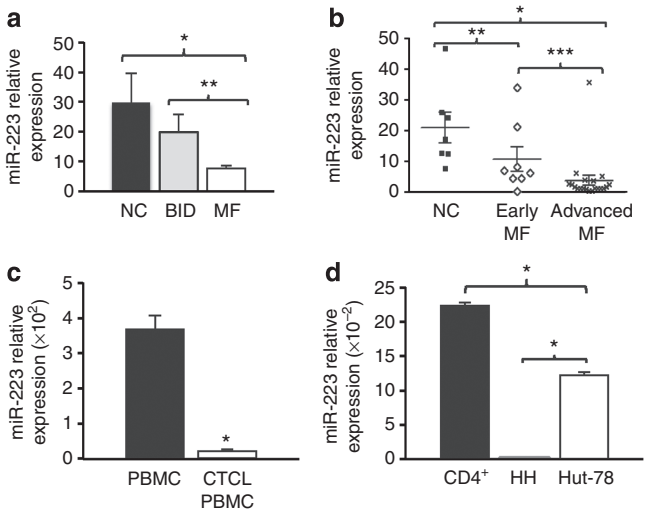

Figure 1. Reduced expression of microRNA (miR)-223 in mycosis fungoides (MF). miR-223 expression levels relative to control small RNA RNU24 levels by quantitative real-time reverse-transcriptase–PCR. (**a**) Skin biopsies from subjects with MF ($n=8$, early stage IA–IIA; $n=20$, advanced stage IIB–IV), normal controls (NC; $n=7$) ($^*P<0.001$), and benign inflammatory dermatoses (BID; $n=6$) ($^{**}P=0.04$). (**b**) Skin biopsies from NC and patients with early- or late-stage MF ($^*P<0.001$, $^{**}P=0.022$, $^{***}P=0.036$). (**c**) Pooled control Red Cross peripheral blood mononuclear cells (PBMCs) and cutaneous T-cell lymphoma (CTCL) PBMCs ($n=6$, $^*P<0.001$). (**d**) CTCL cell lines (HH and Hut-78) and $CD4^+$ NC ($n=2$, $^*P<0.001$).

RESULTS

Decreased miR-223 levels in MF/CTCL

Evidence suggests that miR-223 levels are reduced in CTCL, but its expression in MF has yet to be determined. To evaluate miR-223 levels in MF, we analyzed 28 tissue samples of MF ($n=8$ early stage I–IIA, $n=20$ advanced stage IIB–IV), 6 tissue samples of benign inflammatory dermatoses (BID), and 7 tissue samples of normal skin controls. BID samples were evaluated as they contain activated T cells and therefore serve as a comparison control to malignant activated T cells in MF. Quantitative real-time reverse-transcriptase–PCR (qRT-PCR) revealed a significant decrease in miR-223 in MF as compared with normal controls and BID (*t*-test, $^*P<0.001$, $^{**}P=0.004$, Figure 1a). There was no significant difference in miR-223 expression between the subgroups of BID (*t*-test, $P=0.16$). There was even greater reduction in miR-223 in advanced MF as compared with early-stage MF (*t*-test, $^*P<0.001$, $^{**}P=0.022$, $^{***}P=0.036$, Figure 1b). Peripheral blood mononuclear cells (PBMC) from patients with leukemic MF and Sézary syndrome ($n=6$) also demonstrated reduced miR-223 levels versus a pooled collection of PBMCs from Red Cross donors (*t*-test, $^*P<0.001$, Figure 1c). These data indicate that miR-223 is expressed at a reduced level in both the diseased skin and blood of MF patients as compared with normal controls and that miR-223 diminishes as the clinical stage advances.

To determine whether the decreased miR-223 levels observed in patients with MF were similar in CTCL cell lines, we measured miR-223 levels in two CTCL cell lines, HH and Hut-78. We determined that the miR-223 levels in these two CTCL cell lines were decreased as compared with levels in $CD4^+$ T cells from normal controls ($n=2$, Figure 1d). Specifically, Hut-78 had a greater than 45% reduction in miR-223 expression as compared with control (*t*-test, $^*P<0.001$), and the miR-223 levels were almost undetectable in HH cells (*t*-test, $^*P<0.001$). Therefore, miR-223 is reduced in CTCL lines as well as in patient samples, making the CTCL lines a viable tool to test the effects of miR-223.

miR-223 inhibits CTCL cell growth and clonogenic potential

Although miRNA are altered within MF/CTCL, it is unknown what role these changes have in the malignancy. To assess the consequences of restoring miR-223 levels on CTCL growth, we transfected miR-223 mimic into both HH and Hut-78 cells. The increase in miR-223 levels from the mimic was determined by qRT-PCR (Figure 2a). By using the Trypan Blue Dye exclusion assay, we measured a significant decrease (30%) in viable cell numbers 72 hours post transfection in the HH cells transfected with the miR-223 mimic as compared with an RNA control ($n=5$, *t*-test, $^*P<0.001$, Figure 2b). Similarly, with increased levels of miR-223 from the mimic (Figure 2c), the Hut-78 cells showed a 29% and 20% decrease in viable cell numbers at 48 and 72 hours, respectively, following miR-223 transfection ($n=3$, *t*-test, $^*P<0.001$, Figure 2d). No difference in the number or percentage of dead cells was detected when comparing miR-223 mimic and RNA control samples at any of the times evaluated.

To assess the effect of increased levels of miR-223 on CTCL clonogenic capabilities, we transfected HH and Hut-78 cells with either miR-223 mimic or RNA control and performed

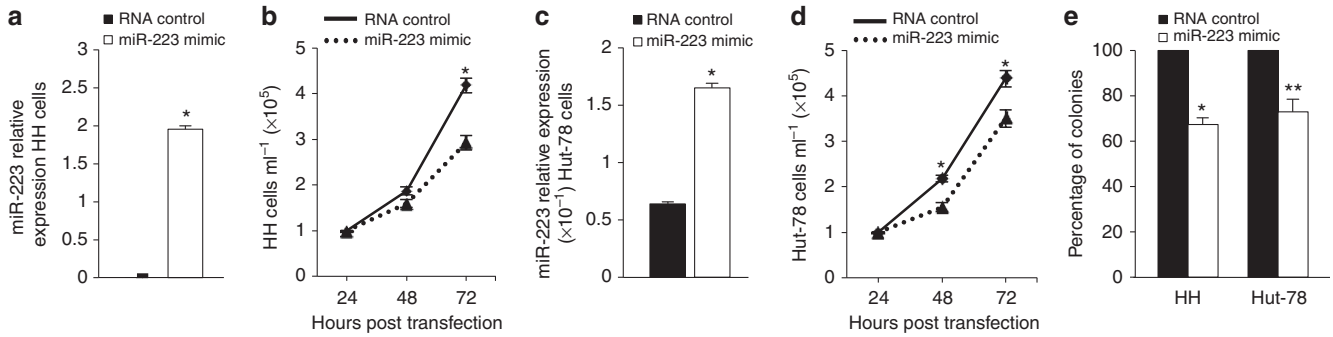

Figure 2. Increased microRNA (miR)-223 inhibits cutaneous T-cell lymphoma cell growth and decreases clonogenic potential. (**a**, **c**) miR-223 levels after transfection with the miR-223 mimic in HH and Hut-78 cells. (**b**) Number of viable HH ($n=5$) and (**d**) Hut-78 ($n=3$) cells transfected with miR-223 mimic or control RNA were determined at intervals by Trypan Blue Dye Exclusion assay (*$P<0.001$). (**e**) Methylcellulose clonogenic assays. Colony numbers in HH and Hut-78 cells transfected with miR-223 mimic are relative to cells transfected with RNA control ($n=3$; *$P<0.001$, **$P=0.003$).

methylcellulose assays. HH cells transfected with the miR-223 mimic had a 32.5% reduction in colony formation and Hut-78 cells had a 27.1% reduction in colony number as compared with RNA control–transfected cells ($n=4$, *t*-test, *$P<0.001$, **$P=0.003$, Figure 2e). We did not detect a difference in colony size between the mimic and RNA control–transfected cells.

To further assess the impact of altered miR-223 levels on CTCL growth, an inhibitor of miR-223 was used to block its function. Transfection of HH and Hut-78 cells with the miR-223 inhibitor led to increased cell growth, as measured by Trypan Blue Dye exclusion and MTS assays (Figure 3a and data are not shown). The Trypan Blue Dye exclusion assay showed a 24% increase in viable cell numbers in the HH cells transfected with miR-223 inhibitor versus the cells transfected with negative control ($n=3$, *t*-test, *$P<0.001$, Figure 3a). The Hut-78 cells also showed a statistically significant increase in viable cell numbers (18%) in miR-223 inhibitor–transfected cells by MTS assay ($n=3$, *t*-test, **$P=0.013$, Figure 3b). Together, our results indicate that miR-223 inhibits CTCL cell growth and clonogenic potential, but does not appear to have an impact on apoptosis.

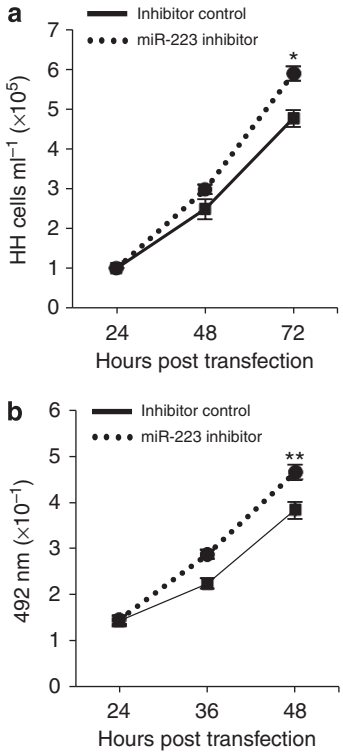

Figure 3. Inhibition of microRNA (miR)-223 enhances cutaneous T-cell lymphoma growth. (**a**) Number of viable HH cells transfected with miR-223 inhibitor or control ($n=3$) were determined at 24-hour intervals by Trypan Blue Dye Exclusion assay (*$P<0.001$). (**b**) Hut-78 cells transfected with miR-223 inhibitor or control ($n=3$) were evaluated by MTS assay (**$P=0.013$).

TOX is a direct target of miR-223

TOX, a protein normally expressed in thymocytes with unclear function, was recently found to have an aberrant increased expression in MF, suggesting a possible role in MF pathogenesis (Zhang *et al.*, 2012). By using online miRNA target databases (www.TargetScan.org and www.miRDB.org), we identified *TOX* as a potential miR-223 target. The predicted highly conserved miR-223-binding site (seed sequence) is within the 3′-UTR of TOX (Figure 4a). To test whether *TOX* is a direct target of miR-223, we generated a luciferase expression plasmid with the 3′-UTR of *TOX* containing the putative miR-223-binding site. We also generated a similar plasmid with a mutated seed sequence, in which miR-223 could not bind. NIH-3T3 cells were co-transfected with the wild-type or mutated *TOX* 3′-UTR luciferase expression plasmid and either miR-223 mimic or control RNA. The cells with the miR-223 mimic and the *TOX* wild-type 3′-UTR had a significant reduction (53.6%) in luciferase activity, compared with the RNA control–transfected cells ($n=3$, *t*-test, *$P<0.001$, Figure 4b). Moreover, the miR-223 mimic did not induce a significant change in luciferase activity in cells with the mutated 3′-UTR of *TOX* (Figure 4b). These results indicate that miR-223 binds to the 3′-UTR of *TOX* and shows that *TOX* is a direct target of miR-223.

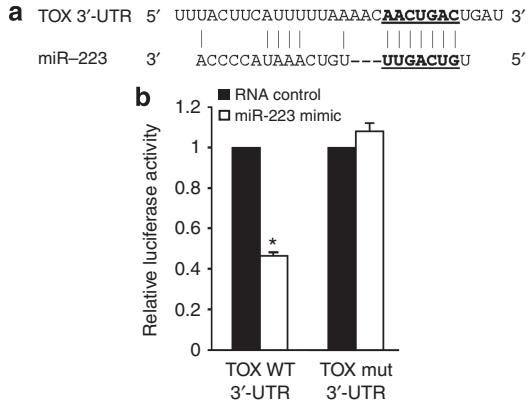

Figure 4. *TOX* is a direct target of microRNA (miR)-223. (**a**) miR-223 binding site within the 3′ untranslated region (3′-UTR) of thymocyte selection–associated high mobility group box (TOX) and miR-223 sequence (seed sequence underlined). (**b**) Luciferase activity was measured in NIH-3T3 cells transfected with miR-223 mimic or RNA control and a pMIR-REPORT plasmid with the wild-type 3′-UTR of *TOX* or a 3′-UTR of *TOX* with a mutated miR-223 seed sequence. Luciferase activity is relative to β-galactosidase activity, which controlled for transfection efficiency for each ($n=3$; $^{*}P<0.001$). WT, wild type.

miR-223 targets *E2F1*, *MEF2C*, and *TOX* in CTCL

We have identified *TOX* as a direct miR-223 target, and miR-223 has been previously shown to target the oncogenic transcription factors *E2F1* and *MEF2C* in myeloid cells (Johnnidis *et al.*, 2008; Pulikkan *et al.*, 2010). To determine whether these miR-223 targets are actually targeted by miR-223 in CTCL and thus could contribute to the pathogenesis of this disease, we first used qRT-PCR to assess the levels of *E2F1*, *MEF2C*, and *TOX* mRNA in CTCL lines. We detected significantly higher levels of *E2F1*, *MEF2C*, and *TOX* in HH cells as compared with $CD4^{+}$ controls ($n=3$; *t*-test, $^{*}P<0.001$, $^{**}P=0.007$, $^{***}P=0.006$, Figure 5a). In addition, increased levels of *E2F1* and *TOX* mRNA were observed in Hut-78 cells ($n=3$, *t*-test, $^{*}P<0.001$, Figure 5a). Immunohistochemistry also revealed increased TOX protein expression in both early and advanced MF patient samples as compared with normal and inflammatory controls (Figure 5b). There is notable staining of TOX in lymphocytes within both Pautrier's microabscesses and MF tumors.

To more directly test the effects of miR-223 on its targets in CTCL, we transfected HH cells with miR-223 mimic or control RNA and evaluated mRNA and protein levels by qRT-PCR and western blot, respectively, for E2F1, MEF2C, and TOX. miR-223 mimic–transfected cells had a statistically significant reduction in *E2F1* (24.5%), *MEF2C* (31.1%), and *TOX* (16.4%) mRNA versus RNA control–transfected cells ($n=4$, *t*-test, $^{*}P=0.005$, $^{**}P=0.003$, $^{***}P=0.019$, Figure 6a). We also assessed the protein levels of these targets. Western blots showed decreased protein expression of E2F1, MEF2C, and TOX in HH cells transfected with the miR-223 mimic compared with the RNA control–transfected cells (Figure 6b). Conversely, when the miR-223 inhibitor was transfected into Hut-78 cells, we observed an increase in protein expression in all three miR-223 targets (Figure 6c). These results suggest that

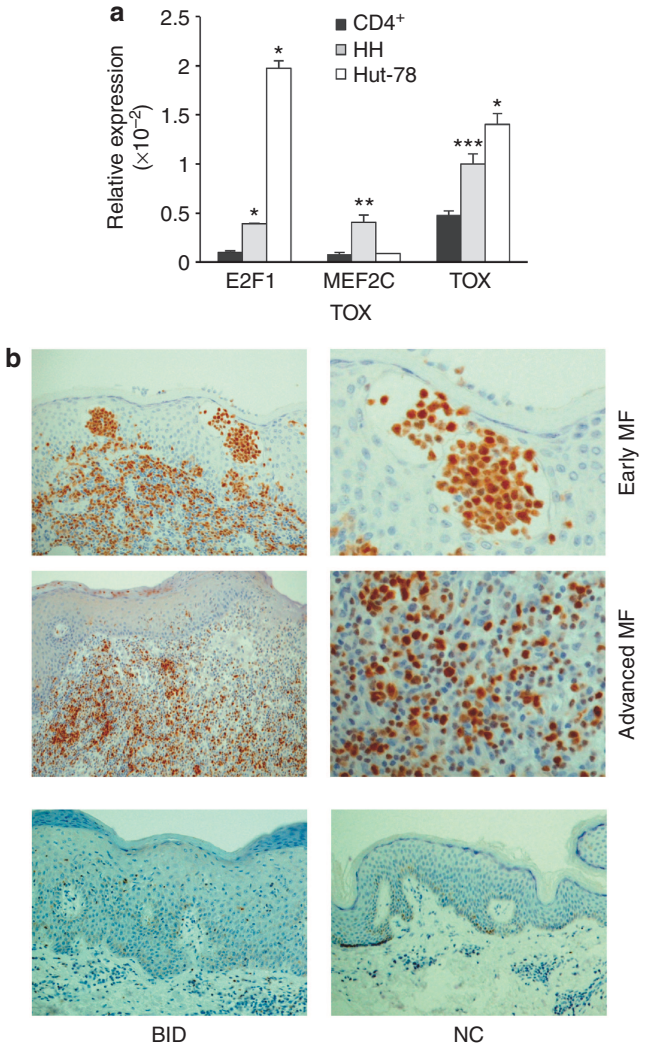

Figure 5. Endogenous expression of microRNA (miR)-223 mRNA targets is increased in cutaneous T-cell lymphoma. (**a**) Quantitative real-time reverse-transcriptase–PCR for miR-223 targets *E2F1*, *MEF2C*, and *TOX* mRNA levels relative to *β-actin* in HH, Hut-78, and $CD4^{+}$ cell controls ($n=3$; $^{*}P<0.001$, $^{**}P=0.007$, $^{***}P=0.006$). (**b**) Immunohistochemical staining of thymocyte selection–associated high mobility group box (TOX) in early-stage mycosis fungoides (MF; stage IA–IIA; $n=10$), advanced-stage MF (stage IIB–IV, $n=5$), benign inflammatory dermatoses (BID; $n=8$), and normal controls (NC; $n=3$) (representative photomicrographs of each shown).

miR-223 targets *E2F1*, *MEF2C*, and *TOX* in CTCL cells, and this likely contributes to MF pathogenesis.

DISCUSSION

miRNA have been shown to be altered within a wide variety of malignancies, including CTCL and MF (Ballabio *et al.*, 2010; Ralfkiaer *et al.*, 2011). It remains unclear whether these miRNA are innocent bystanders, occurring secondarily from other primary oncogenic mutations, or if they function as initiators and/or drivers of the disease. Our current study focused on miR-223 expression in early- and advanced-stage MF and CTCL and on identifying how it may affect oncogenesis

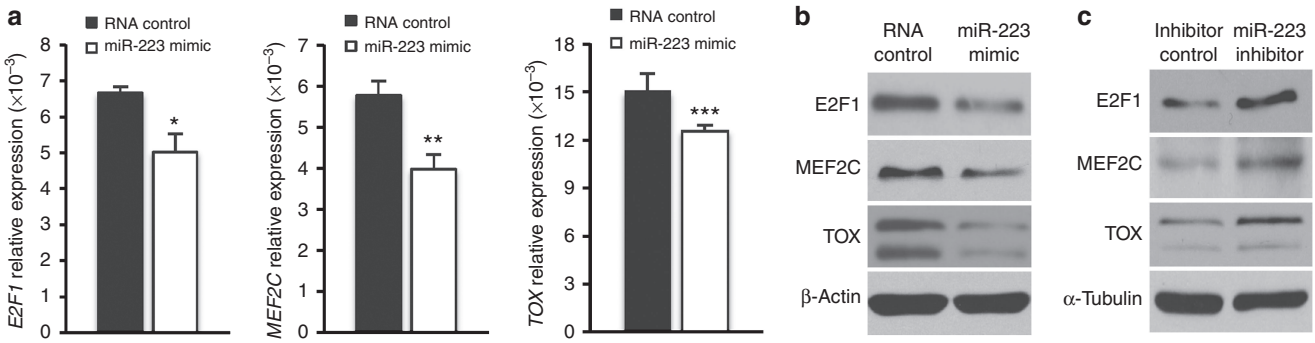

Figure 6. Modulation of microRNA (miR)-223 alters E2F1, myocyte-enhancing factor 2C (MEF2C), and thymocyte selection–associated high mobility group box (TOX) expression. (**a**) Quantitative real-time reverse-transcriptase–PCR of the miR-223 targets *E2F1*, *MEF2C*, and *TOX* relative to β-actin control in HH cells 48 hours after transfection with miR-223 mimic or RNA control ($n = 4$, $^{*}P = 0.005$, $^{**}P = 0.003$, $^{***}P = 0.019$). Western blots of E2F1, MEF2C, TOX, and β-actin or α-tubulin control in (**b**) HH cells after transfection with miR-223 mimic or RNA control and (**c**) Hut-78 cells after transfection with miR-223 inhibitor or inhibitor control.

or disease progression. Most of the research on miR-223 has centered on granulopoesis/myelopoesis, but less is known about miR-223 within T-cell malignancies (Fazi *et al.*, 2005, 2007; Fukao *et al.*, 2007). We demonstrated that miR-223 was significantly reduced in both early- an late-stage MF skin samples as compared with normal and inflammatory skin controls and that the levels decreased further as the clinical stage advanced. In addition, there were lower levels of miR-223 in CTCL PBMCs and cell lines compared with controls. The fact that miR-223 was consistently reduced across a variety of CTCLs and that it diminished as the disease clinically progressed suggests that miR-223 has an important role in the development and/or maintenance of MF/CTCL. Indeed, CTCL cell growth was reduced by increasing miR-223 levels, while inhibiting miR-223 increased growth. Clonogenic assays with miR-223 and CTCL cells revealed similar findings. Our data indicate that miR-223 retards CTCL growth by targeting pro-proliferative genes such as *E2F1* and *MEF2C*, resulting in decreased proliferation. However, we cannot exclude the possibility that elevated miR-223 levels may halt proliferation entirely in some CTCL cells, potentially by inducing senescence. Therefore, our results indicate that alteration of a single miRNA affects cell growth and clonogenic potential in CTCL and provides evidence that miR-223 is likely to have an integral part in disease development and/or progression.

To address how miR-223 is capable of affecting CTCL cell growth, we investigated potential and putative mRNA targets. The oncogenic transcription factor *E2F1* has been shown to be a target of miR-223 in acute myeloid leukemia cells (Pulikkan *et al.*, 2010). E2F1 is a member of the E2F family of transcription factors and is essential for cell cycle progression (Johnson and Degregori, 2006). *E2F1* functions as an oncogene and is amplified and/or overexpressed in many malignancies (Chen *et al.*, 2009). We demonstrated that *E2F1* levels could be modulated by miR-223 in CTCL cells. *MEF2C* was also shown to be targeted by miR-223 in myeloid cells (Johnnidis *et al.*, 2008). MEF2C is a transcription factor elevated in a subset of the T-cell malignancy, T-cell acute lymphoblastic leukemia. It has been shown to work synergistically with the RAS and MYC oncogenes to induce *in vitro* transformation of immortalized fibroblasts (Homminga *et al.*, 2011). We showed that miR-223 was able to regulate MEF2C protein expression. Our data suggest that the reduced levels of miR-223 in MF/CTCL allow for an increased expression of the oncogenic transcription factors E2F1 and MEF2C and may help to explain the dysregulated cell growth in CTCL.

TOX is a small DNA binding protein that is tightly regulated in the thymus during positive selection and is necessary for $CD4^{+}$ T-lymphocyte development. Once T cells have matured, TOX is normally no longer expressed (Aliahmad *et al.*, 2012). However, TOX has recently been shown to be overexpressed in mature $CD4^{+}$ lymphocytes in MF (Zhang *et al.*, 2012), and we have also found that TOX is increased in MF as compared with normal and inflammatory controls. We have demonstrated that *TOX* is a direct target of miR-223, and modulating miR-223 in CTCL cells led to significant changes in TOX protein expression. Although the function of TOX has yet to be established, it has been hypothesized that TOX may influence E-protein transcription factors and/or Id2, both of which are involved in lymphocyte proliferation and differentiation (Aliahmad *et al.*, 2012). Loss of E proteins in T-cell development mimics TOX overexpression in thymocytes (Jones and Zhuang, 2007). Elevated levels of Id2 have been found in CTCL (Cotta *et al.*, 2008), and it has been suggested that Id2 hastens cell cycle progression through interactions with the Rb family (Lasorella *et al.*, 1996). Therefore, miR-223 regulation of TOX should have significant ramifications for MF/CTCL growth.

Overall, MF/CTCL has been shown to have aberrant miRNA expression, including reduced miR-223 levels. Our study indicates that ''correcting'' this aberrancy leads to reduced CTCL cell proliferation and reduced clonogenic potential, which is mitigated through the effects of miR-223 target mRNA/protein. Our data provide evidence that miR-223 contributes to the oncogenesis and/or progression of MF/CTCL and that miR-223 or its targets should be evaluated for the development of new therapeutics.

MATERIALS AND METHODS

Tissue, blood, and cell lines

Frozen, banked PBMCs and 5–6 mm bisected skin biopsies from MF/CTCL patients were identified retrospectively. The diagnosis of MF/CTCL was confirmed through clinical and histological evaluation and flow cytometric analysis. Skin biopsies from patients with BID (e.g., psoriasis $n=3$, spongiotic dermatitis $n=3$) were identified retrospectively through clinical and histological reports. Control normal skin was obtained through discarded normal skin from surgery, and 6-mm biopsies were performed and the samples bisected and frozen. Dr Utpal Dave kindly provided control PBMC RNA from pooled Red Cross donors. The HH (CRL-2105) and Hut-78 (TIB-161) CTCL cell lines and NIH-3T3 cells were cultured as described by the American Type Culture Collection (Manassas, VA). $CD4^+$ cells from normal donors were obtained from Sanguine Biosciences (Santa Monica, CA). This study was approved by the Vanderbilt Institutional Review Board.

RNA isolation and qRT-PCR

RNA was isolated from skin biopsies, PBMCs, and cell lines using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's protocol, with one exception. To enhance the isolation of small RNA, the isopropanol precipitation step was performed overnight at $-20\,^{\circ}C$. Sequences for *β-actin*, *E2F1*, *MEF2C*, and *TOX*-specific qRT-PCR primer pairs were obtained from the Primer Bank (Harvard Medical School) and synthesized by Eurofins MWG Operon (Huntsville, AL). cDNA was generated and qRT-PCR was performed with SybrGreen (SABiosciences, Valencia, CA) in triplicate, as previously reported (Wang *et al.*, 2008). The data are expressed in $2^{-\Delta Ct}$ using *β-actin* as an internal reference. TaqMan qRT-PCR for miRNA used TaqMan MicroRNA Assay (Applied BioSystems, Grand Island, NY) in triplicate and was compared with the expression of RNU24, an internal small RNA control.

Transfection

HH cells (2×10^6 per sample) were prepared using the Nucleofector Kit V (Lonza, Basel, Switzerland) and transfected with the X-005 program in the Nucleofector II instrument (Lonza). Hut-78 cells (2×10^6 per sample) were prepared with the Nucleofector Kit R (Lonza) and transfected with the V-001 program. The miRIDIAN miR-223 mimic RNA oligomer and control RNA (100 nM) and the miRIDIAN miR-223 hairpin inhibitor and inhibitor control (500 nM) were used for transfection (Dharmacon, ThermoScientificBio, Waltham, MA).

Cell viability and growth

Cell numbers and cell viability following the transfection of miR-223 mimic and inhibitor were determined by Trypan Blue Dye exclusion assay and with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI).

Clonogenic assays

CTCL cells ($5\times10^3\,ml^{-1}$) were plated in duplicate in methylcellulose medium containing Iscove's Modified Dulbecco's Medium with L-glutamine and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gibco, Grand Island, NY), 20% fetal bovine serum, and 2.6% methylcellulose (per protocol MethoCult H4100, Stemcell Technologies, Vancouver, Canada). Twenty-four hours before plating, CTCL cells were transfected with 100 nM miR-223 mimic or RNA control as described above. After 7 days (HH) or 10 days (Hut-78), colonies consisting of >30 cells were quantified, and colony numbers were compared between the cells transfected with miR-223 mimic and the RNA control.

Luciferase vector generation and luciferase assay

A 60-mer of the 3′-UTR of *TOX* containing the miR-223 seed sequence and a 60-mer with a mutated (base substitutions) seed sequence designed to prevent miR-223 from binding were cloned into pMIR-REPORT (Invitrogen). NIH-3T3 cells were transfected with 100 ng of pMIR-REPORT wild-type *TOX* 3′-UTR or mutant *TOX* 3′-UTR and 150 nM of miR-223 mimic or control RNA using Lipofectamine 2000 (Invitrogen). The cells were also co-transfected with 100 ng of pMIR-REPORT vector expressing a β-galactosidase control for normalizing transfection efficiency. Luciferase reporter gene activity was assayed 24 hours after transfection using the luciferase assay kit (Promega). β-Galactosidase activity was assayed by combining 50 µl cellular lysate with 50 µl β-galactosidase assay buffer (0.2 M sodium phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.1 M β-mercaptoethanol, ortho-nitrophenyl-β-D-galactopyranoside) for 1 hour, followed by absorbance evaluation at 405 nm.

Immunohistochemistry

Slides were placed on the Leica Bond Max IHC stainer (Leica Microsystems, Buffalo Grove, IL). Slides were deparaffinized, and heat-induced antigen retrieval was performed using the Epitope Retrieval 2 solution for 20 minutes. The slides were then incubated with anti-TOX (Sigma-Aldrich, St Louis, MO; Catalog no. HPA018322) at a 1:500 dilution for 1 hour.

Western blotting

Cell pellets were lysed with radio-immunoprecipitation assay buffer (50 mM Tris, 150 mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate) at 48 or 72 hours after transfection (see above) with miR-223 mimic or RNA control or miR-223 inhibitor or inhibitor control, respectively, and total cellular proteins were western blotted, as previously described (Alt *et al.*, 2003). Antibodies specific for E2F1 (KH-95, Santa Cruz Biotechnology, Santa Cruz, CA), MEF2C (4B10, Abcam, Cambridge, MA), TOX (HPA018322, Sigma-Aldrich), β-actin (Sigma-Aldrich), and α-tubulin (T6074, Sigma-Aldrich) were used.

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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