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miR-223 Deficiency Increases Eosinophil Progenitor Proliferation

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Recently, microRNAs have been shown to be involved in hematopoietic cell development, but their role in eosinophilopoiesis has not yet been described. In this article, we show that miR-223 is upregulated during eosinophil differentiation in an ex vivo bone marrow–derived eosinophil culture system. Targeted ablation of miR-223 leads to an increased proliferation of eosinophil progenitors. We found upregulation of a miR-223 target gene, IGF1R, in the eosinophil progenitor cultures derived from miR-223−/− mice compared with miR-223+/+ littermate controls. The increased proliferation of miR-223−/− eosinophil progenitors was reversed by treatment with an IGF1R inhibitor (picropodophyllin). Whole-genome microarray analysis of differentially regulated genes between miR-223+/+ and miR-223−/− eosinophil progenitor cultures identified a specific enrichment in genes that regulate hematologic cell development. Indeed, miR-223+/+ eosinophil progenitors had a delay in differentiation. Our results demonstrate that microRNAs regulate the development of eosinophils by influencing eosinophil progenitor growth and differentiation and identify a contributory role for miR-223 in this process. The Journal of Immunology, 2013, 190: 1576–1582.

Eosinophils are multifunctional effector cells implicated in the pathogenesis of a variety of diseases including asthma, eosinophil gastrointestinal disorders, and helminth infection (1–3). They differentiate from a common myeloid progenitor in mice through an intermediate granulocyte/macrophage progenitor and then via an eosinophil lineage-committed progenitor (4). The cytokine IL-5 is particularly important in eosinophil lineage development as it promotes the selective differentiation of eosinophils and also stimulates the release of mature eosinophils from the bone marrow (5). Indeed, eosinophil progenitors are a subset of granulocyte/macrophage progenitors that are IL-5Rα+, and IL-5 induces the growth and maturation of eosinophils (4, 6).

MicroRNAs (miRNAs) are short, single-stranded RNA molecules that silence target genes posttranscriptionally by either inhibiting protein translation or facilitating the degradation of target mRNAs. Different hematopoietic lineages have significant differences in their miRNA expression (7). Various miRNAs have been reported to regulate the differentiation and lineage commitment of hematopoietic progenitor cells (8). However, there is a lack of information on how miRNAs regulate the development of hematopoietic cells after lineage commitment. To the best of our knowledge, regulation of eosinophil progenitor cell proliferation by miRNAs has not been reported. Although multiple cytokines (e.g., IL-3, IL-5, and GM-CSF) and transcription factors (e.g., GATA-1, PU.1) have been shown to regulate the growth of eosinophil progenitors (9), other regulatory mechanisms such as miRNAs likely have a role in regulating or fine-tuning this process. Only one recent report has focused on this, showing that miR-21* could regulate the prosurvival effect of GM-CSF on eosinophils (10).

The expression of miR-223 has been shown to be driven by the myeloid transcription factors PU.1 and C/EBP, factors that are important in eosinophilopoiesis (11). In this article, we show that miR-223 was upregulated during eosinophil differentiation in an ex vivo bone marrow–derived eosinophil culture model. Notably, miR-223-deficient eosinophil progenitor cells showed a hyperproliferative capacity. Mechanistic analysis identified a contributory role for the miR-223 target, IGF1R, in mediating eosinophil progenitor cell proliferation. Gene expression analysis followed by systems biological analysis identified a role for miR-223 in hematopoietic development and cellular growth and function. Consistent with this prediction, miR-223+/+ eosinophils had a delay in eosinophil differentiation as assessed by CCR3 expression. Our data suggest that miRNAs can directly regulate the development of eosinophils by influencing the proliferation and differentiation of eosinophil progenitor cells.

Materials and Methods

Mice

The miR-223 gene–targeted mice backcrossed for five generations into the C57BL/6 background was previously described; the mice were kindly provided to us by Dr. Eran Hornstein (Weizmann Institute of Science, Rehovot, Israel) (12). Littermate controls were used for all experiments. All animals used were housed under specific pathogen-free conditions in...
accordance with institutional guidelines. The Institutional Animal Care and Use Committee of the Cincinnati Children’s Hospital Medical Center approved the use of animals in these experiments.

**Bone marrow–derived eosinophil cultures**

Bone marrow cells were collected from femur and tibia of the mice, and the stem/progenitor cell–enriched, low-density fraction was isolated by gradient centrifugation using Histopaque 1083 (Sigma) according to manufacturer’s protocol. The low-density fraction of bone marrow cells was cultured in IMDM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin supplemented with 100 ng/ml stem cell factor and 100 ng/ml FLT-3 ligand (PeproTech) from days 0–4. On day 4, the stem cell factor and FLT-3 ligand were replaced with 10 ng/ml IL-5 and cultured for an additional 10–12 d (6). The culture media was changed every other day, and cells were counted and concentration was adjusted to 1 × 10^6/ml during each media change. Eosinophil maturity was assessed by FACS staining for CCR3 and Siglec-F and/or Diff-Quik staining of cytospin preparations. Eosinophil progenitor proliferation was assessed by counting the cells every 2 d using a hemacytometer.

**Quantitative assessment of miRNA levels**

Total RNA was isolated using mirNeasy mini Kit according to manufacturer’s protocol (Qiagen). Levels of miRNA expression were measured quantitatively by using the TaqMan MicroRNA Assay (Applied Biosystems) following the manufacturer’s protocol and assayed on the Applied Biosystems 7900HT Real-Time PCR System. Normalization was performed using U6 small nuclear RNA. Relative expression was calculated using the comparative threshold cycle method as previously described (13).

**Quantitative RT-PCR for mRNA**

Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). All primer/probe sets were obtained from Applied Biosystems. Samples were analyzed by TaqMan quantitative RT-PCR for Cer3 (Assay ID: Mm01216172_m1) and normalized to Hprt1 (Assay ID: Mm00440968_m1). Relative expression was calculated using the comparative threshold cycle method.

**Flow cytometry analysis of eosinophil surface CCR3 expression**

One million cultured eosinophil progenitor cells were stained with CCR3-FITC (R&D Systems) and Siglec-F-PE (BD Bioscience), as CCR3 and Siglec-F are markers for mature eosinophils. Staining was performed on ice for 30 min in staining buffer (0.5% BSA, 0.01% NaN₃ in 1× HBSS) according to manufacturer’s protocol. Data were acquired on a BD FACS Canto I flow cytometer and analyzed using FlowJo.

**Preparation of total cell lysates and Western blot**

Cells were rapidly washed in PBS and lysed in M-PER mammalian protein extraction reagent ( Pierce) according to manufacturer’s protocol. Protease inhibitor cocktails (Pierce) and phosphatase inhibitor cocktails (Pierce) were added to the M-PER protein extraction reagent immediately before lysis. Western blot analysis was performed as previously described (14). The anti-IGF1R Ab was obtained from Cell Signaling Technology. The anti-GAPDH Ab was from Abcam.

**Analysis of cell proliferation after picropodophyllin treatment**

Bone marrow–derived eosinophils were resuspended at a concentration of 1 × 10^6 cells/ml and treated with DMSO or 2 μM picropodophyllin at day 8 (15). Cell proliferation was determined by cell counting using a hemacytometer on days 10 and 12. Cell lysates were collected on day 10, and levels of IGF1R expression were determined by Western blot. To determine a dose–response curve of picropodophyllin, bone marrow–derived eosinophils were resuspended at a concentration of 1 × 10^6 cells/ml and plated in a 96-well plate at 100 μl/well on day 9. The cells were treated with increasing concentrations of picropodophyllin, and the level of cell proliferation was determined using Cell-Titer Glo luminescent cell viability assay (Promega) according to manufacturer’s protocol.

**Mouse genome-wide mRNA microarray**

The Affymetrix Mouse Gene 1.0ST array was used to compare gene expression profiles between miR-223+/+ and miR-223−/− eosinophil progenitor cultures at days 4, 8, and 12. Microarray data were analyzed using the GeneSpring software (Agilent Technologies). Global scaling was performed to compare genes from chip to chip, and a base set of probes was generated by requiring a minimum raw expression level of 20th percentile out of all probes on the microarray. The resulting probe sets were then baseline transformed and filtered on at least 1.5-fold difference between miR-223+/+ and miR-223−/− eosinophil progenitor cultures. Statistical significance was determined at p < 0.05 with Benjamini–Hochberg false discovery rate correction. The resulting list of genes was clustered using hierarchical clustering, and a heat map was generated. Biological functional enrichment analysis was carried out using Ingenuity Pathway Analysis (Ingenuity Systems) and TopGene/ToppCluster (16, 17). The microarray data have been deposited into the Array Express database (http://www.ebi.ac.uk/arrayexpress) with accession number E-MEXP-3350 in compliance with minimum information about microarray experiment standards.

**Statistical analysis**

Statistical analyses were performed with Student t test or one-way ANOVA with Tukey post hoc test where appropriate. Statistical significance and the p values were indicated on the figures where appropriate. The p values <0.05 were considered statistically significant.

**Results**

**Expression of miR-223 in an ex vivo culture of bone marrow–derived eosinophils**

Bone marrow–derived eosinophils were cultured according to the schematic shown in Fig. 1A. Eosinophils were obtained with high purity as determined by FACS staining for CCR3 and Siglec-F double-positive cells on day 14 (Fig. 1B). There was upregulation of miR-223 during the eosinophil differentiation culture from days 4–14 (Fig. 1C), with the most prominent difference seen between days 8 and 14.

**FIGURE 1.** MiR-223 is induced during eosinophil differentiation. (A) Schematic of ex vivo bone marrow–derived eosinophil culture. (B) Purity of cultured eosinophils after 14 d. Eosinophils are identified as CCR3+ Siglec-F+ cells. (C) Levels of miR-223 during the eosinophil differentiation culture. n = 3 per group; data are represented as mean ± SEM.
Compared with the miR-223 +/+ cultures, the miR-223 IGF1R level was progressively decreased from days 10–14, cultures (Figs. 2A, 3). However, in the miR-223 +/+ cultures, the phils in cultures derived from miR-223+/+ and miR-223+ bone marrow–derived eosinophils were morphologically indistinguishable from each other at day 8, 10, 12, or 14 (Fig. 2B, Supplemental Fig. 1). IGF1R is upregulated in eosinophil cultures derived from miR-223+/+ and miR-223−/− mice compared with littermate controls

MiR-223 has been shown to target IGF1R (12). The IGF1R is the major physiologic receptor for IGF1 (18). Because IGF1 is a major anabolic hormone that stimulates cell proliferation and is a potent inhibitor of programmed cell death, we decided to investigate whether the levels of IGF1R were differentially regulated between eosinophil cultures derived from miR-223−/− mice and miR-223+/+ littermate controls. It is notable that IGF1 has not been previously examined for its impact on eosinophils or their progenitors. The IGF1R is not expressed at day 4 (Fig. 3), indicating that proliferation of progenitor cells under the influence of stem cell factor and FLT-3 ligand is not likely dependent on the levels of IGF1R. However, there were substantial levels of IGF1R expression from days 10–14 of the culture, coinciding with the increased proliferation seen in both the miR-223−/− and miR-223+/+ eosinophil cultures (Figs. 2A, 3). However, in the miR-223+/+ cultures, the IGF1R level was progressively decreased from days 10–14, reflecting that eosinophil progenitors gradually lose their proliferation capacity during the differentiation process (Figs. 2A, 3). Compared with the miR-223+/+ cultures, the miR-223−/− cultures have significantly increased levels of IGF1R at both days 12 and 14 (Fig. 3).

The increased proliferation in miR-223−/− eosinophil cultures can be reversed by treatment with an IGF1R inhibitor

To determine whether the upregulation of IGF1R was responsible for the increased proliferation seen in eosinophil cultures derived from the miR-223−/− mice, we treated eosinophil cultures on day 8 with 2 μM of an IGF1R inhibitor, picropodophyllin, or an equivalent volume of DMSO as a control. The DMSO treatment had no effect on eosinophil proliferation. The miR-223−/− cultures treated with DMSO had significant increases in proliferation compared with DMSO-treated miR-223+/+ cultures (Fig. 4A), confirming our results in Fig. 2B. In contrast, treatment with 2 μM picropodophyllin inhibited the proliferation of both miR-223+/+ and miR-223−/− cultures to a similar extent (Fig. 4A), completely reversing the increased proliferation seen in miR-223−/− cultures. We analyzed the levels of IGF1R expression on day 10 with or without picropodophyllin treatment and found that picropodophyllin induced a nearly complete downregulation of IGF1R in both the miR-223+/+ and miR-223−/− cultures (Fig. 4B), in agreement with previous reports that picropodophyllin could inhibit IGF1R expression (19). Dose–response studies demonstrated that picropodophyllin inhibited miR-223+/+ and miR-223−/− eosinophil progenitor proliferation with similar IC50 (Fig. 4C).

The increased proliferation seen in miR-223−/− eosinophil progenitor cultures is associated with a delay in differentiation

Interestingly, we identified a delayed upregulation of CCR3 in miR-223−/− eosinophil progenitor cultures compared with miR-223+/+ eosinophil progenitor cultures. We performed quantitative RT-PCR analysis of the Ccr3 level on day 8, 10, and 12 eosinophil progenitor cultures and found that miR-223−/− eosinophil progenitors had decreased upregulation of Ccr3 compared with miR-223+/+ eosinophil progenitors at all three time points (Fig. 5A), indicative of delayed maturation of the miR-223−/− eosinophil progenitor cells. To determine the surface expression of CCR3 during the eosinophil progenitor culture, we performed FACS analysis of CCR3 and Siglec-F expression from days 8–16 of eosinophil culture. The mature eosinophils are CCR3+ and Siglec-F+. There are <2% CCR3+Siglec-F− cells during the eosinophil culture on day 8, indicating that nearly all cells are in the progenitor stage in both the miR-223+/+ and miR-223−/− cultures (Fig. 5B). Although CCR3+Siglec-F− cells begin to appear on day 10, the CCR3+Siglec-F− cells in the miR-223−/− cultures were substantially less than that in the miR-223+/+ cultures. This difference is most pronounced during days 10 and 12 of the eosin-
miR-223 expression in miR-223+/+ and miR-223−/− measured by cell counting using a hemacytometer. (IGF1R inhibitor) or an equivalent volume of DMSO. Cell proliferation is data are represented as mean ± SEM. The full gene list is provided in Supplemental Table I.

**Discussion**

In this report, we show that miR-223 regulates the proliferation and differentiation of eosinophil progenitors. We found upregulation of miR-223 in an ex vivo eosinophil differentiation culture model. Targeted ablation of this single miRNA led to a major effect on eosinophil progenitor cells as miR-223−/− cells had a markedly increased proliferation in response to the eosinophil growth factor IL-5. Furthermore, miR-223 deficiency led to a defect in eosinophil maturation as indicated by a delayed upregulation of surface CCR3 expression.

We found upregulation of the miR-223 target gene IGF1R in the miR-223−/− eosinophil cultures compared with miR-223+/+ cultures (12). The upregulation of IGF1R coincides with the onset of the increased proliferation seen in the miR-223−/− eosinophil culture. We subsequently demonstrated that the increased proliferation in the miR-223−/− culture could be reversed by treatment with an IGF1R inhibitor. Similarly, the proliferation of miR-223+/+ eosinophil progenitors could also be blocked by an IGF1R inhibitor. This indicates that the increased proliferation seen in miR-223−/− eosinophil cultures has not bypassed the IGF1R pathway. To our knowledge, these data are the first to show that IGF1R is involved in eosinophil development. Several IGF1R inhibitors are currently under development for the treatment of various types of cancer (20). Our data indicate that the IGF1R inhibitors could potentially also be used to treat patients with eosinophilia, such as those with hypereosinophilic syndrome (21).

Furthermore, we have identified miR-223 as a regulator of eosinophil IGF1R levels. Although the upregulation of IGF1R likely has a contributory role in the increased proliferation seen in the miR-223−/− eosinophil progenitor cultures, we do not preclude the involvement of additional pathways. In particular, our microarray analysis identified multiple additional growth- and proliferation-related genes differentially regulated between miR-223+/+ and miR-223−/− cultures. These include downregulation of NAD(P)H: quinone oxidoreductase 1 where NAD(P)H:quinone oxidoreductase 1–deficient mice have been found to have a significant increase in blood granulocytes including eosinophils (22). We also noted downregulation of inhibitor of DNA binding 2, whose knockdown has been shown to cause increased eosinophil progenitor growth and delayed eosinophil progenitor differentiation (23).

We found a delayed upregulation of CCR3 in miR-223−/− eosinophil progenitor cultures, likely representing delayed eosinophil maturation. This is associated with the increased expression of IGF1R, raising the possibility that IGF1 could negatively regulate the expression of CCR3. We did not find significant differences in the mRNA expression of eosinophil granule proteins. We measured the blood eosinophil level in vivo and did not find any difference between the miR-223+/+ and miR-223−/− mice (Supplemental Fig. 3A). This is likely due to in vivo compensation at the stage where multipotent progenitors are differentiated into eosinophil lineage-committed progenitors. Indeed, when we measured the level of ILSRα/CCR3+ eosinophil lineage-committed progenitors in vivo, we found a decreased level of eosinophil lineage-committed progenitors in the miR-223−/− mice (Supplemental Fig. 3B). This likely compensated...
for the increased proliferative capacity of the miR-223−/− eosinophil progenitor cells.

MiR-223 has been found to be overexpressed in asthma, eosinophilic esophagitis, and atopic dermatitis, in which eosinophils are implicated in the disease pathogenesis to varying degrees (24–29). We have recently found that both miR-223 and miR-21 were upregulated in patients with eosinophilic esophagitis (28). They are the top two miRNAs correlated with eosinophil levels in pa-

**FIGURE 5.** Analysis of eosinophil progenitor culture expression of CCR3. (A) CCR3 expression at days 8, 10, and 12 of the eosinophil progenitor culture was determined in miR-223−/− cultures compared with miR-223+/+ cultures by quantitative RT-PCR normalized to HPRT1. n = 3 per group; data are represented as mean ± SEM. (B) Levels of CCR3 expression during eosinophil differentiation culture determined by FACS staining of surface CCR3 and Siglec-F levels. Mature eosinophils are identified as CCR3’Siglec-F’ cells.
FIGURE 6. Heat map of differentially regulated genes between miR-223+/+ and miR-223−/− eosinophil progenitor cultures at days 8 and 12 with their associated top biological functions. (A) Heat map of differentially regulated genes at day 8 of the eosinophil differentiation culture. Red represents up-regulated in miR-223−/− eosinophil progenitor culture compared with miR-223+/+ eosinophil progenitor culture; blue represents down-regulated in miR-223−/− eosinophil progenitor culture compared with miR-223+/+ eosinophil progenitor culture. (B) Functional enrichment analysis of differentially regulated genes in the eosinophil progenitor cultures at day 8. The networks are shown as Cytoscape graph networks generated from ToppCluster network analysis. (C) Heat map of differentially regulated genes at day 12 of the eosinophil differentiation culture. Red represents up-regulated in miR-223−/− eosinophil progenitor culture compared with miR-223+/+ eosinophil progenitor culture; blue represents down-regulated in miR-223−/− eosinophil progenitor culture compared with miR-223+/+ eosinophil progenitor culture. (D) Ingenuity analysis of the most significant biological functions represented by the differentially regulated genes between miR-223+/+ and miR-223−/− eosinophil progenitor cultures at day 12.
tient esophageal biopsies. Using systems biology analysis, we found that miR-223 and miR-21 regulated a set of interacting target genes involved in eosinophil proliferation and differentiation (28). MiR-21 has been known to promote cell proliferation in various cell types by downregulating a variety of proapoptotic genes both directly and indirectly (30). The upregulation of miR-223 likely provides a check and balance in the system given the ability of miR-223 to promote eosinophil maturation. Furthermore, a recent report showed that miR-21*, a complementary miRNA of miR-21, was upregulated after GM-CSF treatment and could inhibit the apoptosis of eosinophils (10). This indicates that the minor miRNAs could also have a role in regulating the proliferation of eosinophil progenitors, adding another level of complexity. Future therapies targeting miRNAs, including miR-21 and miR-223 and their minor miR* forms, will likely allow fine-tuning of the eosinophil level in various diseases.

In summary, we have identified miR-223 as a regulator of eosinophil progenitor proliferation. We found that IGF1R is upregulated during eosinophil development, and miR-223 is a regulator of IGF1R levels. Further elucidating and understanding the roles and regulations of miRNAs during eosinophil development may lead to novel therapeutic targets for eosinophilic disorders.

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Disclosures
M.E.R has equity interest in reslizumab through Cephalon, is a consultant for and Chief Scientific Officer of Immune Pharmaceuticals, is on the American Partnership for Eosinophilic Disorders Medical Advisory Board, and is on the International Eosinophil Society’s Executive Council. The other authors have no financial conflicts of interest.

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