Commentary

Divergent roles of *miR-126* in normal and malignant stem cells

Harold K. Elias[1], **Mona Khalaj**[1,2], **Christopher Y. Park**[1,2,3]

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, NY, USA; [2]Weill Graduate School of Medical Sciences, Cornell University, NY, USA; 3Pathology and Laboratory Medicine, Memorial Sloan Kettering Cancer Center, NY, USA

Correspondence to: Christopher Y. Park, M.D., Ph.D. Departments of Pathology and Clinical Laboratories, Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA. Email: parkc@mskcc.org.

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Malignant blasts in acute myeloid leukemia (AML) are organized in a hierarchal manner resembling normal hematopoiesis, with leukemic stem cells (LSCs) at the apex, giving rise to more differentiated progeny with reduced capacity to self-renew (1). Functionally, LSCs demonstrate the ability to propagate disease upon transplantation (demonstrating leukemia-initiating cell, or LIC, activity), as well as serial transplantation. With each transplantation, LSCs cells are capable of retaining properties of the original leukemia, which reflects their unique capacity to self-renew, similar to normal hematopoietic stem cells (HSCs). Clinically, LSCs are thought to mediate disease relapse and chemoresistance, but few validated LSC-targeted therapies have been successfully translated to the clinic (2). To identify key regulators of LSC function, several groups have performed gene-expression analyses on LSC-enriched cells from AML patient samples (3-5). These studies have demonstrated that LSCs share a similar gene expression signature to HSCs, and that gene signatures from these populations can reliably prognosticate clinical outcomes in normal karyotype AML (3). While several microRNAs (miRNAs), including *miR-125a/b* and *miR-29a*, have been shown to maintain HSCs (4,6-8), studies demonstrating functional roles for miRNAs in LSC function in AML are limited (9).

Recently, John Dick and colleagues identified *miR-126* as a regulator of LSC function (10). Considering functional LSCs may exist within different immunophenotypically defined leukemia blast populations (11,12), the authors initially sought to identify miRNAs highly associated with LIC activity and therefore fractionated total leukemic blasts into 4 populations based on the presence or absence of CD34 and CD38 surface expression. Subsequently, each population was assessed for LIC activity using xenotransplantation assays. They performed miRNA expression profiling studies on the same populations (10) and identified several miRNAs enriched in the experimentally validated LIC-enriched fractions, including one miRNA known to regulate HSC function (*miR-125*) (6,8). Of note, *miR-99*, another miRNA shown to be highly expressed in HSCs (6), displayed the highest level of enrichment in LSCs. We have recently shown that *miR-99* plays a critical role in maintaining LSCs in both primary mouse and human AML (manuscript in preparation), which attests to the robustness of the LSC miRNA signature. Additional miRNAs included in the LIC signature included *miR-155*, which is compatible with previous publications suggesting it serves as an oncogene in AML (13), as well as *miR-126*, an unexpected finding given that the same group previously demonstrated that it is a negative regulator of HSC function (4). The signature was evaluated in an AML patient cohort and shown to prognosticate overall survival in a univariate analysis, providing additional support for a role for *miR-126* in LSC function. A more recent study corroborated these findings, demonstrating that higher *miR-126* levels predict poor overall survival in older AML patients (>60 years) with normal karyotype (14).

As *miR-126* had been previously shown by the same group to regulate HSCs (4) and others had shown a potential role in LSCs (4,15), the authors sought to explore the functional role of *miR-126* in LSCs. The investigators devised a lentiviral GFP reporter system to assess for biologically active *miR-126* in patient AML blasts. Using

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this system, the authors showed that *miR-126* activity is enriched in LIC-enriched populations. However, the correlation between repression of the *miR-126* reporter and LIC activity could not be clearly established in all AML samples tested, suggesting that *miR-126* may not regulate LIC activity in all AML subtypes.

To circumvent the technical limitations of working with primary AML blasts, the authors took advantage of a cell line they established from an AML patient (referred to as 8227) that exhibited several important properties resembling primary AMLs, including a hierarchal organization with CD34+CD38- blasts being the most primitive and exhibiting the highest LSC activity. *miR-126* overexpression (OE) in 8227 cells resulted in decreased proliferation, with CD34+CD38- blasts exhibiting increased quiescence as evidenced by decreased BrdU incorporation. In addition, about half the *miR-126* OE AML samples exhibited decreased CD15+ differentiation *in vitro*, and xenotransplantation assays utilizing three primary AML samples transduced with *miR-126* OE showed an increased LIC frequency observed in secondary transplantations, establishing *miR-126*'s role as a positive regulator of LSC activity. In contrast, *miR-126* knockdown (KD) using a "sponge" strategy induced increased cycling among CD34+CD38- blasts, enhanced clonogenicity, and induced a small degree of differentiation in primary samples *in vitro*, but engraftment of *miR-126* KD blasts in primary and secondary xenotransplant assays was not impaired. While this may have been due to technical difficulties including the degree of *miR-126* KD or the particular samples tested, the authors did not comment on these observations, and therefore the explanation for this finding remains unclear. Follow-up studies using larger number of samples with attempts to correlate outcomes to molecular/cytogenetic subsets would help improve our understanding of the role of *miR-126* in LSC function.

The authors next attempted to identify downstream effectors of *miR-126* function. As they previously showed that *miR-126* inhibits various proteins within the PI3K-AKT-mTOR axis in HSCs (16), they investigated this pathway in LSCs. Mass spectrometry analysis and gene expression profiling in 8227 cells following *miR-126* OE and KD identified all three isoforms of AKT and CDK3 (a well-known downstream target of PI3K-AKT) as potential targets of *miR-126*. As cyclin C forms a complex with CDK3 to promote cell cycle entry (17,18), this target could explain the effects of *miR-126* on LSC quiescence. To test whether *miR-126* KD effects on LSCs are mediated through induction of CDK3, the authors sought to determine whether CDK3 is necessary and/or sufficient to mimic *miR-126* KD effects. While wild type CDK3 OE induced increased proliferation and clonogenicity of both CD34+CD38- and CD34+CD38+ blasts, mutant CDK3 did not, thereby confirming the functional significance of CDK3 kinase activity. As additional cyclin C-CDK complexes influence important LSC-associated signaling pathways mediated by Notch and Wnt (19), it would be interesting to determine whether these pathways mediate *miR-126*'s effects on LSC self-renewal. It is not clear whether or not concurrent mRNA expression data was generated for the blast populations evaluated in this study; however, the same group did generate a mRNA signature for LSCs (3), which may be useful to identify *miR-126* targets since such targets might display inverse expression patterns compared to *miR-126* in LSCs *vs*. non-LSCs.

As chemoresistance in AML is associated with quiescence/dormancy of LSCs (2) and *miR-126* OE induced reduced cell cycling, the authors tested whether or not *miR-126* OE may confer resistance to standard AML chemotherapies (daunorubicin and cytarabine). The authors not only showed that *miR-126* OE confers resistance to 8227 cells as predicted, but that overexpression of CDK3 partially reverses resistance. To further support *miR-126*'s potential importance in mediating therapy responses, blasts from refractory patients following induction chemotherapy showed higher expression of *miR-126*. Overall, these data underscore *miR-126*'s role as a mediator of chemo-resistance through its direct inhibition of CDK3 and arrest of G0-G1 exit.

Developing stem cell-directed therapies in AML is challenging given that LSCs share many determinants of stemness with normal HSCs (3). Thus, the studies by Dick and colleagues demonstrating divergent roles for *miR-126* in LSC and HSC function credential *miR-126* as a candidate therapeutic target in AML. Others have recently investigated the potential of targeting *miR-126* in LSCs using nanoparticles (14). After observing that AML patients with normal cytogenetics and relapsed/refractory disease express higher levels of *miR-126* in LSC-enriched cells, the investigators used nanoparticles to deliver antagomirs against *miR-126*. Targeting *miR-126* in primary human AML depleted quiescent CD34+ blasts, and loss of LSC activity was demonstrated by decreased self-renewal and increased survival in secondary recipients of *miR-126* antagomiR-treated CD34+ AML cells. Morever, the treatment had no negative effects on mouse HSC reconstitution potential,

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suggesting a potential therapeutic window. To further test the possibility of therapeutically targeting *miR-126 in vivo*, the authors treated *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/ITD}$ double knockin mice with their antagomiR strategy. Treatment resulted in improved survival similar to their xenotransplant experiments. While this result was more robust than those observed with the experiments of Dick and colleagues, this may have been due to differences in the extent of gene silencing using of a lentiviral 'sponge' strategy versus a traditional antagomir strategy. Understanding the basis of this difference will be important for potential future development of therapies targeting *miR-126*.

In summary, studies of *miR-126* highlight its unique functional roles in AML LSCs and normal HSCs. Given its profound impact on LSC function and its contribution to chemoresistance, partly by reactivation of the PI3K-AKT-CDK3 axis, the data support the development of therapies that target *miR-126* or its downstream targets. As translation of antagomir therapeutic strategies remains difficult (20) and given *miR-126*'s potential importance as a therapeutic target in AML, future studies can help identify cis-acting elements and transcription factors that regulate *miR-126* expression, as these may become potential alternative therapeutic targets in the future.

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