MicroRNA-128-3p is a novel oncomiR targeting *PHF6* in T-cell acute lymphoblastic leukemia

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ABSTRACT

T-cell acute lymphoblastic leukemia arises from the leukemic transformation of developing thymocytes and results from cooperative genetic lesions. Inactivation of the *PHF6* gene is frequently observed in T-cell acute lymphoblastic leukemia, suggesting an important tumor suppressive role for *PHF6* in the pathobiology of this leukemia. Although the precise function of *PHF6* is still unknown, this gene is most likely involved in chromatin regulation, a strongly emerging theme in T-cell acute lymphoblastic leukemia. In this context, our previous description of a cooperative microRNA regulatory network controlling several well-known T-cell acute lymphoblastic leukemia tumor suppressor genes, including *PHF6*, is of great importance. Given the high frequency of *PHF6* lesions in T-cell acute lymphoblastic leukemia and the integration of *PHF6* in this microRNA regulatory network, we aimed to identify novel oncogenic microRNAs in T-cell acute lymphoblastic leukemia which suppress *PHF6*. To this end, we performed an unbiased *PHF6* 3'UTR-microRNA library screen and combined the results with microRNA profiling data of samples from patients with T-cell acute lymphoblastic leukemia and normal thymocyte subsets. We selected miR-128-3p as a candidate *PHF6*-targeting, oncogenic microRNA and demonstrated regulation of *PHF6* expression upon modulation of this microRNA in T-cell acute lymphoblastic leukemia cell lines. *In vivo* evidence of an oncogenic role of this microRNA in T-cell acute lymphoblastic leukemia was obtained through accelerated leukemia onset in a NOTCH1-induced T-cell acute lymphoblastic leukemia mouse model upon miR-128-3p over-expression. We conclude that miR-128-3p is a strong novel candidate oncogenic microRNA in T-cell acute lymphoblastic leukemia which targets the *PHF6* tumor suppressor gene.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a hematologic malignancy arising from leukemic transformation of developing thymocytes and resulting from cooperative genetic lesions. Collectively, these genetic aberrations affect processes such as self-renewal, proliferation and survival, and block differentiation of precursor T cells.[1] Inactivation of the *Plant Homeodomain Finger 6* (*PHF6*) gene is frequently observed in T-ALL. More specifically, deletions and loss-of-function mutations in *PHF6* were found in about 16% of children and 38% of adults with T-ALL, indicating that *PHF6* plays an important tumor suppressive role in the pathobiology of T-ALL.[2-6] Although the function of *PHF6* remains largely unknown, the presence of two plant homeodomain (PHD) domains in the *PHF6* protein and the recent association of the protein with components of the nucleosome remodeling and deacetylase (NuRD) complex suggests a role in chromatin remodeling and regulation of gene expression.[7,8]

MicroRNAs (miRNAs) represent an important class of small non-coding RNA molecules implicated in the post-transcriptional regulation of gene expression. Mechanistically, miRNAs suppress the expression of their target genes by guiding the RNA-induced silencing complex (RISC) to binding sites complementary to the miRNA's seed sequence within the 3' untranslated region (3'UTR) of mRNAs. This interaction will result in mRNA degradation or inhibition of translation. Notably, many miRNAs are able to control expression of multiple targets, and inversely, many genes can be regulated by multiple miRNAs, underscoring the complexity of miRNA-mRNA interaction networks.[9] During the last decade, it has been comprehensively shown that deregulated miRNA expression can contribute to cancer formation.[10,11] In the context of T-ALL development, we recently established a cooperative miRNA-tumor suppressor gene network controlling several well-known T-ALL tumor suppressor genes, including *PHF6*, which drives malignant transformation of developing thymocytes.[12]

 doi:10.3324/haematol.2013.099515
The online version of this article has a Supplementary Appendix.
Manuscript received on October 12, 2013. Manuscript accepted on May 6, 2014.
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Given the high frequency of inactivating *PHF6* lesions in T-ALL and the involvement of this gene in the miRNA regulatory network, we aimed to identify novel miRNAs that have oncogenic potential in T-ALL through suppression of *PHF6*. To this end, we integrated the results from an unbiased *PHF6* 3'UTR-miRNA library screen with miRNA profiling data from T-ALL patients' samples and subsets of normal thymocytes at different stages of T-cell maturation. Following this approach, we identified and validated miR-128-3p as a novel miRNA with a potential oncogenic role in T-ALL through targeting of *PHF6*.

Methods

Full details of the methods are provided in the *Online Supplementary Methods*.

PHF6 *3'UTR-miRNA library screens*

A complete description is provided in the *Online Supplementary Methods*. Briefly, HEK-293T cells were co-transfected with a reporter vector containing wild-type *PHF6* 3'UTR cloned downstream of Firefly luciferase and the pRL-TK control vector containing Renilla luciferase together with a library of 470 miRNA mimics. Forty-eight hours after transfection, luciferase reporter gene activities were assayed and Firefly activities were normalized to Renilla values, log-transformed and subsequently robust z-scores were calculated and median centered to the distribution of robust z-scores of 36 analogous screens for other genes on a per miRNA basis to remove potential systematic bias. The resulting interaction scores are thus more negative for miRNAs that interact with the 3'UTR. In order to determine the interaction score cutoff, the scores for a set of miRNA interactions validated in literature and re-evaluated in the analogous screens were used together with the scores for a set of negative control interactions from an empty 3'UTR vector miRNA library screen to perform receiver operating characteristic (ROC)-curve analysis and determine the point of highest accuracy (interaction score cutoff = -1.94, accuracy = 91%, specificity = 99%, sensitivity = 51%). *PHF6* 3'UTR-miRNA library screen results were replicated in three independent experiments.

MicroRNA nomenclature and annotation

During the course of this study, official nomenclature and sequence annotation as put forward by miRBase changed for the miRNAs investigated here. Briefly, at the start of our study, miR-574 and both miR-128a and miR-128b were annotated in miRBase (v9.2), and were included in the miRNA libraries for the 3'UTR screens and miRNA profiling of the T-ALL and thymocyte samples. However, at the start of our validation studies, miRBase merged the records of miR-128a and miR-128b into a single record, miR-128, of which the mature sequence is one nucleotide shorter. Furthermore, miR-574 was re-annotated to miR-574-3p, which is one nucleotide longer at the 3' end. We used these newly annotated miR-128 and miR-574-3p sequences in our follow-up experiments. In the most recent release of miRBase (v20), the human mature miR-128 was renamed miR-128-3p. In this manuscript, we use the most up-to-date names at time of publication for miR-128-3p and miR-574-3p. For the miRNA over-expression studies in mouse, the human pre-miR-128-2 sequence was used, which gives rise to the mature miR-128-3p.

T-cell acute lymphoblastic leukemia mouse model

All animal experiments were performed in accordance with protocols that were approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering. A complete description of the experiments can be found in the *Online Supplementary Methods*. Briefly, mouse fetal liver cells were retrovirally transduced with ICN1 expression vectors in combination with miR-128-3p expression vectors or empty vector controls, and subsequently injected in the tail vein of lethally irradiated mouse recipients. Leukemia onset in these mice was then monitored by analysis of lymphoblast counts in blood smears and physical appearance. At diagnosis, several tissues were fixed for histological evaluation (see *Online Supplementary Methods*). Time-to-leukemia onset data were analyzed using the Kaplan-Meier method and the log-rank (Mantel-Cox) test for statistical significance.

Results

An unbiased PHF6 *3'UTR-miRNA library screen identifies 23 miRNAs potentially targeting* PHF6

Most of the functionally validated miRNA-target gene interactions are currently biased towards miRNAs that are predicted to target the gene under investigation. A major drawback of these *in silico* prediction algorithms is the large degree of false positive results and their inability to predict the full complement of miRNAs targeting a given gene of interest. In order to address the shortcomings of *in silico* approaches, we developed an unbiased, *in vitro* screening strategy to identify novel miRNAs targeting our gene of interest, *PHF6*. More specifically, a high-throughput miRNA library screen was performed, exploring possible interactions between 470 miRNA mimics and the 3'UTR of *PHF6*. In brief, HEK-293T cells were co-transfected with a reporter construct, containing the *PHF6* 3'UTR downstream of a luciferase gene, and each of the miRNA mimics in the library. Based on the relative luciferase activities for all tested miRNA-*PHF6* combinations in three independent library screen experiments, an interaction score was calculated for each miRNA (see Methods). We identified 23 miRNAs with a high probability of targeting *PHF6* (interaction score < -1.94, see Methods) (Figure 1 and *Online Supplementary Table S1*), with only 11 of these 23 hits being predicted from a total

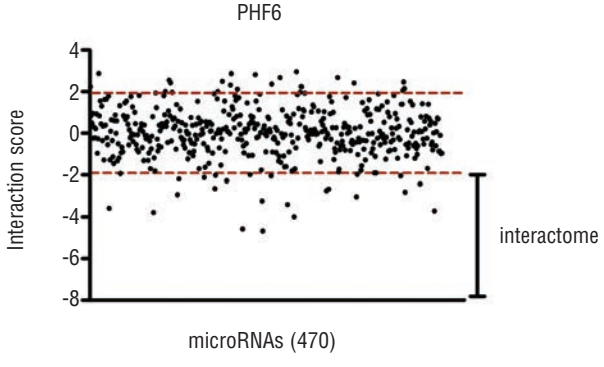

Figure 1. An unbiased *PHF6* 3'UTR-miRNA library screen revealed 23 miRNAs potentially targeting *PHF6*. Interaction scores were calculated based on three independent screens and are plotted (Y-axis) for each tested miRNA (X-axis). The lower the interaction score, the higher the probability of a true interaction between the miRNA and the 3'UTR of *PHF6*. Twenty-three miRNAs (see *Online Supplementary Table S1* for the complete list) have interaction scores lower than the interaction score cutoff of -1.94 (see Methods).

of 93 as determined through various target prediction programs (TargetScan, PicTar, miRanda, MirTarget2, Diana microT; see *Online Supplementary Table S1*). This effort was part of a large-scale 3'UTR screening in which the interactions of 470 miRNAs with 17 selected cancer/disease genes were probed (Van Peer *et al.*, unpublished data). Interaction score cutoff values were determined as such to minimize false positive hits at the expense of missing a few true interactions (false negative results). This probably explains why miR-20a and miR-26a, the only reported *PHF6*-targeting miRNAs,[12] were not retained as positive hits in our screen. However, our results underscore the value of an unbiased screening method to detect novel, predicted as well as non-predicted, miRNA-target gene interactions.

Integrated analyses identify miR-128-3p and miR-574-3p as top candidate PHF6-targeting miRNAs in T-cell acute lymphoblastic leukemia

As miRNA-target gene interactions can be highly tissue- and cell-type-specific, and as the 3'UTR-miRNA library screens were performed in HEK-293T cells, we aimed to select candidate oncogenic miRNAs targeting *PHF6* in the specific context of T-ALL cells. We, therefore, evaluated miRNA expression levels in a genetically well-characterized cohort of patients (n=50) with T-ALL as well as in subsets of normal, flow sorted thymocytes (data previously reported data by Mavrakis *et al.*[12] and see Methods). Following the assumption that miRNAs with a potential oncogenic effect should show significant expression in primary T-ALL samples, we identified seven of the 23 miRNAs selected from the miRNA library screen which were among the top 50% expressed miRNAs in the T-ALL patients' samples (*Online Supplementary Table S2*). Next, we reasoned that miRNAs with a putative oncogenic role in T-ALL would be more highly expressed in T-ALL samples than in normal T-cell counterparts. Using rank products analysis, we identified 37 miRNAs that were significantly (percentage of false predictions-value <0.05) more highly expressed in T-ALL than in normal thymocyte subsets (*Online Supplementary Table S3*). Based on the miRNA expression results and those from the 3'UTR-miRNA library screen, we prioritized miR-128b and miR-574 as top candidate *PHF6*-targeting miRNAs with a possible oncogenic role in T-ALL (Figure 2 and *Online Supplementary Figure S1*). During the course of this study, the annotation (and mature sequence) of miR-128b was updated in miRBase to miR-128 and later to miR-128-3p (latest miRBase release v20). Similarly, the annotation (and sequence) of miR-574 was updated to miR-574-3p. We, therefore, performed all further experiments with the updated miR-128-3p and miR-574-3p sequences (see Methods).

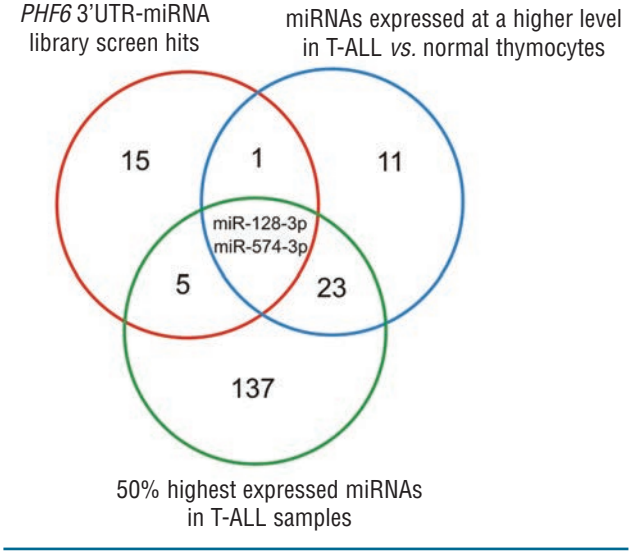

Figure 2. MicroRNA-128-3p and miR-574-3p are top-candidate miRNAs targeting *PHF6* in T-ALL. The selection was based on (i) all candidate miRNAs that target *PHF6* from the *PHF6* 3'UTR-miRNA library screen (red circle; see also *Online Supplementary Table S1*), (ii) miRNAs that were among the 50% highest expressed miRNA in the T-ALL samples (green circle; see also *Online Supplementary Table S2*), and (iii) miRNAs expressed at significantly (rank products analysis, percentage of false predictions-value < 0.05) higher levels in T-ALL *versus* normal thymocyte subsets (blue circle; see also *Online Supplementary Table S3*).

MicroRNA-128-3p interacts directly with the 3'UTR of PHF6, whereas miR-574-3p targets PHF6 through a non-canonical mechanism

In order to confirm the results from the high-throughput miRNA library screen for *PHF6* (Figure 1), we performed independent single luciferase reporter assays for both miR-128-3p and miR-574-3p. Co-transfection of miR-128-3p mimics and the *PHF6* 3'UTR luciferase reporter construct in HEK-293T cells resulted on average in a 50% down-regulation of luciferase activity as compared to a non-targeting miRNA control (Figure 3A). The *PHF6* 3'UTR sequence was found to contain three distinct 7mer-m8 target sites and one offset 6mer target site for miR-128-3p (Figure 3B). To further validate the specificity of this interaction, we performed site-directed mutagenesis to generate a *PHF6* 3'UTR reporter construct in which all three 7mer-m8 and the offset 6mer binding sites were mutated. Upon transfection of this mutant construct, down-regulation of luciferase activity by miR-128-3p was completely rescued, demonstrating the validity of these binding sites for interaction (Figure 3C). Together, these results confirm that miR-128-3p interacts directly with its complementary seed regions in the 3'UTR of *PHF6*. For miR-574-3p, we also observed significant, yet weaker, down-regulatory effects in repeated independent luciferase assays with the wild-type *PHF6* 3'UTR construct (Figure 3D), confirming the results from the miRNA library screen. However, the 3'UTR of *PHF6* does not contain any canonical target sites for miR-574-3p, indicating that miR-574-3p possibly targets the 3'UTR of *PHF6* through an unknown, non-canonical mechanism.

Evaluation of PHF6 mRNA and protein regulation by miR-128-3p and miR-574-3p in HEK-293T and T-cell acute lymphoblastic leukemia cells

In order to measure effects of miR-128-3p and miR-574-3p on the regulation of endogenous *PHF6* expression, we transfected HEK-293T cells with either miR-128-3p or miR-574-3p mimics and measured the effect on PHF6 mRNA and protein levels. Quantitative real-time polymerase chain reaction gene expression analysis revealed that both miRNAs were able to reduce PHF6 mRNA levels at both 48 h and 72 h after transfection (Figure 4A). In

addition, western blot analysis confirmed that PHF6 protein levels were suppressed by both miRNAs (Figure 4B). Notably, although both miRNAs were able to regulate *PHF6* expression in HEK-293T cells, the effect for miR-128-3p was more profound than that for miR-574-3p, in accordance with the observed effects in the luciferase reporter assays (Figure 3A,D).

Next, we investigated effects of miR-128-3p and miR-574-3p over-expression on *PHF6* expression in the context of T-ALL cells. To this purpose, we selected two different *PHF6* wild-type T-ALL cell lines, Jurkat and ALL-SIL. Over-expression of miR-128-3p resulted in a significant down-regulation of PHF6 mRNA and protein in both Jurkat cells (Figure 5A,B) and ALL-SIL cells (*Online Supplementary Figure S2*). On the other hand, we were not able to demonstrate significant repressive effects of miR-574-3p on PHF6 mRNA expression or protein levels in repeated experiments in both T-ALL cell lines (*Online Supplementary Figure S3*). Therefore, miR-574-3p was not studied further in the context of T-ALL.

Finally, we performed miR-128-3p knockdown experiments to demonstrate regulation of PHF6 upon inhibition of endogenous miR-128-3p in T-ALL cells. More specifically, we transfected Jurkat cells with oligonucleotides of locked nucleic acids (LNA) directed against endogenous miR-128-3p and observed up-regulation of PHF6 protein levels as compared to levels in control LNA-transfected cells (Figure 5C). Together, the miR-128-3p over-expression and knockdown experiments provide evidence that miR-128-3p is able to regulate PHF6 expression in T-ALL cells.

Evaluation of possible regulation of other validated and predicted target genes by miR-128-3p in T-cell acute lymphoblastic leukemia cells

On the basis of the results of the above experiments, we speculated that miR-128-3p acts as an oncomir in T-ALL by targeting the *PHF6* tumor suppressor gene. However, as many miRNAs can target multiple genes, we checked whether miR-128-3p could target other genes in the context of T-ALL. Considering the known miRNA-target gene network acting in T-ALL, we investigated possible additional interactions of miR-128-3p with other *in silico* predicted T-ALL tumor suppressor genes, including *FBXW7*,

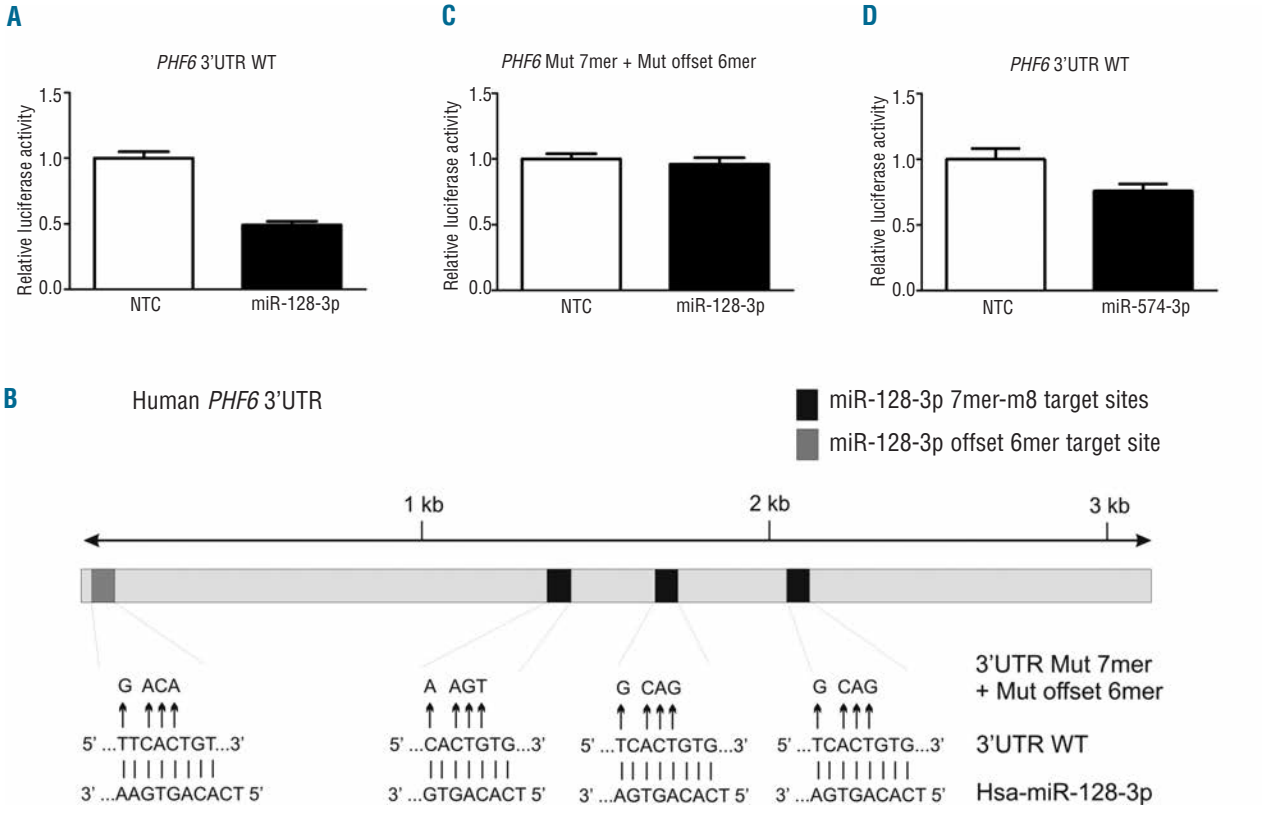

Figure 3. MicroRNA-128-3p and miR-574-3p target the 3'UTR of *PHF6*. (A) Transient over-expression of miR-128-3p reduces the luciferase activity of the wild-type *PHF6* 3'UTR reporter in HEK-293T cells. Bar graphs show the mean (± SD) of five independent experiments relative to the non-targeting miRNA transfection control (NTC). (B) Schematic representation of the human *PHF6* 3'UTR. The sequences indicated include three 7mer-m8 target sequences (black boxes) and one offset 6mer target sequence (dark gray box) for miR-128-3p in the wild-type 3'UTR reporter construct and their corresponding mutated sequences upon site-directed mutagenesis in the quadruple mutant reporter. The 3'UTR of *PHF6* does not contain seed regions for miR-574-3p. (C) Mutation of the 7mer-m8 and offset 6mer target sites for miR-128-3p in the *PHF6* 3'UTR construct completely rescues the down-regulatory effect of miR-128-3p on luciferase activity. Bar graphs show the mean (± SD) of three independent experiments, relative to NTC. (D) Transient over-expression of miR-574-3p reduces the luciferase activity of the wild-type *PHF6* 3'UTR reporter in HEK-293T cells. Bar graphs show the mean (± SD) of three independent repeats, relative to NTC.

NF1, *IKZF1* and *SUZ12*. However, miR-128-3p overexpression did not significantly suppress the expression levels of these four genes in T-ALL cell lines (*Online Supplementary Figure S4*). In addition, we tested other validated miR-128-3p target tumor suppressor genes in other tumor entities including *BAX*[13] and *E2F5*[14] (and *SUZ12*[15] and *NF1*[16]). Again, no significant or clear effects on the expression of the latter genes by miR-128-3p were observed in T-ALL cells (*Online Supplementary Figure S4*), suggesting that miR-128-3p has oncogenic effects in T-ALL predominantly through targeting *PHF6*.

MicroRNA-128-3p accelerates leukemia formation in a NOTCH1-induced T-cell acute lymphoblastic leukemia mouse model

Given the consistent *in vitro* effects of miR-128-3p on *PHF6* in T-ALL cell lines, and the fact that miRNA profiling data suggest a potential oncogenic role for miR-128-3p, we aimed to demonstrate the function of miR-128-3p in T-ALL oncogenesis *in vivo*. More specifically, we used a mouse model of NOTCH1-induced T-ALL,[17] in which fetal liver cells (hematopoietic progenitors) are transduced with ICN1 (active NOTCH1) in combination with either a miR-128-3p expression construct or an empty vector control (co-expressing GFP). Subsequently, the transduced cells are injected into the tail vein of lethally irradiated recipient mice after which leukemia onset is monitored (Figure 6A). We observed significantly faster leukemia

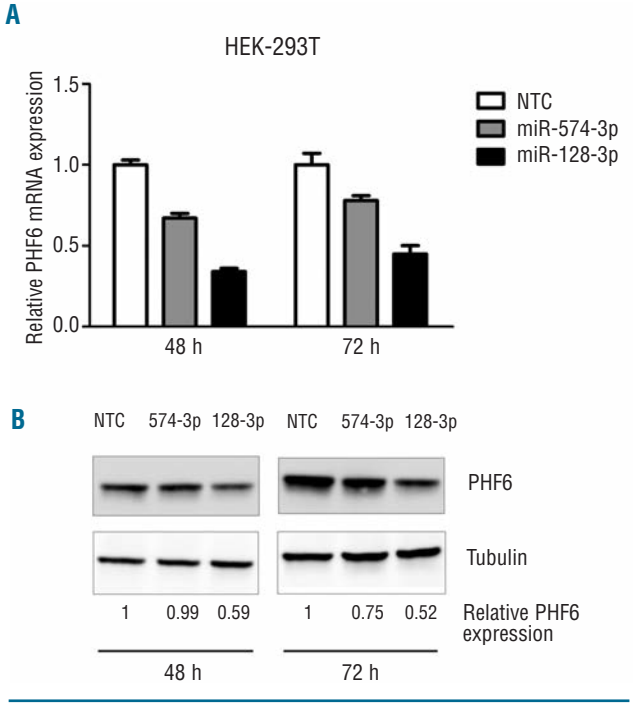

Figure 4. MicroRNA-128-3p and miR-574-3p regulate *PHF6* expression in HEK-293T cells. Forty-eight and 72 h after transfection of miR-128-3p and miR-574-3p mimics, RNA and protein fractions were isolated in parallel. Subsequently, quantitative real-time polymerase chain reaction (RT-qPCR) and western blot analyses were performed to evaluate the effects of miRNA over-expression on PHF6 mRNA (A) and protein (B) levels, respectively. Results are shown from one experiment, representative of two independent biological replicates. (A) PHF6 mRNA expression was normalized against three reference genes. Normalized PHF6 expression values (± SE for two technical RT-qPCR measurements) are shown relative to the NTC control for each time point. (B) PHF6 protein levels were quantified using Image J software, normalized to α-tubulin levels, and compared to relative PHF6 levels in the NTC controls for each time point.

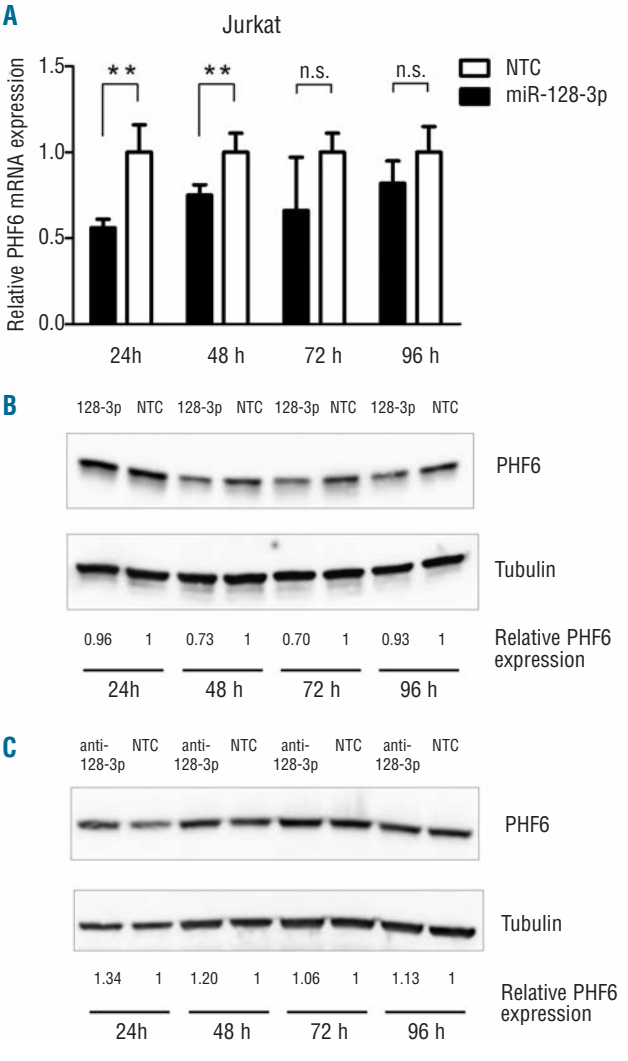

Figure 5. MicroRNA-128-3p regulates *PHF6* expression in T-ALL. Twenty-four, 48, 72, and 96 h after electroporation of Jurkat cells with miR-128-3p mimics, RNA and protein fractions were isolated in parallel. Subsequently, quantitative real-time polymerase chain reaction (RT-qPCR) and western blot analyses were performed to evaluate the effects of miR-128-3p over-expression on PHF6 mRNA (A) and protein (B) levels, respectively. Results are shown from one experiment, representative of two independent biological replicates. (A) Each data point represents the mean (± SD) of four independent electroporation reactions (performed in parallel for each time point and for miR-128-3p/NTC). PHF6 mRNA expression was normalized against three reference genes (see Methods) and compared to relative PHF6 expression in the NTC controls for each time point. Significance levels (t-test comparing 4 replicates of each condition per time point): $^{**}P<0.01$; n.s.: not significant. (B) PHF6 protein levels were quantified using Image J software, normalized to α-tubulin levels, and compared to relative PHF6 levels in the NTC controls for each time point. (C) Twenty-four, 48, 72, and 96 h after electroporation of Jurkat cells with specific oligonucleotides of locked nucleic acids (LNA) antagonizing miR-128-3p function (anti-128-3p) or non-targeting LNA controls (NTC), protein fractions were isolated. Subsequently, western blot analysis was performed to evaluate the effects of endogenous miR-128-3p down-regulation on PHF6 protein levels. PHF6 protein levels were quantified using Image J software, normalized to α-tubulin levels, and compared to relative PHF6 levels in the NTC controls for each time point. Results are shown for one experiment, representative of two independent biological replicates.

development in the mice over-expressing miR-128-3p (n=5, mean latency = 40 days, $P<0.05$) than in the vector control group (n=6, mean latency = 57 days) (Figure 6B), providing evidence for an oncogenic role of miR-128-3p in T-ALL development *in vivo*. Of note, all miR-128-3p-driven leukemia samples (from NOTCH1 and miR-128-3p co-transduced cells) showed GFP expression at the time of disease (*data not shown*). Pathological analysis of the mice demonstrated aggressive leukemia development with infiltration of highly proliferative Ki67-positive lymphoblasts in liver, kidneys, spleen and lungs (Figure 6C and *Online Supplementary Figure S5*). No drastic differences could be observed in the number of splenic Ki67-positive cells or in the systemic distribution and organ involvement upon miR-128-3p over-expression (Figure 6C and *Online Supplementary Figure S5*). Additional western blot analysis of the mouse tumors showed that Phf6 was down-regulated in the miR-128-3p-expressing mice compared to in the controls (Figure 6D). Notably, these results are in line with our previous observations that endogenous Phf6 knock-down by RNAi results in accelerated onset of leukemia in the NOTCH1-induced T-ALL mouse model.[12] Together, these results further strengthen our hypothesis that miR-128-3p exerts its oncogenic effects in T-ALL at least partly through targeting the *PHF6* tumor suppressor.

Discussion

In recent years, many miRNAs have been implicated in a wide variety of developmental processes as well as diseases including cancer.[18-22] MicroRNAs can function as tumor suppressor genes or oncogenes, depending on their regulation and their respective target genes in cancer

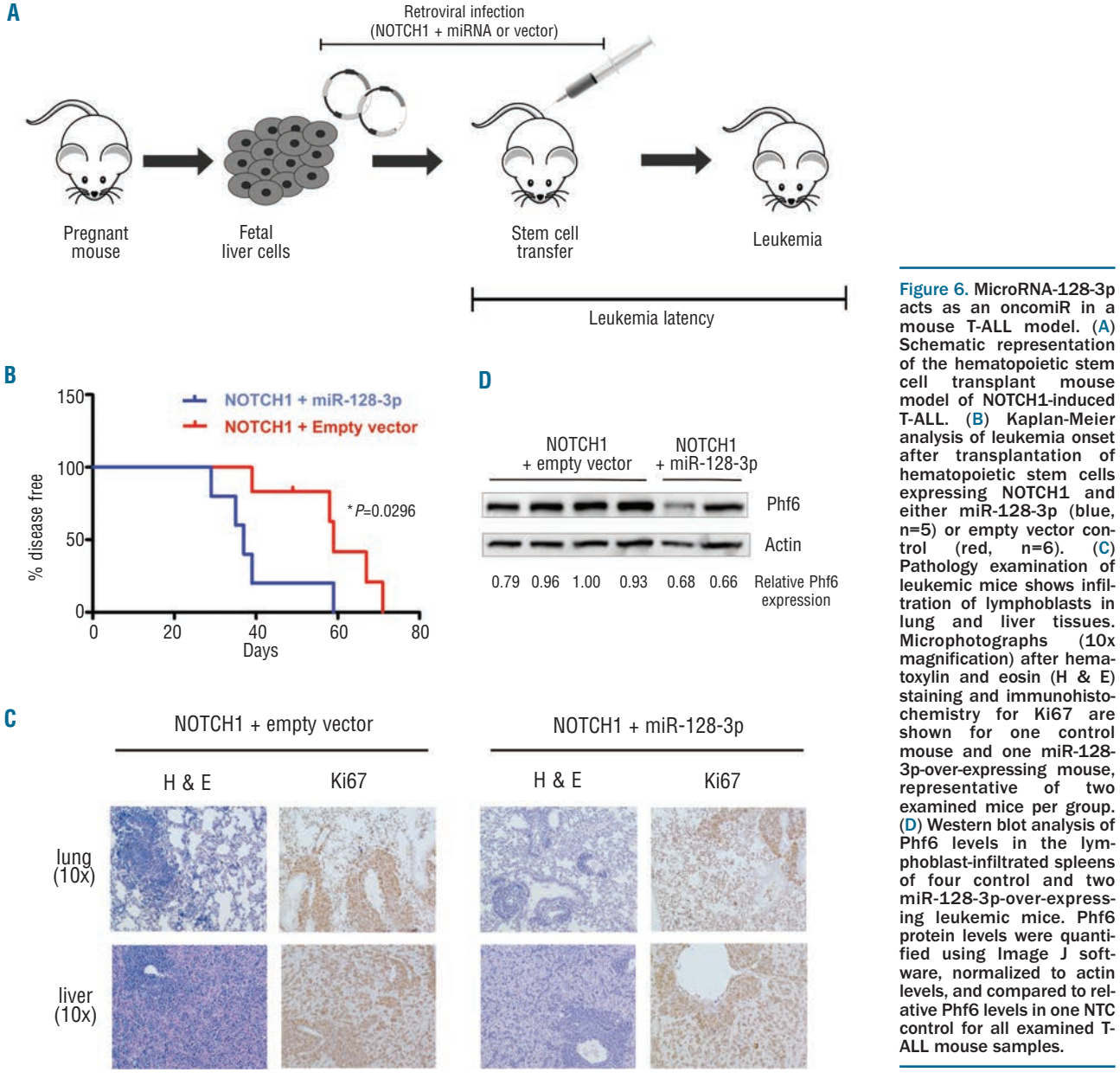

Figure 6. MicroRNA-128-3p acts as an oncomiR in a mouse T-ALL model. (A) Schematic representation of the hematopoietic stem cell transplant mouse model of NOTCH1-induced T-ALL. (B) Kaplan-Meier analysis of leukemia onset after transplantation of hematopoietic stem cells expressing NOTCH1 and either miR-128-3p (blue, n=5) or empty vector control (red, n=6). (C) Pathology examination of leukemic mice shows infiltration of lymphoblasts in lung and liver tissues. Microphotographs (10x magnification) after hematoxylin and eosin (H & E) staining and immunohistochemistry for Ki67 are shown for one control mouse and one miR-128-3p-over-expressing mouse, representative of two examined mice per group. (D) Western blot analysis of Phf6 levels in the lymphoblast-infiltrated spleens of four control and two miR-128-3p-over-expressing leukemic mice. Phf6 protein levels were quantified using Image J software, normalized to actin levels, and compared to relative Phf6 levels in one NTC control for all examined T-ALL mouse samples.

cells.[10,11] In a previous study, we demonstrated that miRNAs and protein-coding genes form intricate networks that are perturbed in cancer, as illustrated by the complex interaction between several oncogenic miRNAs and known tumor suppressor genes in T-ALL. This network included *PHF6*, which is a frequently affected tumor suppressor in T-ALL.[12] Understanding more complete interactive miRNA-mRNA regulatory networks is currently hampered by the limitations of *in silico* miRNA-target prediction algorithms, which often fail to reveal all true targets and predict many false positive interactions. Moreover, these algorithms do not take into account the tissue-specificity and physiological relevance of most miRNA-target gene interactions. To obtain a more comprehensive picture of miRNAome-wide regulation of *PHF6* in T-ALL, we performed an unbiased 3'UTR-miRNA library screen for *PHF6* and integrated the results with the miRNA expression landscape from primary T-ALL samples and normal thymocyte subsets to identify novel oncogenic miRNAs targeting *PHF6* in the context of T-ALL.

The miRNAome-wide 3'UTR screen, which was designed to generate a minimum of false positive hits, yielded a total of 23 hits for the *PHF6* gene. Almost half of these hits (11/23) could not be predicted by different target prediction algorithms: this may be because of the presence of non-canonical binding sites, which are generally not predicted by *in silico* algorithms. Based on integrated data from an empty vector screen in our 3'UTR-miRNA library screen experiments (see Methods), we have evidence to rule out the possibility that the luciferase open reading frame is targeted by these specific miRNA hits. Together, these data underscore the power of an unbiased screening method to identify novel candidate interactions (that would not have been identified by prediction algorithms).

In order to refine the selection of candidate miRNAs relevant in the context of T-ALL from these 23 hits, we looked for miRNAs differentially expressed between primary T-ALL samples and normal thymocyte precursors. Using this approach, we selected two novel candidate *PHF6*-targeting miRNAs with a potential oncogenic function in T-ALL, namely miR-128-3p (formerly miR-128b) and miR-574-3p. Individual 3'UTR luciferase assays and rescue experiments upon mutagenesis of miRNA binding sites confirmed direct interaction of miR-128-3p with the 3'UTR of *PHF6*. Interestingly, the 3'UTR of *PHF6* does not contain canonical binding sites for miR-574-3p, but multiple independent luciferase assays confirmed down-regulation of the *PHF6* 3'UTR luciferase reporter by miR-574-3p, suggesting that miR-574-3p potentially targets the 3'UTR of *PHF6* through an unknown, non-canonical mechanism. One possibility is that miR-574-3p targets *PHF6* through 'seedless' binding, a mechanism described for miR-24.[23] Regardless of the mechanism of interaction involved, this observation further demonstrates that unbiased screening of miRNA-target gene interactions is able to identify novel interactions that would have remained undetected by current *in silico* prediction algorithms, most of which predict canonical interactions.

Whereas *in vitro* assays after over-expression of miR-128-3p and miR-574-3p confirmed that both miRNAs are able to target endogenous *PHF6* in HEK-293T cells, only miR-128-3p was found to regulate *PHF6* expression in the investigated T-ALL cell lines. Possibly, the miR-574-3p interaction with *PHF6* is subtle or transient, or does not exist in T-ALL cells. We, therefore, further focused our attention on the *in vivo* oncogenic potential of miR-128-3p by using a NOTCH1-induced T-ALL mouse model.[17] Mice over-expressing miR-128-3p showed accelerated leukemia formation as compared to control mice, demonstrating that miR-128-3p is a candidate novel oncomiR that contributes to T-ALL oncogenesis *in vivo*.

Previous studies have shown that miR-128(-3p) acts as an oncogene in malignancies such as acute leukemia,[24,25] breast cancer[13,26-28] and lung cancer.[14] However, this miRNA has also been described to have tumor suppressive functions in other cancer entities (for an overview, refer to *Online Supplementary Table S4*). Such a context-dependent role has been described in cancer for several miRNAs as well as for protein-coding genes. In this study, we demonstrate that miR-128-3p could act as an oncomiR in T-ALL. Other studies reported in literature support a possible oncogenic function for miR-128-3p in ALL, as miR-128(-3p) was found to be significantly more highly expressed in ALL samples than in acute myeloid leukemia samples and normal control samples.[24,25,29] Possible mechanisms that could underlie miR-128-3p over-expression in T-ALL remain to be explored in further detail. Although to the best of our knowledge chromosomal aberrations or mutations specifically affecting miR-128 loci have not been reported so far, there is evidence that miR-128-3p expression could be affected by perturbed epigenetic regulation [hypomethylation of miR-128 promoter(s)] in T-ALL cells.[25,30] Finally, miR-128 over-expression was also demonstrated to result in increased resistance of Jurkat/R (T-ALL) cells to Fas-mediated apoptosis,[30] indicating that miR-128(-3p) can have oncogenic properties in T-ALL cells. In line with these studies, we have demonstrated, using T-ALL mouse models, that miR-128-3p can indeed have oncogenic properties in T-ALL development *in vivo*.

Furthermore, we speculate that miR-128-3p exerts its oncogenic effects in T-ALL through targeting the tumor suppressor *PHF6*. However, as it is well known that many miRNAs are able to control multiple target genes, we investigated whether miR-128-3p could target other genes in the context of T-ALL. First of all, in view of the already established regulatory miRNA-target gene network acting in T-ALL, we investigated possible additional interactions of miR-128-3p with other *in silico* predicted T-ALL tumor suppressor genes, including *FBXW7*, *NF1*, *IKZF1* and *SUZ12*. However, no significant effects on the expression levels of these four genes were detected upon miR-128-3p over-expression in T-ALL cell lines (*Online Supplementary Figure S4*), thus excluding miR-128-3p from broader interaction in the T-ALL regulatory network. Next, we also looked at other validated miR-128-3p target tumor suppressor genes in other tumor entities including *BAX*[13] and *E2F5*[14] (as well as *SUZ*[12,15] and *NF1*[16]), but no such interaction was observed in T-ALL cell lines (*Online Supplementary Figure S4*). From these results, we hypothesize that miR-128-3p exerts its oncogenic effects in T-ALL predominantly (or at least partly) through targeting of the *PHF6* tumor suppressor gene. This hypothesis is further strengthened by the observation that Phf6 levels are down-regulated in mice over-expressing miR-128-3p in the NOTCH1-sensitized T-ALL model, and our prior studies demonstrating that endogenous Phf6 knockdown by RNAi resulted in accelerated onset of leukemia in this model.[12] MicroRNA-128-3p over-expression thus mimics the phenotype of Phf6 knockdown in the NOTCH1-induced T-ALL model. Nevertheless, we cannot exclude

the possibility that miR-128-3p has additional, yet unidentified targets that may explain its synergism in NOTCH1-driven murine T-ALL.

In summary, we identified miR-128-3p as a strong novel candidate oncomiR targeting *PHF6* in T-ALL, thereby extending the established cooperative miRNA-tumor suppressor gene network that is involved in T-ALL. In addition, our study adds T-ALL to a growing number of cancer entities in which miR-128(-3p) is implicated.

Acknowledgments

The authors would like to thank the following funding agencies: the Fund for Scientific Research Flanders ('FWO Vlaanderen' research projects G.0202.09, G.0869.10N to FS, 3GA00113N to PVV and G0B2913N and 3G002711 to TT, research grant 1.5.210.11N to PR; PhD grant to JVdM; postdoctoral grants to TT, PM, PVV, SG and PR; BP is a senior clinical investigator), the Belgian Foundation against Cancer [FS, JV (SCIE 2010-177) and SG], the Flemish League against Cancer ('VLK') (PhD grant to JVdM); Ghent University (GOA grant 12051203 to FS; BOF10/PDO/140 to PR; BOF01D35609 to GVP), the Cancer Plan from the Federal Public Service of Health (FS), the Children's Cancer Fund Ghent (FS) and the Belgian Program of Interuniversity Poles of Attraction (IUAP P7/03 and P7/07). Additional funding was provided by the NCI (R01-CA142798-01 and U01CA105492-08 to HGW), the Leukemia Research Foundation (HGW), the Experimental Therapeutics Center at MSKCC (HGW), the American Cancer Society (HGW) and the Geoffrey Beene Cancer Center (HGW). We also would like to thank Aline Eggermont for excellent technical assistance.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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