Stop the dicing in hematopoiesis: What have we learned?

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What have we learned?

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MicroRNAs (miRNAs) belong to an abundant class of highly conserved small (22nt) non-coding RNAs. MiRNA profiling studies indicate that their expression is highly cell type-dependent. DICER1 is an essential RNase III endoribonuclease for miRNA processing. Hematopoietic cell type- and developmental stage-specific *Dicer1* deletion models show that miRNAs are essential regulators of cellular survival, differentiation and function. For instance, miRNA deficiency in hematopoietic stem cells and progenitors of different origins results in decreased cell survival, dramatic developmental aberrations or dysfunctions in mice. We recently found that homozygous *Dicer1* deletion in myeloid-committed progenitors results in an aberrant expression of stem cell genes and induces a regained self-renewal capacity. Moreover, *Dicer1* deletion causes a block in macrophage development and myeloid dysplasia, a cellular condition that may be considered as a preleukemic state. However, *Dicer1*-null cells do not develop leukemia in mice, indicating that depletion of miRNAs is not enough for tumorigenesis. Surprisingly, we found that heterozygous *Dicer1* deletion in myeloid-committed progenitors, but not *Dicer1* knockout, collaborates with *p53* deletion in leukemic progression and results in various types of leukemia. Our data indicate that *Dicer1* is a haploinsufficient tumorsuppressor in hematopoietic neoplasms, which is consistent with the observed downregulation of miRNA expression in human leukemia samples. Here, we review the various hematopoietic specific *Dicer1* deletion mouse models

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and the phenotypes observed within the different hematopoietic lineages and cell developmental stages. Finally, we discuss the role for DICER1 in mouse and human malignant hematopoiesis.

Introduction

DICER1 is an evolutionarily conserved member of the RNase III family of endoribonucleases. The gene encoding DICER1 is located on human chromosome 14q32 and mouse chromosome 12E. DICER1 is a complex protein and contains three N-terminal Helicase domains (HEL1, HEL2i, HEL2), a DUF283 domain, which is presumably involved in binding of double-stranded RNA (dsRNA), a Platform domain, the pre-miRNA binding domain PAZ, RNase IIIa, RNase IIIb and a C-terminal dsRNA binding domain (dsRBD).[1-3] The RNase III domains of DICER1 cleave double-stranded RNA (dsRNA) substrates and specific precursor hairpin sequences, including so-called pre-miRNAs, into small 5'-phosphorylated RNAs of typically 21–23 nucleotides called miRNA.[4] Deep sequencing of 5'-phosphorylated short RNAs in ES cells showed that the miRNA is the only class of short RNAs to be fully DICER1-dependent.[5] However, the premature *miR-451* is the single well-conserved miRNA-containing sequence known to bypass DICER1 processing and is matured by an Argonaute-2 (Ago-2)-dependent mechanism.[6-9] The DICER1-generated short RNAs bind to Argonaute proteins in the so-called RNA-induced silencing complex (RISC). This complex induces degradation or inhibits translation of homologs target mRNAs.

Moreover RISC triggers gene silencing via chromatin modifications at target promoters under specific conditions such as cellular senescence.[10,11]

Genetic studies in plants, zebrafish and mice show that *Dicer1* is essential for normal development.[12-14] For instance, genetic deletion of *Dicer1* in mice results in early embryonic mortality due to depletion of the Oct-4-positive pluripotent embryonic stem cell pool at embryonic day (E) 6-E7.[14] *Dicer1*-null ES cells are incapable of processing miRNA hairpins or dsRNAs.[5,15,16] However, *Dicer1* is dispensable for the siRNA-mediated gene silencing response.[16] Although a role for *Dicer1* in centromeric silencing has been suggested, deep sequencing of small RNAs in *Dicer1*-null and *Dicer1* wild type ES cells indicates that the production of miRNAs is the sole catalytic function of DICER1 in these cells.[5] To bypass embryonic lethality and to enable investigation of *Dicer1* functions in adult tissues in mice, a floxed *Dicer1* allele (*Dicer1*[fl]) has been generated that allows conditional deletion of *Dicer1* in a cell type- and developmental stage-specific fashion.[17] To address the overall role of miRNAs in the development and function of hematopoietic cells, different hematopoietic cell stage and lineage-specific conditional *Dicer1* deletion strains have been used. First, we will review the phenotypic consequences of *Dicer1* deletion at different stages of hematopoiesis and cell types. Second, we discuss what we have learned from these models about miRNA-controlled pathways in hematopoiesis. Finally, we show evidence for *Dicer1* haploinsufficient tumorsuppressor activity in mouse leukemia and discuss the role for *DICER1* in human AML.

The Role of *Dicer1* in T-Lymphocyte Development

In one of the first studies addressing the role of *Dicer1* in hematopoiesis in vivo, floxed *Dicer1* alleles were deleted by CRE in lymphocyte-specific protein tyrosine kinase (Lck)-positive cells. In this model, *Cre* is active at the double-negative (DN) CD4$^-$CD8$^-$ T cell developmental stage and results in *Dicer1*-null CD44$^-$CD25$^-$ (DN4), CD4$^+$CD8$^+$ and CD4$^+$CD8$^-$, CD4$^-$CD8$^-$ cells (**Fig. 1A**).[18] *Dicer1* seems

to be essential for the generation and survival of αβ T-cells. However, in the surviving T-cells, *Dicer1* is dispensable for CD4$^+$ and CD8$^+$ single positive lineage commitment.[18] These results strongly suggest that *Dicer1* deletion does not affect normal T-cell lineage-specific gene expression programs. In these cells, the transcriptional repression of centromeric satellite repeats and features of facultative heterochromatin are maintained in the absence of *Dicer1*,[18] suggesting that survival of immature T-cells is regulated directly by a miRNA-controlled mechanism.

The *CD4-Cre* transgenic mouse model enables investigation of the consequences of *Dicer1* deletion at a later stage of T cell development (**Fig. 1B**). These mice show four major phenotypes: (1) *Dicer1* is required for basic cellular processes, such as proliferation and survival, as also proposed by Cobb et al., and therefore *Dicer1* deficiency results in decreased number of T-cells.[18,19] (2) *Dicer1* deletion appears to favor T-cell lineage production from CD4$^+$CD8$^+$ double-positive stage toward CD4$^+$ single-positive peripheral T-cells over CD8$^+$ single-positive cells. However, this phenotype was less obvious from thymic T-cell lineage analysis. This discrepancy may be explained by the fact that *CD4-Cre*-driven deletion of *Dicer1* does not result in complete depletion of all miRNAs, presumably due to high miRNA stability and limited cell divisions of a small fraction of CD4$^+$ T-cells, which may be different for *Dicer1*-null CD8$^+$ T-cells. (3) *Dicer1*-null CD4$^+$ T-cells produce increased levels of IFN-γ, a pro-Th1 cytokine, indicating that *Dicer1* controls Th1-lineage commitment.[19] (4) *CD4-Cre*; *Dicer1*^{fl/fl} mice show a more than 2-fold decreased proportion of Foxp3$^+$ regulatory T cells (Treg).[20] Interestingly, these mice developed a splenomegaly, and their lymph nodes were severely enlarged at the age of 3 to 4 mo. Moreover, organs such as colon, lung and liver were affected by immune pathology caused by an overactive immune system, which is less severe as compared with *Foxp3*-knockout mice lacking functional Tregs.[20] However, this phenotype suggests that *Dicer1*-deficient Tregs are functionally aberrant as well.

Two studies revealed the role of *Dicer1* more specifically in the function of mature

Tregs, using a *Foxp3-Cre* knock-in mouse (**Fig. 1C**).[21,22] Under steady-state conditions, Foxp3-controlled deletion of *Dicer1* has minimal effects on Treg cell development, cellular proliferation and survival in the peripheral compartments.[22] However, a diminished fitness of *Dicer1*-deficient Treg cells in the periphery was observed in a competitive experiment in mice.[21] Under inflammatory conditions, the immune-repressive capacity of the mutant Treg cells is markedly reduced and results in rapid fatal autoimmunity and complete failure of immune suppression activity.[21,22] Moreover, *Dicer1* deletion in Treg cells leads to the progression of fatal lymphoproliferative autoimmune syndrome with an early onset, which is indistinguishably comparable to T-cell-specific Foxp3 deficiency.[21] The expression of putative suppressor effector molecules, including CTLA4, IL-10, EBV-induced gene 3 (Ebi-3) and granzyme B, was decreased by still-unidentified miRNA-controlled mechanisms.[21] Tregs express a specific set of miRNAs, including *miR-223*, *miR-155* and *miR-146*, which is distinct from naïve CD4$^+$ T-cells.[20] Therefore, the expression of these miRNAs may be under direct or indirect control of the transcription factor Foxp3.[20] Identification of the targets that are controlled by these miRNAs in Tregs may provide new insights about the molecular pathways involved in the activity of these cells.

The role of miRNAs in invariant Natural Killer T (iNKT) cells was studied in a mouse strain by *Tie2-Cre*-mediated disruption of *Dicer1*.[23] The Tie2 kinase is specifically expressed in hematopoietic progenitors and endothelial cells.[24] Similar to the immune phenotypes in *CD4-Cre;Dicer1*^{fl/fl} and *Lck-Cre;Dicer1*^{fl/fl}, these mice show reduced numbers of iNKT cells in the thymus, spleen and liver. Moreover, *Dicer1* deletion results in developmental abnormalities of iNKT cells.[23,25] In addition, *Dicer1*-deficient peripheral iNKT cell numbers are decreased and displayed profound defects in α-GalCer, phorbol myristate acetate (PMA) and ionomycin-induced cellular activation and production of cytokines such as IL-4 and IFN-γ.[23] Together, these data indicate that *Dicer1* controls survival at the early T-cell developmental stage. At the later

stage, *Dicer1* is critical for the balance of Th1/Th2 lineage production and controls functions such as immune-repression and specific cellular activity.

Dicer1 Function During B Cell Development

Ablation of *Dicer1* in early B cell progenitors, mediated by the *Mb1-Cre* allele, which is expressed at the earliest stage of B-cell development, blocks B-cell development almost completely at the pro-B-cell (B220^{low}, c-kit^{+} CD25^{-}) to pre-B-cell (B220^{int}, c-kit^{-}, CD25^{+}) transition (**Fig. 1D**).[26] This block in B-cell development is caused by a strong induction of apoptosis and results in total depletion of B cells in the BM and the peripheral lymphoid organs in mice.[26] Gene expression profiling of Abelson virus (v-Abl)-transformed *Dicer1*-null pro-B-cells revealed that *miR-142–3p* and different members of the *miR-17-92* family of miRNA, such as *miR-17*, *miR-19*, *miR-20* and *miR-92*, are the most active at the pro-B-cell stage.[26] Derepression of the proapoptotic protein BIM, a confirmed target of *miR-17-92*, was shown to be mainly responsible for the failure of the cells to respond to survival signals.[26] In full agreement, Ventura A and colleagues have demonstrated that deletion of the *miR-17~92* in mouse hematopoietic stem cells leads to a cell development arrest at the pro-B to pre-B transition that is highly reminiscent of what has been observed in the *Dicer1*-deficient mice.[27]

The role of miRNAs in terminal B cell differentiation is addressed by the analysis of *CD19-Cre* driven *Dicer1*-deletion mouse model (**Fig. 1E**).[28] In contrast to early *MB1-Cre* driven *Dicer1* deletion, depletion of *Dicer1* with *CD19-Cre* in immature B220[+] IgM^{+} cells does not induce cell death and allowed analysis of the role for *Dicer1* in mature B cells in peripheral tissues.[28] In the absence of *Dicer1*, transitional and marginal zone B cells are overrepresented, and the generation of follicular B cells is impaired.[28] The *miR-185* is abundantly expressed in follicular B-cells and controls the expression of B cell antigen receptor (BCR) signaling effector Bruton tyrosine kinase (BtK) in activated B cells.[28] *Dicer1*-deficient B cells
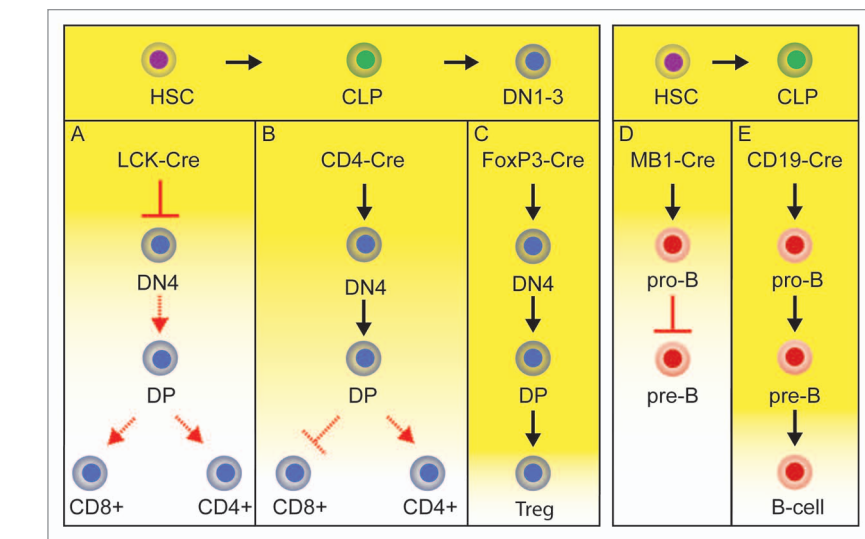

Figure 1. Schematic overview of the phenotypic characteristics of different CRE-mediated *Dicer1*-deletion models in lymphopoiesis (A) HSCs develop via different progenitors toward mature CD4^{+} or CD8^{+} single positive cells. The effects of *LCK-Cre*-mediated *Dicer1* deletion are depicted. The apparent level of *Dicer1* expression is indicated by the yellow background color (yellow, normal endogenous levels; white, no Dicer1 expression). HSC, hematopoietic stem cells; CLP, common lymphoid progenitor; DN1–3, double-negative stage 1 to 3 (CD4^{-}CD8^{-}); DN4, double-negative stage 4, DP: double-positive CD4^{+}CD8^{+} cells. Phenotypic characteristics are indicated by the red arrows and lines. Dashed lines indicate less cells than in wild-type situation (B). See also (A). The effects of *CD4-Cre*-mediated *Dicer1* deletion are depicted. Phenotypic characteristics are indicated by the red arrow and lines (C). See also (A). The effects of *FoxP3-Cre*-mediated *Dicer1* deletion results in normal numbers of regulatoty T-cells (Tregs), but these cells are functionally aberrant. (D) HSCs develop via indicated progenitors toward mature B-cells. The effects of *MB1-Cre*-mediated *Dicer1* deletion are indicated by the red lines and arrow and result in developmental block from the pro-B-cell to the pre-B-cell stage. Pro-B: earliest stage of progenitor B-cell development, pre-B-cell, precursor stage of B-cell development (E). See also (D). *CD19-Cre*-mediated deletion of *Dicer1* results in mature B-cells which are functionally aberrant.

produce high titers of autoreactive antibodies and as a result cause autoimmune disease in aged female mice.[28] However, the miRNAs that control autoreactivity are still unidentified.

To investigate the role for *Dicer1* in antigen-activated, but not naive B cells, an activation-induced cytadine deaminase *(Aicda)-Cre*-mediated *Dicer1* deletion mouse model has been generated.[29] This mouse model showed that *Dicer1* is required for the production of antigen-specific high-affinity antibodies during a T-cell-dependent immune response.[29] Also, the formation of germinal center B cells is drastically impaired in *Dicer1*-deficient mice.[29] These mutant mice fail to generate memory B and long-lived plasma cells after immunization with a T cell-dependent antigen. This study provides evidence for *Dicer1*-controlled cell proliferation of activated germinal center B-cells by strong repression of cell cycle

inhibitory genes, such as *Cdkn1c* (*p57[Kip2]*), *Cdkn2b* (*p16[INK4a]*), *Cdk1a* (*p21[Cip1]*) and *Cdkn1b* (*p27[Kip]*).[29] Furthermore, *Dicer1* deletion in B-cells leads to massive induction of apoptosis due to derepression of the proapoptotic protein BIM1 as described for early stages of B-cell development.[29] Together, these data show that *Dicer1* controls survival of B-cells at different stages of B-cell development, regulates cellular proliferation and is critical for proper B- and plasma cell functions.

The Role for *Dicer1* in NK Cell Function

Bezman et al. induced ablation of conditional *Dicer1* alleles with a tamoxifen-inducible Cre recombinase (human estrogen receptor *(ER[T2])-Cre*) and studied the effects of miRNA depletion in NK cells.[30] This non-specific model revealed a role for *Dicer1* in the maintenance of
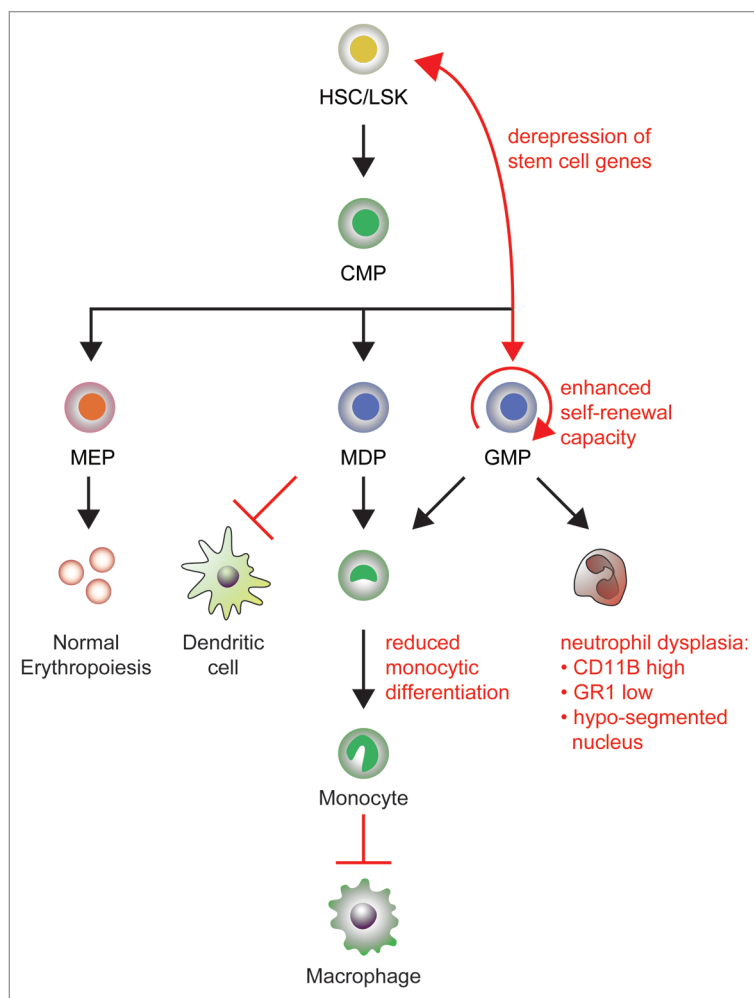
Figure 2. Schematic overview of the results of *C/ebpa-Cre*-mediated deletion of *Dicer1* in myeloid-committed progenitors. Phenotypic characteristics are indicated in red. In short, deletion of *Dicer1* results in derepression of stem cell genes in myeloid progenitors and an enhanced self-renewal capacity. Furthermore, MDPs and GMPs are blocked in macrophage and dendritic cell development. In addition, *Dicer1* deletion results in neutrophil dysplasia with cells that are characteristic for Pelger-Huet anomaly. HSC/LSK, hematopoietic stem cells/Lin⁻;Scal⁺; Kit⁺; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocytic-erythroid progenitor; MDP, macrophage-dendritic cell progenitor; CD11B, pan marker for myeloid cells; GR-1, marker for mature granulocytes.

survival and function of NK cells.[30] They found that in response to a viral infection with mouse cytomegalovirus (MCMV), the expansion of NK cells, but not the IFN-γ production, is *Dicer1*-dependent, suggesting that survival but not activity of NK cells is affected by *Dicer1* deficiency.[30] Similarly, *HCD2-Cre;Dicer1^fl/fl* mice, which enable a lymphocyte-restricted *Dicer1* deletion at the early stage of NK cells development, also showed reduced NK cell maturation and survival.[30,31] However, *Dicer1*-null NK cells showed enhanced degranulation and IFN-γ production in response to cytokines such as IL-15 and IL-12, tumor target cells, activating NK cell receptor ligation as well as during acute MCMV infection in vivo.[31] The *miR-15/16* family of miRNAs is potentially contributing to IFN-γ suppression and may control dampening of NK cell functions.[31]

Dicer1 Deletion in Myeloid-Committed Progenitors Revealed an Unexpected Function in Hematopoiesis

The consequences of *Dicer1* deletion in hematopoietic stem and progenitor cells

(HSPCs) was first studied by breeding *Dicer1^fl/fl* with *Mx-Cre* mice.[32] These mice express the Cre-recombinase in response to interferons and are highly efficient in recombination of floxed alleles in the hematopoietic system in vivo via peritoneal injection of polyI:polyC (pIpC).[32] *Dicer1* ablation in these mice depletes functional HSCs, induces rapid apoptosis in HSPCs and consequently causes total disruption of hematopoiesis.[32] In addition, miRNA-depleted HSCs are unable to reconstitute hematopoiesis in mice.[32] In full agreement, *Dicer1^fl/fl* HSCs containing the *VAVi-Cre* transgene that is highly active in HSCs and efficient in deletion of floxed alleleles[33] are incapable to reconstitute lethally irradiated recipient mice (Erkeland SJ et al., unpublished data). Together, these data show that *Dicer1* is essential for HSCs survival. Interestingly, *miR-125a* controls the expansion of HSCs in vivo through targeting the proapototic gene *Bak1*. Whether *miR-125a* as a single miRNA can rescue *Dicer1*-null HSC survival and functions remains elusive, but it is more likely that multiple miRNAs are critical at this stage.

To address the question whether miRNAs play a role in early myeloid-lineage decisions, we deleted *Dicer1* in CCAAT/enhancer-binding protein α (C/EBPA)-positive myeloid-committed progenitors in vivo (**Fig. 2**).[34] In striking contrast to the results in HSCs and early lymphoid progenitors, we recently found that miRNA depletion does not affect the number of myeloid-committed progenitor cells in mice.[34] However, isolated *Dicer1*-deficient granulocyte-macrophage progenitors (GMPs) were defective in myeloid development and exhibited an increased self-renewal potential.[34] In mice, *Dicer1* deletion by *C/ebpa-Cre* blocked monocytic differentiation, depleted macrophages and myelo-dendritic cells and caused myeloid dysplasia with morphological features of Pelger-Huet anomaly[34] (**Fig. 2**). Strikingly, monocytes express low levels of proteins involved in miRNA processing and functions such as DROSHA, AGO1 and AGO2 compared with the levels found in T-cells, and are deficient for DICER1, unless the cells are forced to differentiate toward macrophages.[35,36] The presence of some miRNAs in the

monocytic and *Dicer1*-deficient cell line U937 suggests that some miRNAs can be generated by proteins other than DICER1, such as PIWIL4,[35] but this hypothesis still needs proper validation. However, the fact that *Dicer1*-null monocytes are blocked in their differentiation in vivo indicates that *Dicer1* is essential at this stage, and its function cannot be bypassed by other miRNA processing mechanisms.

MiRNA profiling of wild type GMPs showed that 104 miRNAs are abundantly expressed at this stage, of which at least 20 miRNA families are potentially active by reducing their target mRNA abundance.[34] Interestingly, of the derepressed miRNA targets in *Dicer1*-null GMPs, 27% are normally exclusively expressed in HSCs or are specific for multi-potent progenitors and erythropoiesis.[34] Unlike the results from HSCs and lymphoid progenitors showing functions of *Dicer1* mainly in survival pathways, these results provide evidence for a miRNA-controlled switch of a hematopoietic stem cell program of self-renewal and expansion toward myeloid differentiation (**Fig. 2**).[34]

The Role for *Dicer1* in Leukemia

Human cancer including different types of leukemia is characterized by a global reduction in miRNA expression.[37] The first experimental evidence for a role of global downregulation of miRNAs in cellular transformation and tumorigenesis has been presented in a *K-Ras*-induced mouse model for lung cancer.[38] Further studies of the role for *Dicer1* in human cancer development in immune-deficient mice strongly suggested that *Dicer1* is a haploinsufficient tumorsuppressor.[39] In this model, homozygous deletion of *Dicer1* is tolerated by the tumor cells; however, lack of miRNAs abrogates tumor outgrowth due to strongly reduced cell proliferation capacity of the DICER1-null cells.[39] In agreement, heterozygous deletion of *Dicer1*, but not *Dicer1*-knockout, accelerated tumor formation on a retinoblastoma-sensitized background.[40] In mouse B-cells, *Dicer1* is required for Myc-induced B-cell lymphomagenesis and survival of B-cell lymphomas.[41] However, in this model *Dicer1* is not a haploinsufficient tumor suppressor, as heterozygous deletion of *Dicer1* does not affect lymphoma latency and overall survival.[41] This discrepancy may indicate that the tumorsuppressing activity of DICER1 is cell type-dependent.

We asked whether *Dicer1* deletion enhances myeloid leukemia development in mice. In hematopoietic cells, *C/ebpa* starts to be expressed in early myeloid-committed progenitors, making it a suitable promoter to drive *Dicer1* deletion for studying the role of miRNA depletion in myeloid leukemias.[34,42] To circumvent prenatal lethality, we transplanted fetal liver cells from mutant and control embryos into lethally irradiated recipient mice. While heterozygous deletion of *Dicer1* in myeloid-committed progenitors does not affect myeloid development, homozygous *Dicer1* deletion results in block of macrophage/dendritic cell development and myeloid-dysplasia, a cellular condition that may be considered as a preleukemic state[34] (**Fig. 2**). However, mice transplanted with either heterozygous floxed *Dicer1* or homozygous floxed *Dicer1* cells survived devoid of any signs of myelo-proliferative disease or leukemia development within a year of observation, indicating that loss of *Dicer1* in myeloid-committed progenitors is not sufficient to initiate short-term leukemogenesis in mice[34] (**Fig. 3A**). To further investigate whether depletion of miRNAs accelerates myeloid leukemia development in a tumor susceptible model, we crossed *Dicer1* floxed (*Dicer1*[fl]) alleles with *p53*^{fl/fl} mice and transplanted fetal liver cells from double mutants and control embryos into lethally irradiated recipient mice. *C/ebpa-cre* driven deletion of *p53* and hemizygous deletion of *Dicer1* in mice caused development of various types of leukemias in half of the reconstituted mice with a latency of approximately 6 mo (**Fig. 3**). Only one out of eight *Dicer1*^{fl/fl};*p53*[f/f] recipient mice developed a leukemia with a latency of 9 mo (**Fig. 3A**). However, PCR analysis on genomic DNA isolated from the *Dicer1*[f/f] tumor cells in liver and spleen showed that the *Dicer1* floxed alleles were incompletely recombined (**Fig. 3B**). These results are in full agreement with data published by Kumar et al. and strongly suggest that only reduced levels of *Dicer1*, but not bialleleic loss of *Dicer1*, may play a functional role in leukemia development.[39,40] However, the fact that total depletion of miRNAs does not affect the viability of myeloid progenitors in mice may suggest that no negative selection due to reduced survival or proliferation by lack of miRNAs occurs in these cells. Together, these data provide evidence for a model in which reduced level of miRNAs is an oncogenic event in the development of leukemia but that activity of at least some miRNA species is essential for oncogenic transformation (**Fig. 4**). This is in full agreement with experimental data showing tumor suppressing and oncogenic activities of investigated miRNAs, such as *miR-17~92* and *miR-125*.[43] Moreover, miRNA expression profiling data of human cancer and AML samples are consistent with this hypothesis, as a small subset of miRNAs, including e.g., *miR-9*, *miR-125* and *miR-17~92*, are highly expressed, whereas most other miRNAs are downregulated.[44]

Dicer1 Mutations in Human Leukemia

To date, the mechanism behind the reduced miRNA expression in subsets of human myeloid leukemia samples still remains elusive. One possibility is that the widespread silencing of miRNAs is the result of a defect in miRNA biogenesis caused by mutations in the gene encoding DICER1. For instance, data from Cancer Genome Project at the Wellcome Trust Sanger Institute (www.sanger.ac.uk/cosmic) show that somatic *DICER1* mutations occur in different human tumors, including lung carcinoma, malignant melanoma and ovarian cancer.[45] Recently, Hill et al. found *DICER1* mutations in familial pleuro-pilmonary blastoma.[46] In addition, a recent study in human non-epithelial ovarian cancers revealed mutations in the codons encoding metal-binding sites within the RNase IIIb catalytic centers of *DICER1* in 30 of 102 (29%) of the tumors.[47] These authors also detected mutations in 1 out of 14 non-seminomatous testicular germ-cell tumors, in 2 of 5 embryonal rhabdomyosarcomas, and in 1 of 266 epithelial ovarian and endometrial carcinomas.[47] The RNase III domains of DICER1 are essential for miRNA maturation, and introduced mutations in the RNase IIIa and in RNase IIIb abrogate in vitro processing of the 3p and 5p miRNAs,
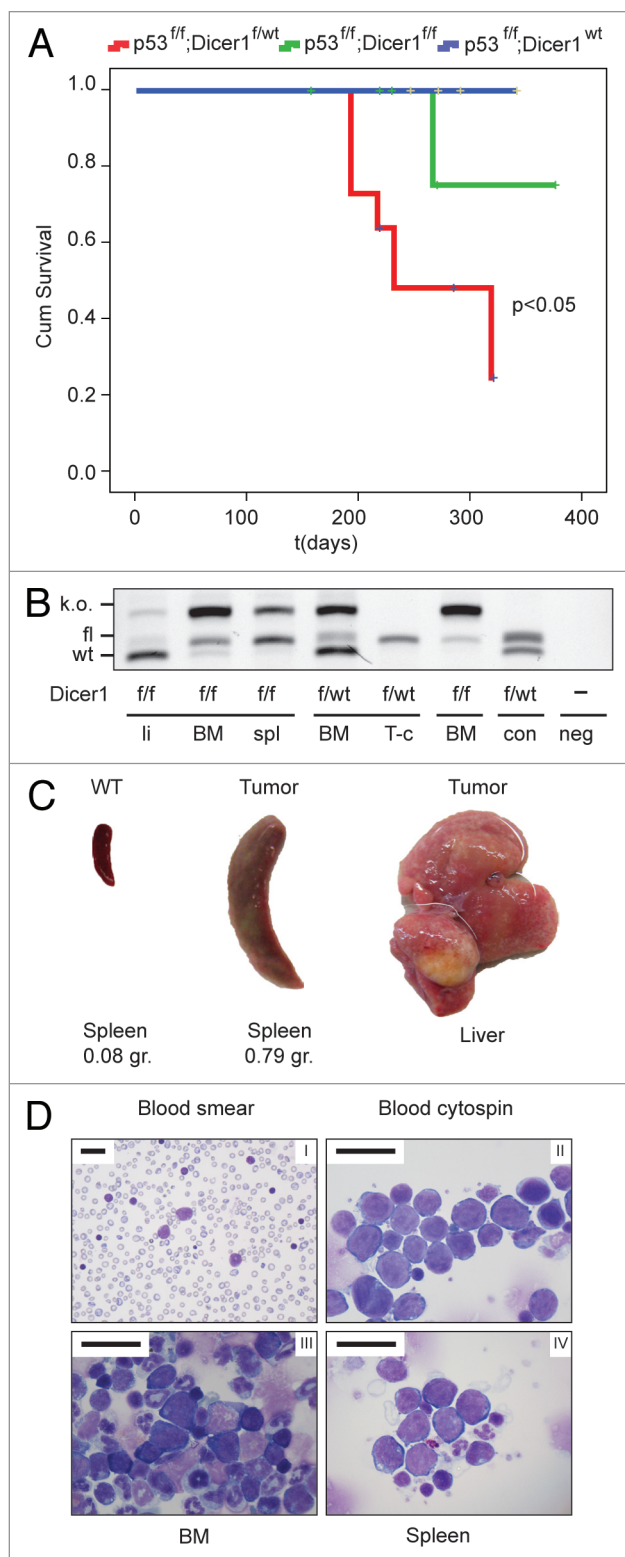
Figure 3. Leukemia developed from *C/ebpa-Cre; p53^{f/f}; Dicer1^{f/wt}* HSCs. (A) Cumulative survival of mice transplanted with HSCs from fetal livers of *C/ebpa-Cre; p53^{f/f}; Dicer1 ^{wt}* (n = 8), *C/ebpa-Cre; p53^{f/f}; Dicer1 ^{f/wt}* (n = 12) and *C/ebpa-Cre; p53^{f/f}; Dicer1 ^{f/f}* (n = 8) embryos. Significance: p < 0.05 (log-rank Mantel-Cox test). (B) PCR on genomic DNA extracted from tumor cells. K.O., knockout allele; fl, floxed allele; wt, wild type allele; li, liver; BM, bone marrow; spl, spleen; con, control DNA heterozygous floxed *Dicer1*; neg, loading control. (C) Example of tumor infiltration in liver and spleen of leukemic mice transplanted with *C/ebpa-Cre; p5 ^{f/f}; Dicer ^{f/wt}* HSCs. (D) Micrographs showing morphology of tumor cells in blood, bone marrow and spleen. Bar indicates 10 μm.

respectively.[47,48] In agreement, transient expression experiments of mutant human *DICER1* constructs in murine *Dicer1*-null mesenchymal stem cells showed that inactivation of the RNase IIIb domain by mutation of D1709, results in complete loss of, particularly, 5p-derived mature miRNAs, including the tumor-suppressive *Let-7* family of miRNAs.[49] This mutation is found in subsets of nonepithelial ovarian cancers. Indeed, the identified *Dicer1* hot spot mutations in cancer result in reduced RNase IIIb activity but retain RNase IIIa activity, strongly suggesting a positive selection for the mutations that reduces *Let7*-tumorsuppressing activity in cancer development.[47]

In a first attempt to gain more functional insight into the mechanisms behind the reduced miRNA expression in AML, a panel of 45 AML samples, characterized by activation of the oncogene EVI-1 due to t(3;3)(q21;q26) or inv(3)(q21q26) and poor prognosis, and five AML cell lines, including U937, MOLM1, MUTZ3, KASUMI-3 and F36P, were sequenced. In this panel of high-risk AML samples, no mutation in *Dicer1* coding sequences and untranslated regions were identified (unpublished data, Erkeland S.J., Valk P., Delwel H., Sanders M.A., Groschel S. and Hoogenboezem R., 2012). Despite the limited set of data, this result suggests that other mechanisms are involved in deregulation of miRNA expression in human AML.

Different Mechanisms of DICER1 Activity Reduction in Human Leukemia

The expression of miRNAs may be deregulated by different mechanisms in human cancer.[50] For instance, the activity of DICER1 may be reduced, as *DICER1* is frequently deleted in various human cancers.[39] In addition, low expression of *DICER1* independently predicted poor outcomes in ovarian cancer patients.[51] In chronic lymphocytic leukemia (CLL), low expression of *DICER1* has been correlated with increased aggressiveness of the disease, shorter overall survival as well as reduced treatment-free survival.[52] Notably, no such correlation between *DICER1* transcript levels and disease

outcome were found in human AML.[53] However, there is evidence for regulation of *DICER1* expression by miRNAs such as *miR-15a* and *miR-16* in a cohort of del(13q14) in CLL,[52] *miR-9* in Hodgkin lymphoma,[54] *miR-125* in human megakaryoblastic leukemia[55] and *miR-106a* in the undifferentiated primary monocytes.[35] Interestingly, *miR-9, miR-125* and *miR-106a* are frequently aberrantly expressed at high levels in human AML[44,56] (and review[43]) and may control *DICER1* translation, leaving mRNA levels intact. Thus, aberrant miRNA biogenesis in human AML may occur via direct miRNA-controlled feedback mechanisms on translation of DICER1 transcripts, but this hypothesis still needs proper experimental confirmation.

Reduction of miRNA expression may be controlled by other mechanisms as well. This hypothesis is supported by recently described mutations in the TAR RNA-binding protein 2 (TARBP2), a critical protein for processing miRNAs in sporadic and hereditary carcinomas, and the inactivating mutations in *Exportin-5*, which results in trap of pre-miRNAs in the nucleus in human cancer cells.[57-59] Other possible mechanisms behind aberrant miRNA expression are single nucleotide polymorphisms (SNPs) that influence processing of miRNAs[60] or RNA editing of miRNA precursors that blocks cleavage by DICER1.[61,62] Sequencing of factors involved in the biogenesis of miRNAs or a better understanding of miRNA expression regulation by, e.g., transcription factors, epigenetic events or miRNA stability, are needed to unravel the mechanisms behind the reduced miRNA activity in human AML.

Methods

Mice and reconstitution experiments. To generate the different mouse lines of interest, we first crossed *C/ebpa-Cre;R26-LSL-Eyfp;Dicer1$^{wt/fl}$* mice[34] with mice that contain floxed *p53* conditional alleles (Jackson Laboratories). Finally, *C/ebpa-Cre;R26-LSL-Eyfp;Dicer1$^{wt/fl}$/Dicer1$^{fl/fl}$;p53 $^{fl/fl}$* mice were obtained from breeding *C/ebpa-Cre;Dicer1$^{wt/fl}$; p53$^{fl/wt}$* mice with *R26-LSL-Eyfp;Dicer1$^{fl/fl}$;p53 $^{fl/fl}$* mice. Fetal livers were obtained on
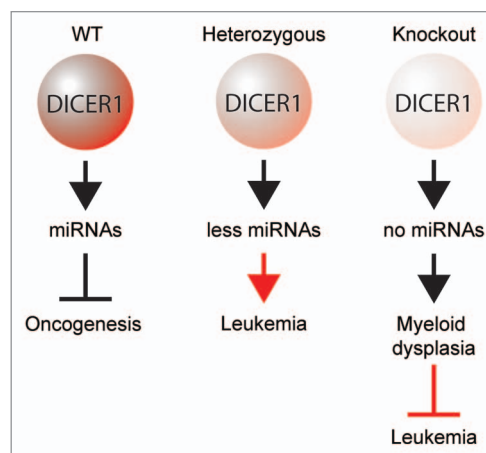

Figure 4. Model for the role of *Dicer1* in leukemia development. *Dicer1* knockout and as a result total loss of miRNA biogenesis, lead to myeloid dysplasia but not leukemia in a p53 knockout background. In contrast, heterozygous loss of *Dicer1* conserves the expression of a set of miRNAs needed for normal differentiation. Furthermore, our model suggests that at least some miRNA activity is needed for oncogenic transformation.

embryonic day (E) 13.5. Genotyping of *Dicer1; p53; C/ebpa-Cre;R26-LSL-Eyfp* embryos was performed by PCR assays of DNA from tail or foot biopsies. Sequences of primers are available upon request. All primers were obtained from Biolegio BV. For transplantation, 8-week-old recipient mice C57Bl/6, (Jackson Laboratories) were irradiated (8.5 Gy) and tail-vein injected with fetal liver single-cell suspensions. Typically, cells from each fetal liver were transplanted into two recipient mice. Tumorigenicity was subsequently monitored by daily examination of the transplanted mice. Mice were euthanized when moribund. All animal experiments were approved by the Animal Welfare/Ethics Committee of the Erasmus Medical Center.

Antibodies, cell staining, flow cytometry and cytospins. Peripheral blood was obtained by heart puncture at the moment of euthanasia. Bone marrow cell suspensions were prepared as described previously.[34] Tumor samples were prepared as single-cell suspension for cytospins or FACS analysis. For morphological analysis of the cells, cytospins were stained with May-Grünwald-Giemsa and examined with a Leica DMLB microscope (100x and 40x objectives) and Leica Application Suite software version 2.7.1 R1.

Statistics. Kaplan-Meier survival curves were plotted using SPSS software (SPSS, PASW, 17.0.2), and log-rank

Mantel-Cox test was used to determine statistical significance.

Conclusion

Recent data show that *Dicer1* is an essential factor at different stages of normal hematopoiesis. A limitation of the *Dicer1*-deletion models is the global depletion of miRNAs that presumably results in disruption of many cellular pathways simultaneously, which hampers the identification of the functions of individual miRNAs. Although some studies show evidence for only a few miRNAs making dominant contributions, such as *miR-17~92* in B-cell development, this may be different for other cell types or even be developmental stage-dependent. Overall, *Dicer1* mainly controls survival and expansion at the early stages of lymphoid development and controls cellular activities at the terminal maturation stage. The function of *Dicer1* is different in myelopoiesis at the earliest developmental stage as *Dicer1* is not essential for cell viability but instead controls essential steps in switching from the stem cell stage toward myeloid lineage development. Although the functions of some miRNAs, such as *miR-17/20/93/106* and *miR-223,* are well-described in immature and mature myeloid cells, respectively,[56,63] the miRNA-controlled pathways that are involved at different stages of myelopoiesis are still largely elusive. Therefore,

tissue and developmental stage-specific miRNA-add-back in the *Dicer1*-deficient models and experimental target identification approaches may be of help for the understanding of the miRNA activities in hematopoiesis.

Acknowledgments

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