PERSPECTIVE

Life beyond cleavage: the case of Ago2 and hematopoiesis

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A recent article in *The Economist* (June 16, 2007) describes research on RNA as the 21st century's Big Bang in biology. The "stars" of the RNA universe are small regulatory RNAs known as microRNAs (miRNAs). These ~22-nt-long RNAs were discovered in 1993 (Lee et al. 1993; Wightman et al. 1993), but their role in biology remained obscure until 2001, when the Tuschl, Ambros, and Bartel laboratories identified hundreds of miRNAs in different organisms (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Functional analyses of these miRNAs and their associated complexes subsequently led to the discovery of RNA-guided gene-silencing pathways as important novel principles in controlling gene expression. miRNAs indeed regulate the expression of a large proportion of eukaryotic protein-coding genes at the post-transcriptional level by sequence-specific binding to the 3' untranslated region (3' UTR) of mRNAs (Lim et al. 2005). To date, RNA silencing is known to play an important role in diverse biological phenomena such as development, stem cell maintenance, cell proliferation, survival, differentiation, metabolism, and cancer (Kloosterman and Plasterk 2006).

The primary transcripts of miRNA genes are mainly processed by the nuclear RNase III enzyme Drosha into stem-loop-structured miRNA precursors (pre-miRNAs) (Lee et al. 2003; Ruby et al. 2007), which are transported via the Exportin-5 receptor to the cytoplasm (Yi et al. 2003). The cytoplasmic RNase III enzyme Dicer cleaves the pre-miRNAs into double-stranded (ds) RNAs of ~22 nt (Bernstein et al. 2001; Hutvagner et al. 2001). One strand of these dsRNA intermediates associates as a mature miRNA with a member of the Argonaute protein family to generate an active ribonucleoprotein complex known as RNA-Induced Silencing Complex (RISC). Within this effector complex, both the miRNA and the Argonaute protein fulfill specific roles. The single-stranded miRNA recognizes the target mRNA with full or partial base-pair complementarity. The Argonaute protein helps the miRNA in searching for its target

mRNA and, upon recognition, can either cleave it or remain tethered to the mRNA to repress its translation and/or regulate its stability. While the biochemistry of the miRNA-guided cleavage reactions is well understood (Martinez and Tuschl 2004; Schwarz et al. 2004), it is still controversial to what extent RISC interferes with the initiation or elongation of translation or controls mRNA stability by sequestration into cytoplasmic processing bodies (P-bodies) (Kiriakidou et al. 2007; Peters and Meister 2007; Pillai et al. 2007).

The Argonaute (Ago) proteins can be subdivided into Ago-like and Piwi-like subfamilies. The mammalian Piwi-like proteins are specifically expressed during spermatogenesis, where they bind a novel set of small RNAs termed Piwi-interacting RNAs (piRNAs) (Aravin et al. 2006; Seto et al. 2007). In contrast, the Ago proteins are broadly expressed in somatic cells, associate with miRNAs and are key actors in different RNA silencing pathways (Peters and Meister 2007; Tolia and Joshua-Tor 2007). The Ago gene family consists of four (*AGO1–AGO4*) and five (*Ago1–Ago5*) members in human and mouse, respectively (Peters and Meister 2007). However, only the Ago2 protein displays endonucleolytic or "Slicer" activity and can therefore execute miRNA-directed cleavage of target mRNA, provided that the base-pairing between the Ago2-associated miRNA and the mRNA sequence is perfect (Liu et al. 2004; Meister et al. 2004). In case of partial complementarity, the Ago2 protein fails to cleave, but instead interferes with translation of the target mRNA via its translational repression activity. In addition to Ago2, other mammalian Ago proteins are also part of miRNA effector complexes that mediate translational inhibition of target mRNAs (Liu et al. 2004; Meister et al. 2004). Gene disruption in the mouse demonstrated that the Ago2 protein is essential for embryonic development (Liu et al. 2004). To study the function of Ago2 in adult hematopoiesis, O'Carroll et al. (2007) have used conditional gene inactivation to bypass the embryonic lethality and report in this issue of *Genes & Development* that Ago2 is a key regulator of B-lymphoid and erythroid development. Retroviral rescue experiments surprisingly revealed that the Slicer endonuclease activity, which is a unique and defining feature of Ago2, is dispensable for hematopoietic develop-

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ment as well as for miRNA biogenesis. Here we discuss these novel data in the context of what is known about the function of miRNAs in hematopoiesis.

MicroRNAs in hematopoietic development

The function of miRNAs has been studied either by gain- and loss-of-function analyses of individual miRNAs or more globally by preventing the biogenesis of all miRNAs (Table 1). The latter approach relies on the inactivation of Dicer (*Dcr*), the key enzyme, which is required for the processing of pre-miRNAs into mature miRNAs (Hutvagner et al. 2001). As Dicer is essential for mouse embryogenesis (Bernstein et al. 2003; Yang et al. 2005), its *in vivo* role in adult hematopoiesis can only be studied by conditional mutagenesis. This strategy has so far been applied to T cell development (Table 1), which proceeds in the thymus from the CD4⁺CD8[−] double-negative (DN) progenitors via CD4⁺CD8⁺ double-positive (DP) thymocytes to CD4⁺CD8[−] and CD4⁺CD8⁺ single-positive (SP) T cells that migrate to, and further mature in, peripheral lymphoid organs. The early-acting *Lck-cre* transgene initiates Cre-mediated excision of the floxed (*fl*) *Dcr* allele in DN cells, thus resulting in complete deletion during the transition to DP thymocytes. As a consequence, the cellularity of the thymus is 10-fold decreased in *Lck-cre Dcr^{fl/fl}* mice, which indicates an essential role for Dicer and, by inference, for miRNAs in the generation and survival of normal numbers of DP thymocytes (Cobb et al. 2005). Dicer appears, however, to be dispensable for CD4 and CD8 lineage commitment and the implementation of their lineage-specific gene expression programs (Cobb et al. 2005). Although the later-acting *Cd4-cre* transgene inactivates *Dcr* in DP thymocytes, mature miRNAs are significantly depleted only in peripheral T cells of *Cd4-cre Dcr^{fl/fl}* mice, resulting in a moderate two- to fourfold decrease of splenic T cells

(Muljo et al. 2005). Dicer-generated miRNAs are therefore required for optimal maturation and homeostasis of peripheral T lymphocytes. CD4⁺ T cells are able to differentiate into four different helper T cell subtypes, i.e., into T_H1, T_H2, T_H17, and regulatory T (T_{reg}) cells. Dicer deficiency is compatible with T_H1 and T_H2 cell differentiation (Muljo et al. 2005). However, the absence of Dicer compromises naïve CD4⁺ and T_H2 cells in their ability to repress the T_H1-specific cytokine IFN γ , suggesting a role for miRNAs in the repression of the T_H1 gene expression program (Muljo et al. 2005). Like all T cells, T_{reg} cells also originate in the thymus, but then patrol peripheral organs to prevent autoimmunity by suppressing autoreactive T cells (Zheng and Rudensky 2007). Early *Dcr* inactivation in *Lck-cre Dcr^{fl/fl}* mice interferes with the development of T_{reg} cells in the thymus, whereas later deletion in *Cd4-cre Dcr^{fl/fl}* mice results in approximately threefold lower numbers of peripheral T_{reg} cells in the spleen (Cobb et al. 2006). This moderate decrease in peripheral T_{reg} cells is, however, sufficient to lead to a late onset of autoimmune disease in *Cd4-cre Dcr^{fl/fl}* mice (Cobb et al. 2006). It is, however, surprising to see that many aspects of T cell differentiation are relatively normal in the absence of Dicer, which may reflect the longevity of mature miRNAs and/or strong counterselection against *Dcr* deletion. Conditional *Dcr* inactivation in hematopoietic stem cells and progenitors leads, however, to a dramatic phenotype, as the transplantation of Dicer-deficient bone marrow is unable to radioprotect lethally irradiated recipient mice (D. O'Carroll and A. Tarakhovsky, pers. comm.).

miRNA cloning and profiling has identified several miRNA genes that are differentially expressed during hematopoietic development (Chen et al. 2004; Monticelli et al. 2005; Table 1). For instance, miR-181a is preferentially expressed in B cells of the bone marrow and in DP thymocytes (Chen et al. 2004; Neilson et al. 2007).

Table 1. miRNA pathways in hematopoiesis

miRNA	Candidate targets	Function	References
miR-150	c-Myb	B cell differentiation and responses	Zhou et al. (2007); Xiao et al. (2007)
miR-155	c-Maf	Control of immune responses	Rodriguez et al. (2007); Thai et al. (2007)
		Lymphomagenesis	Costinean et al. (2006)
miR-181a	Tcra, Cd69	Lymphopoiesis	Chen et al. (2004); Neilson et al. (2007)
miR-221, miR-222	c-Kit	Erythropoiesis	Felli et al. (2005)
miR-223	NFI-A	Granulopoiesis	Chen et al. (2004); Fazi et al. (2005)
Gene	Cre line	Phenotype	References
Dicer	<i>Lck-cre</i> (DN T cells) ^a <i>Cd4-cre</i> (DP T cells) ^a	Thymocytes reduced, T _{reg} cells lost Peripheral T cells decreased Autoimmune disease	Cobb et al. (2005, 2006) Muljo et al. (2005) Cobb et al. (2006)
Ago1	Knock-out	No obvious hematopoietic defect	O'Carroll et al. (2007)
Ago2	<i>Mx-cre</i> (HSCs) ^b ^a	Early block of erythroid and B cell development Reduced miRNA expression	O'Carroll et al. (2007)
Ago3	Knock-out	No obvious hematopoietic defect	O'Carroll et al. (2007)

^aThe first developmental stage in the hematopoietic system, where deletion by the indicated Cre line is observed, is shown in parentheses.

^b(HSC) Hematopoietic stem cells.

Forced retroviral expression of miR-181a in hematopoietic stem cells, followed by bone marrow transplantation, results in an increase of B cells at the expense of T cells in peripheral blood, suggesting that miR-181a is a positive regulator of B cell development (Chen et al. 2004). Alternatively, ectopic miR-181a expression may interfere with the generation or trafficking of SP T cells, given the recent identification of the 3' UTRs of the *Tcra* and *Cd69* genes as functional miR-181a targets (Neilson et al. 2007). miR-150 is specifically expressed in mature B and T cells, and its premature expression in hematopoietic progenitors strongly impairs B cell development at the transition from the pro-B to pre-B cell stage (Zhou et al. 2007; Xiao et al. 2007). This developmental block is caused by the down-regulation of the transcription factor c-Myb, which was genetically identified as a functional miR-150 target (Xiao et al. 2007). c-Myb is required not only for early B cell development, but also for the generation of one of the mature B cell types, the so-called B1 cells (Thomas et al. 2005). Inactivation of the miR-150 gene leads to the increased generation of B1 cells due to enhanced c-Myb expression, whereas ectopic expression of miR-150 interferes with B1 cell development (Xiao et al. 2007). Hence, miR-150 primarily mediates its effect on B-lymphopoiesis by regulating c-Myb expression. miR-155 is processed from the noncoding RNA of the *bic* gene, which is highly expressed in activated macrophages, dendritic, T, and B cells as well as in a variety of human B cell lymphomas (van den Berg et al. 2003; Eis et al. 2005; Kluiver et al. 2005). Interestingly, Eμ-miR155 transgenic mice develop B cell lymphomas, further implicating miR-155 in tumorigenesis (Costinean et al. 2006). Gene disruption and conditional activation experiments revealed an important role for miR-155 in the immune system by regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response (Rodriguez et al. 2007; Thai et al. 2007). miR-155 controls these immune functions, at least in part, by regulating the expression of cytokines (Rodriguez et al. 2007; Thai et al. 2007), which is further corroborated by the identification of c-Maf, a potent transactivator of the *Il4* gene, as a miR-155 target (Rodriguez et al. 2007). The two miRNAs miR-221 and miR-222 are down-regulated during erythroid differentiation of hematopoietic progenitors, which correlates inversely with the expression of the essential tyrosine kinase receptor c-Kit (Felli et al. 2005). Both miRNAs bind indeed to a functional target sequence in the 3' UTR of the *c-kit* mRNA (Felli et al. 2005). As a consequence, ectopic expression of miR-221 or miR-222 leads to reduced proliferation and accelerated maturation of differentiating erythroblasts, which is mediated, at least in part, by down-regulation of the c-Kit receptor (Felli et al. 2005). Finally, the miR-223 gene with its restricted myeloid expression pattern (Chen et al. 2004) controls granulopoiesis as part of a negative feedback loop involving the two transcription factors C/EBPα and NFI-A, which bind in a mutually exclusive manner to the same regulatory element of the miR-223 promoter (Fazi et al. 2005). During granulocyte differen-

tiation, the potent activator C/EBPα is up-regulated and the negative regulator NFI-A is down-regulated, resulting in increased miR-223 expression. As miR-223 binds to the 3' UTR of NFI-A, it represses its own negative regulator, thereby maintaining its expression in granulocytes (Fazi et al. 2005). The loss of miR-223 function furthermore inhibits granulocytic differentiation, thus indicating an important role for miR-223 in granulopoiesis (Fazi et al. 2005). In summary, individual miRNAs have potent regulatory functions controlling different aspects of hematopoiesis.

Essential function of Ago2 in erythroid and B cell development

As Argonaute proteins mediate the silencing function of RISC (Peters and Meister 2007; Tolia and Joshua-Tor 2007), their inactivation provides yet another genetic approach for studying the function of miRNA pathways (Table 1). Whereas germline mutation of the *Ago2* gene results in embryonic lethality (Liu et al. 2004), *Ago1*^{-/-} and *Ago3*^{-/-} mice survive to adulthood without obvious developmental abnormalities and hematopoietic defects (O'Carroll et al. 2007; D. O'Carroll and A. Tarakhovsky, pers. comm.). To study the unique function of Ago2 in hematopoiesis, O'Carroll et al. (2007) generated mice that lack Ago2 specifically in the hematopoietic system (referred to as *Ago2*^{-/-} mice). Using the *Mx-cre* transgene, they inducibly deleted a conditional *Ago2* allele in bone marrow progenitors, which were then transplanted into lethally irradiated mice to reconstitute their hematopoietic system in the absence of Ago2. These *Ago2*^{-/-} mice give rise to all hematopoietic cell lineages, although the development of erythrocytes and B cells is grossly abnormal. B-lymphopoiesis is impaired at the pro-B to pre-B cell transition and at all subsequent developmental stages (O'Carroll et al. 2007). Erythropoiesis is even more severely blocked, as early basophilic erythroblasts accumulate in large numbers in the bone marrow and spleen, leading to erythroid hyperplasia and splenomegaly. In contrast, later erythroblasts and functional red blood cells are strongly reduced, causing severe anemia (O'Carroll et al. 2007). Despite its severity, the Ago2 deficiency results in a milder phenotype than similar inactivation of Dicer in blood stem cells and their progeny, which causes complete hematopoietic failure (see above). Hence, other members of the Argonaute family must fulfill important redundant functions in hematopoiesis.

Retroviral restoration of Ago2 expression in *Ago2*-deleted bone marrow cells prior to transplantation rescues all B-lymphoid and erythroid defects, including splenomegaly and anemia (O'Carroll et al. 2007). Surprisingly however, retroviral expression of the Slicer-deficient Ago2^{D669A} protein also restores, with similar efficiency, all aspects of B cell and erythroid development in *Ago2*^{-/-} mice. Hence, the unique and defining feature of Ago2, its Slicer endonuclease activity, is dispensable for Ago2's role in hematopoiesis. Ago2 thus functions as a critical regulator of erythropoiesis and B cell develop-

Control of miRNA biogenesis by Argonaute proteins

The diagram illustrates two pathways for miRNA biogenesis. In the top pathway, pre-miRNA is processed by Dicer (with Ago2 and TRBP) into mature miRNA, which then loads into Ago2 to form miRISC. In the bottom pathway, pre-miRNA is processed by Dicer (with Ago2m and TRBP) into mature miRNA, which then loads into Ago2m to form miRISC. A dashed line labeled 'Ago2 loss' connects the two pathways, indicating that in the absence of Ago2, the Ago2m pathway is used.

ing in vivo (O'Carroll et al. 2007) consistent with the finding that Dicer in a complex with the Slicer-inactive Ago2^{D669A} protein has normal endonuclease activity in vitro (Maniataki and Mourelatos 2005). Ago2 deficiency reduces but does not eliminate miRNA production (O'Carroll et al. 2007), and hence the residual pre-miRNA processing activity is likely contributed by Ago1 and Ago3, which are coexpressed with Ago2 in mouse fibroblasts (Liu et al. 2004). The expression of miRNAs is, however, normal in *Ago1*^{-/-} and *Ago3*^{-/-} fibroblasts, indicating that Ago2 is the dominant Argonaute family member participating in pre-miRNA processing in this cell type (O'Carroll et al. 2007). Ago2 could potentially control two different steps of miRNA biogenesis (Fig. 1). Ago2 may be required in vivo for the assembly and/or stabilization of the miRLC complex or for regulating Dicer's endonuclease activity in the complex. Prior to

the study of O'Carroll et al. (2007), Ago2 was considered to be a unique member of the Argonaute family due to its Slicer endonuclease activity. Now we know that the Slicer activity makes only a minor contribution to the biological function of Ago2. Instead, Ago2 is a unique family member because of its nonredundant role in development and miRNA biogenesis, which will warrant future studies of this important member of the RNA silencing machinery.

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