Long-lived microRNA–Argonaute complexes in quiescent cells can be activated to regulate mitogenic responses

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Cellular proliferation depends on the integration of mitogenic stimuli with environmental conditions. Increasing evidence suggests that microRNAs play a regulatory role in this integration. Here we show that during periods of cellular quiescence, mature microRNAs are stabilized and stored in Argonaute protein complexes that can be activated by mitogenic stimulation to repress mitogen-stimulated targets, thus influencing subsequent cellular responses. In quiescent cells, the majority of microRNAs exist in low molecular weight, Argonaute protein-containing complexes devoid of essential components of the RNA-induced silencing complex (RISC). For at least 3 wk, this pool of Argonaute-associated microRNAs is stable and can be recruited into RISC complexes subsequent to mitogenic stimulation. Using several model systems, we demonstrate that stable Argonaute protein-associated small RNAs are capable of repressing mitogen-induced transcripts. Therefore, mature microRNAs may represent a previously unappreciated form of cellular memory that allows cells to retain posttranscriptional regulatory information over extended periods of cellular quiescence.

signaling | Ago2 | GW182

Cellular proliferation is a highly regulated process that is driven by mitogenic stimuli and fueled by available nutrients. Mitogenic stimuli (growth factors, cytokines, etc.) reprogram cellular metabolism to facilitate increased macromolecular synthesis required to replicate cells. This metabolic reprogramming causes proliferating cells to rely heavily on uptake of extracellular nutrients, particularly glucose and amino acids (1, 2). When cells are deprived of growth-factor signaling or essential nutrients, they withdraw from the cell cycle and adopt long-term survival strategies based on fatty acid oxidation and macroautophagy (3, 4). Subsequent recovery into the cell cycle depends on restoration of growth-factor signaling and/or depleted nutrients. Over the last several years we have examined factors that influence this recovery.

MicroRNAs are small (20–25 nt), noncoding RNAs that regulate gene expression by targeting nascent transcripts to the RNA-induced silencing complex (RISC), where mRNA degradation and/or translational repression occurs (5). Mounting evidence suggests that microRNAs play important roles in the regulation of mitogen-induced gene expression (6–8) and are required for cells to deal with associated transcriptional stress (9). We and others have recently identified Arsenite resistance protein 2 (Ars2) as a mitogen-induced component of the nuclear cap-binding complex that stimulates expression of a large subset of microRNAs in proliferating cells (10–12). In addition to its role in microRNA biogenesis, Ars2 is capable of modulating histone mRNA processing (11) and can act to regulate transcription in neural stem cells (13).

In the current study, we set out to use siRNA to examine the role of Ars2 in the mitogen-stimulated proliferation of quiescent cells. As was the case in constitutively proliferating cells, blocking expression of Ars2 with siRNA inhibited mitogen-stimulated cell proliferation. During the course of these studies we found that Ars2 siRNAs used were capable of target repression weeks after being transfected into quiescent cells. This observation led us to hypothesize that endogenous microRNAs might also be stable in quiescent cells and capable of subsequent repression of mitogen-induced targets. Data presented here demonstrate that microRNAs are stabilized in quiescent cells and stored in low molecular weight, Argonaute protein complexes that lack GW182, a protein required for microRNA function. Upon mitogenic stimulation of quiescent cells high molecular weight microRNA–Argonaute complexes are reassembled and the microRNAs that have persisted are able to repress mitogen-stimulated targets. Together our data reveal previously unappreciated properties of mammalian microRNA machinery that may allow cells to retain regulatory information over long periods of cellular quiescence to modulate subsequent mitogenic responses.

Results

Knockdown of Ars2 by Long-Lived siRNAs. In cells with constitutive mitogenic signaling, Ars2 is required to maintain proliferation and cotranscriptional processing of microRNAs and histone mRNAs (10–12). Whether or not lack of Ars2-dependent microRNA or histone mRNA processing contributes to the ability of Ars2 to promote cellular proliferation remains unclear. Because mitogenic stimulation of quiescent cells is able to enhance expression of Ars2 (10), microRNAs (6), and histone mRNAs (14), we devised a system to test the role of Ars2 during cellular quiescence and subsequent mitogen-stimulated growth and proliferation. Immortalized interleukin-3 (IL-3)-dependent hematopoietic cells derived from $bax^{-/-}$,$bak^{-/-}$ embryos [IL-3 double knockout (DKO) cells; ref. 3] were transfected with two independent siRNA oligonucleotides targeting Ars2 [Ars2-1 (10) and Ars2-4; Fig. S1] and cultured in the absence of IL-3 for 1, 2, or 3 wk followed by IL-3 restimulation (Fig. 1*A*). The absence of the core proapoptotic proteins, Bax and Bak, in IL-3 DKO cells allows them to survive long periods in the absence of mitogenic stimulation, during which cells do not proliferate, maintaining their viability through macroautophagy and fatty acid oxidation (3, 15). Upon restimulation with IL-3, cells immediately begin to grow in size and resume proliferation 1 to 14 d later depending on the length of withdrawal. When cells transfected with Ars2

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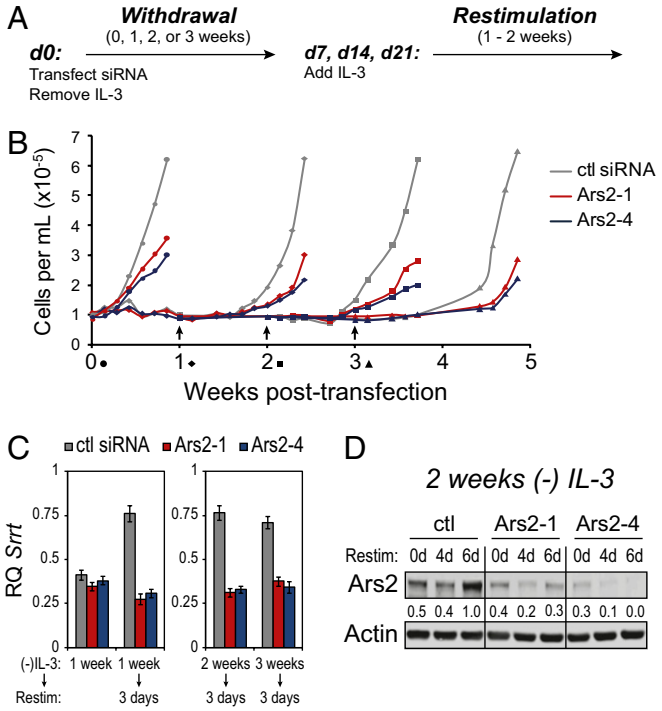

Fig. 1. Stable siRNAs repress Ars2 and proliferation upon growth-factor restimulation of quiescent cells. (*A*) Schematic depicting experiments performed to test Ars2 function over long-term growth-factor withdrawal and restimulation. (*B*) Proliferation of IL-3 DKO cells transfected with two independent Ars2 siRNAs or control siRNA and then withdrawn from IL-3 for 0, 1, 2, or 3 wk followed by 1–2 wk of IL-3 restimulation. Arrows indicate time points at which IL-3 was added to cells (circle, no withdrawal; diamond, 1 wk IL-3 withdrawal; square, 2 wk IL-3 withdrawal; and triangle, 3 wk IL-3 withdrawal). A representative experiment is shown and has been repeated more than three times. (*C*) Expression of *Srrt* mRNA, which codes for Ars2 protein, in IL-3 DKO cells transfected with control or Ars2 siRNAs and then withdrawn from IL-3 for 1, 2, or 3 wk followed by 3 d of IL-3 restimulation. Bars represent expression relative to IL-3 DKO cells immediately following transfection (RQ) measured by qPCR in quadruplicate using the ΔΔCt method ±95% confidence interval of the mean. *Eif2c2* was used as an endogenous control. (*D*) Expression of Ars2 protein in IL-3 DKO cells transfected with control or Ars2 siRNAs and then withdrawn from IL-3 for 2 wk (0 d of restimulation) followed by 4 or 6 d of IL-3 restimulation. Quantification of Ars2 protein (normalized to actin) relative to cells maintained in complete medium is shown.

siRNAs were examined, they displayed a greater than 24 h delay in proliferation following IL-3 restimulation compared with control transfectants, even 3 wk after the transient transfection of Ars2 siRNA (Fig. 1*B*). Delayed proliferation following IL-3 restimulation was accompanied by repression of mitogen-stimulated mRNA expression of *Srrt*, the gene encoding Ars2 (Fig. 1*C*), and of Ars2 protein (Fig. 1*D*).

MicroRNA Stability in Quiescent Cells. In order for Ars2 siRNAs to repress their target 3 wk following transfection, they must be extraordinarily stable in quiescent cells. However, modifications made to commercially available siRNAs are designed to enhance their stability, which could account for these observations. To address this issue, two highly expressed endogenous microRNAs, miR-26a and miR-23a, were examined in quiescent IL-3 DKO cells. Quantitative real-time PCR (qPCR) analysis revealed that both miR-26a and miR-23a were enriched over endogenous control RNA (U6 snRNA) in response to growth-factor withdrawal and remained elevated for at least 3 wk in the absence of IL-3 (Fig. 2*A*, *Left*). In contrast to the stable increase in expression of mature miR-26a and miR-23a, primary transcripts containing miR-26a or miR-23a (Fig. 2*A*, *Right*) decreased relative to endogenous control RNA (*Eif2c2*) within one week of IL-3 withdrawal. Care was taken when selecting endogenous controls so that their Ct (cycle threshold) values remain constant over the course of 3 wk of IL-3 withdrawal (Fig. S2*A*). Furthermore, persistent expression of miR-23a did not result from enhanced nuclear processing because previous work has shown that cotranscriptional processing of miR-23a was significantly inhibited by the absence of Ars2 (11) and knockdown of Ars2 at the time of growth-factor withdrawal had no effect on the persistence of miR-23a (Fig. S2*B*).

Increased expression of mature microRNAs and concomitant decrease of their primary transcripts suggests that endogenous microRNAs can be stably expressed over long periods of growth-factor withdrawal-induced quiescence. To independently confirm that increased detection of mature microRNAs in growth-factor–withdrawn cells resulted from their being more stable than the endogenous control used for qPCR, U6 snRNA, Northern blots were performed. Blots for miR-26 loaded with an equal amount of total RNA per lane confirmed qPCR data in that the relative abundance of miR-26 increased substantially (15- to 20-fold) following IL-3 withdrawal from IL-3 DKO cells (Fig. 2*B*, *Left*) or serum withdrawal from immortalized mouse embryonic fibroblasts derived from $bax^{-/-}$,$bak^{-/-}$ embryos (DKO MEFs) (16) (Fig. S2*C*, *Left*). When RNA extracted from an equal number of cells was loaded per lane, Northern blots revealed that expression of miR-26 varied by roughly 2-fold over a 2-wk period of IL-3 withdrawal from IL-3 DKO cells (Fig. 2*B*, *Right*) or 5 d of serum withdrawal from DKO MEFs (Fig. S2*C*, *Right*). Thus, decreased expression of total RNA per cell (Fig. S2*D*) coupled with the stability of miR-26 accounted for the apparent 15- to 20-fold increase in miR-26 levels when equal amounts of RNA were compared between growth-factor–replete and growth-factor–deprived cells.

Ago2 Stability and Complex Formation in Quiescent Versus Mitogen-Stimulated Cells. Both the stability and function of microRNAs depend on their association with the Argonaute proteins, Ago1, Ago2, Ago3, and Ago4 in mammals (17–19). Therefore, the effects of IL-3 withdrawal on Argonaute protein expression and function were examined. Ago2 remained stably expressed over long periods in the absence of IL-3 (Fig. 3*A*). Ago1 also remained

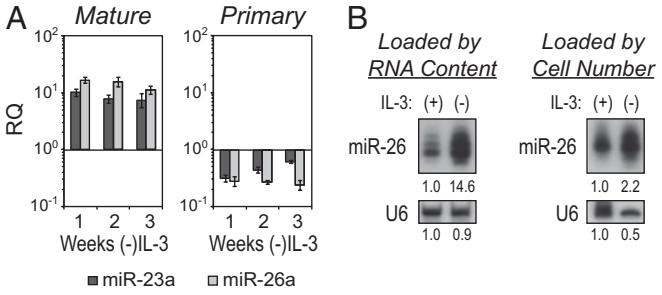

Fig. 2. Stabilization of microRNAs in quiescent cells. (*A*) Expression of mature miR-23a and miR-26a (*Left*) or the primary transcript containing miR-23a or miR-26a (*Right*) in IL-3 DKO cells maintained in the absence of IL-3 for 1, 2, or 3 weeks. Bars represent change in expression (RQ) relative to day 0 of withdrawal measured in quadruplicate using the ΔΔCt method ±95% confidence interval of the mean. U6 snRNA was used as an endogenous control for mature microRNAs and *Eif2c2* was used as an endogenous control for primary transcripts. (*B*) Northern blot for mature miR-26 and U6 snRNA using RNA isolated from IL-3 DKO cells in the presence of IL-3 (+) or following 2 wk of IL-3 withdrawal (–). Either 10 μg of RNA per lane (*Left*) or 2×10^5 cell equivalents of RNA per lane (*Right*) was loaded on 15% urea-PAGE gels for Northern blot analysis. Blots were quantified using ImageJ software and relative band intensities are displayed below each blot.

highly expressed following growth-factor withdrawal (Fig. S3*A*), whereas Ago3 or Ago4 were undetectable using commercially available antibodies. To determine if stability or de novo translation of Ago2 could account for its maintained expression following growth-factor withdrawal, cyclohexamide was added to cultures and 5 d later Ago2 expression was measured by Western blot (Fig. S3*B*). These blots revealed that Ago2 has a half-life of greater than 5 d regardless of growth-factor availability.

The function of microRNA–Argonaute complexes depends on their integration into larger macromolecular RISCs that contain proteins required for translational repression and/or mRNA degradation (20). Gel filtration (Superose 6) revealed a dramatic change in the size distribution of Ago2-containing macromolecular complexes when IL-3 was removed from the medium of IL-3 DKO cells (Fig. 3*B*). These data demonstrate that a fundamental change in the composition of the Ago2-RISC occurs in growth-factor–deprived cells. Furthermore, expression of the RISC component GW182 substantially decreased in IL-3–withdrawn DKO cells (Fig. 3*C*).

To determine if these findings could be extended to a more physiologically relevant model of mitogenic stimulation, freshly isolated mouse T cells were stimulated with beads coated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2. Consistent with previous findings (21), GW182 increased when resting T cells were subjected to mitogenic stimulation (Fig. 3*D*). Moreover, the gel filtration pattern of Ago2 in resting T cells mimicked that seen in growth-factor–withdrawn IL-3 DKO cells (Fig. 3*E*, *Top*). Upon mitogenic stimulation of these T cells, a large fraction of Ago2 shifted to high molecular weight complexes (Fig. 3*E*, *Bottom*). These data demonstrate that mitogenic stimuli regulate the expression of the RISC component GW182 and the association of Ago2 with large macromolecular complexes.

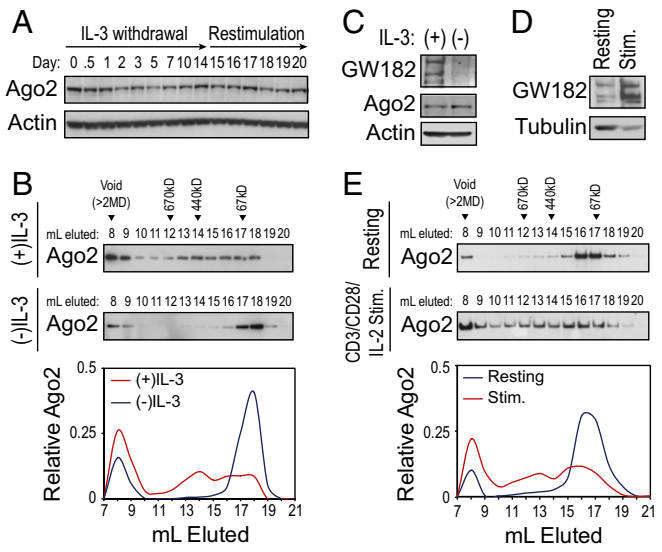

Fig. 3. Mitogenic stimuli promote assembly of high molecular weight Ago2-containing complexes. (*A*) Western blot analysis of Ago2 over the course of 14 d of IL-3 withdrawal followed by 6 d of IL-3 restimulation. (*B*) Superose 6 fractionation followed by Western blot for Ago2 on day 0 (*Top*) or day 14 (*Middle*) as in *A*. (*C*) Western blot analysis for GW182 in IL-3 DKO cells maintained in IL-3–replete medium or IL-3–deficient medium for 2 wk. (*D*) Western blot analysis for GW182 in resting splenic T cells or splenic T cells stimulated for 3 d with beads coated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 (30 units/mL). (*E*) Superose 6 fractionation followed by Western blot for Ago2 in resting splenic T cells or splenic T cells stimulated as in *D*. For *B* and *E*, fractions 1–7 were consistently devoid of protein and fractions 8–21 consistently contained cellular proteins, with fraction 8 containing the void of the column. Ago2 expression was quantified using ImageJ software and the relative amount of Ago2 in each fraction was plotted (*Bottom*).

miR-21 Stabilization in the Absence of Glucose. To determine if cells made quiescent in the presence of growth-factor signaling also exhibited alterations in microRNA stability and Ago2 complex assembly, DKO MEFs were cultured in serum-replete medium that lacked glucose for 5 d followed by addition of glucose to the serum-replete medium for 2 d. Unlike IL-3–dependent DKO cells that grow in suspension, studies of DKO MEFs in medium lacking nutrients or growth factor are limited to 5 d, after which cells begin to detach from culture plates. As was the case for growth-factor–withdrawn cells, expression of several mature microRNAs increased, whereas their primary transcripts decreased following glucose withdrawal (Fig. S4). Of these, miR-21 was examined further. The relative abundance of mature miR-21 increased following glucose withdrawal from serum-replete medium and this enrichment was maintained until glucose was added to the medium 5 d later, at which point relative miR-21 expression returned to the level it had been before withdrawal (Fig. 4*A*, red line). In contrast, the primary transcript containing miR-21 decreased over the first 2 d of glucose withdrawal and remained repressed until glucose was added to the medium (Fig. 4*A*, blue line). To determine if miR-21 stability was altered in glucose-deprived cells, DKO MEFs were cultured in complete or glucose-free medium containing actinomycin D and miR-21 levels were determined by Northern blot over the course of 5 d (Fig. 4*B*). Quantification of blots revealed that miR-21 had a half-life of ∼2.6 d in the presence of glucose and a half-life of greater than 5 d in the absence of glucose (Fig. 4*C*).

Ago2 Stability and Complex Formation in Glucose-Fed Versus -Starved Cells. In addition to exhibiting microRNA stabilization similar to that observed in growth-factor–withdrawn cells, expression of GW182 decreased in glucose-withdrawn cells and could be recovered by restimulation of cells with glucose, whereas Ago2 expression remained constant during glucose withdrawal and restimulation (Fig. 4*D*). Although GW182 expression rapidly decreased following glucose withdrawal, a sufficient amount of protein remained following overnight glucose deprivation to determine that it was excluded from the low molecular weight fractions into which Ago2 shifted (Fig. 4*E*). Moreover, microRNAs remain associated with Ago2 despite its shift to low molecular weight GW182-deficient complexes (Fig. S5). Because the interaction of Ago2 and GW182 is required for microRNAs to repress their targets (22, 23), these data suggest that in quiescent cells the majority of Ago2–microRNA complexes are not localized in functional RISCs.

Reincorporation of Ago2–MicroRNA Complexes into Functional RISCs. Restoration of high molecular weight Ago2-RISCs following restimulation could result from incorporation of preexisting or de novo synthesized microRNA–Ago2 complexes into high molecular weight RISCs. To distinguish between these possibilities, an exogenous oligonucleotide duplex with sequence complementarity to the 3′ UTR of *CXCR4* mRNA (24) was transfected into DKO MEFs that were subsequently starved of glucose for 5 d followed by glucose restimulation for 2 d (Fig. 5*A*). Similar to endogenous microRNAs, *CXCR4* siRNAs were enriched in low molecular weight complexes during glucose withdrawal and progressively shifted to high molecular weight complexes upon glucose restimulation (Fig. 5*B*). Because *CXCR4* siRNAs are not made endogenously, these data confirm that persistent Argonaute-associated small RNAs are reassembled into high molecular weight RISCs. Furthermore, *CXCR4* siRNA was able to repress reporters (24) transfected just before glucose restimulation in both an siRNA-like (perfect) and microRNA-like (bulge) manner, despite being transfected 5 d earlier and subjected to glucose withdrawal

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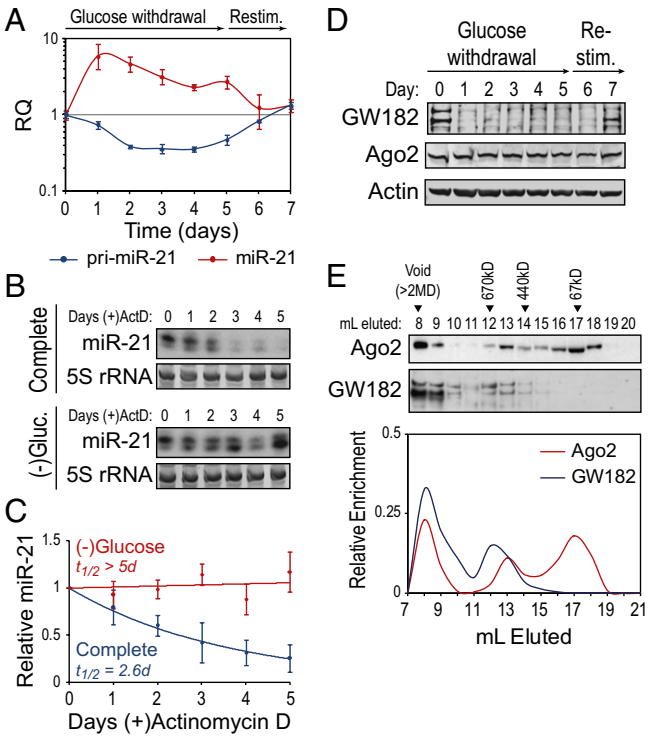

Fig. 4. Glucose availability determines microRNA stability and association of Ago2 with GW182. (*A*) Expression of primary (blue line) and mature miR-21 (red line) in DKO MEFs over the course of 5 d of glucose withdrawal followed by 2 d of glucose restimulation. Change in expression ±95% confidence interval of the mean relative to day 0 was determined using the ΔΔCt method with U6 snRNA or 18S rRNA for mature or primary transcripts, respectively, as endogenous controls. (*B*) Northern blot analysis of miR-21 in DKO MEFs treated with actinomycin D (5 μg/mL) in complete medium (*Upper*) or medium lacking glucose (*Lower*) over the course of 5 d. (*C*) Quantification of Northern blots from three independent experiments as in *B*. Each point represents the mean miR-21 signal normalized to 5S rRNA ±SD. (*D*) Western blot analysis of GW182 and Ago2 in DKO MEFs over the course of 5 d of glucose withdrawal followed by 2 d of glucose restimulation. (*E*) Superose 6 fractionation followed by Western blot for Ago2 or GW182 from lysates of MEFs maintained in glucose-free medium overnight. Ago2 and GW182 expression was quantified using ImageJ software and the relative amount of Ago2 and GW182 in each fraction was plotted (*Lower*).

and restimulation (Fig. 5*C*). These data were confirmed using additional reporters responsive to let-7 (25) (Fig. S6). Moreover, the ability of *CXCR4* siRNA to repress microRNA and siRNA reporters following mitogenic restimulation could be extended for up to 3 wk in IL-3 DKO cells (Fig. 5*D*).

Repression of Mitogen-Induced Targets by Stable miR-21. Data presented to this point suggest that microRNAs are stabilized and stored in inactive Argonaute complexes during periods of cellular quiescence and can subsequently repress targets following mitogenic restimulation. Stabilization, storage, and subsequent reactivation of microRNAs may therefore represent a mechanism by which cells regulate mitogen-induced protein expression. To test this idea, regulation of the mitogen-induced expression and function of Programmed Cell Death 4 (PDCD4) by miR-21 was examined (Fig. 6*A*). PDCD4 is a robust miR-21 target known to regulate MAPK signaling via suppression of AP-1 activity (26, 27). When miR-21 was transfected just before IL-3 withdrawal it did not affect PDCD4 protein expression in quiescent cells, despite a stable increase in miR-21 level (Fig. 6*B*). Upon restimulation of cells with IL-3, PDCD4 expression rapidly increased (Fig. S7*A*). The magnitude of PDCD4 induction was reduced approximately twofold by persistent miR-21 that had been transfected 2 wk earlier (Fig. 6*C*). Quantification of PDCD4 protein revealed that induction peaked between 30 min and 2 h following IL-3 restimulation and that the magnitude of this peak could be significantly inhibited ($P = 0.0025$) by persistent miR-21 transfected 2 wk earlier (Fig. 6*D*). No difference in *Pdcd4* mRNA expression was observed between control and miR-21 transfected cells (Fig. S7*B*), suggesting that miR-21–mediated translational repression could account for the observed change in PDCD4 protein induction. As expected, the miR-21–induced decrease in PDCD4 induction was associated with increased AP-1 activity following IL-3 restimulation (Fig. 6*E*). Examination of additional miR-21 targets (27, 28) revealed that reversion-inducing cysteine-rich protein with kazal motifs (RECK) expression was significantly decreased ($P = 0.02$) following restimulation of miR-21

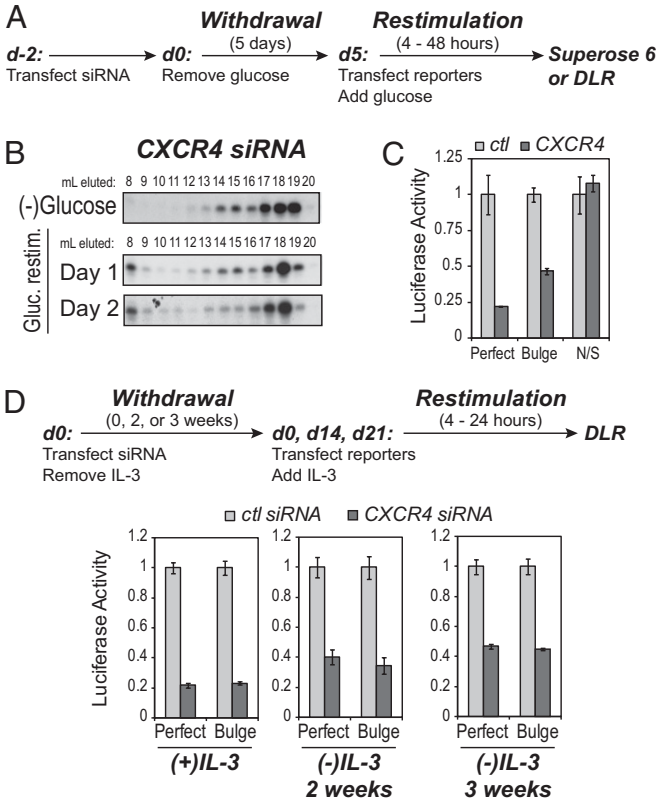

Fig. 5. CXCR4 siRNA is activated to repress microRNA and siRNA reporters following mitogenic stimulation of quiescent cells. (*A*) Schematic depicting experiments performed to test CXCR4 siRNA function over the course of glucose withdrawal and restimulation. (*B*) Superose 6 fractionation followed by Northern blot for *CXCR4* siRNA from DKO MEFs transfected with *CXCR4* siRNA followed by 5 d of glucose withdrawal (*Upper*) and 1 or 2 d of subsequent glucose restimulation (*Lower*). (*C*) Dual luciferase (DLR) assays performed on DKO MEFs transfected with control or *CXCR4* siRNA before 5 d of glucose withdrawal and subsequent transfection with renilla luciferase reporters (24) containing a 3′ UTR with one perfectly complementary *CXCR4* siRNA binding site (perfect), six *CXCR4* siRNA binding sites with imperfect complementarity (bulge), or three *let-7* binding sites (N/S). Following transfection of reporters DKO MEFs were restimulated with glucose for 4–24 h before determination of luciferase activity. (*D*) DLR assays performed using the CXCR4 siRNA system described in *C* transfected into IL-3 DKO cells following 0, 2, or 3 wk of IL-3 withdrawal. Following transfection of reporters, IL-3 DKO cells were restimulated for 4–24 h before determination of luciferase activity. For *C* and *D*, bars represent mean normalized luciferase activity obtained from four independent cultures ±SD. pGL3 control vectors were cotransfected and luciferase activity was calculated as renilla luciferase RLU ÷ firefly luciferase RLU and normalized to control siRNA transfection.

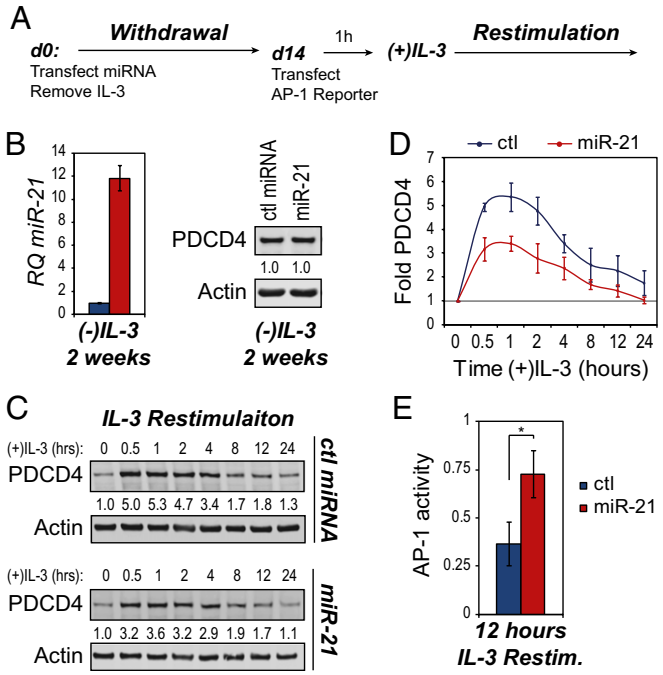

Fig. 6. Stable miR-21 represses PDCD4 and enhances AP-1 activity upon growth-factor restimulation. (*A*) Schematic depicting experiments performed to test miR-21 function over long-term growth-factor withdrawal and restimulation of IL-3 DKO cells. (*B*) Level of miR-21 following 2 wk of IL-3 withdrawal from IL-3 DKO cells transfected with control microRNA (blue bar) or miR-21 (red bar) (*Left*). Bars represent relative quantification (RQ) of mature miR-21 using the ΔΔCt method normalized to control microRNA transfection ±95% confidence interval of the mean. U6 snRNA was used as an endogenous control. Western blot for the miR-21 target PDCD4 following 2 wk of IL-3 withdrawal from IL-3 DKO cells transfected with control microRNA or miR-21 (*Right*). ImageJ software was used to quantify PDCD4 expression normalized to actin. (*C*) Western blot for PDCD4 in cells transfected with a control microRNA or miR-21 followed by 2 wk of IL-3 withdrawal (0 h) or at the indicated times following IL-3 restimulation. (*D*) Quantification of PDCD4 protein expression from three independent experiments, as in *C*, ±SD. ANOVA analysis of the time course of IL-3 restimulation revealed a significant ($P < 0.005$) decrease in the magnitude of PDCD4 induction in miR-21 transfected cells. (*E*) AP-1 activity measured 12 h following IL-3 restimulation, as in *C*, using a reporter containing 6 tandem AP-1 binding sites upstream of firefly luciferase. Bars represent the average AP-1 activity (firefly luciferase RLU ÷ renilla luciferase RLU) from three independent experiments performed in triplicate ±SD. *$P < 0.0005$ as determined by paired *T* test.

transfected cells, whereas expression of mitogen-activated protein kinase kinase 3 (MKK3) was not affected by persistent miR-21 (Fig. S7 *C* and *D*).

Discussion

Regulated cellular proliferation is essential to the development and survival of multicellular organisms. Whereas most cells lose their ability to proliferate upon terminal differentiation, many cells, including those that comprise the immune system, retain the ability to respond to mitogenic stimuli with rapid growth and proliferation. Moreover, under normal circumstances a majority of immune cells exist in quiescent states. The current study reveals a mechanism to retain regulatory information over prolonged periods of cellular quiescence based on the stability of microRNA–Argonaute complexes. Data demonstrate that Argonaute-associated small RNAs are stabilized during periods of cellular quiescence and retained in low molecular weight complexes devoid of GW182, a protein required for microRNA-mediated repression of targets (22, 23). Upon mitogenic restimulation, persistent small RNA–Argonaute complexes are recovered into high molecular weight RISCs and are capable of repressing mitogen-induced targets. In this way, quiescent cells can retain previously produced microRNAs to influence subsequent mitogen-induced protein expression.

Several potential advantages exist when considering the use of such a system of regulation. First, stabilization of existing microRNA–Argonaute complexes obviates the need for their de novo synthesis during quiescence, a cell state in which cellular bioenergetics is maintained at a minimal level. Preexisting microRNA–Argonaute complexes also have the potential to repress rapidly induced targets, as was the case for PDCD4 repression by miR-21 (Fig. 6). Whether this repression absolutely depends on reincorporation of low molecular weight microRNA–Argonaute complexes into high molecular weight RISCs or by a different mechanism remains to be determined. Further characterization of the components of high molecular weight complexes containing Ago2 and microRNAs may help resolve this issue. A potential consequence of blocking the translation of certain proteins is that mRNAs not targeted by microRNAs may be preferentially translated when translational resources are limited. Such properties of stable microRNA–Argonaute complexes could provide cells a mechanism to buffer against rapid changes in their environment by reducing the magnitude of induced expression of certain proteins while enhancing the ability of other proteins to be translated.

Based on gel filtration, Ago2 exists in what appears to be three distinct complexes, all of which contain microRNAs. In mitogen-stimulated cells, expression of GW182 is elevated and Ago2 is predominantly found in two high molecular weight complexes which elute in the void of the gel filtration column (Superose 6) or around fraction 13. This elution pattern is similar to the elution pattern seen for GW182 (Fig. 4*E*). Ago2 complexes found in stimulated cells likely represent RISC loaded onto mRNA and associated RNA binding proteins (void, >2MDa) or assembled RISC not bound to mRNA (fraction 13, ~500 kDa). In quiescent cells a small portion of Ago2 remains in the void fraction (bound to mRNA), whereas the majority Ago2 elutes around fraction 17. Because the 67-kDa standard also elutes around this fraction and Ago2 is 97 kDa, the majority of Ago2 is likely free of association with other proteins in quiescent cells. MicroRNAs remain bound to Ago2 in quiescent cells as determined by immunoprecipitation and by coelution from gel filtration columns (Fig. S5). Elution of Ago2–microRNA complexes at high molecular weight correlates with increased GW182 expression (Figs. 3 and 4) and total cellular RNA content (Fig. S2*D*), supporting the conclusion that high molecular weight complexes are RISC assembled on mRNA targets. Overall, these data suggest that assembly of RISCs on mRNA is decreased in quiescent cells where transcription and translation are limited, likely as a result of a paucity of cytoplasmic target mRNA, GW182, or both.

Several recent studies have suggested microRNA turnover can be affected by cell state or cell type. In the mouse heart, miR-208 has a half-life of greater than 12 d (29) and in liver miR-122 levels remain stable despite decreased primary transcript levels (30), whereas in neural tissue microRNA half-lives are measured in hours, not days (31, 32). Additionally, the stability of specific microRNAs can be affected by the cell cycle (33, 34) and cell signaling pathways (35, 36). The current study focused on a few of the most abundant microRNAs found during quiescence in the cell types examined. Genome-wide studies are required to fully appreciate the extent of microRNA–Argonaute complex stabilization in quiescent cells and to determine the consequences of such stabilization on mitogen-induced target expression. It will be interesting to determine if distinct microRNAs are stabilized in different cell types or following different methods of inducing quiescence. For example, initial observations indicate that miR-21 stability is selectively responsive to glucose availability (Fig. 4 *A*–*C*). Perhaps miR-21 turnover is an active process that requires a product of glucose metabolism to complete. If microRNA

stability is regulated by target availability, however, it is possible that miR-21 targets are more rapidly degraded in the absence of glucose. Whereas several possibilities exist to explain changes in microRNA stability in quiescent cells, uncovering physiological conditions in which microRNA turnover is altered will likely aid in determining mechanisms that regulate microRNA stability.

Although the field of microRNA biology is nearly 20 y old, many unanswered questions regarding the function and biological significance of microRNAs remain. Somewhat unexpectedly, most single microRNA knockout animals generated to date show no gross developmental or phenotypic changes until subjected to stressful conditions (reviewed in ref. 37). This observation could be explained by functional redundancy among microRNAs and/or other compensatory changes in gene expression. Our data suggest that external stimuli (growth factors, glucose, etc.) are necessary to fully activate microRNAs. In the absence of such stimulation microRNA activity may not be sufficient to alter gross phenotypes, especially when compensatory mechanisms of gene regulation are in place. Continued investigation into the regulation of microRNA stability, function, processing, and expression by extracellular signals is necessary to gain a more complete picture of the physiological role(s) of microRNAs.

Materials and Methods

Small RNA Transfection. Small RNAs used in these studies are listed in Table S1. Stealth RNAi siRNAs targeting Ars2 and control stealth RNAi siRNAs were obtained from Life Technologies. *CXCR4* siRNA and *let-7* were ordered from Dharmacon. Allstars Negative Control siRNAs (Qiagen) were used as controls for *CXCR4* siRNA and *let-7* experiments. PremiR hsa-miR-21 and control PremiR mimic were obtained from Ambion. Small RNAs were transfected at 10 pM per 1×10^6 cells. Transfection of small RNAs into IL-3 DKO cells was accomplished using an Amaxa 4D-Nucleofector as follows. Cells were resuspended in Cell Line 4D-Nucleofector Solutions SF at 1×10^7 per 100 μL, added to cuvettetes or 16-well Nucleocuvette strips, and transfected using program EH-100. Cells were then allowed to rest for 5 min before being transferred to prewarmed medium at a concentration of $1–2 \times 10^6$ cells per milliliter. Following 4 h of culture at this concentration, cells were counted and their concentration adjusted to 0.2×10^6 cells per milliliter. DKO MEFs were reverse transfected using Lipofectamine RNAiMAX. Lipofectamine RNAiMAX was combined with 20 μM small RNAs at a 4:3 ratio (vol:vol) in Opti-MEM and incubated for 20 min at room temperature. Trypsinized cells were added to culture dishes containing siRNAs and Lipofectamine RNAiMAX at 3.8×10^4 cells per centimeter squared. Three volumes of complete medium were added to culture dishes and cells were incubated for 1–2 d before glucose or serum withdrawal.

Size Exclusion Chromatography. Size exclusion chromatography was performed using a Superose 6 10/300 GL prepacked column (GE Healthcare) equilibrated with sup6-150 buffer (150 mM NaCl, 10 mM Tris·HCl pH 7.5, 2.5 mM $MgCl_2$, 0.01% Triton X-100). To prepare total extracts cells were trypsinized, washed once with PBS, and spun down at $300 \times g$. Dry cell pellets were snap frozen in liquid nitrogen and stored at −80 °C. Pellets were then resuspended in sup6-150 buffer, incubated for 10 min on ice, and cleared by centrifugation at $20,000 \times g$. A total of 400 μL (1.5–2 mg) of precleaned total extracts were loaded on the column. Flow rate was adjusted to 0.3 mL/min and 1-mL fractions were collected. Fractions 1–7 were consistently devoid of protein, whereas fraction 8 contained the void of the column. Following fractionation, recombinant GFP protein was added to each fraction as an internal control at 25 ng/mL. Proteins were then acetone precipitated and resuspended in 20 μL loading buffer, boiled, and run on Novex NuPAGE SDS/PAGE gels for Western blot analysis. RNA was extracted from 800 μL of each fraction with phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7), precipitated with isopropanol and resuspended in Gel Loading Buffer II (Ambion) for Northern blotting.

Additional Materials and Methods. Additional information can be found in *SI Materials and Methods*.

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