miR-34a is essential for p19^{Arf}-driven cell cycle arrest

Nida Iqbal[1,†], Jie Mei[1,2,†], Jing Liu[1], and Stephen X Skapek[1,3,*]

[1]Division of Hematology/Oncology; Department of Pediatrics; University of Texas Southwestern Medical Center; Dallas, TX USA; [2]College of Fisheries; Key Laboratory of Freshwater Animal Breeding; Ministry of Agriculture; Huazhong Agricultural University; Wuhan, China; [3]Center for Cancer and Blood Disorders; Children's Medical Center; Dallas, TX USA

[†]These authors contributed equally to this work.

The *Arf* tumor suppressor gene product, p19^{Arf}, regulates cell proliferation in incipient cancer cells and during embryo development. Beyond its commonly accepted p53-dependent actions, p19^{Arf} also acts independently of p53 in both contexts. One such p53-independent effect with in vivo relevance includes its repression of Pdgfrβ, a process that is essential for vision in the mouse. We have utilized cell culture-based and mouse models to define a new role for miR-34a in this process. Ectopic expression of *Arf* in cultured cells enhanced the expression of several microRNAs predicted to target Pdgfrβ synthesis, including the miR-34 family. Because miR-34a has been implicated as a p53-dependent effector, we investigated whether it also contributed to p53-independent effects of p19^{Arf}. Indeed, in mouse embryo fibroblasts (MEFs) lacking p53, *Arf*-driven repression of Pdgfrβ and its blockade of Pdgf-B stimulated DNA synthesis were both completely interrupted by anti-microRNA against miR-34a. Ectopic miR-34a directly targeted Pdgfrβ and a plasmid reporter containing wild-type Pdgfrβ 3′UTR sequence, but not one in which the miR-34a target sequence was mutated. Although miR-34a expression has been linked to p53—a well-known effector of p19^{Arf}—*Arf* expression and its knockdown correlated with miR-34a level in MEFs lacking p53. Finally, analysis of the mouse embryonic eye demonstrated that *Arf* controlled expression of miR-34a, and the related miR-34b and c, in vivo during normal mouse development. Our findings indicate that miR-34a provides an essential link between p19^{Arf} and its p53-independent capacity to block cell proliferation driven by Pdgfrβ. This has ramifications for developmental and tumor suppressor roles of *Arf*.

Introduction

Cell proliferation is governed by 2 major pathways: the p16^{Ink4a}/RB pathway and the p19^{Arf}/p53 pathway.[1,2] The p19^{Arf} tumor suppressor was originally discovered as a protein encoded at the mouse (and human) *Cdkn2a* locus in an alternate reading frame (*Arf*) when compared with p16^{Ink4a}, the first tumor suppressor identified at that locus.[3,4] The simplest paradigm describing p19^{Arf} biology posits that its expression is induced by oncogenic stimuli, at which point it sequesters and inactivates Mdm2, a negative regulator of p53.[5-7] The resulting p53 elevation fosters numerous anti-cancer events, such as cell cycle arrest and the induction of pro-apoptotic or DNA damage repair pathways.[8]

Soon after its initial discovery, it became clear that the seemingly linear pathway stemming from *Arf* induction to p53-dependent tumor suppression was incomplete. In the first compelling challenge to that prevailing model, members of the Zambetti laboratory showed that ectopic expression of p19^{Arf} could still induce cell cycle arrest in mouse embryo fibroblasts (MEFs) lacking *Arf*, *Mdm2*, and *p53* (TKO MEFs).[9] Although

the arrest in TKO MEFs was slower than when *Arf* was expressed in *Arf*^{/–} MEFs retaining *Mdm2* and *p53*, the findings argued for p53-independent activities of p19^{Arf}. Over the ensuing years, the portfolio of p53-independent biochemical effects of p19^{Arf} grew to now include: (1) inhibition of rRNA processing;[10] (2) physical interactions with E2F1[11] and c-Myc[12,13] to repress their trans-activating potential; (3) blockade of the RelA subunit of NFκB;[14] (4) blunting of Pdgfrβ expression;[15] (5) promotion of p53-independent sumoylation of Mdm2, nucleophosmin, and other proteins;[16,17] and recently, post-transcriptional repression of Drosha, a microRNA-processing enzyme.[18]

The relevance of the p53-independent effects of p19^{Arf} for cancer suppression is supported by several observations in mouse models. For example, mice that lack *p53*, *Arf*, or both develop tumors spontaneously, but mice lacking both genes develop tumors more rapidly and include a broader range of histological subtypes than in singly mutant animals.[19,20] In an experimental model of skin cancer, the effect of *Arf* deficiency on early papilloma growth exceeds the effect of *p53* deficiency, but the rate of progression from papilloma to malignancy is similar in

the absence of either gene.[21] Further, in the RIP–Tag2 model of pancreatic islet cell neoplasia, *Arf* loss accelerates the angiogenic switch by a mechanism that is not strictly p53-dependent.[22] Notably, which of the above-mentioned p53-independent effects contribute to tumor suppression remains a mystery.

Beyond tumor surveillance, p19Arf plays an essential role for vision as it guides vascular involution in the vitreous space during late stages of mouse eye development.[15,23,24] It accomplishes this by limiting the proliferation of perivascular cells flanking the hyaloid vascular system (HVS). Without *Arf*, primary vitreous hyperplasia and failed HVS regression in the newborn period render the animals sightless.[24,25] This developmental function also cannot be attributed solely to p53, because most *p53^{-/-}* mice have normal eyes.[26] Genetic and biochemical studies show that p19Arf acts in a cell-intrinsic way to control the expression of *Pdgfr*β mRNA and protein,[27] and that Pdgfrβ is essential for primary vitreous hyperplasia that develops without *Arf*.[15,28] Because p19Arf can repress Pdgfrβ protein expression in TKO MEFs, understanding how it can accomplish this will shed light on the p53-independent mechanisms used by p19Arf to control cell cycle arrest in development, and possibly also as a tumor suppressor.

microRNA species have emerged as factors that finely tune the expression of many proteins during development and in disease processes.[29-31] It has recently been demonstrated that p19Arf controls the expression of microRNAs in a cell type-specific manner,[32] or more broadly by repressing the translation of Drosha, an RNase III endonuclease required for microRNA processing.[18] Following this lead, we used a candidate-based approach to determine whether microRNAs might contribute to p53-independent cell cycle control and Pdgfrβ repression by p19Arf. Our results reveal a previously unrecognized capacity for p19Arf, acting without p53, to guide the expression of miR-34 family microRNAs previously linked to p53.[33-37] Using complementary cell culture-based and mouse models, we demonstrate that *Arf* status dictates miR-34a, b, and c expression in cells and in vivo; that miR-34a is sufficient to block Pdgfrβ expression; and that this microRNA is required for *Arf*-driven, p53-independent cell cycle arrest and Pdgfrβ repression. These findings help to clarify certain confusing aspects of miR-34 biology, increase our understanding of miR-34 regulation, and underline the role that miR-34a can play as a p53-independent effector of p19Arf in normal development and disease.

Results

Arf regulates expression of certain miRNAs independently of p53

We recently demonstrated that p19Arf uses p53-independent mechanisms to block Pdgfrβ protein expression without influencing the level of its mRNA. Considering whether microRNAs might play a role in the post-transcriptional regulation of this protein, we used a candidate-based approach to prioritize potential microRNA regulators of Pdgfrβ. We employed TargetScan and miRDB to generate a list of microRNAs that may target the 3' untranslated region (UTR) of Pdgfrβ. We narrowed the list by only focusing on those microRNAs that are highly conserved or

expressed in the developing eye (**Fig. 1A**).[38] We utilized quantitative real-time PCR (qRT-PCR) to measure how the expression of 9 of these changed when p19Arf was ectopically expressed in TKO MEFs. Ectopic p19Arf expression significantly induced 3 microRNAs—miR-29a, miR-34a, and miR-34b—whereas one, miR-222, decreased in *Arf*-expressing TKO MEFs (**Fig. 1B**). We focused further studies on miR-34a, because it exhibited the greatest p53-independent induction by p19Arf and had previously been implicated as a tumor suppressor.[39]

miR-34a is required for Pdgfrβ repression and cell proliferation arrest by p19Arf

p19Arf can block cell proliferation stimulated by Pdgf-B in cultured MEFs, including TKO MEFs lacking endogenous *Arf*, *Mdm2*, and *p53*.[15,28] In order to understand if miR-34a is necessary for p19Arf to blunt Pdgfrβ expression, we utilized TKO MEFs transduced with retrovirus vectors expressing p19Arf or Gfp as a control. In this context, p19Arf substantially decreased Pdgfrβ; however, transfection of an anti-microRNA to miR-34a reversed this effect (**Fig. 2A**).

As an additional functional test of miR-34a importance in the p53-independent effects of p19Arf, we considered whether it was needed for *Arf* expression to overcome cell cycle progression stimulated by Pdgf-B. Similar to previous work,[15] exogenous Pdgf-B increased the S-phase fraction—measured by either propidium iodide staining or BrdU incorporation—in cultured TKO MEFs, and ectopic p19Arf expression blunted that effect (**Fig. 2B and C**, left 2 lanes). Transfection of an anti-microRNA targeting miR-34a nullified the ability of p19Arf to block Pdgf-B-driven cell proliferation (**Fig. 2B and C**, right 2 lanes). These results indicate that p53-independent Pdgfrβ repression and cell cycle arrest imposed by p19Arf both depend on miR-34a.

miR-34a directly targets Pdgfrβ

miR-34a has a number of protein targets that are known to be important for cell cycle regulation, including Cdk4/6, N-Myc and c-Met (reviewed in Hermeking et al.[40]). Previous mRNA-based surveys for miR-34a targets did not find *Pdgfr*β;[37] however, the 3'UTR of mouse *Pdgfr*β actually contains 2 miR-34a target sequences (**Fig. 1A**). To determine whether this transcript could be a direct miR-34a target, we sub-cloned wild-type and mutant versions of 1 of the putative miR-34a targets from the mouse *Pdgfr*β 3'UTR (**Fig. 3A**) into the pMIR-REPORT plasmid. Ectopic expression of miR-34a by transient transfection of HEK293T cells demonstrated that this microRNA blunted expression of the *Luciferase* cDNA containing the Pdgfrβ wild-type 3'UTR target, but not the mutated sequence (**Fig. 3B**).

To understand if miR-34a expression is sufficient to diminish endogenous Pdgfrβ expression, we examined changes in *Pdgfr*β mRNA and protein following retroviral expression of miR-34a in TKO MEFs. Although *Pdgfr*β mRNA expression was not altered by miR-34a, western blot revealed Pdgfrβ protein to be significantly lower (**Fig. 3C and D**). Taken together, these data indicate that miR-34a is sufficient to repress Pdgfrβ translation.

miR-34a expression depends on p19Arf in cells and in the developing mouse eye

If miR-34a acts downstream of p19Arf to mediate its anti-proliferative activities without p53, we considered whether

endogenous levels of p19[Arf] could influence miR-34a expression in cultured cells and in vivo. We addressed this using wild-type MEFs, as well as *Arf*-deficient MEFs derived from *Arf*[Gfp/Gfp][41] or *Arf*[lacZ/lacZ] mice.[42] In both of these lines, the first exon in *Arf* is replaced by cDNA encoding 1 of the 2 reporters. At passages 1, 3, and 5, miR-34a expression was significantly lower in *Arf*-deficient MEFs as compared with the wild-type cells (**Fig. 4A**). p53 is known to regulate miR-34a expression in human colorectal[33] and lung carcinoma cells,[34,35] and in MEFs.[36] We considered, then, whether decreased miR-34a in the absence of *Arf* might simply reflect the decreased p53 activity.[5,7] That miR-34a expression was even lower in TKO MEFs than *Arf*-deficient MEFs supports the fact that p53 does play a role in our model (**Fig. 4A**, right panel). However, examining miR-34a expression in *p53*^{−/−} MEFs expressing shRNA directed at *Arf* allowed us to determine whether a component of miR-34a regulation was independent of p53. With approximately 60% reduction of endogenous *Arf* mRNA in *p53*^{−/−} MEFs, we observed a quantitatively similar decrease in miR-34a (**Fig. 4B**). Of note, miR-34b and c were similarly diminished when *Arf* expression was knocked down in these MEFs (**Fig. 4C**). Hence, endogenous *Arf* drives the expression of miR-34 family microRNAs even in MEFs lacking p53.

In order to investigate whether miR-34a is controlled by p19[Arf] in vivo, we utilized laser capture microdissection (LCM) to isolate discrete compartments of the mouse eye at embryonic day (E) 13.5 (**Fig. 5A**), when *Arf* is first robustly expressed in the vitreous.[15] At this point in development, we already observe *Arf*-dependent alterations in *Pdgfr*β mRNA and increased cell proliferation, but there are few other anatomic differences between the wild-type and *Arf*^{−/−} animal;[15] as such, any *Arf*-dependent change in miR-34a would not be obscured by dramatic ocular pathology. By utilizing qRT-PCR, we demonstrated that miR-34a was most highly expressed in the primary vitreous compartment (**Fig. 5B**), which is the only anatomic site where *Arf* is measurably expressed.[23,28]

Broadening the scope of our query, we noted that other members of the miR-34 microRNA family were more highly expressed in the vitreous than miR-29a or miR-221, 2 other microRNAs predicted to target the Pdgfrβ 3′UTR (**Figs. 1A and 5C**). We then inquired if these microRNAs were influenced by the presence or absence of *Arf* by comparing their expression in the vitreous in eyes taken from E13.5 wild-type and *Arf*[Gfp/Gfp] embryos. Consistent with our analyses of MEFs, the absence of *Arf* significantly diminished the expression of miR-34a, b, and c, as well as miR-29a, which was also induced by ectopic p19[Arf] in MEFs. The expression of miR-221 was not significantly influenced by *Arf* expression in vivo (**Fig. 5D**) or in cultured MEFs (**Fig. 1B**). The *Arf*-dependent expression in the vitreous of the miR-34 microRNAs supports
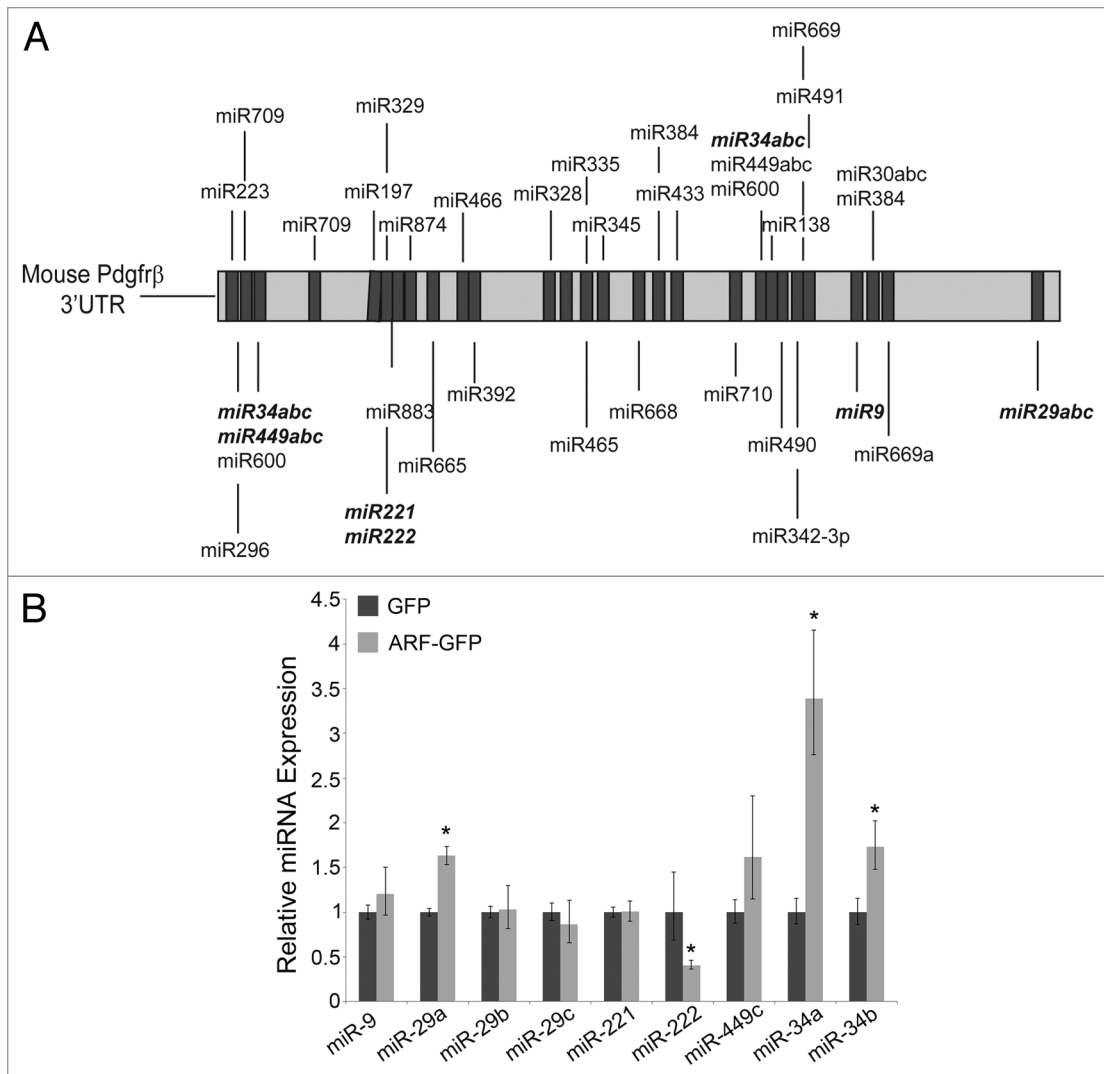

Figure 1. p19[Arf] regulates miRNAs independently of p53. (**A**) Schematic diagram depicting candidate miRNAs expressed in the developing eye with predicted binding sites in mouse *Pdgfr*β 3′UTR. Pursued candidates are in bold. (**B**) Quantitative analysis of expression of candidate microRNAs, measured by qRT-PCR, in TKO MEFs transduced with retrovirus encoding p19[Arf] or GFP (control). (*$P < 0.05$)

their candidacy as physiological regulators of Pdgfrβ during mouse eye development.

Discussion

Although initially described as a tumor suppressor that acts through p53,[5-7] p19[Arf] clearly has functional capabilities that do not depend on this downstream effector. Such activities include its capacity to interfere with rRNA processing,[10] perhaps by translational repression of Drosha;[18] inhibition of signaling to NFκB;[14] blockade of Myc-driven transcriptional activation;[12,13] and fostering sumoylation of Mdm2 and other nuclear proteins.[16,17] Perhaps the best example supporting the in vivo relevance of p53-independent biochemical activities relates to repression of Pdgfrβ by p19[Arf]. In this instance, deregulated proliferation of perivascular cells in the primary vitreous of *Arf*^{−/−} mice leads to severe ocular developmental defects and blindness; that the primary vitreous hyperplasia associated with this phenotype is reversed in *Arf*^{−/−}, *Pdgfr*β[−/−] animals[28] demonstrates that this biochemical pathway is crucial for a normal developmental process. Such a developmental role for p19[Arf] cannot be easily explained by its activation of p53, because *p53*^{−/−} mice usually have normal eyes (see more below).[23,26] Further, cell culture-based studies indicate that *Arf* expression represses Pdgfrβ independently of p53.[28] Given that in vivo relevance is already established, understanding how Pdgfrβ repression is accomplished becomes an important matter.

In this report, we illustrate how p19[Arf] can block Pdgfrβ protein synthesis without affecting its mRNA level and without engaging Mdm2 and p53. First, we have established that *Arf* expression elevates miR-34a, among several others microRNAs with the predicted capacity to target Pdgfrβ. This can be achieved even in MEFs that lack Mdm2 and p53, which is novel, because miR-34a is largely recognized as a p53 target.[33,34,36,37] Second, we show that miR-34a can directly target the 3′UTR of mouse *Pdgfr*β. Although this does not diminish *Pdgfr*β mRNA level, miR-34a expression is sufficient to decrease Pdgfrβ protein. The absence of a measurable effect on the transcript may help to explain why larger, RNA-based surveys did not identify Pdgfrβ as a miR-34a target.[37] Third, an essential role for miR-34a in *Arf*-driven Pdgfrβ repression is indicated, because anti-microRNA targeting miR-34a completely blocks the ability of p19[Arf] to repress Pdgfrβ and to blunt Pdgf-B-driven cell proliferation. Although it remains

possible that other closely related miRNAs with the same seeding sequence may also contribute, our findings do discount the possible roles for microRNAs targeting other regions of the *Pdgfr*β 3′UTR (see **Fig. 1A**). Finally, we demonstrate that miR-34 family members are highly expressed in the primary vitreous—the only region in the eye with detectable expression of *Arf*[23,28]—and they are repressed in *Arf*^{−/−} embryos. This represents the first in vivo evidence in which expression of miR-34a is directly correlated with a developmental disease. Further, providing in vivo evidence for *Arf* regulation of this microRNA supports the validity of our analyses of cultured MEFs.
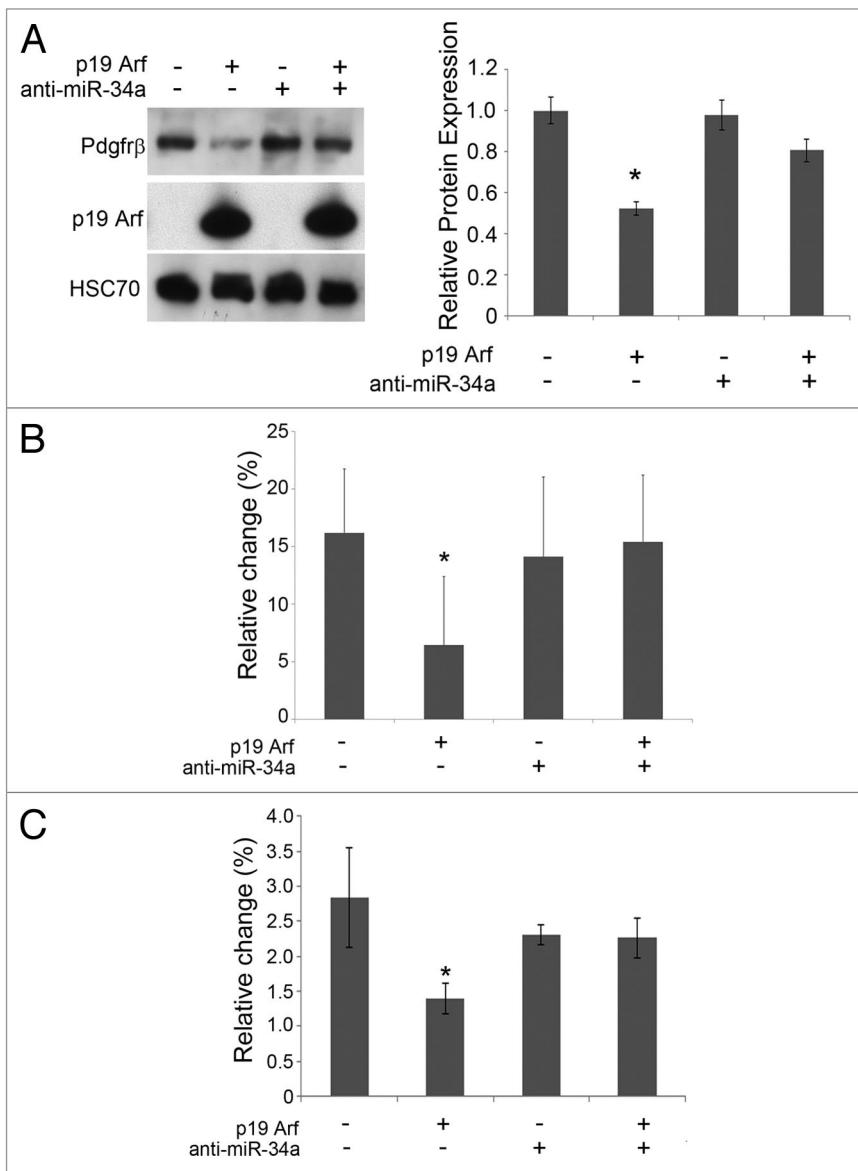

Figure 2. miR-34a is required for p19[Arf] driven repression of Pdgfrβ. (**A**) Representative western blot showing Pdgfrβ, p19[Arf] and HSC-70 protein expression in lysates prepared from cells transduced with p19[Arf] retrovirus or control and plus or minus miR-34a hairpin inhibitor. Quantification, on right, is normalized to HSC-70. (**B and C**) Change in S-phase fraction in TKO MEFs, stimulated by Pdgf-B, is assessed by propidium iodide staining and FACs (**B**) or by BrdU incorporation (**C**) in cells transduced with p19[Arf] or GFP control (+ and −, respectively); and miR-34a hairpin inhibitor or control (+ and −, respectively). (*$P < 0.05$, when compared with baseline control)

Our new findings allow us to pose a model by which p19[Arf] controls Pdgfrβ expression by 2 mechanisms: p53-dependent transcriptional repression and post-transcriptional repression via miR-34a, a process that can be separated from p53 (**Fig. 6**). This model offers new insight into the confusing role that p53 seems to play during mouse embryo eye development, which is heavily influenced by mouse genetic background.[23,26,43] In most genetic backgrounds, *p53*^{−/−} mice have normal eyes. When bred into pure C57BL/6 and pure BALB/c backgrounds, *p53*^{−/−} mice can develop primary vitreous hyperplasia, mimicking that observed without *Arf*.[23,26,43] However, in a mixed C57BL/6 × 129/Sv lineage, the eyes are usually normal.[23] We previously speculated that this might be due to a p53-independent capacity for p19[Arf] to block Pdgfrβ translation, and its capacity to do so might vary with genetic background.[28] We can now attribute that capacity to *Arf*-dependent regulation of miR-34a and related microRNAs in the eye.

Our model also helps to resolve another surprising paradox. Although miR-34a is well-known to be a transcriptional target of p53,[33,34,36] and it exerts anti-proliferative effects in a number of tumor models,[35,39] mice lacking all 3 members of the miR-34 microRNA family are seemingly normal, with no overt developmental defects or cancer susceptibility.[44] One might expect the eyes of miR-34a (and miR-34a, b, c)-null animals to be normal, because *Arf*-driven, p53-dependent transcriptional repression of *Pdgfr*β mRNA will still be intact (**Fig. 6**). However, loss of miR-34 family members might unmask an eye development defect in certain lines of *p53*-deficient mice.

Based on current understanding, we can propose at least 2 reasonable mechanisms by which p19[Arf] regulates miR-34a without p53. First, it is possible that in vivo, like in cultured MEFs, its expression is dually controlled by p53 and p19[Arf]. Interestingly, we previously showed that *Arf* mRNA increases in the vitreous of *p53*^{−/−} animals.[28] As such, effects of p19[Arf] on miR-34a should be magnified without p53. A second possibility stems from a recent report by Kuchenreuther and Weber demonstrating that p19[Arf] represses Drosha expression in cultured MEFs, and loss of the *Arf-Drosha* pathway substantially increases or decreases a number of microRNAs.[18] miR-34a was not listed among them, but their analysis focused only on a subset of 147 expressed more highly. Here, *Arf* might normally repress Drosha, enhancing miR-34a expression and blocking primary vitreous hyperplasia.

Finally, our findings shed light on p53-independent tumor suppression by *Arf*. Differences in tumor susceptibility in mice lacking either *Arf* or *p53* and in *Arf*^{−/−}, *p53*^{−/−} double knockout

mouse lines underscore the importance of an alternative pathway for p19[Arf] to block tumor formation.[19,20] Tumorigenesis studies in the *RIP-Tag2* mouse model,[22] as well as earlier studies of intestinal adenomas arising in *Arf, Ink4a* double knockout mice bred into the *Min* mouse,[45] indicate that p19[Arf] might influence tumor vascular biology. In neither case is a molecular explanation clear. Indeed, Ulanet and Hanahan performed a broad array of molecular analyses, including RNA-based analysis of a panel of angiogenesis-related genes, and they found no significant differences.[22] Given our findings that *Arf* induction of miR-34a can influence Pdgfrβ protein without influencing its mRNA, and recent findings that other angiogenesis-related proteins like Pdgf-B and Vegf-A are controlled post-transcriptionally by p19[Arf],[46] protein-based assays might be more revealing as one explores possible anti-angiogenic effects and p53-independent tumor suppression by p19[Arf].
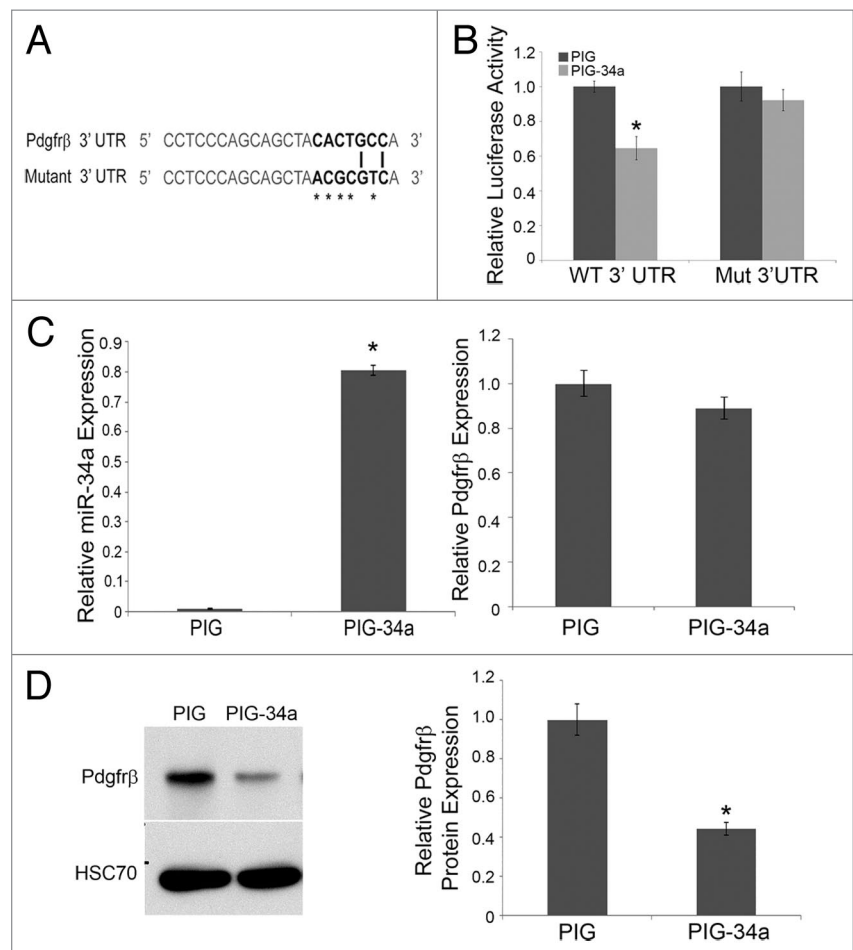
Figure 3. Pdgfrβ is a direct target of miR-34a. (**A**) miR-34a target sequence from *Pdgfr*β 3′ UTR. Seed sequence is in bold, and asterisks highlight mutated nucleotides. (**B**) Quantitative analysis of luciferase reporter in HEK293T cells, transiently transfected with expression plasmids for miR-34a (PIG-34a) or control (PIG), showing that miR-34a represses the reporter linked to the wild-type (WT) but not mutant (MUT) 3′ UTR of Pdgfrβ. Luciferase activity, normalized to β-galactosidase from co-transfected reporter, is presented relative to control for each reporter. (**C**) Quantitative analysis by qRT-PCR for miR-34a (left) or *Pdgfr*β mRNA (right) expression upon transduction of TKO MEFs with retroviral vectors expressing miR-34a or empty vector control, as indicated. (**D**) Representative western blot (left panel) and quantitative analysis, relative to HSC70, (right panel) for Pdgfrβ protein expression in lysates from TKO MEFs transduced as described in (**C**). (*$P < 0.05$, compared with baseline control)

Materials and Methods

Plasmids and other recombinant DNA reagents

Anti-miR-34a and scrambled controls for in vitro studies (obtained from Dhamacon) were used at a final concentration 200 nM and transfected into MEFS using the Dharmafect transfection reagent. Retroviral plasmids, pMSCV-PIG and pMSCV-PIG-34A, were provided by Joshua Mendell at UT Southwestern Medical Center. MSCV-based bicistronic retrovirus vectors mouse *Arf* and/or *Gfp* or *Rfp* were prepared and used as previously described.[15] Cells were infected with retrovirus shRNA targeting p19[Arf47] on day 1 and day 2, and then selected with 2 μg/mL puromycin for 6 d prior to harvest. Transduction efficiency was monitored by GFP expression.

Mouse models and cell lines

Arf[Gfp/Gfp] mice[41] and *Arf*[lacZ/lacZ42] were maintained in a mixed C57BL/6 × 129/Sv genetic background. Experimental protocols were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Primary MEFs from *Arf*[lacZ/lacZ]; *Arf*[Gfp/Gfp]; $p53^{-/-}$, $Mdm2^{-/-}$; and $p53^{-/-}$, $Arf^{-/-}$, $Mdm2^{-/-}$ (TKO) mutant and wild-type mice were cultivated as previously described.[15] TKO MEFs were provided by G Zambetti (St. Jude Children's Research Hospital).[9]

Laser-capture microdissection (LCM)

LCM was performed as previously described.[28] Briefly, mouse embryos were harvested at E13.5, and heads were immediately embedded in OCT freezing medium without fixation. Fourteen-μm sections were cut on a CryoStar NX70 cryostat, mounted on PEN Membrane Metal Slides (Applied Biosystems), and stained with hematoxylin and eosin (Molecular Machines and Industries AG). LCM was performed on the Arcturus Veritas Microdissection System. At least 10 micro-dissected sections from the vitreous, lens, retina, and sclera were pooled from each embryo.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from MEFs using the miRNeasy mini kit (Qiagen). For qRT-PCR, 1 μg of total RNA was reverse transcribed using *NCode miRNA* First-Strand Synthesis (Invitrogen) and Fast SYBR Green Master Mix (Applied Biosystem). qRT-PCR was performed in a 96-well plate using ABI 7900HT instrument. The PCR program consisted of 20 s at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. Primer quality was analyzed by dissociation curves. The expression of miRNAs and *Pdgfr*-β was normalized to *U6* and *Hprt*, respectively.

Luciferase reporter assay

The 362 bp 3′UTR of *Pdgfr*β gene was amplified by PCR with primers 5′-GTACTAGTCT CTGGCTGAAG CAGAGGAC and 5′-CGAAGCTTAC CACCGTACAG TCGTGGAT. The PCR product was digested by *Spe*I/*Hind*III and inserted into *pMIR-REPORT* vector (Ambion). To create this mutant we replaced the seed sequence CACTGCC with ACGCGTC through site-directed mutagenesis using QuickChange kit (Stratagene). HEK293T cells were transiently transfected using Fugene 6 reagent (Roche) in 96-well plates, as previously reported.[48] pCMV-LacZ was used as a control to monitor transfection efficiency of the luciferase reporter assay.

Western blotting

Protein expression was examined by Western-blotting according to a standard procedure. The following antibodies were used: anti-p19[Arf] (Ab80, Abcam, 1:1000), anti-Pdgfrβ (AF1042, R&D, 1:1000), anti-Hsc70 (Sc-1059, Santa Cruz, 1:5000). Band intensity was quantified using NIH ImageJ software.

Cell cycle analysis

Cell cycle analysis was assessed in TKO MEFs transduced with p19[Arf]-expressing retrovirus, with or without transfection with anti-miR-34a reagent (Dharmacon), and with or without stimulation using Pdgf-B (50 ng/mL) (R&D) for 12 h. Relative change in S-phase fraction was assessed in 2 ways: first, propidium iodide (PI) (sigma) staining was performed after cells were harvested by trypsin-EDTA and fixed in 70% ethanol. Fixed cells were washed in PBS and centrifuged at 1200 rpm for 5 min. Cells were resuspended in 0.3 ml PBS and RNaseA (Sigma) was added to the suspension to final concentration of 0.5 mg/mL. After 1 h of incubation at 37 °C, PI was added to the suspension to final concentration of 10 μg/mL. PI-stained cells were analyzed for DNA content with a BD Calibur flow cytometer. Cell sorting results were analyzed with FlowJo
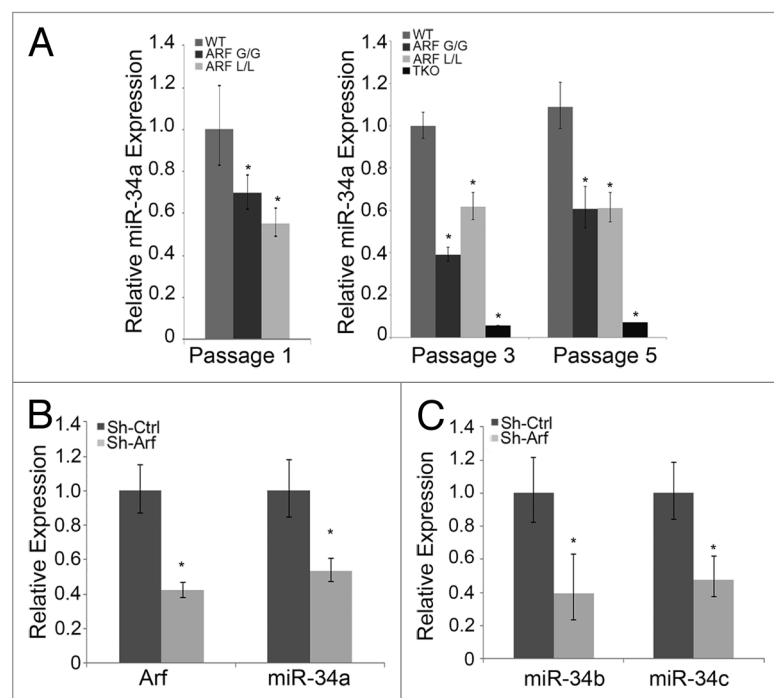

Figure 4. miR-34a expression correlates with p19[Arf] status. (**A**) Quantitative analysis of miR-34a expression, measured by qRT-PCR, in MEFs that are derived from WT, *Arf*[Gfp/Gfp] (ARF G/G), *Arf*[lacZ/lacZ] (ARF L/L), and $Arf^{-/-}$, $p53^{-/-}$, $MDM2^{-/-}$ (TKO) mice and cultivated for 1, 3, or 5 passages. (**B and C**) Quantitative RT-PCR analyses of *Arf*, and miR-34a, b, and c, mRNA expression, as indicated, in $p53^{-/-}$ MEFs transduced with retroviral vectors targeting p19[Arf] (Sh-Arf) or control (Sh-Ctrl). In each case, data are expressed relative to baseline control. (*$P < 0.05$, when compared with baseline control)

Software using a cell cycle platform and Watson Pragmatic Model to calculate the distribution of cells in G_1, S, and G_2 cell cycle phases. In some experiments, cells were incubated with BrdU (10 µM) for 6 h prior to fixing with 2% paraformaldehyde. BrdU was assessed by immunofluorescence staining using a FITC-conjugated anti-BrdU antibody (BD 347583, BD Biosciences, 1:100) and quantified by determining the fraction of DAPI-positive with detectable BrdU in at least 5 fields from replicate plates.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was determined using a 2-tailed Student t test. A P value of < 0.05 was considered significant. We conducted each experiment in triplicate.
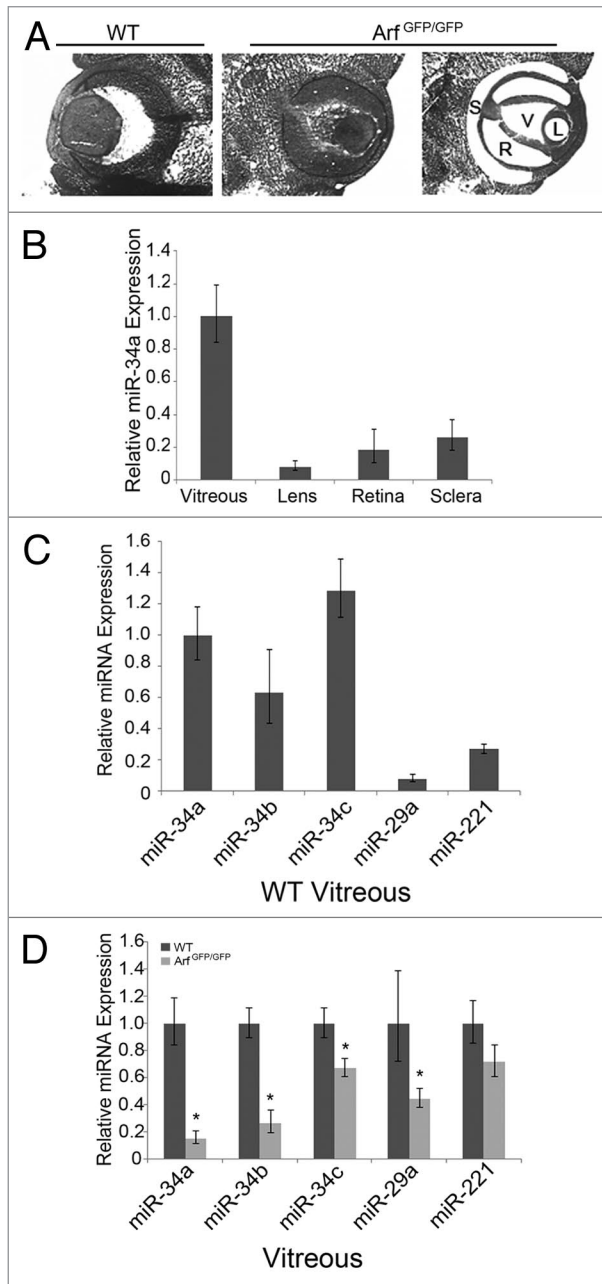
Figure 5. miR-34a expression in vivo is dependent upon p19^{Arf}. (**A**) Representative photomicrographs of E13.5 mouse embryo eye from wild-type (WT) or $Arf^{Gfp/Gfp}$ embryo, as indicated, showing laser capture microdissection (LCM) of sclera (S), retina, (R), vitreous (V), and lens (L). (**B**) Quantitation of mature miR-34a expression, measured by qRT-PCR, in LCM specimens from different parts of the wild-type E13.5 mouse eye. Expression is normalized to *U6* snRNA and arbitrarily displayed relative to that in the vitreous fraction. (**C and D**) Quantitative analysis shows relative expression of different microRNAs in the vitreous of E13.5 wild-type (WT) and $Arf^{Gfp/Gfp}$ mouse embryos. In each case, expression is normalized to *U6* snRNA and shown relative to miR-34a expression in the wild-type vitreous (**C**) or relative to each microRNA expression in wild-type embryos (**D**). (*$P < 0.05$, when compared with wild-type embryos)
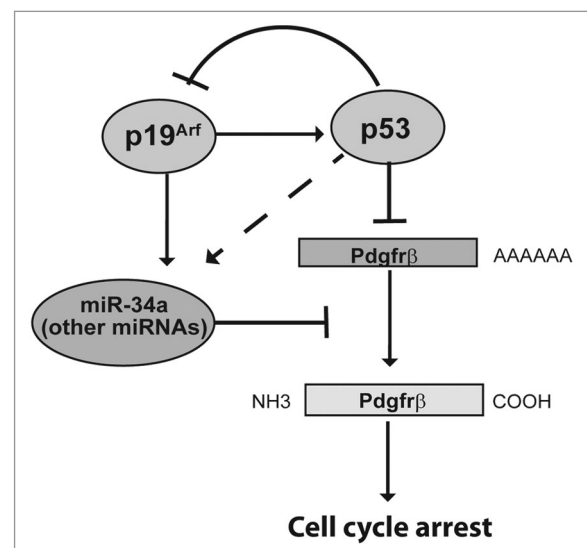

Figure 6. Schematic diagram shows dual mechanisms for *Arf* dependent control of Pdgfrβ expression. p19^{Arf} mediates transcriptional repression of Pdgfrβ by stabilizing p53 and post-transcriptional repression of Pdgfrβ protein levels via induction of miR-34a and potentially other microRNAs. Although *Arf* expression can induce miR-34a and other microRNAs independently of p53 in cultured MEFs (solid arrow), p53 may contribute to miR-34a expression in the developing mouse eye (dashed arrow).

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