Arf-dependent regulation of Pdgf signaling in perivascular cells in the developing mouse eye

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We have established that the *Arf* tumor suppressor gene regulates mural cell biology in the hyaloid vascular system (HVS) of the developing eye. In the absence of *Arf*, perivascular cells accumulate within the HVS and prevent its involution. We now demonstrate that mural cell accumulation evident at embryonic day (E) 13.5 in *Arf*^{-/-} mice was driven by excess proliferation at E12.5, when *Arf* expression was detectable in vitreous pericyte-like cells. Their expression of *Arf* overlapped with Pdgf receptor β (Pdgfr β), which is essential for pericyte accumulation in the mouse. In cultured cells, p19^{Arf} decreased Pdgfr β and blocked Pdgf-B-driven proliferation independently of *Mdm2* and *p53*. The presence of a normal *Arf* allele correlated with decreased Pdgfr β in the embryonic vitreous. Pdgfr β was required for vitreous cell accumulation in the absence of *Arf*. Our findings demonstrate a novel, *p53*- and *Mdm2*-independent function for p19^{Arf}. Instead of solely sensing excessive mitogenic stimuli, developmental cues induce *Arf* to block Pdgfr β -dependent signals and prevent the accumulation of perivascular cells selectively in a vascular bed destined to regress.

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Introduction

Cellular and molecular interactions between mural cells and underlying endothelial cells are key determinants of vascular integrity and remodeling (reviewed in Hanahan and Folkman, 1996; Yancopoulos *et al*, 2000). Mural cells are often broadly referred to as either pericytes, defined by their direct apposition to the abluminal side of endothelial cells beneath the endothelial cell basement membrane, or vascular

smooth muscle cells (reviewed in Sims, 1986; Beck and D'Amore, 1997; Gerhardt and Betsholtz, 2003). Mural cell accumulation largely depends on endothelial sources of platelet-derived growth factor (Pdgf)-B (Hellstrom *et al*, 1999; Gerhardt *et al*, 2003; Lindblom *et al*, 2003) and the expression of Pdgf receptor β (Pdgfr β) in mural cells or their progenitors (Benjamin *et al*, 1999; reviewed in Hoch and Soriano, 2003). The critical importance of Pdgf signaling is evidenced by the fact that mice lacking *Pdgf-B* or *Pdgfr β* have an embryonic lethal phenotype due to microvascular hemorrhage and edema caused by lack of pericytes (Leveen *et al*, 1994; Soriano, 1994; Lindahl *et al*, 1997; Hellstrom *et al*, 2001).

The preponderance of evidence supports the notion that mural cell investment of maturing vessels governs a 'plasticity window'—in their absence, existing vessels can regress (Benjamin *et al*, 1998) or remodel by forming angiogenic sprouts (Gee *et al*, 2003). Mural cells preserve vascular stability by providing mechanical support to underlying endothelial cells (reviewed in Sims, 1986) and by preventing endothelial cell proliferation (Orlidge and D'Amore, 1987; Hellstrom *et al*, 2001) and migration (Sato and Rifkin, 1989). Surprisingly, pericyte coverage of individual blood vessels varies widely (reviewed in Sims, 1986), perhaps to confer greater or less plasticity. Understanding regulatory mechanisms for mural cell recruitment may reveal how certain vascular beds are stabilized (or destabilized) during development and how this can go awry in disease.

We recently uncovered evidence that a key mammalian tumor suppressor p19^{Arf} controls perivascular cell accumulation in the postnatal mouse eye (McKeller *et al*, 2002; Martin *et al*, 2004). p19^{Arf}, encoded by the *Arf* gene resident at the *Ink4a/Arf* locus in the human and murine genomes, was initially demonstrated to physically interact with and inhibit the function of *Mdm2*, a negative regulator of the *p53* tumor suppressor. The net effect of *Arf* expression is *p53* activation to induce cell proliferation arrest or apoptosis (reviewed in Sherr, 1998). More recent findings indicate that p19^{Arf} activity does not entirely depend on *p53*. For example, ectopically expressed p19^{Arf} arrests proliferation in mouse embryo fibroblasts (MEFs) lacking *p53* and *Mdm2* (smaller effects are observed in cells with *Mdm2*) (Weber *et al*, 2000). The tumor spectrum also differs in *p53*^{-/-} (lymphoma predominance) (Donehower *et al*, 1992; Jacks *et al*, 1994) and *Arf*^{-/-} (sarcoma predominance) (Kamijo *et al*, 1997, 1999) mice and in mice lacking both *Arf* and *p53* (broader types; multiple tumors) (Weber *et al*, 2000). p19^{Arf} can inhibit ribosomal RNA processing (Sugimoto *et al*, 2003), NF- κ B activation (Rocha *et al*, 2003), and Myc-mediated transactivation (Qi *et al*, 2004) independently of *p53*. The relative importance of these activities to the tumor suppression by p19^{Arf} is not yet known.

Our previous finding that p19^{Arf} blocks perivascular cell accumulation within the vasa hyaloidea propria (VHP), the vitreous component of the hyaloid vascular system (HVS),

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was somewhat surprising. Existing paradigms hold that $p19^{Arf}$ is an oncogene sensor induced by abnormal or excessive mitogenic stimuli (de Stanchina *et al*, 1998; Zindy *et al*, 1998, 2003; Palmero *et al*, 1999). *Arf* expression is undetectable or very low through most of mouse embryo development (Zindy *et al*, 1997, 2003), consistent with the idea that active *Arf* repression prevents untoward $p19^{Arf}$ -mediated effects during embryogenesis. That *Arf*-deficient mice have a developmental eye disease evident at birth implies that *Arf* is not universally repressed in the developing mouse embryo and that its gene product functions beyond tumor suppression.

The present work elucidates one such function. We show that *Arf* is expressed in a subset of perivascular cells in the eyes of developing mouse embryos. Its restricted expression suggests that the *Arf* promoter is not only induced by aberrant mitogens but also responds to specific developmental cues. During embryonic development, $p19^{Arf}$ controls perivascular cell accumulation in the vitreous by blocking proliferation driven by $Pdgfr\beta$ in the *Arf*-expressing cells. Our *in vivo* and *in vitro* analyses show that $p19^{Arf}$ may accomplish this by dampening $Pdgfr\beta$ expression, even in cells lacking *Mdm2* and *p53*. Hence, our results extend existing paradigms for $p19^{Arf}$ biology by showing that *Arf* is expressed in a strikingly restricted pattern to couple developmental signals to *p53*-independent effects and block $Pdgfr\beta$ -driven mural cell accumulation in a vascular bed destined to regress in the postnatal period.

Results

Increased cell proliferation in the developing vitreous in *Arf*^{-/-} mouse embryos

We previously detected *Arf* mRNA by RT-PCR and *in situ* hybridization in the postnatal eye (McKeller *et al*, 2002; Martin *et al*, 2004), but *Arf* expression during embryonic eye development has not been reported. In the present analyses, we took advantage of an engineered mouse in which cDNA encoding the green fluorescent protein (Gfp) replaces *Arf* exon 1 β (Zindy *et al*, 2003). Like the original *Arf*^{-/-} mouse (McKeller *et al*, 2002), *Arf*^{Gfp/Gfp} (effectively *Arf*^{-/-}) mice have eye disease while *Arf*^{Gfp/+} mice do not (Zindy *et al*, 2003). Gfp expression accurately reflects *Arf* mRNA in *Arf*^{Gfp/+} mice and MEFs derived from them (Zindy *et al*, 2003). To elucidate putative developmental functions for $p19^{Arf}$, we used the *Arf*^{Gfp/+} mouse to define the temporal and spatial expression pattern of *Arf* in the eye and the earliest developmental defects in *Arf*^{-/-} embryos.

In the immediate postnatal period, direct green fluorescence was evident in the retrolental mass in unfixed eyes taken from *Arf*^{Gfp/Gfp} mice (Zindy *et al*, 2003), but relatively weak green fluorescence in developing embryos prevented direct visualization of Gfp. In contrast, antibody-based detection readily showed Gfp expression in the vitreous of *Arf*^{Gfp/+} and *Arf*^{Gfp/Gfp} but not wild-type mice evaluated from embryonic day (E) 11.5 to E18.5 (Figure 1A; some data not shown). Occasional Gfp-positive cells were observed along the developing optic tract at E11.5 and the number of vitreous cells with strong Gfp expression seemed to increase from E11.5 to E12.5 in heterozygous embryos (Figure 1A). Gfp-expressing cells were scattered throughout the vitreous from E11.5 to E13.5 and appeared to form linear structures by

Figure 1 Vitreous cells express the *Arf* promoter from E11.5 of mouse embryo development. (A, C) Representative immunofluorescence photomicrographs of eyes taken from *Arf*^{Gfp/+} (A, C) and *Arf*^{Gfp/Gfp} (C) mouse embryos at the indicated stages of embryonic development (A) and E14.5 (C). Sections stained with DAPI and α -Gfp antibody (red (A); green (C)) as indicated. Note Gfp-positive cells along developing optic tract at E11.5 (arrow) and in the vitreous at each stage. (B) Representative immunofluorescence photomicrographs of E11.5 wild-type mouse eye, following staining with α - $p19^{Arf}$ antibody. Original magnification: $\times 200$ (A) and $\times 400$ (B, C).

E18.5 (Figure 1A), consistent with the fact that *Arf*-expressing cells are perivascular in postnatal mice (Martin *et al*, 2004). That the Gfp reporter accurately reflected protein expression was verified by immunofluorescence staining using a rat monoclonal antibody directed against $p19^{Arf}$ (Bertwistle *et al*, 2004), which labeled vitreous cells in wild-type mice as early as E11.5 (Figure 1B).

In postnatal *Arf*^{-/-} eyes, the principal abnormality is excess accumulation of perivascular cells within the VHP in the retrolental vitreous (McKeller *et al*, 2002). Relatively few *Arf*-expressing perivascular cells are present in the VHP in wild-type or *Arf*^{Gfp/+} mice in the postnatal period, whereas

the bulk of the cells in the retrolental mass in the *Arf^{Gfp/Gfp}* mice express the *Arf* promoter (Zindy *et al*, 2003; Martin *et al*, 2004). Similarly, additional Gfp-expressing cells were evident in developing *Arf^{Gfp/Gfp}* versus *Arf^{Gfp/+}* embryos as early as E14.5 (Figure 1C). This implied that p19^{Arf} functioned early during eye development to check vitreous cell accumulation.

We determined whether the earliest evidence of vitreous cell accumulation correlated with *Arf* promoter activity in the embryo. Between E12.5 and E13.5, fibroblast-like cells accumulated and persisted in the vitreous of *Arf^{-/-}* embryos (Figure 2A), resulting in increased cellular density as compared to heterozygous and wild-type mice (Figure 2B; some data not shown). Differences in cellular density in *Arf^{-/-}* and *Arf^{+/-}* mice were not apparent at E12.5 (Figure 2A and B). Absence of *Arf* did not appreciably alter the morphology of the fibroblast-like cells nor did it affect the lens and neuroblastic tissue. Increased cellular density was not likely due to decreased vitreous volume: the vitreous area in sections from E13.5 *Arf^{-/-}* eyes ($0.42 \pm 0.11 \text{ mm}^2$) was only slightly smaller

than in *Arf^{+/-}* and wild-type eyes ($0.54 \pm 0.17 \text{ mm}^2$) ($P=0.05$). Hence, the increased vitreous cell density primarily represented increased cell number, which became apparent in *Arf^{-/-}* mice at E13.5.

Because p19^{Arf} regulates cell proliferation by both p53-dependent and -independent mechanisms (Kamijo *et al*, 1997; Weber *et al*, 2000), we determined whether it arrested vitreous cell proliferation between E12.5 and E13.5. Immunohistochemical staining for the cell proliferation marker Ki67 labeled vitreous cells in wild-type, *Arf^{+/-}*, and *Arf^{-/-}* eyes at E12.5 and E13.5 (Figure 2C; some data not shown), but the relative number of Ki67-positive cells was significantly higher in *Arf^{-/-}* eyes (Figure 2D). With rare exception (see below), the *Arf*-expressing cells in *Arf^{Gfp/+}* heterozygous embryos were negative for Ki67 (Figure 2E). Few apoptotic cells were identified in the vitreous at E12.5 (three of 120 nuclei counted were TUNEL positive in *Arf^{+/-}* versus 0 of 162 in *Arf^{-/-}* embryos) (Figure 2C and data not shown). We conclude that p19^{Arf} controlled vitreous cell

Figure 2 Cellular density and cell proliferation increase in the vitreous of *Arf^{-/-}* mice between E12.5 and E13.5. (A) Representative photomicrographs of midline, hematoxylin and eosin (H&E)-stained section of eyes taken from E12.5 and E13.5 *Arf^{+/-}* or *Arf^{-/-}* mouse embryos. (B) Quantitative analysis of average cellular density (and standard deviation) in the vitreous at E12.5 and E13.5 in *Arf^{+/-}* versus *Arf^{-/-}* mice. Differences at E13.5 (*) were statistically significant ($P=0.0002$) whereas those at E12.5 were not ($P=0.55$). (C) Representative photomicrographs of vitreous cells from E13.5 *Arf^{-/-}* (left) and *Arf^{+/-}* (middle) and E12.5 *Arf^{+/-}* (right) eyes following staining for Ki67 and TUNEL (arrow), as indicated. (D) Quantitative analysis shows average percentage (and standard deviation) of Ki67-positive cells within vitreous of wild-type, *Arf^{+/-}*, or *Arf^{-/-}* eyes at E12.5 and E13.5. Differences between *Arf^{-/-}* and *Arf^{+/-}* or *Arf^{+/+}* were statistically significant at each time point ($P<0.002$), while differences between *Arf^{+/-}* and *Arf^{+/+}* at E13.5 were not ($P=0.27$). (E) Representative immunofluorescence photomicrograph of vitreous cells from E13.5 *Arf^{Gfp/+}* mouse depicts Gfp (a), DAPI (b), Ki67 (c), and dual Gfp and Ki67 (d). Arrows show Gfp-positive cells, negative for Ki67; arrowheads show Gfp-negative cells, positive for Ki67. Lens (asterisk) provides internal negative control for Gfp and Ki67 stains. Original magnification: $\times 400$ (A, C, E).

accumulation primarily by arresting proliferation from E12.5 although less pronounced proapoptotic effects might also have contributed.

Quantitative analysis at E13.5 suggested a trend toward increased Ki67-positive cells in *Arf*^{+/-} versus wild-type mice (Figure 2D). Although no pathology was evident before E13.5 or in postnatal eyes (McKeller *et al*, 2002), inspection of the *Arf*^{+/-} vitreous at E14.5 demonstrated clusters of fibroblast-like cells, present in all heterozygous eyes (*n* = 6) (Figure 3B, arrowhead) but not in wild-type eyes (*n* = 8) (Figure 3A) examined in a blinded fashion. Cells often clustered near linear structures (Figure 3B, arrow) thought to represent developing hyaloid vessels. Dual immunofluorescence labeling for Gfp and CD31, an endothelial cell marker, in *Arf*^{Gfp/+} eyes at E14.5 and E15.5 confirmed that the cellular clusters were composed of *Arf*-expressing cells (Figure 3C and D, arrowhead) adjacent to CD31-positive vessels (Figure 3C, arrow). Occasional Gfp-expressing cells in the clusters expressed Ki67 (Figure 3D). Hence, *Arf* haploinsufficiency provided a sufficient genetic insult to allow localized accumulation of *Arf*-expressing cells adjacent to some developing hyaloid vessels, which may provide a mitogenic signal (see below). But loss of one *Arf* allele was not sufficient to allow the unchecked vitreous cell proliferation and obvious eye pathology that occurs uniformly in *Arf*^{-/-} eyes.

Arf is expressed in mural cells within the developing hyaloid vessels

The timing and localization of *Arf*-expressing cells in the vitreous and their clustering near developing endothelial tubes in *Arf*^{Gfp/+} eyes suggested that they may represent progenitors of the hyaloid vasculature. We used immunofluorescence staining to determine whether the Gfp-positive cells in the embryonic vitreous were of endothelial or mural cell lineage.

In the postnatal period, cells expressing the *Arf* promoter in the vitreous of heterozygous or null mice do not express CD31 (Martin *et al*, 2004). Similarly, expression of Gfp and CD31 did not overlap in the eyes of E15.5 embryos (Figure 4Ab) nor was there coexpression of Gfp and the Vegf receptor Flk-1 in E13.5 *Arf*^{Gfp/Gfp} embryos (AC Martin and SX Skapek, unpublished). Thus, we found no evidence that *Arf*-expressing cells in the vitreous were of endothelial cell lineage.

Mural cells lack true lineage-defining markers, but their anatomic localization and the expression of either NG2 chondroitin sulfate proteoglycan (Ozerdem *et al*, 2001) or Pdgfrβ (Benjamin *et al*, 1999; reviewed in Hoch and Soriano, 2003) can place them in this lineage. As in the postnatal mouse eye (Martin *et al*, 2004), we observed that some Gfp-positive cells in both *Arf*^{Gfp/+} and *Arf*^{Gfp/Gfp} embryos coexpressed NG2 (Figure 4B; some data not shown). Gfp expression also overlapped with Pdgfrβ in vitreous cells from E11.5 through at least postnatal day 1 (Figure 4C and D; additional data not shown). Of note, not all of the Pdgfrβ-positive cells in the eye expressed the *Arf* promoter (Figure 4C, arrows). Moreover, at E11.5, when relatively few Gfp-positive cells were evident, Pdgfrβ was readily detectable (Figure 4D). Although the degree of overlap varied somewhat in *Arf*^{Gfp/+} and *Arf*^{Gfp/Gfp} embryos at E13.5 (see below), the findings indicated that the *Arf*-expressing cells in the embryonic vitreous likely represented mural cells or their progenitors.

Figure 3 *Arf*-expressing cells cluster near developing hyaloid vessels in *Arf*^{Gfp/+} embryos. Representative photomicrographs of H&E-stained paraffin sections (A, B) and immunofluorescence-stained cryostat sections (C, D) through the midline sections of eyes of wild-type (A) and heterozygous E14.5 (B, C) and E15.5 (D) mouse embryos. Note vessel-like structure (arrow (B, C)) near the cluster of vitreous cells (arrowhead (B–D)) in *Arf*^{Gfp/+} eyes. Vessel-like structure expresses CD31 (arrow (C)). Clustered cells express Gfp (green (C); red (D)). Several Gfp-expressing cells in the cluster are Ki67 positive (green (D)). Sections in panels B–D are from separate *Arf*^{Gfp/+} embryos. Original magnification: × 100 (A, B) and × 400 (C, D).

p19^{Arf} can regulate Pdgfrβ and block Pdgf-B-driven cell proliferation in pericyte-like cells and MEFs independent of Mdm2 and p53

Pdgf-B/Pdgfrβ signaling is essential for pericyte and VSMC recruitment and proliferation during mouse development (reviewed in Hoch and Soriano, 2003). Because of overlapping *Arf* and Pdgfrβ expression, we considered whether

Figure 4 Coexpression of the *Arf* promoter and mural cell proteins in the developing vitreous. (**A, B**) Representative immunofluorescence photomicrographs of E15.5 (**A**) and E13.5 (**B**) *Arf*^{Gfp/+} eyes stained for Gfp (green (**A** and **B**, **c**)), CD31 (red (**A**, **a**)), NG2 (red (**B**, **a**)), and DAPI (blue (**A** and **B**, **d**)). Panel **b** shows dual red/green fluorescence. Note lack of overlapping CD31/Gfp expression (**A**, **b**) and cell membrane staining for NG2 in some Gfp-expressing cells (arrows (**B**)). Original magnification: $\times 400$. (**C**) Representative immunofluorescence (**a–c**, **e–g**) and phase-contrast (**d**) photomicrographs from cryostat sections of an E13.5 *Arf*^{Gfp/Gfp} mouse. Gfp expression (green (**a**, **e**)) is confined to vitreous cells where it overlaps with Pdgfr β (red (**b**, **f**); dual red/green (**g**)). Note that other Pdgfr β -expressing cells adjacent to pigment epithelial cells (arrow (**b**)) do not express Gfp (arrow (**a**)). DAPI stain (**c**). Original magnification: $\times 100$ (**a–d**) and $\times 400$ (**e–g**). (**D**) Representative confocal photomicrographs of E11.5 *Arf*^{Gfp/+} mouse embryo following dual immunofluorescence labeling for Pdgfr β (red (**a**)) and Gfp (green (**a**)) and DAPI (**b**). Relatively few vitreous cells behind the lens (**L**) express Gfp (green, arrow) compared to abundant Pdgfr β -expressing cells (red, arrowhead). Original magnification: $\times 400$.

p19^{Arf} may influence Pdgf-B signaling to control mural cell accumulation. We first addressed this *in vitro* using mouse 10T1/2 cells. These are embryonic fibroblast-like cells that express Pdgfr β , *Ang-1*, and *Vegf* (SX Skapek, unpublished data), suggesting that they have pericyte-like properties. Moreover, they migrate toward cocultured endothelial cells growing as a monolayer (Hirschi *et al*, 1998) or in tube-like structures on Matrigel (JD Thornton and SX Skapek, unpublished data) and they localize to blood vessel walls when

injected into mice (Hirschi *et al*, 1998). Southern blotting showed they had biallelic deletion of the *Arf* gene (SX Skapek, unpublished data), but they were efficiently transduced with retrovirus to express p19^{Arf} (Figure 5A).

To mimic what occurs in the vitreous, we evaluated whether Pdgf-B promoted 10T1/2 cell accumulation *in vitro* and whether *Arf* expression could block it. Ectopically expressed *Arf* was functional in these cells, as it decreased their accumulation in response to 10% FBS by 20–40% over a

Figure 5 *Arf* expression blocks Pdgf-B-driven cell proliferation in 10T1/2 pericyte-like cells. (A) Immunofluorescence photomicrographs of 10T1/2 cells transduced with *Arf*-expressing (Arf) or control (Gfp) retrovirus and stained using anti-p19^{Arf} antibody or DAPI as indicated. Note focal subnuclear localization of ectopic p19^{Arf} (arrowhead). (B, C) Quantitative analysis of 10T1/2 cell number following transduction with *Gfp* (G)- or *Arf* (A)-encoding virus, with or without exposure to Pdgf-B, as indicated. Cells were harvested on day 1 (24 h) or day 2 (48 h) as indicated. Lanes 1 and 2, and 3–6 (B) and lanes 1–6 and 7–18 (C) represent separate experiments. Cell numbers are presented as average percentage (and standard deviation) of transduced cells not treated with Pdgf-B from triplicate samples. (D) Representative histograms (top) and cumulative data (bottom) from FACS analysis of PI-stained cells following 24 h exposure of cells to 50 ng/ml Pdgf-B. Data are presented as average percent S-phase fraction (and standard deviation) using duplicate samples from two or three representative experiments.

24–48 h period (Figure 5B, compare lanes 2, 4, and 6 with 1, 3, and 5). The addition of 5 or 50 ng/ml of Pdgf-B augmented 10T1/2 cell accumulation and this effect was blunted in cells expressing p19^{Arf} (Figure 5C, lanes 1–3, 7–9, and 13–15 versus 4–6, 10–12, and 16–18). We performed quantitative analyses to determine whether the effects of Pdgf-B and p19^{Arf} were primarily due to effects on the cell cycle or cell survival. Exposure to Pdgf-B for 24 h increased the fraction of 10T1/2 cells in S phase (Figure 5D, lanes 2 and 6 versus 1 and 5). *Arf* transduction prevented cell cycle phase changes (Figure 5D, lanes 4 and 8 versus 3 and 7). Interestingly, exposure of *Arf*-expressing cells to 50 ng/ml Pdgf-B further limited 10T1/2 cell accumulation as compared to *Arf*-transduced cells cultured without or with only 5 ng/ml Pdgf-B (Figure 5C, compare lanes 6, 12 and 18 with lanes 4, 5, 10, 11, 16, and 17). This may have resulted from slightly enhanced apoptosis. The sub-G₁ fractions in repeated experiments

were as follows: 1.74 and 0.71% in untreated *Gfp*-transduced cells; 0.43 and 0.83% in *Gfp*-transduced cells treated with 50 ng/ml Pdgf-B; 1.54 and 1.73% in untreated *Arf*-transduced cells; and 3.43 and 3.78% in *Arf*-transduced cells treated with 50 ng/ml Pdgf-B. Hence, *in vitro* studies of 10T1/2 cells recapitulated *in vivo* findings in that *Arf* expression limited Pdgf-B-driven pericyte-like cell accumulation by arresting cell proliferation while smaller effects on apoptosis also occurred.

Because *Arf* promoter activity only partially overlapped with Pdgfr β expression in the developing vitreous and Pdgfr β may decrease with mural cell maturation (Benjamin *et al*, 1998; Hellstrom *et al*, 1999), we tested whether ectopic p19^{Arf} modulated Pdgfr β expression in cultured 10T1/2 cells. Pdgfr β expression decreased after transduction with *Arf*-encoding retrovirus (Figure 6A, lanes 4–6 versus 1–3). Decreased Pdgfr β protein on day 3 in *Arf*- versus

Figure 6 Downregulation of *Pdgfrβ* mRNA and protein by p19^{Arf}. (A) Representative Western blots for indicated proteins in 10T1/2 cells transduced with *Gfp*- or *Arf*-expressing retrovirus. (B) Representative Northern blot (top) and phosphoimage densitometry (bottom) for *Pdgfrβ* and β -Actin in 10T1/2 cells transduced with retrovirus and harvested as indicated. (C) Representative Western blots for indicated proteins and cell cycle analysis of 10T1/2 cells cultivated for 3 days as a subconfluent (SC) or confluent (C) monolayer.

Gfp-transduced cells correlated with decreased mRNA (Figure 6A, lane 6 versus 3 and Figure 6B, lane 4 versus 3) while *Pdgfrβ* protein and mRNA were similar in *Arf*- versus *Gfp*-transduced cells on day 1 (Figure 6A, lane 4 versus 1 and Figure 6B, lane 1 versus 2). Dampening of *Pdgfrβ* expression was not simply secondary to the fact that *Arf*-transduced cells were growth arrested because *Pdgfrβ* expression was similar in proliferating 10T1/2 cells versus those arrested by confluence (Figure 6C).

We used genetically engineered MEFs to determine whether *Arf*-dependent arrest of Pdgf-B-driven cell proliferation and downregulation of *Pdgfrβ* depended on *p53*, which is

intact in 10T1/2 cells (Syljuasen *et al*, 2001). First, we verified that the effects of ectopic *Arf* expression on *Pdgfrβ* were similar in *Arf*^{-/-} MEFs and 10T1/2 cells (Figure 7A, lanes 1 and 3 versus 2 and 4). The apparent difference in the kinetics of *Pdgfrβ* downregulation in 10T1/2 cells versus *Arf*^{-/-} MEFs likely reflects the fact that additional factors can control its expression in cultured cells (see, for example, Vaziri and Faller, 1995). In contrast to these cells, ectopic p19^{Arf} did not downregulate *Pdgfrβ* in *Arf*^{-/-}, *p53*^{-/-} (DKO) MEFs, nor did it block Pdgf-B-mediated cell proliferation on day 3, despite high transduction efficiency and p19^{Arf} expression (Figure 7B, lanes 1 and 3 versus 2 and 4; some data not shown). Interestingly, concurrent loss of *Mdm2* restored the capacity of ectopically expressed *Arf* to dampen *Pdgfrβ* in *Arf*^{-/-}, *Mdm2*^{-/-}, *p53*^{-/-} (TKO) MEFs (Figure 7C, lanes 1, 3, and 5 versus 2, 4, and 6) and to block cell proliferation stimulated by Pdgf-B (Figure 7D, lanes 1–3 versus 4–6). Our findings indicated that *Arf*-dependent reduction in *Pdgfrβ* could be achieved independent of *p53* in TKO MEFs and suggested that the presence of endogenous *Mdm2* might interfere with p19^{Arf} when expressed in DKO MEFs.

p19^{Arf} blocks *Pdgfrβ* signaling to limit vitreous cell accumulation

We considered whether endogenous p19^{Arf} was associated with decreased expression of *Pdgfrβ*. A strict correlation between the presence of p19^{Arf} and the level of *Pdgfrβ* was not always observed in serial-passed wild-type and *Arf*^{-/-} MEFs (AC Martin and SX Skapek, unpublished). To better assess this *in vivo*, we determined whether the presence of one functional *Arf* allele correlated with loss of *Pdgfrβ* in the vitreous of E13.5 *Arf*^{Gfp/+} embryos. At this stage, nearly all *Gfp*-positive vitreous cells also expressed *Pdgfrβ* in *Arf*^{Gfp/Gfp} mice while *Arf*^{Gfp/+} mice had significantly fewer double-positive cells ($P=0.001$) (Figure 8A and B). This decrease was offset both by more *Pdgfrβ*-positive cells lacking detectable *Arf* expression and by more *Arf*-expressing cells with no detectable *Pdgfrβ* (schematically depicted in Figure 8C). Increased *Pdgfrβ* single-positive cells in heterozygous animals may be due to dampening of the *Arf* promoter in cells with functional p19^{Arf} or it may simply reflect lower *Gfp* protein in mice heterozygous for the reporter gene. Increased *Gfp*-positive (*Arf*-expressing), *Pdgfrβ*-negative cells in *Arf*^{Gfp/+} mice supports the concept that endogenous p19^{Arf} dampens *Pdgfrβ* *in vivo*. The relatively small number of *Gfp*-positive, *Pdgfrβ*-negative cells may be due to suboptimal repression of the receptor as *Arf* heterozygous mice have subtle defects in mural cell accumulation (Figure 3). Alternatively, it could reflect 'disappearance' of *Arf*-expressing cells if the *Arf* promoter decreases with further mural cell maturation.

To establish a causal relationship between *Pdgfrβ* and vitreous cell accumulation, we tested whether loss of *Pdgfrβ* lessened the phenotype in *Arf*^{-/-} embryos. We analyzed *Arf*^{-/-} embryos with heterozygous or homozygous *Pdgfrβ* deficiency at E14.5 and E15.5 because *Pdgfrβ*^{-/-} embryos manifest developmental defects after E16 (Soriano, 1994). The average number of vitreous cells in *Arf*^{-/-}, *Pdgfrβ*^{-/-} embryos (1.64 ± 1.11 cells/2500 μ m²) ($n=10$) was significantly decreased as compared to *Arf*^{-/-}, *Pdgfrβ*^{+/-} embryos (7.46 ± 1.40 cells/2500 μ m²) ($n=8$) ($P<0.001$) (Figure 8D). These findings indicated that *Pdgfrβ* was

Figure 7 Downregulation of Pdgfr β by p19^{Arf} can occur in the absence of Mdm2 and p53 in MEFs. (A–C) Representative Western blots for indicated proteins in Arf^{-/-} (A), Arf^{-/-}, p53^{-/-} (B), and Arf^{-/-}, Mdm2^{-/-}, p53^{-/-} (C) MEFs transduced with Gfp- or Arf-expressing retrovirus. (D) Quantitative analysis of the relative fraction of cells that are BrdU positive in Arf^{-/-}, Mdm2^{-/-}, p53^{-/-} MEFs transduced with the indicated retrovirus and harvested on day 2 following 1.5 h exposure to 5 or 50 ng/ml Pdgf-B. Results are presented as percent (and standard deviation) of triplicate samples from a representative experiment.

required to drive vitreous cell accumulation in the absence of Arf.

Discussion

Regulation of the Pdgfr-B/Pdgfr β axis is particularly important in the vitreous because excess cells accumulate in mice expressing a mutated form of Pdgfr-B that lacks the retention motif to limit its mitogenic effects (Lindblom *et al*, 2003). Our experiments have addressed whether the Arf tumor suppressor gene product regulates this signaling pathway. We have established that Arf was expressed in and blocked the proliferation of mural cells within the vitreous early during its development. That the phenotype in Arf^{-/-} embryos was corrected in those also lacking Pdgfr β demonstrated a requirement for p19^{Arf} to control Pdgfr β -dependent effects in the eye. Without p19^{Arf} protein, nearly all of the vitreous cells expressing the Arf promoter also expressed Pdgfr β while the number of Arf-expressing cells with detectable Pdgfr β decreased in the presence of one normal Arf allele. Arf expression in cultured pericyte-like cells and MEFs suppressed Pdgfr β and blocked Pdgfr-B-driven proliferation in a manner separable from Mdm2 and p53. Taken together, our findings show that Arf plays an essential role to check Pdgfr β signals driving vitreous accumulation (Figure 8E).

In vivo and *in vitro* analyses indicated that one mechanism by which p19^{Arf} might accomplish this is by downregulating Pdgfr β expression. It must be emphasized, though, that p19^{Arf} can control proliferation by multiple, p53-dependent and -independent mechanisms operating with differing kinetics (Weber *et al*, 2000). Indeed, we observed this in 10T1/2 cells in which Arf-mediated cell cycle arrest preceded Pdgfr β downregulation (compare Figures 5D and 6A). The early arrest was likely secondary to p53-dependent induction of the cyclin-dependent kinase inhibitor p21^{Waf1} evident on day 1 (Supplementary Figure 1). How Arf-dependent down-

regulation of Pdgfr β relates to other recognized p53-independent functions of p19^{Arf} is not yet known. Moreover, whether downregulation of Pdgfr β is the sole mechanism by which p19^{Arf} functions in the eye must be critically evaluated, for example, by testing if transgenic expression of Pdgfr β under the control of the Arf promoter recapitulates the Arf^{-/-} eye phenotype.

Certain mechanistic aspects of Arf-dependent dampening of Pdgfr β signaling merit emphasis. First, a number of observations allowed us to conclude that decreased Pdgfr β was not merely secondary to p19^{Arf}-dependent cell cycle arrest or decreased confluence as follows: (1) Pdgfr β did not measurably change in proliferating cells versus those arrested by confluence (Figure 6C) nor did it change in Gfp-expressing cells over the course of an experiment (Figure 6A, lanes 1–3), even though the S-phase fraction decreased due to changes in confluence (Figure 5D, compare lanes 1 and 5). (2) Pdgfr β expression was dampened by p19^{Arf} on day 3 (Figure 6A, lane 6 versus 3) at a point when the S-phase fraction was similar in Gfp- and Arf-transduced cells (Figure 5D, compare lanes 7 and 5). (3) As mentioned above, 10T1/2 cells transduced with Arf are arrested and less confluent than Gfp-transduced cells on day 1, but little difference in Pdgfr β is evident (Figure 6A, lane 4 versus 1). Second, the parallel decrease in Pdgfr β mRNA and protein (Figure 6A and B) suggested that p19^{Arf} influenced its transcription or message stability. The former possibility is intriguing because p73, a p53-related protein, represses the Pdgfr β promoter when expressed in cultured NIH 3T3 cells but p53 cannot (Hackzell *et al*, 2002). A functional relationship between p19^{Arf} and p73, though, has not been established. Finally, depression of Pdgfr β in Arf-expressing MEFs could occur in the absence of p53 as long as the MEFs also lacked Mdm2. That endogenous Mdm2 can hinder p19^{Arf} in p53^{-/-} cultured MEFs was previously proposed (Weber *et al*, 2000), but its significance for eye development is not clear.

Figure 8 Pdgfr β expression correlates with *Arf* status in the developing mouse vitreous. (A) Representative confocal photomicrographs of cryostat sections from E13.5 *Arf^{Gfp/+}* (a) and *Arf^{Gfp/Gfp}* (b) mice following dual immunofluorescence staining for Gfp (green) and Pdgfr β (red). Note some cells express only Gfp (arrowhead) or only Pdgfr β (arrow) in the heterozygous eye. (B) Quantitative analysis of Gfp- and Pdgfr β -expressing cells stained as in (A), expressed as average percent (and standard deviation) of single- or double-positive cells. (C) Schematic diagram depicting potential model in which p19^{Arf} may both (1) check the expression of its own promoter to block the accumulation of Arf-, Pdgfr β -double-positive cells and (2) promote the subsequent downregulation of Pdgfr β expression with mural cell maturation. (D) (a) Documentation of *Arf* and *Pdgfr β* genotype in embryos and (b) photomicrographs of H&E-stained sections of E14.5 and E15.5 mouse embryo eyes of the indicated genotypes. Data are representative of 10 eyes of each genotype examined from three separate litters. (E) Schematic diagram showing that p19^{Arf} blocks Pdgfr β -driven vitreous cell accumulation in a manner that is not strictly Mdm2 or p53 dependent. Original magnification: $\times 400$ (A) and $\times 100$ (D).

We previously established that *Arf* is required for VHP regression in the mouse and that its failed involution causes secondary lens and retina defects that mimic the human eye

disease, persistent hyperplastic primary vitreous (PHPV) (McKeller *et al*, 2002). The disease has apparently complete penetrance in *Arf^{-/-}* mice and complete absence in *Arf^{+/+}*

mice despite the subtle embryonic phenotype reported here. Whether human *ARF* defects contribute to PHPV pathogenesis is not yet proven. Consistent with our mouse model, eye abnormalities were not reported in two small kindreds with *ARF* exon 1 β haploinsufficiency (Randerson-Moor *et al*, 2001; Hewitt *et al*, 2002). Abnormalities in p16^{Ink4a}, also encoded at the *Arf/Ink4a* locus, do not result in PHPV in *Ink4a*^{-/-} mice (Martin *et al*, 2004) or in humans with heterozygous or homozygous *INK4A* mutations (Yakobson *et al*, 2000; Della *et al*, 2001; Pavel *et al*, 2003).

The role of *p53* in VHP involution has been harder to understand. Certain inbred *p53*^{-/-} mice develop a PHPV-like process with variable severity and penetrance (Reichel *et al*, 1998; Ikeda *et al*, 1999). We and others reported that *p53*^{-/-} mice in a mixed C57BL/6 $\times$ 129/Sv genetic background usually have normal eyes (Ikeda *et al*, 1999; McKeller *et al*, 2002) and we proposed that p19^{Arf} might regulate parallel, *p53*-dependent and -independent pathways to control vitreous cell accumulation (Martin *et al*, 2004). Dampening of Pdgfr β signaling may represent one such *p53*-independent pathway.

That we did not observe *Arf* promoter activity widely in mural cells enveloping other vessels implies that *Arf*-dependent modulation of mural cell biology might somehow contribute to the 'transient' nature of the VHP. The simplest mechanism by which p19^{Arf} might destabilize this vasculature is by limiting mural cell coverage. Integrity of the underlying vessels would then depend on other sources of angiogenic factors like Vegf derived from the retina (Martin *et al*, 2004) or lens (Mitchell *et al*, 1998). Dampening of Pdgfr β signals by p19^{Arf} might also influence other aspects of mural cell biology like their expression of angiogenic factors to more directly destabilize the vessels. Either concept has potential implications for tumor suppression by p19^{Arf} because there is some evidence that pericytes influence tumor vascular biology (Benjamin *et al*, 1999; Bergers *et al*, 2003; Gee *et al*, 2003; Jain and Booth, 2003). Conceivably, *Arf*-dependent regulation of perivascular cells—to prevent their accumulation or alter their expression of angiogenic factors—might destabilize developing tumor vasculature and prevent the 'angiogenic switch' required for tumorigenesis (Hanahan and Folkman, 1996).

Perhaps our most surprising finding was the localized expression of *Arf* in the developing eye. Induction by excessive mitogenic or oncogenic signals does not fully explain its selective expression in a subset of cells in the relatively homogenous embryonic vitreous. The temporal and spatial correlation of Pdgfr β and *Arf* expression suggest that Pdgf-dependent signals may induce the *Arf* promoter. But these are likely not sufficient because Pdgf-B is generally required for mural cell accumulation in developing blood vessels (reviewed in Hoch and Soriano, 2003) whereas *Arf* is not widely expressed in the embryo (AC Martin and SX Skapek, unpublished data). Elucidating the specific developmental cues driving *Arf* expression may help predict settings where it has tumor suppressor effects: if *Arf* is only induced by certain mitogens or in particular cells or cellular environments, its tumor suppressor properties may be similarly restricted. Understanding how it is controlled during eye development may ultimately lead to ways to re-activate its expression in that subset of human cancers where the gene is intact but its expression is repressed.

Materials and methods

Mice and cell lines

Mice in which *Arf* exon 1 β was inactivated (Kamijo *et al*, 1997) or replaced by a reporter gene encoding Gfp (Zindy *et al*, 1998) were bred to maintain a mixed C57BL/6 $\times$ 129/Sv genetic background. Pdgfr β -deficient mice (Soriano, 1994) were provided by P Soriano (Fred Hutchinson Cancer Research Center). Genotyping methods have been described (Soriano, 1994; Kamijo *et al*, 1997; Zindy *et al*, 2003). Animal studies were approved by the St Jude Children's Research Hospital Animal Care and Use Committee.

Mouse 10T1/2 pericyte-like cells (CCL-226) (ATCC) were maintained in DMEM with 10% FBS supplemented with L-glutamine and penicillin/streptomycin (pen/strep). Primary MEFs from wild type; *Arf*^{-/-}; *Arf*^{+/-}; *p53*^{-/-}; and *Arf*^{+/-}, *Mdm2*^{-/-}, *p53*^{-/-} mice (provided by Charles J Sherr and Martine F Roussel, St Jude Children's Research Hospital) were cultivated as previously described (Zindy *et al*, 1997).

Histology studies

Embryos removed from euthanized, pregnant mice were fixed in 4% paraformaldehyde (PFA) in PBS and processed for paraffin or cryostat sections as described previously (Martin *et al*, 2004). Immunofluorescence staining for Gfp in *Arf*^{+/-Gfp} and *Arf*^{Gfp/Gfp} mouse embryo eyes was performed as previously described (Martin *et al*, 2004). Dual immunofluorescence staining was carried out using rabbit α -Gfp and either rat α -CD31 or goat α -Pdgfr β . Staining for p19^{Arf} in wild-type embryos was accomplished with a rat monoclonal antibody directed against mouse p19^{Arf} (Bertwistle *et al*, 2004). Primary antibodies were detected using species-specific secondary antibodies (Jackson Immunoresearch Laboratories). Cell proliferation was assessed by staining for Ki67 proliferating cell nuclear antigen. Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Digital photomicrographs were obtained with an Olympus BX60 microscope equipped with a SPOT RT Slider camera (Diagnostic Instruments) or with a Zeiss 510 NLO multiphoton/confocal laser-scanning microscope. (Additional details for histology studies are provided in Supplementary data.)

Quantitative analysis of vitreous cell density in embryos of different genotypes was performed by counting the total number of cells (excluding nucleated red blood cells) within the vitreous in comparable midline eye sections of stained slides. Cell number was presented relative to the area of the vitreous. TUNEL-, Ki67-, and Pdgfr β -positive cells were presented as a percent of the total number of vitreous cells. Pdgfr β expression data were determined using confocal microscopy, whereas others were assessed using light microscopy ($\times$ 400 magnification in all cases). Summary data (Figures 2B, D, and 8B) are from analyses of 6–17 embryos from six timed pregnancies, presented as mean value and standard deviation. Statistical significance was assessed by *t*-test.

Retroviral production

Retrovirus was produced as described previously (Zindy *et al*, 1998), using the control pMSCV-IRES-Gfp plasmid or one containing mouse *Arf* cDNA 5' to the IRES (provided by Martine F Roussel, SJCRH).

Cell proliferation analysis

Subconfluent 10T1/2 cells or MEFs were infected with *Arf*-encoding or control retrovirus (Zindy *et al*, 1998). At 16 h after infection, the culture media were replaced. After 20 h, 5 or 50 ng/ml of Pdgf-B (R&D Systems) was added to the media; this point represents day 0 for experiments using transduced cells. Pdgf-B was replenished each day. Cells from triplicate wells were removed and counted using a hemocytometer on days 1, 2, and 3. Cell cycle phase was assessed in *Arf*- or control-transduced cells by flow cytometry (FACScan, Becton Dickinson) of Gfp-sorted cells that were stained with propidium iodide (PI). For cell cycle analyses, Pdgf-B was added 1.5 or 24 h prior to analysis. S phase was also monitored by cultivating cells in 10 μ M BrdU followed by staining with the APC-BrdU Flow kit (BD Biosciences Pharmingen).

Gene expression studies in cultured cells

Western analyses were conducted using subconfluent cells, transduced with *Arf*-encoding or control retrovirus and harvested

on days 1, 2, and 3 for protein extraction as described (Zindy *et al*, 1998). Proteins were detected by chemiluminescence using α -Pdgfr β , α -p19^{Arf}, or α -Hsc70 primary with species-specific secondary antibodies. The Northern-Max kit (Ambion) was used to perform Northern blotting of total RNA isolated from 10T1/2 cells harvested at days 1 and 3. The blot was probed with ³²P-labeled Pdgfr β and β -actin probes and scanned using Storm Phosphorimager (Amersham Pharmacia Biotech). Pdgfr β mRNA abundance was normalized using β -actin transcript. mRNA extracted from cultured 10T1/2 cells was used for RT-PCR. (See Supplementary data for details of Western blotting, PCR primer design, and cDNA probe synthesis.)

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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