MECHANISMS OF $p19^{ARF}$- MEDIATED REGULATION OF PERIVASCULAR CELL BIOLOGY DURING MAMMALIAN EYE DEVELOPMENT

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I dedicate this work to my mother, my father, my brother and in memory of my grandparents for teaching me the value of family and a strong education.

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Since its discovery in 1995, p19^{Arf} has been under critical interrogation for its role as a potent cell cycle regulator and tumor suppressor. In the last decade, there has been considerable evidence describing an essential function for p19^{Arf} during mammalian eye development. In this context, p19^{Arf} is required for the ultimate involution of the hyaloid vasculature system that exists in the primary vitreous space and serves to nourish the lens and retina. Knock-out mouse models for p19^{Arf} demonstrate that in the absence of *Arf,* there is an abnormal accumulation of cells that persist into the adult secondary vitreous and cause detrimental ocular defects including blindness, retinal detachment and lens opacity. It has been further demonstrated that p19^{Arf} enacts a dual mechanism to inhibit the accumulation of the perivascular cells that occupy the vitreous space during development and lead to the clearing of these cells followed by eventual involution of the underlying vasculature system. Platelet-derived growth factor receptor β (Pdgfrβ) is required for the accumulation of cells in the absence of *Arf* and while it is clear that p19^{Arf} utilizes a

p53-dependent mechanism to inhibit *Pdgfrβ* transcription, the mechanism by which it can inhibit Pdgfrβ protein in the absence of p53 is not well defined. Further, the biological consequences of *Arf* expression have, to date, only been studied in a context in which *Arf* is not normally expressed, such as during tumor progression and culture shock. This work addresses these two open questions. First, I will discuss a novel capacity for $p19^{Arf}$ to employ microRNAs outside of the p53 pathway to lead to repression of Pdgfrβ protein. Next I will describe an *ex vivo* cell culture system to study unexplored facets of *Arf* biology in a context in which it is endogenously expressed.

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Iqbal, N.S, Mei, J, Devitt, C.C, Xu, L, Liu, J, Skapek, S.X. Through the eyes of *Arf*: $p19^{Arf}$ controls cell proliferation in the primary vitreous. Mechanisms and Models of Cancer, Cold Spring Harbor Laboratories, August 2014.

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LIST OF DEFINITIONS

ARF/ $p19^{Arf}$ – Alternate reading frame

ANG – angiopoietin

BrdU – 5-bromo 2'-deoxyuridine

cDNA – complementary deoxyribonucleic acid

CTL – control

EMT – epithelial to mesenchymal transition

FACS – fluorescence activated cell sorting

FPKM – fragments per kilobase of exons per million fragments mapped

GFP – green fluorescent protein

GO – gene ontology

HA – hyaloid artery

HVE – hyaloid vascular endothelium

HVS – hyaloid vascular system

KEGG – Kyoto encyclopedia of genes and genomes

MDM2 – mouse double minute 2

MEF – mouse embryonic fibroblast

PBS – phosphate buffered saline

PDGF – platelet derived growth factor

PFV – persistent fetal vasculature

PHPV – persistent hyperplastic primary vitreous

PVC – primary vitreous cells

PVR – proliferative vitreoretinopathy

qRT-PCR – quantitative reverse-transcriptase polymerase chain reaction

RISC – RNA induced silencing complex

RFP – red fluorescent protein

RNA-seq – RNA sequencing

RPE – retinal pigment epithelium

shRNA – small hairpin ribonucleic acid

TGFβ – transforming growth factor beta

TUNEL – terminal deoxy-nucleotidyl transferase mediated nick-end labeling

TKO – triple knockout

TVL – tunica vasculosa lentis

UTR – untranslated region

VEGF – vascular endothelial growth factor

VHP – vasa hyaloidea propria

WT – wild type

CHAPTER 1:

An Introduction

Mammalian Eye Development

The vascular primary vitreous, a transitory structure that exists between the lens and retina, functions to satisfy the metabolic needs of the developing eye (Goldberg, 1997; Ito and Yoshioka, 1999). While much has been investigated tracking the evolution of eyes, the appearance and selective pressures concerning the development of the vitreous space are sparse and unclear. The most primitive structures resembling light-sensing organs can be found in animals that appeared over 500 million years ago. The explosion of multicellular organisms in the Cambrian period led to the development from the earlier primitive photoreceptor system to a more complex organ for the purposes of vision. It is not until later in vertebrate evolution that the presence of the optic cup, the emergence of the lens and appearance of eyelids that a vitreous-like structure is observed (reviewed in (Lamb et al., 2009)). In mammals, the developing vascular primary vitreous is composed of arteries termed the hyaloid vascular network which is formed by a continuous layer of endothelial cells, juxtaposed with a discontinuous layer of pericytes and hyalocytes within the vitreous matrix (Zhu et al., 1999). Once developed, the network of vessels regress giving rise to the largely acellular and permanent secondary vitreous, providing a clear path for light.

Timing of hyaloid vessel development and regression

The hyaloid vascular network includes the hyaloid artery (HA), the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL) (Goldberg, 1997; Ito and Yoshioka, 1999). The HA enters the vitreous through the fetal fissure and branches to generate the VHP and finally anastomoses with the dense TVL network that encapsulates the developing lens (Goldberg, 1997; Ito and Yoshioka, 1999) (Figure 1.1). These vessels consist of only arteries whereas veins of the choroidal vasculature system at the anterior edge of the optic cup allow for removal of metabolic waste (Saint-Geniez and D'Amore, 2004). In mice, this network begins developing at E10.5 and is fully developed by E18 (Ito and Yoshioka, 1999). Clearance of these vessels begins early postnatally but does not complete until after the first few weeks of birth. The VHP regresses between postnatal day 12 and 16 in mice while the TVL and HA are gradually lost between P14 and P30 (Ito and Yoshioka, 1999). In humans, appearance of the hyaloid artery is evident at the 10mm stage or fourth week of development and the entire embryonic system is cleared before birth (Goldberg, 1997). The regression of the hyaloid vascular system is an imperative event in mammalian development as it produces a transparent vitreous body which is required for proper vision. While the timing of regression is well defined, mechanisms regulating the clearing of this network remain largely unclear. It is known that

apoptosis, expression of antiangiogenic factors, inhibition of survival factors and macrophages all play a role in guiding hyaloid vascular regression.

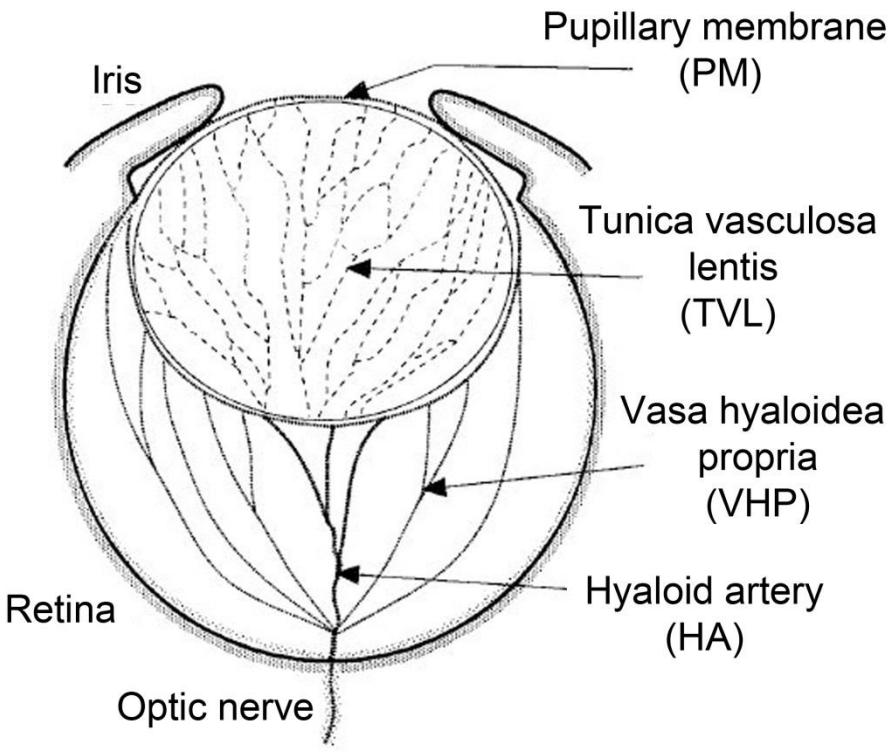

Figure 1.1: Schematic diagram of the intraocular vessels of the mouse eye. The hyaloid artery branches to the vasa hyaloidea propria (VHP) and tunica vasculosa lentis that envelopes the lens. The pupillary membrane sits at the anterior part of the lens. These vessels sequentially regress during postnatal mouse development and before birth in humans (Modified from (Ito and Yoshioka, 1999)).

Mechanisms of hyaloid vascular regression

Apoptosis is required for the remodeling of many tissues and organs during normal animal development including digit individualization, maintenance of optimal brain function in nervous system development and elimination of transient blood vessel networks such as the hyaloid vascular system (Hernandez-Martinez and Covarrubias, 2011; Kim and Sun, 2011; Zhu et al., 1999). Terminal deoxy-nucleotidyl transferase mediated nick-end labeling (TUNEL) staining shows that apoptosis of the hyaloid vascular endothelium (HVE) begins to occur as early as E17.5 in mouse and continues through P5 (Mitchell et al., 1998). Further, deletion of *bcl-2* pro-apoptotic family members *bak* and *bax* results in retention of the mouse fetal vascular bed in the primary vitreous, suggesting apoptosis is required for proper involution of hyaloid vessels (Hahn et al., 2005).

Hyalocytes, specialized macrophages of the vitreous, have been suggested to induce apoptosis of endothelial cells. Specific ablation of macrophages in this environment results in failure of regression (Lang and Bishop, 1993). Further, hyalocytes transiently upregulate the expression of *Ninjurin* which increases cell-cell contacts between the macrophages and endothelial cells while simultaneously stimulating the expression of Wnt7b within

the macrophages (Lee et al., 2009; Lobov et al., 2005). In conjunction with modulating the expression of *Angiopoietins (Ang)* on pericytes such that Ang1 is downregulated and Ang2 expression is increased, vitreous macrophages mediate the induction of endothelial cell death (Diez-Roux and Lang, 1997).

Of course the reciprocal mechanism to apoptosis required for the clearing of this space is inhibition of survival signals. Vegf is expressed in various components of the developing eye including the lens and acts as a growth factor to initiate ocular angiogenesis (Mitchell et al., 1998). Physical separation of Vegf from flk-1 receptor expressing cells within the TVL occurs during normal hyaloid regression and disruption of Vegf levels leads to abnormalities in vitreous vasculature (Mitchell et al., 1998; Rutland et al., 2007). This is also in part mediated by platelet-derived growth factor (Pdgf) signaling. Expression of Pdgf receptor β (Pdgfrβ) on pericytes and concomitant secretion of PDGF-B ligand from endothelial cells is critical for accumulation of pericytes and their localization to blood vessels (Hellstrom et al., 1999; Hoch and Soriano, 2003). The importance of Pdgf signaling to pericytes is made clear by the fact that deletion of Pdgfrβ or PDGF-B results in lethality of mice due to hemorrhaging caused by lack of pericytes (Leveen et al., 1994; Lindahl et al., 1997; Soriano, 1994). Inappropriate Pdgf signaling is associated with ocular diseases and overexpression of PDGF-B under the control of the *nestin* promoter causes

delayed regression of the hyaloid vessels, among other ocular defects (Lei et al., 2010; Niklasson et al., 2010)

Regression of the hyaloid vasculature is critical for proper development of the vitreous and retina as well as other surrounding structures in the eye. Failure of these vessels to regress leads to severe detrimental defects in ocular anatomy and physiology. A debilitating pathology associated with vessel regression defects is persistent hyperplastic primary vitreous (PHPV), also known as persistent fetal vasculature (PFV).

Persistent hyperplastic primary vitreous

PHPV, or PFV, is a rare congenital disorder of the eye that develops as a consequence of vessel regression failure (Goldberg, 1997). PHPV is characterized by the presence of a fibrovascular mass adjacent to the lens and can be classified into anterior and posterior types depending on the location of the mass (Goldberg, 1997; Haddad et al., 1978). The aberrant retrolental mass can be surrounded by pigmented cells in certain situations and in severe forms can interact with the inner part of the neuroretina and cause retinal detatchment (Brue et al., 2012). While some cases of bilateral and inherited PHPV have been described, most cases are unilateral and sporadic suggesting somatic events

giving rise to the disease (Haddad et al., 1978; Lin et al., 1990; Shastry, 2009; Wang and Phillips, 1973; Yu and Chang, 1997). Additionally, PHPV is often associated with presence of other ocular defects and pathologies including micropthalmia, retinal folding, intraocular hemorrhage and cataracts (Goldberg, 1997; Haddad et al., 1978; Pollard, 1997). Currently, the standard of care for patients with PHPV is surgical removal of the mass. This is not always successful and can lead to further blindness (Shastry, 2009). In order to better understand PHPV and perhaps develop novel therapeutics, many mouse models have been generated that provide insight into the manifestations of this disease.

Mouse models of PHPV

Several genetic mouse models have clarified the mechanisms of normal primary vitreous regression and elucidated key factors that drive pathogenesis of PHPV. For example, conditional deletion of *Wnt* family member *Frizzled5* (*Fzd5*) in the retina, results in the formation of a highly pigmented hyperplastic primary vitreous and causes defects in retinal morphogenesis. This suggests a cell-nonautonomous role for *Fzd5* in regulating hyaloid system regression (Zhang et al., 2008). *Ang2* $^{-/-}$ mice display delayed and incomplete development of the retinal vascular bed as well as defects in hyaloid vascular regression implying a role for Ang2 and Tie2 signaling in promoting retinal angiogenesis in addition to

vascular regression (Hackett et al., 2002). Overexpression of Vegf in the lens leads to endothelial cell hyperplasia as well as persistence of the intraocular vessels (Ash and Overbeek, 2000). Other models that can lead to defects in hyaloid vessel regression include *Fzd4* $^{-/-}$, *norrin* $^{-/-}$ *and collagenXVIII* $^{-/-}$ mice (Fukai et al., 2002; Richter et al., 1998; Xu et al., 2004).

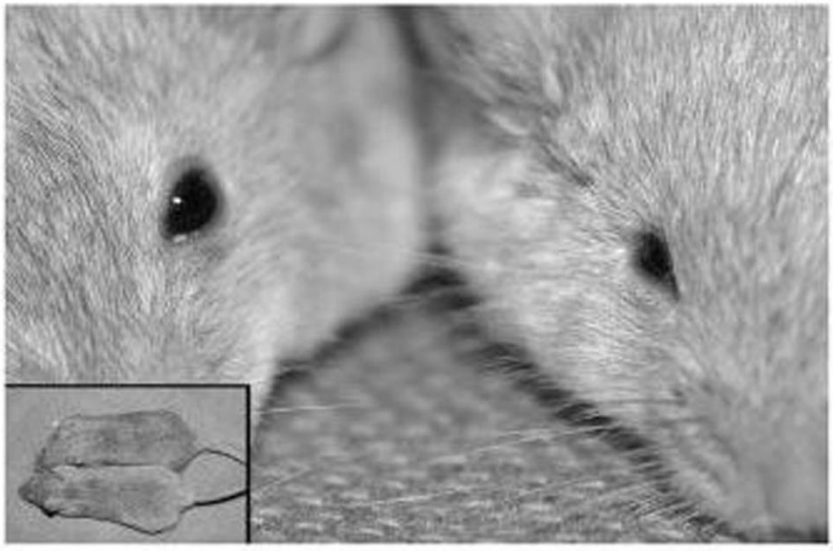

Figure 1.2: *Arf* $^{-/-}$ mice display signs of PHPV. *Arf*-null mice (seen on right side of image) have smaller eyes than their WT littermates (on left). Micropthalmia is a common indication of PHPV. (Adapted from (Martin et al., 2004))

Deletion of *Arf*, an upstream regulator of p53 signaling, also results in an ocular phenotype that closely resembles aspects of PHPV. *Arf* $^{-/-}$ mice have smaller eyes than their WT counterparts, which was the initial indication that this tumor suppressor may be associated with PHPV pathology (Figure 1.2). Indeed,

closer examination of their eyes revealed the presence of a pigmented hyperplastic mass in the vitreous space that persists into adulthood (McKeller et al., 2002). The phenotype observed in the *Arf* $^{-/-}$ mice strongly recapitulates what is observed in human patients with posterior PHPV (Table1.1). However, there are two distinctions: *Arf* $^{-/-}$ mice display a uniformly penetrant, bilateral pathology where as in humans, PHPV is often varied and unilateral (Goldberg, 1997; Haddad et al., 1978). However, graded somatic deletion of *Arf* by generating mouse chimeras more closely reflects the full range of disease severity seen in humans (Mary-Sinclair et al., 2014). These data and others suggest that *Arf* is imperative for normal eye development and its abrogation leads to the progression of an ocular pathology which models aspects of the human eye disease PHPV. Because *Arf* is largely studied in the context of cancer progression, in order to better gain insight into normal developmental functions it is important to clearly discuss what is known about this tumor suppressor.

Clinical manifestations of posterior PHPV	Phenotype of *Arf* $^{-/-}$ mice
Vascular tissue at posterior surface	✔
Contracture of retrolental tissue	✔
Micropthalmia	✔
Persistence of hyaloid artery	✔
Retinal detachment	✔
Lenticular opacification	✔

Table 1.1: Clinical manifestations of human PHPV observed in *Arf* $^{-/-}$ mice

p19^{Arf} Expression and Function

An alternate gene product from the *Cdkn2a* locus was first described in adjacent reports published in a 1995 issue of *Cancer Research.* Both groups were interested in identifying a melanoma susceptibility gene within the human 9p21 region and predicted α and β forms of *p16Ink4*, one of the genes encoded at this locus (Mao et al., 1995; Stone et al., 1995). A separate publication from the Sherr group, later that year, was the first to compellingly demonstrate that the β transcript produced from this gene encodes for a functional, 169 amino acid protein, that is distinct from the product arising from the α transcript, but also capable of inhibiting cell proliferation (Quelle et al., 1995). This marked the beginning of a series of manuscripts exploring the tumor suppressive functions of the infamous and quite elusive protein, p19^{Arf}.

The Cdkn2a locus

The *Cdkn2a* gene on mouse chromosome 4 is ostensibly unassuming. It spans a mere 20kb of the genome, consists of only four exons and is largely silenced during normal animal development. Closer examination, however, reveals the truly unique architecture of this locus. In humans, *CDKN2A* represents one of only three confirmed dual coding genes from which there are

two proteins produced. Unlike *GNAS1* and *XBP1*, *CDKN2A* uses alternative splicing to generate two distinct protein products that bear no amino acid homology (Figure 1.3). Because *p16Ink4a* was identified first, it is arbitrarily designated the constitutive gene while *Arf* (*Alternate Reading Frame*) gets its name from being the second transcript within this dual coding pair.

p16Ink4a transcription begins at exon 1α and continues through exons 2 and 3. This generates a 507 bp mRNA which yields a 16-18kda protein highly homologous to $p15^{Ink4b}$ produced from the neighboring *Cdkn2b* gene. *Arf* transcription starts at exon1β and splices to the identical acceptor site at exon2 as *p16Ink4a*. Because there is a shift in the open reading frame due to the mutually exclusive AUG sequences within each exon1, a single locus is able to produce two distinct products. The majority of $p19^{Arf}$ sequence and function can be attributed to exon1β and indeed, deletion of exon1β is sufficient to disrupt $p19^{Arf}$ function without affecting $p16^{Ink4a}$ (Kamijo et al., 1998; Quelle et al., 1997; Weber et al., 1999).

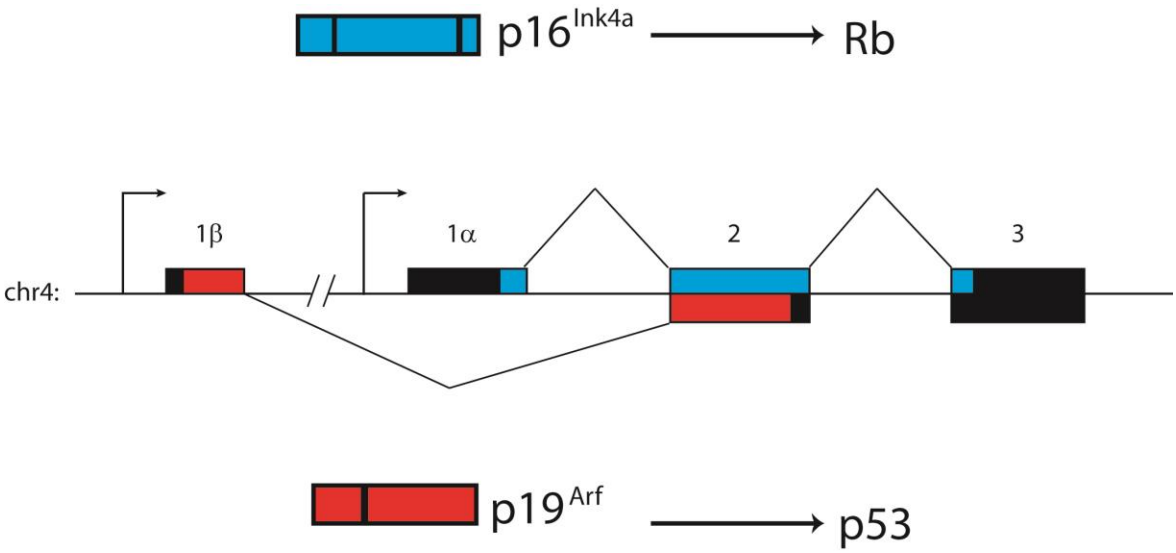

Figure 1.3: Schematic diagram of *Ink4a/Arf* locus. The *Ink4a/Arf* locus on mouse chromosome 4 encodes for $p16^{Ink4a}$ and $p19^{Arf}$. *Arf* transcription starts at exon1β while *p16Ink4a* transcription begins at exon1α. Both genes splice to the same acceptor site at exon2 but in alternate reading frames so that they do not share any amino acid homology. The gene products from this locus indirectly regulate the activities of Rb and p53.

The most enigmatic feature of the *Cdkn2a* locus is its evolutionary history. Not only is it a fairly recent addition to the genome, it is difficult to explain the propensity this region has for accruing mutations. *Arf* is highly susceptible to nonsynonymous mutations suggesting that it is under diversifying selection, incongruous to the fact that *Ink4a* largely accumulates synonymous mutations (Szklarczyk et al., 2007). The rapid and rather asymmetric evolution observed at this locus is contradicted by the fact that the gene products encoded here are at the nexus of two critical signaling networks – the Retinoblastoma (Rb) and p53 pathways.

Cdk2na and cancer

The critical function of the *Ink4a/Arf* locus is made abundantly clear in human malignancy, as loss of function of these genes or downstream components of the signaling networks is evident in most if not all cancers (Hainaut et al., 1997; Ruas and Peters, 1998). In fact, this locus was first discovered by cancer biologists and is still largely studied in the context of tumor progression. Disruption of either $p16^{Ink4a}$ or $p19^{Arf}$ does not cause gross anatomical defects during mouse development but results in spontaneous tumor formation in adult mice (Kamijo et al., 1999; Zindy et al., 1997a). $p19^{Arf}$ null mice

are more prone to murine cancer while $p16^{Ink4a}$ seems to be more often silenced in human tumors versus $p19^{Arf}$ alone.

That there was a driving force to select for two critical cell cycle regulatory genes to exist in such close proximity suggests that, either, there is a requirement for these genes to respond to signaling events in a coordinated manner or tumor formation is really not a strong pressure to select against this type of dependent transcriptional relationship. Further, inactivation of this locus in human cancers usually occurs such that the entire locus is lost, making it difficult to determine if selection against tumor suppression derives from the individual or both genes (reviewed in (Sherr, 2001a, b)). To better understand the evolutionary prerogative of the *Arf* gene, one must consider the first organisms where *Arf* is present as well as discuss the current understanding of the ancient and developmental functions of this infamous tumor suppressor.

Arf expression in other organisms

The oldest known organism interrogated for the presence of an *Arf*-like gene is the puffer fish. *Fugu rubripes* is a poisonous, ray-finned fish with a compact genome of only 365mb. Even though it is just a fraction of the size of the human genome, sequencing of *Fugu* revealed that approximately 75% of

protein-coding genes expressed in humans have orthologues in the puffer fish (Gilligan et al., 2002). In 2001, Jonathan Gilley and Mike Fried attempted to identify *Ink4a* and *Arf* orthologues in Fugu using degenerate PCR and probe hybridization methods. They were able to detect a single *Ink4* gene, suggesting that the duplication event giving rise to distinct *Ink4a* and *Ink4b* genes occurred sometime following the branching of the mammalian lineage from fish (Gilley and Fried, 2001). Unfortunately, an *Arf*-like gene was not identified and has not been detected in any other species of fish to date.

The literature currently suggests that the insertion of exon1β within the ancient *Ink4* locus occurred later in metazoan evolution, but before the duplication of *Ink4* (as it is detected in birds but not fish). Chickens (*Gallus gallus)* express a truncated form of *Arf* encoded entirely by exon1β but lack an equivalent exon1α. Moreover, chicken $p7^{Arf}$ is functionally capable of inducing cell cycle arrest, further corroborating evidence observed in mouse and human that exon1β is sufficient for essential, or at least known, canonical functions of Arf (Kamijo et al., 1998; Kim et al., 2003; Quelle et al., 1997; Weber et al., 1999).

Several points must be addressed about the current understanding of *Arf* in organisms outside of human and mouse. First, since evidence indicates that *Arf* does not come up until late in animal history and a p53-like gene has been

evolving for over one billion years, it is possible that the insertion of exon1β at the *Ink4* locus is a refinement of existing regulation to mediate p53-dependent functions and tumor suppression. Of course it is also conceivable that Arf, which does have p53-independent activities, was repurposed in mammals for improved regulation of this pathway. Second, chicken $p7^{Arf}$ indicates that exon2 is dispensable for Arf function but this leaves an open question about the selective pressures that led to the incorporation of exon2 in mammals. Did splicing to exon2 occur randomly and hence could have utilized any neighboring gene? Or is the use of *p16Ink4a* exon2 required for downstream effects of $p19^{Arf}$? Are there aspects of Arf biology that depend on sequence information from exon2? There is a wealth of knowledge that has been gained from use of human cell culture systems and mouse models to distinguish p53 dependent and independent functions of *Arf* that may help clarify the more puzzling aspects of Arf biology.

p53-dependent and independent functions of Arf

The most abundant descriptions of $p19^{Arf}$ functions are in the context of the p53 pathway. A relationship between these two tumor suppressors was first indicated with the observation that expression of the *Arf* transcript was markedly increased in *p53*-deficient BALB 3T3 cells (Quelle et al., 1995). Early papers

also showed that ectopic expression of *Arf* stabilizes p53 in addition to inducing p53-mediated cell cycle arrest (Kamijo et al., 1997; Pomerantz et al., 1998). Careful biochemistry has revealed that $p19^{Arf}$ physically associates with Mdm2 and blocks its E3 ubiquitin ligase activity to stabilize p53 and additionally causes nucleolar localization of Mdm2 (Honda and Yasuda, 1999; Weber et al., 1999). Stabilization of p53 elicits numerous anti-cancer events including the induction of pro-apoptotic and DNA repair pathways as well as cell cycle arrest (reviewed in (Ko and Prives, 1996)).

In the first compelling challenge to the prevailing model describing a seemingly linear pathway stemming from *Arf* activation, members of the Zambetti laboratory showed that ectopic expression of $p19^{Arf}$ in mouse embryonic fibroblasts (MEFs) lacking *Arf*, *Mdm2*, and *p53* (TKO MEFs) could still induce cell cycle arrest (Weber et al., 2000). 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 $p19^{Arf}$ has grown to include a) inhibition of ribosomal RNA processing; b) physical interactions with E2F1 and c-Myc to repress their *trans*-activating potential; c) blockade of the RelA subunit of NFκB; d) blunting of Pdgfrβ expression; e) promotion of p53-independent sumoylation of Mdm2, nucleophosmin, and other proteins; and

recently, post-transcriptional repression of Drosha, a microRNA processing enzyme (Datta et al., 2004; Eymin et al., 2001; Kuchenreuther and Weber, 2013; Qi et al., 2004; Rizos et al., 2005; Rocha et al., 2003; Silva et al., 2005; Sugimoto et al., 2003; Tago et al., 2005) (Figure 1.4).

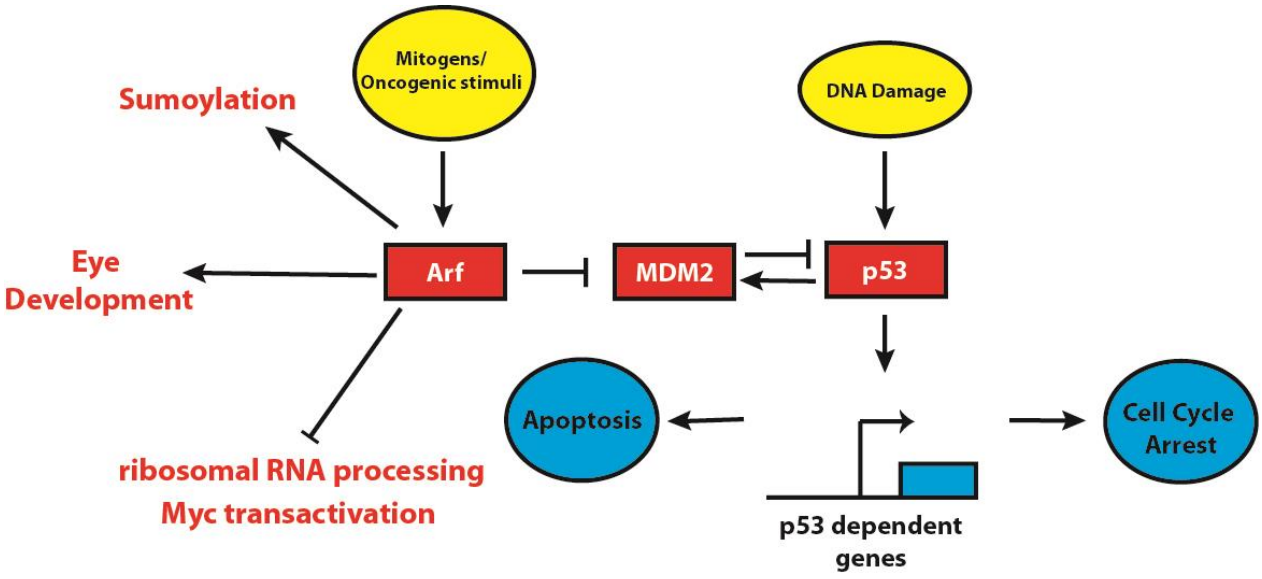

Figure 1.4: p53 dependent and independent functions of $p19^{Arf}$. Mitogenic signals activate $p19^{Arf}$ to sequester the E3 ubiquitin ligase Mdm2 to the nucleolus and thereby stabilize p53. p53 stabilization allows for it to enact its transcriptional network and induce cell cycle arrest and apoptosis, among other effects. Additionally, $p19^{Arf}$ functions that are not strictly p53 dependent include ribosomal RNA processing, Myc transactivation, promoting sumoylation of some of its binding partners and mediation of hyaloid vessel regression during murine eye development.

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 of a broader range of histological subtypes than in singly mutant animals (Jacks et al., 1994; Kamijo et al., 1999). 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 (Kelly-Spratt et al., 2004). 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 (Ulanet and Hanahan, 2010).

Beyond tumor surveillance, $p19^{Arf}$ plays an essential role for vision as it guides vascular involution in the vitreous space during late stages of mouse eye development (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005). $p19^{Arf}$ inhibition of perivascular cell proliferation within the hyaloid vascular system (HVS) also cannot be attributed solely to p53 because most *p53* $^{-/-}$ mice have normal eyes (Ikeda et al., 1999). While *Arf* $^{-/-}$ mice develop PHPV regardless of background, *p53* $^{-/-}$ mice only display characteristics of PHPV when kept in a pure C57BL/6 and pure BALB/c background (Ikeda et al., 1999; McKeller et al., 2002; Reichel et al., 1998). Further, early studies suggested that $p19^{Arf}$ is largely

silenced during normal development as expression is not detected by northern blot at any embryonic stage examined (Zindy et al., 1997b). Indeed, *Arf* $^{-/-}$ mice do not have gross anatomical defects which was surprising in light of its critical functions as a tumor suppressor. The use of a mouse model in which Gfp replaces exon1β of the *Arf* coding locus revealed that $p19^{Arf}$ is expressed in perivascular cells that support the hyaloid vasculature system. Pericytes are required for blood vessel stability and perhaps *Arf* expression in pericytes reveals tumor suppressive functions of *Arf* in regulating the angiogenic program.

Blood Vessel Development and Maintenance

Like many other organs, the eye depends on the formation of vasculature. Blood vessels are one of the first systems to emerge in vertebrates and are essential for providing nutrients and oxygen to developing tissues. Formation of blood vessels largely occurs through vasculogenesis and angiogenesis. Although arteriogenesis is also recognized as important for the development of the circulatory system, only vasculogenesis and angiogenesis will be discussed for the purposes of this review.

Factors that control angiogenesis and vasculogenesis

Following gastrulation, *de novo* formation of blood vessels gives rise to the primary vascular plexus, a process termed vasculogenesis. Endothelial cells proliferate and differentiate to form primitive tubular structures in an otherwise avascular space (Drake and Fleming, 2000; Risau and Flamme, 1995). Angiogenesis, then, is the remodeling and pruning of these vessels that gives rise to the complex, mature blood vessel network seen in the adult animal (Carmeliet, 2000; Risau, 1997). It is not completely clear whether hyaloid vasculature development depends on angiogenesis or vasculogenesis but the prevailing model favors that this system utilizes angiogenesis to develop. While

these two processes are distinct and have fundamental differences, there are a number of regulatory proteins and features shared by both.

Pericytes of the hyaloid vasculature express Arf

During vascular development and remodeling, interactions between pericytes and endothelial cells occur in a reciprocal manner. Pdgfrβ expressing perivascular cells are recruited to endothelial cells that secret PDGF-B. Injection of anti-Pdgfrβ antibody ablates vascular remodeling by inhibiting recruitment of pericytes (Uemura et al., 2002). Further, pericytes also express Ang1 that signals to the Tie2 receptor on endothleial cells (Nishishita and Lin, 2004; Satchell et al., 2001; Suri et al., 1996b). Through proximity, as pericytes are juxtaposed to the abluminal side of the endothelial cell membrane, and secretion of factors, pericytes can influence endothelial cell proliferation and differentiation.

Several pieces of evidence indicate that *Arf* expression in the developing eye is localized to perivascular cells. *Arf*-expressing cells cluster near endothelial cell tubes but do not express CD31 or flk-1, suggesting they are not endothelial cells. Rather, Pdgfrβ is expressed in perivascular cells that also display *Arf* promoter activation and all Pdgfrβ expressing cells co-express Gfp (Silva et al., 2005). Further, deletion of *Pdgfrβ* in the *Arf* $^{-/-}$ background

ameliorates perivascular cell accumulation in the absence of *Arf,* suggesting that Pdgfrβ is required for the hyperplasia that occurs in *Arf* $^{-/-}$ mice (Widau et al., 2012). Genetic and biochemical studies show that $p19^{Arf}$ acts in a cell intrinsic way to control the expression of Pdgfrβ mRNA and protein (Silva et al., 2005; Thornton et al., 2007; Widau et al., 2012). Further, while $p19^{Arf}$ repression of *Pdgfrβ* mRNA requires p53, $p19^{Arf}$ inhibition of Pdgfrβ protein is p53-independent. $p19^{Arf}$ does not affect Pdgfrβ protein stability or polysome loading of *Pdgfrβ* mRNA, a previously described function of $p19^{Arf}$ (Widau et al., 2012) (Figure 1.5). These data led to the attractive hypothesis that $p19^{Arf}$ has the capability of engaging microRNAs to post-transcriptionally regulate Pdgfrβ protein expression.

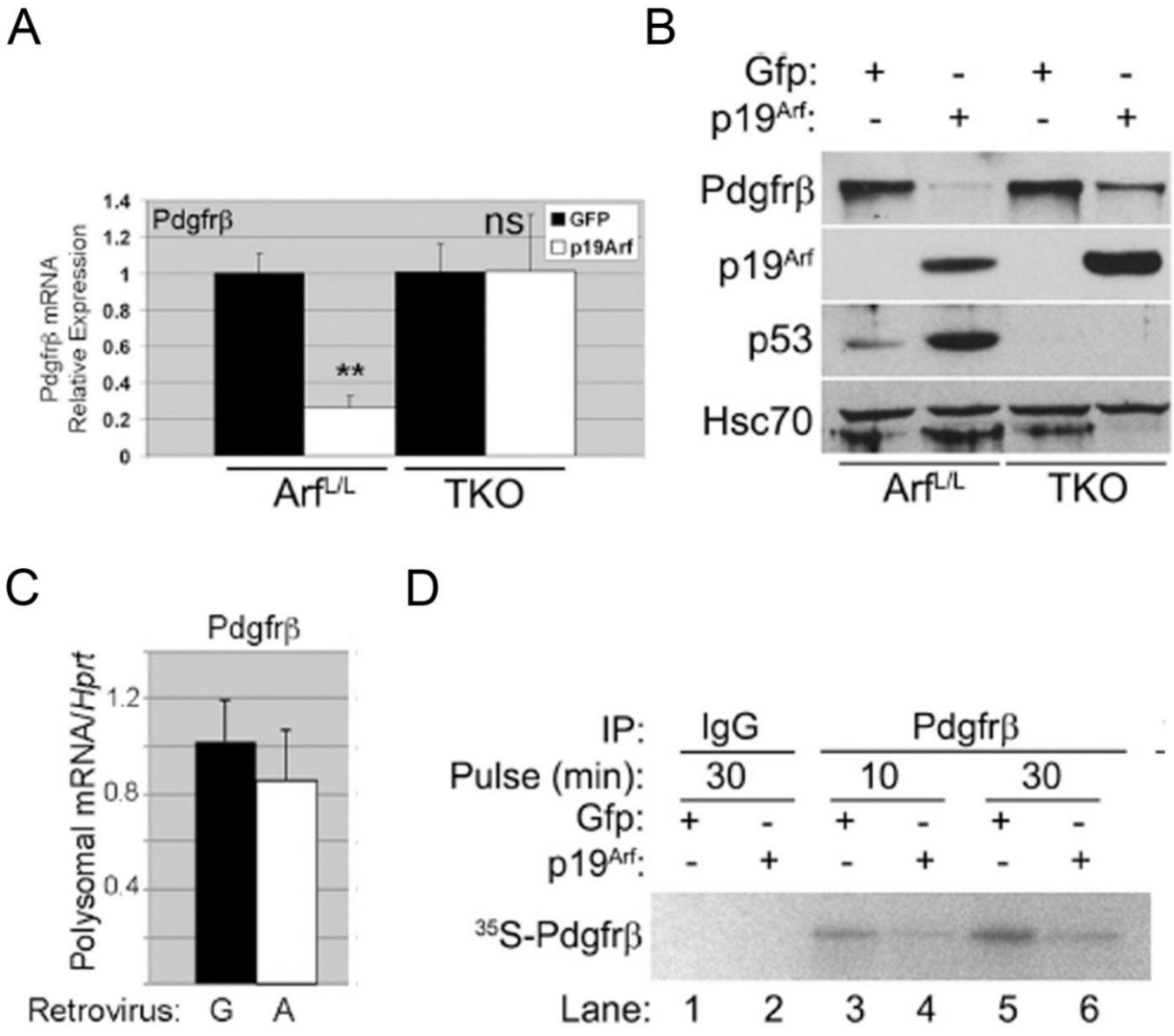

Figure 1.5: p19^{Arf} inhibits Pdgfrβ protein expression independently of p53. A) Ectopic expression of p19^{Arf} by retroviral transduction in *Arf* $^{lacZ/lacZ}$ MEFs (null for p19^{Arf}) suppresses *Pdgfrb* mRNA expression but not in the absence of *p53* (TKO MEFs). B) p19^{Arf} inhibits Pdgfrβ protein expression even in the absence of *p53*. C) There is not a difference in *Pdgfrβ* loading to polysomes in the presence of ectopic p19^{Arf} D) p19^{Arf} does not stabilize Pdgfrβ protein detected by ^{35}S incorporation and chase. (Adapted from (Widau et al., 2012))

microRNA Biogenesis and Function

Since their discovery in the 1990's, microRNAs have emerged as powerful mediators of fine-tuning gene expression (Moss et al., 1997; Reinhart et al., 2000). These small non-coding RNAs have been shown to be required for physiological processes like heart development and body patterning, as well as implicated in driving pathology of disease such as in cancer (He et al., 2005; Liu et al., 2011; Pauli et al., 2011). To date, over 2500 mature microRNAs have been annotated in the human genome and 1915 in mouse (miRBase). They are often tissue specific and expressed at low levels, but can have profound effects on modulating biological activity.

Biogenesis, Processing and Effects

In the context of the genome, microRNA sequences can be situated within introns, as poly-cistronic transcriptional units or even in exonic regions (Lee et al., 2004). microRNAs are RNA polymerase II-transcribed and are subject to a series of specialized processing events. Upon transcription, the primary microRNA (pri-miRNA) is cleaved by the nuclear enzyme Drosha III as a part of the microprocessor complex such that the generally 1kb long stem-loop structure yields a ~ 65 nucleotide short hairpin in which the mature microRNA sequence is

embedded. This pre-miRNA is then exported to the cytoplasm where it is further cleaved at the terminal loop by the enzyme Dicer. Finally, the small RNA duplex is loaded onto the Argonaute protein and forms the RNA-induced silencing complex (RISC) in order to find 3'UTR of target genes (Bartel, 2004).

Recently, evidence from the Weber lab described a negative correlative relationship between *Arf* expression and Drosha. They showed that Arf suppresses Drosha mRNA and knockdown of *Arf* in MEFs alters the expression of a subset of microRNAs (Kuchenreuther and Weber, 2013). Further, $p19^{Arf}$ regulates miR-205 expression in embryonic stem (ES) cells to facilitate formation of the extraembryonic endoderm (Li et al., 2013). These data and others led me to hypothesize that $p19^{Arf}$ inhibition of perivascular cell proliferation depends on microRNAs.

Summary of work

In this work, I will describe a novel capacity for $p19^{Arf}$ to induce miR-34a, a known component of the p53 transcriptional network, independently of p53. I will then describe an *ex vivo* cell culture system that will be useful to explore novel developmental facets of *Arf* biology. The culmination of these data may provide insight into $p19^{Arf}$ contribution to hyaloid vessel regression in addition to pathogenesis of certain eye diseases.

CHAPTER 2:

miR-34a is required for Arf downstream functions

Introduction

Cell proliferation is governed by two major pathways: the $p16^{Ink4a}$/RB pathway and the $p19^{Arf}$/p53 pathway (Levine, 1997; Weinberg, 1995). The $p19^{Arf}$ tumor suppressor was originally discovered as a protein encoded at the mouse (and human) *Cdkn2a* locus in an Alternate Reading Frame when compared to $p16^{Ink4a}$, the first tumor suppressor identified at that locus (Kamb et al., 1994; Quelle et al., 1995). 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 (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998). 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 (Ko and Prives, 1996).

Beyond tumor surveillance, $p19^{Arf}$ plays an essential role for vision as it guides vascular involution in the vitreous space during late stages of mouse eye development (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005). 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 (Gromley et al., 2009; Martin et al., 2004). This developmental function also

cannot be attributed solely to p53 because most *p53* $^{-/-}$ mice have normal eyes (Ikeda et al., 1999). Genetic and biochemical studies show that $p19^{Arf}$ acts in a cell intrinsic way to control the expression of *Pdgfrβ* mRNA and protein and that Pdgfrβ is essential for primary vitreous hyperplasia that develops without *Arf* (Silva et al., 2005; Widau et al., 2012). Because $p19^{Arf}$ can repress Pdgfrβ protein expression in TKO MEFs, understanding how it can accomplish this will shed light on the p53-independent mechanisms used by $p19^{Arf}$ 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 (Ambros et al., 2003; Bentwich et al., 2005; Lagos-Quintana et al., 2001). It has recently been demonstrated that $p19^{Arf}$ controls the expression of microRNAs in a cell-type specific manner (Li et al., 2013) or more broadly by repressing the translation of Drosha, an RNAse III endonuclease required for microRNA processing (Kuchenreuther and Weber, 2013; Li et al., 2013). Following this lead, I used a candidate-based approach to determine whether microRNAs might contribute to p53-independent cell cycle control and Pdgfrβ repression by $p19^{Arf}$. These results reveal a previously unrecognized capacity of $p19^{Arf}$, acting without p53, to guide the expression of miR-34a, a microRNA previously linked to p53 (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Raver-Shapira et al.,

2007; Tarasov et al., 2007). The use of complementary cell culture-based and mouse models demonstrates that *Arf* status dictates miR-34a 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-34a biology, increase our understanding of its regulation, and underline the roles that it can play as a p53-independent effector of $p19^{Arf}$ in normal development and disease.

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 200nM and transfected into MEFS using the Dharmafect transfection reagent. Retroviral plasmids, pMSCV-PIG and pMSCVPIG-34A, were provided by Joshua Mendell at UT Southwestern Medical Center. MSCV-based bicistronic retroviral vectors for mouse *Arf* and/or *Gfp* or *Rfp* were prepared and used as previously described (Silva et al., 2005). Cells were infected with retrovirus shRNA targeting $p19^{Arf}$ on day 1 and day 2, and

then selected with 2μg/mL puromycin for 6 d prior to harvest. Transduction efficiency was monitored by Gfp expression.

Mouse models and cell lines

Arf $^{Gfp/Gfp}$ mice and *Arf* $^{lacZ/lacZ}$ mice were maintained in a mixed C57BL/6 × 129/Sv genetic background (Freeman-Anderson et al., 2009; Zindy et al., 2003). 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 (Silva et al., 2005). TKO MEFs were provided by G Zambetti (St. Jude Children's Research Hospital) (Weber et al., 2000).

Laser-capture microdissection (LCM)

LCM was performed as previously described (Widau et al., 2012). 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 ten microdissected 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 microRNAs 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′-GTACTAGTCTCTGGCTGAAGCAGAGGAC and 5′-CGAAGCTTAC CACCGTACAGTCGTGGAT. The PCR product was digested by *Spe*I/*Hind*III and inserted into *pMIR-REPORT* vector (Ambion). To create this mutant the

seed sequence CACTGCC was replaced 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 (Mei et al., 2011). 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 two 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 Software using a cell cycle platform and Watson Pragmatic Model to calculate the distribution of cells in G1, S, and G2 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. Each experiment was conducted in triplicate.

Results

Arf regulates expression of certain microRNAs independently of p53

Work from the Skapek laboratory recently demonstrated that $p19^{Arf}$ 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, I used a candidate-based approach to prioritize potential microRNA regulators of Pdgfrβ. I employed TargetScan and miRDB to generate a list of microRNAs that may target the 3' untranslated region (UTR) of Pdgfrβ. I narrowed the list by only focusing on those microRNAs that are highly conserved or expressed in the developing eye (Karali et al., 2010) (Figure 2.1A). I utilized quantitative real-time PCR (qRT-PCR) to measure how the expression of nine of these changed when $p19^{Arf}$ was ectopically expressed in TKO MEFs. Ectopic $p19^{Arf}$ expression significantly induced three microRNAs – miR-29a, miR-34a and miR-34b – whereas one, miR-222, decreased in *Arf*-expressing TKO MEFs (Figure 2.1B). Further studies were focused on miR-34a because it exhibited the greatest p53-independent induction by $p19^{Arf}$ and had previously been implicated as a tumor suppressor (Welch et al., 2007)

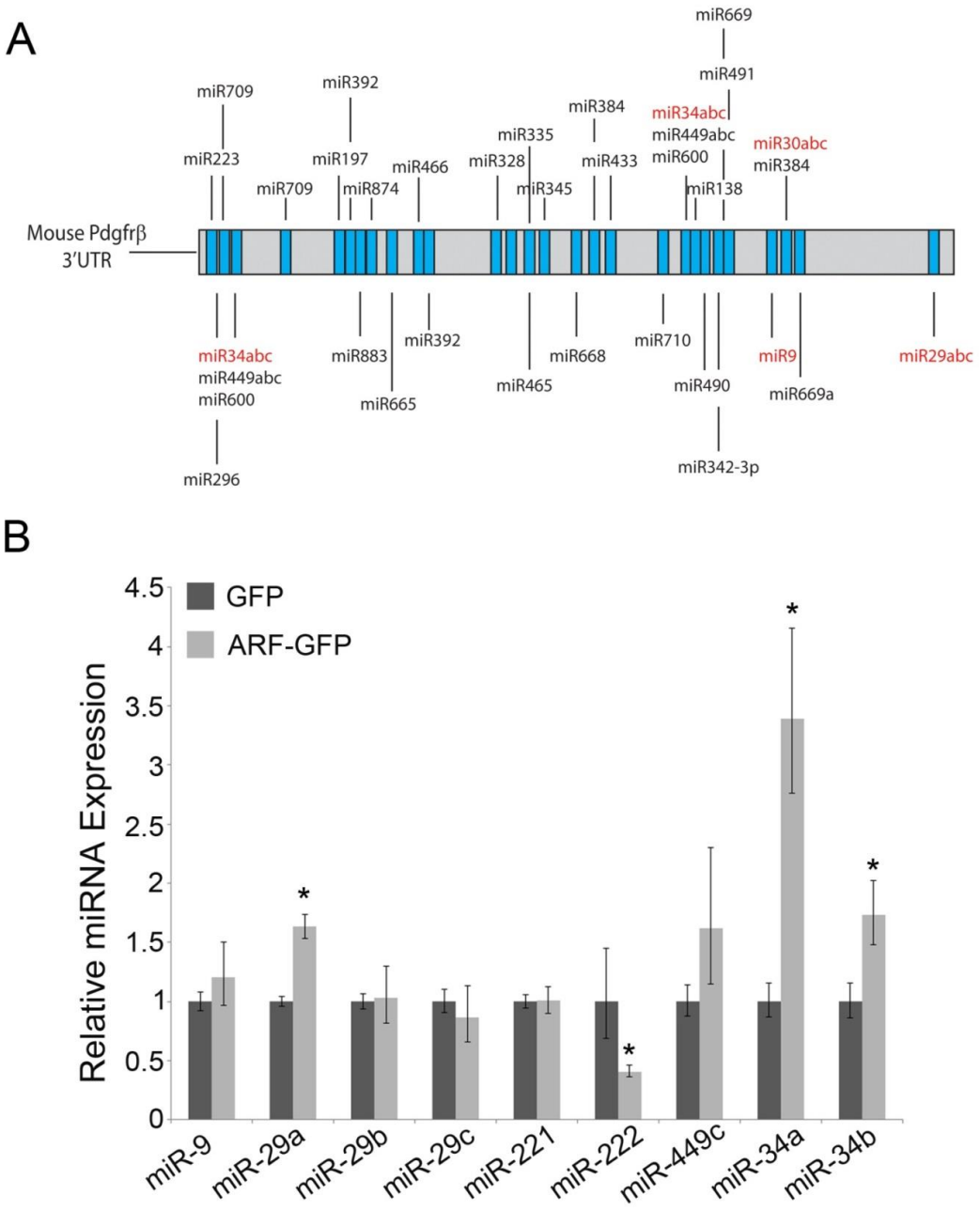

Figure 2.1: p19^{Arf} regulates microRNAs independently of p53. (A) Schematic diagram depicting candidate microRNAs expressed in the developing eye with predicted binding sites in mouse *Pdgfr*β 3′UTR. Pursued candidates are in red. (B) Quantitative analysis of expression of candidate microRNAs, measured by qRT-PCR, in TKO MEFs transduced with retrovirus encoding p19^{Arf} or Gfp control. (*$P < 0.05$)

miR-34a is required for Pdgfrβ repression and cell proliferation arrest by p19^{Arf}

p19^{Arf} can block cell proliferation stimulated by PDGF-B in cultured MEFs, including TKO MEFs lacking endogenous *Arf*, *Mdm2* and *p53* (Silva et al., 2005; Widau et al., 2012). In order to understand if miR-34a is necessary for p19^{Arf} to blunt Pdgfrβ expression, I utilized TKO MEFs transduced with retroviral vectors expressing p19^{Arf} or Gfp as a control. In this context, p19^{Arf} substantially decreased Pdgfrβ; however, transfection of an anti-microRNA to miR-34a reversed this effect (Figure 2.2A).

As an additional functional test of miR-34a importance in the p53-independent effects of p19^{Arf}, I considered whether it was needed for *Arf* expression to overcome cell cycle progression stimulated by PDGF-B. Similar to previous work, exogenous PDGF-B increased the S-phase fraction – measured by either propidium iodide staining or BrdU incorporation – in cultured TKO MEFs, and ectopic p19^{Arf} expression blunted that effect (Silva et al., 2005) (Figure 2B and C, left two lanes). Transfection of an anti-microRNA targeting miR-34a nullified the ability of p19^{Arf} to block PDGF-B-driven cell proliferation (Figure 2.2B and C, right two lanes). These results indicate that p53-independent Pdgfrβ repression and cell cycle arrest imposed by p19^{Arf} both depend on miR-34a.

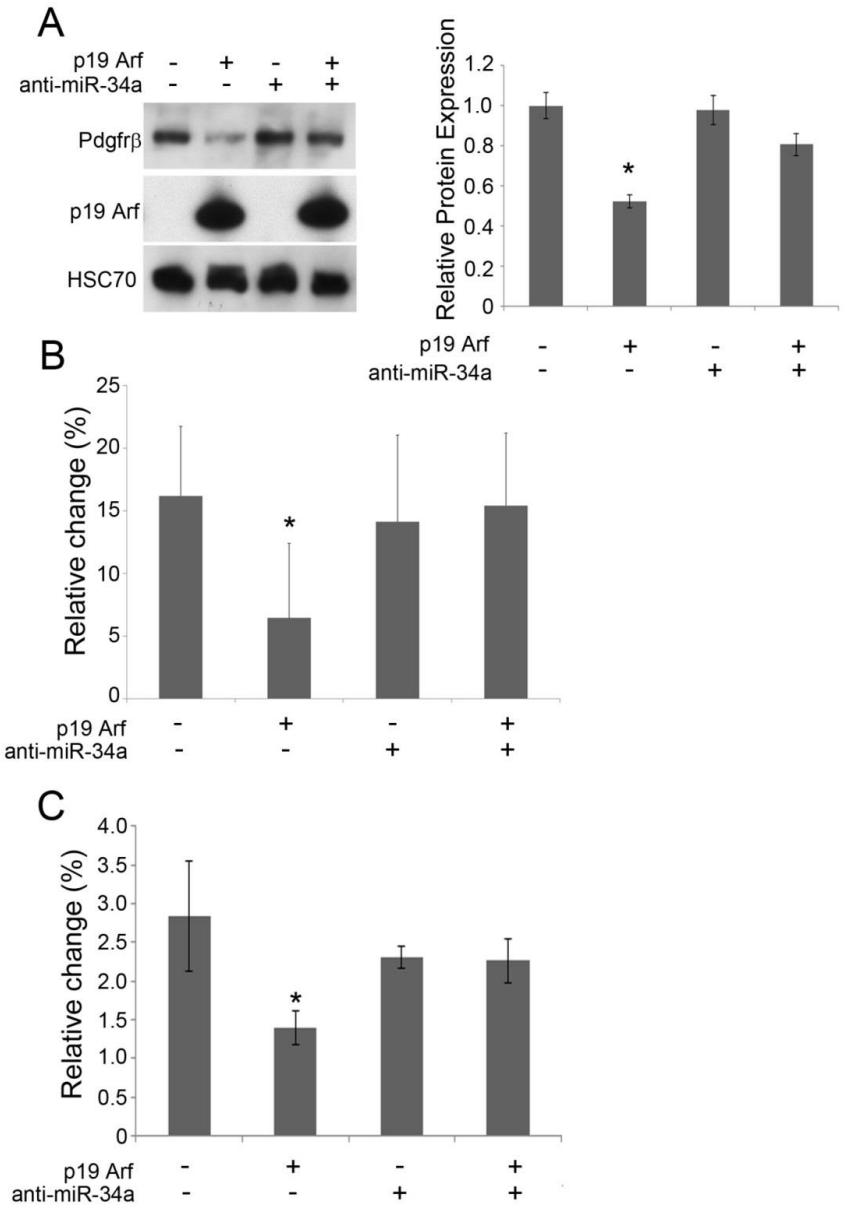

Figure 2.2: miR-34a is required for p19^{Arf} driven repression of Pdgfrβ. (A) Representative western blot showing Pdgfrβ, p19^{Arf} and HSC-70 protein expression in lysates prepared from cells transduced with p19^{Arf} retrovirus or control and +/- miR-34a hairpin inhibitor. Quantification (right) is normalized to HSC-70. (B and C) Change in S-phase fraction in TKO MEFs, stimulated by PDGF-B, assessed by PI staining and FACs (B) or by BrdU incorporation (C) in cells transduced with p19^{Arf} or Gfp control (+/−, respectively); and miR-34a hairpin inhibitor or control (+ and −, respectively). (*P < 0.05, when compared with baseline control) (Performed with Jie Mei)

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, 2010)). Previous mRNA-based surveys for miR-34a targets did not find *Pdgfrβ*; however, the 3'UTR of mouse *Pdgfrβ* actually contains two miR-34a target sequences (Bommer et al., 2007) (Figure 2.1A). To determine whether this transcript could be a direct miR-34a target, I sub-cloned wild type and mutant versions of one of the putative miR-34a targets from the mouse *Pdgfrβ* 3'UTR into the pMIR-REPORT plasmid (Figure 2.3A). 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 (Figure 2.3B).

To understand if miR-34a expression is sufficient to diminish endogenous Pdgfrβ expression, I examined changes in *Pdgfrβ* mRNA and protein following retroviral expression of miR-34a in TKO MEFs. Although *Pdgfrβ* mRNA expression was not altered by miR-34a, western blot revealed Pdgfrβ protein to be significantly lower (Figure 2.3C and 2.3D). Taken together, these data indicate that miR-34a is sufficient to repress Pdgfrβ translation.

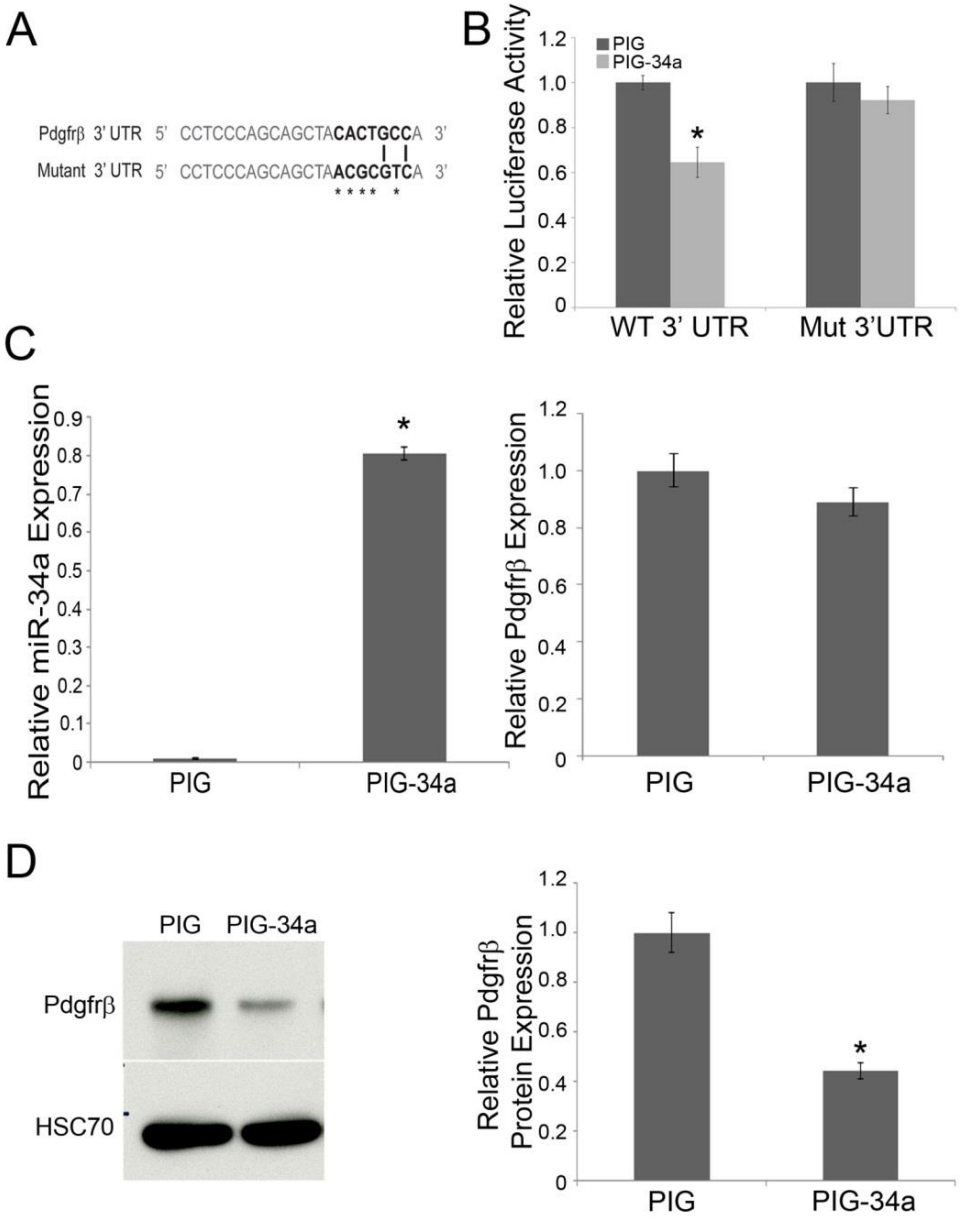

Figure 2.3: Pdgfrβ is a direct target of miR-34a. (A) miR-34a target sequence from *Pdgfr*β 3′ UTR. Seed sequence is in bold, and asterisks highlight mutated nucleotides. (B) Quantitative analysis of luciferase reporter in HEK293T cells, transiently transfected with expression plasmids for miR-34a (PIG-34a) or control (PIG). 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) (Performed with Jie Mei)

miR-34a expression depends on p19^{Arf} in cells and in the developing mouse eye

If miR-34a acts downstream of p19^{Arf} to mediate its anti-proliferative activities without p53, I considered whether endogenous levels of p19^{Arf} could influence miR-34a expression in cultured cells and *in vivo*. I addressed this using wild type MEFs, as well as *Arf*-deficient MEFs derived from *Arf* $^{Gfp/Gfp}$ or *Arf* $^{lacZ/lacZ}$ mice (Freeman-Anderson et al., 2009; Zindy et al., 2003). In both of these lines, the first exon in *Arf* is replaced by cDNA encoding one of the two reporters. At passages 1, 3, and 5, miR-34a expression was significantly lower in *Arf*-deficient MEFs as compared to the wild type cells (Figure 2.4A). p53 is known to regulate miR-34a expression in human colorectal and lung carcinoma cells, and in MEFs (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). I considered, then, whether decreased miR-34a in the absence of *Arf* might simply reflect the decreased p53 activity (Kamijo et al., 1998; Pomerantz et al., 1998). 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 (Figure 2.4A, right panel). However, examining miR-34a expression in *p53* $^{-/-}$ MEFs expressing shRNA directed at *Arf* allowed me to determine whether a component of miR-34a regulation was independent of p53. With approximately 60% reduction of endogenous *Arf* mRNA in *p53* $^{-/-}$ MEFs, I observed a quantitatively similar decrease in miR-34a (Figure 2.4B). Of note, miR-34b and c

were similarly diminished when *Arf* expression was knocked down in these MEFs (Figure 2.4C). Hence, endogenous *Arf* drives the expression of miR-34 family microRNAs even in MEFs lacking p53.

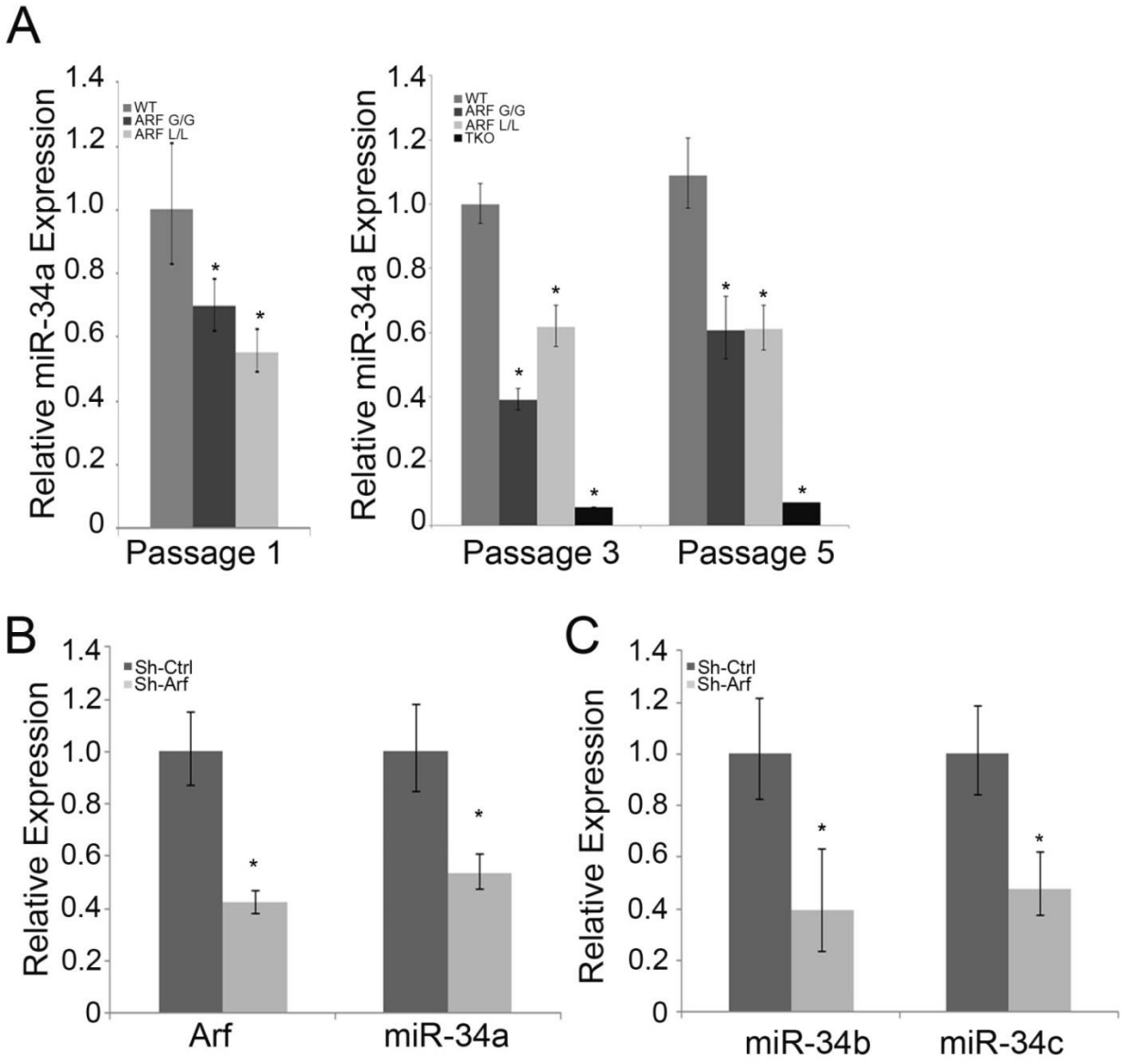

Figure 2.4: miR-34a expression correlates with p19^{Arf} status. (A) Quantitative analysis of miR-34a expression, measured by qRT-PCR, in MEFs that are derived from WT, *Arf* $^{Gfp/Gfp}$ (ARF G/G), *Arf* $^{lacZ/lacZ}$ (ARF L/L), and *Arf* $^{-/-}$, *p53* $^{-/-}$, *MDM2* $^{-/-}$ (TKO) mice and cultivated 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)

In order to investigate whether miR-34a is controlled by $p19^{Arf}$ *in vivo*, I utilized laser capture microdissection (LCM) to isolate discrete compartments of the mouse eye at embryonic day (E) 13.5, when *Arf* is first robustly expressed in the vitreous (Silva et al., 2005) (Figure 2.5A). At this point in development, *Arf*-dependent alterations in *Pdgfrβ* mRNA and increased cell proliferation are already observed, but there are few other anatomic differences between the wild type and *Arf* $^{-/-}$ animal; as such, any *Arf*-dependent change in miR-34a would not be obscured by dramatic ocular pathology (Silva et al., 2005). By utilizinq qRT-PCR, I demonstrated that miR-34a was most highly expressed in the primary vitreous compartment, which is the only anatomic site where *Arf* is measurably expressed (McKeller et al., 2002; Widau et al., 2012) (Figure 2.5B). Broadening the scope of our query, I noted that other members of the miR-34 microRNA family were more highly expressed in the vitreous than miR-29a or miR-221, two other microRNAs predicted to target the Pdgfrβ 3'UTR (Figures 2.1A and 2.5C). I 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 the 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* or in cultured MEFs (Figure 2.5D and 2.1B). The *Arf*-dependent expression

in the vitreous of the miR-34 microRNAs supports their candidacy as physiological regulators of Pdgfrβ during mouse eye development.

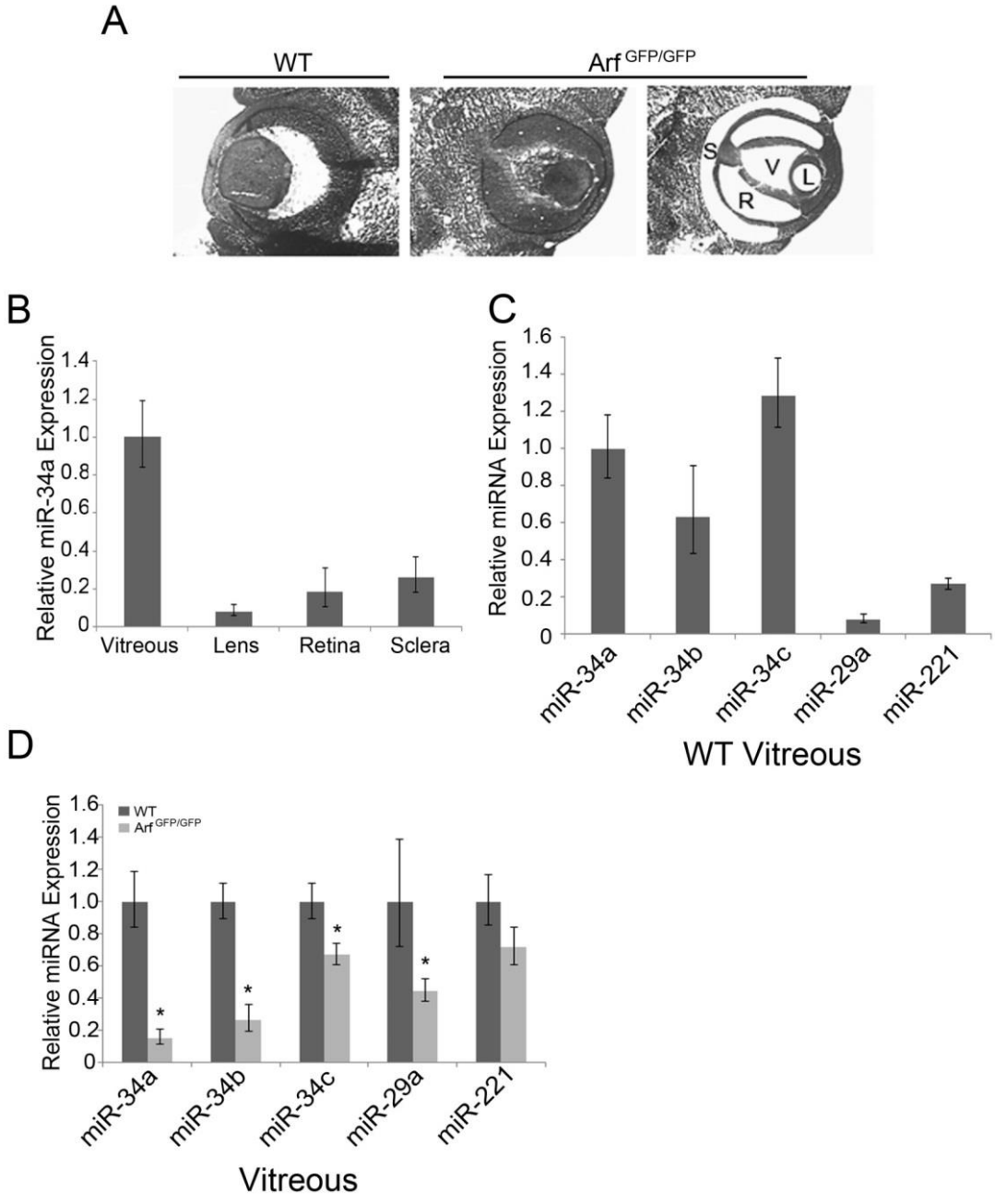

Figure 2.5: miR-34a expression *in vivo* is dependent upon p19^{Arf}. (A) Representative photomicrographs of E13.5 mouse embryo eye from wild-type (WT) or *Arf* $^{Gfp/Gfp}$ embryo, as indicated, showing laser capture microdissection (LCM) of sclera (S), retina, (R), vitreous (V), and lens (L). (B) Quantification of mature miR-34a expression, measured by qRT-PCR, in LCM specimens from

different parts of the wild-type E13.5 mouse eye. (C and D) Quantitative analysis shows relative expression of different microRNAs in the vitreous of E13.5 wildtype (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 wildtype embryos (D). *$P < 0.05$, when compared with wild-type embryos

Concluding Remarks

In this report, I illustrate how $p19^{Arf}$ can block Pdgfrβ protein synthesis without affecting its mRNA level and without engaging Mdm2 and p53. First, I have established that *Arf* expression elevates miR-34a, among several other 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 (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Tarasov et al., 2007). Second, I show that miR-34a can directly target the 3'UTR of mouse *Pdgfrβ*. Although this does not diminish *Pdgfrβ* 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 (Bommer et al., 2007). 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 microRNAs with the same seeding sequence may also contribute, these findings do discount the possible roles for microRNAs targeting other regions of the *Pdgfrβ* 3'UTR (see Figure 2.1A). Finally, I demonstrate that miR-34 family members are highly expressed in the primary vitreous – the only region in the eye with detectable expression of

Arf – and they are repressed in *Arf* $^{-/-}$ embryos (McKeller et al., 2002; Widau et al., 2012). 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 the analyses of cultured MEFs.

CHAPTER 3:

Isolation and characterization of cells expressing the *Arf* promoter during development

Introduction

Despite wide-spread importance of perivascular cells to support vascular integrity, robust *Arf* expression is only observed in the perivascular cells flanking the hyaloid vessels and the internal umbilical artery, both of which represent vascular beds that are not necessary beyond embryonic development (Freeman-Anderson et al., 2009). Even though it is clear that the developmental function of $p19^{Arf}$ is imperative to the animal, as in the absence of $p19^{Arf}$, Pdgfrβ accumulates and leads to hyperplasia of cells in the vitreous space, little is known about the particular perivascular cells that normally express the tumor suppressor gene (Gromley et al., 2009; Silva et al., 2005).

In order to better understand $p19^{Arf}$ function during development, I isolated cells that normally activate the *Arf* promoter from the vitreous compartment of the eye. I was able to purify cells endogenously expressing the *Arf* promoter by fluorescence activated cell sorting (FACS) and examine them in culture. In this report, I describe the isolation and *in vitro* culture of *Arf* expressing primary vitreous cells (PVCs) and compare them to previously established cell culture models for global transcriptome analysis, I gain clearer insight into the identity of the PVCs and further demonstrate the utility of these cells by examining expected and novel molecular changes upon reintroduction of *Arf*. The

availability and use of the *Arf* $^{Gfp/Gfp}$ PVCs holds great potential for better understanding the role of $p19^{Arf}$ in mammalian development and how these functions are abrogated in human ocular and cardiovascular disease as well as during tumorigenesis.

Methods

Animals

Mice in which *Arf* exon 1β is replaced by a reporter gene encoding green fluorescence protein (Gfp) were maintained in a mixed C57BL/6 × 129/Sv genetic background (Zindy et al., 2003). Primary MEFs from *Arf* $^{lacZ/lacZ}$ mice were derived as previously described (Silva et al., 2005). Animal studies were accomplished at the University of Texas Southwestern Medical Center, with approval of the animal care and use committees. Eyes were isolated from *Arf* $^{Gfp/Gfp}$ mice, euthanized and decapitated at postnatal days (P) 0 - 4. The eyelid was incised using a No. 11, straight surgical blade (Feather Safety Razor Co.) to expose the eye. While holding the eyelid open, the scalpel blade was used to transect extra-ocular muscles and other connective tissue between the globe and the bony orbit. Small angled forceps (Fine Science Tools) were inserted between the orbit and globe, grasping the optic nerve/ophthalmic

vessels firmly and gently lifting out the intact eye. Enucleated eyes were submerged in ice-cold PBS and stabilized under PBS by holding the optic nerve stub. Small spring scissors (Fine Science Tools) were used to cut along the circumference of the eye at the equator. The cornea/anterior part of the sclera were lifted off, leaving the optic cup and lens together under PBS. The retina was then removed in piecemeal fashion, leaving the lens with attached retrolental mass.

Cell Isolation and Culture

The lens/retrolental mass tissue from 60 individual eyes were pooled in a 1.5 mL microcentrifuge tube and digested in M2 media with 300 μg/mL hyaluronidase and 1 mg/mL collagenase (all from Sigma-Aldrich) at 37° C for 15 minutes. The tissue was briefly triturated and further incubated at 37° C for 10 minutes. Digested material (including undigested lenses) was centrifuged, washed with D-MEM with 20% FBS, and then resuspended in D-MEM/20% FBS with penicillin/streptomycin. Resuspended cells (including PVCs) were passed through a 35 μM filter into polystyrene tube for FACS. Gfp-positive PVCs were collected using the MoFlo (Dakocytomation) cell sorter. Sorted PVCs were plated (6,000 cells/well) in a 96-well plate with Pericyte Medium (PM) (ScienCell). Cells were passed (1:4) using trypsin/EDTA every 3 days. *Arf* $^{lacZ/lacZ}$ MEFs,

10T1/2 fibroblasts and PVCs were used for RNA-Seq analysis (Freeman-Anderson et al., 2009). Briefly, cells were plated at a density of 1×10^6 cells/ 10 cm plate and cultivated in PM until ~80% confluence, at which point cells were harvested for RNA extraction.

Whole Transcriptome Sequencing (RNA-Seq)

Total RNA was isolated using the miRNeasy mini kit (Qiagen) and treated with RNase free DNaseI to remove genomic DNA (Qiagen). RNA integrity and purity was determined using the Bioanalyser Pico Chip (Agilent), assuring that each sample had a RIN score of 10. RNA (1 μg) from two biological replicates of each cell type was fragmented in the UT Southwestern Next-Generation Sequencing core, converted to cDNA, and amplified by PCR according to the Illumina RNA-Seq protocol (Illumina, Inc. San Diego, CA). The Illumina HiSeq 2000 (San Diego, CA) instrument was used to generate 50 bp single-end sequence reads. RNA-Seq read quality was evaluated in the core using the Illumina purity filter and distribution of base quality scores at each cycle.

Sequence reads for each sample were aligned to the UCSC mm10 version of the mouse reference genome assembly using Bowtie 2.1.0 and the splicing-aware aligner TopHat 2.0.8. The alignment allows only uniquely aligned

reads and up to two mismatches per read. All other parameters were set to the default values. The quality of the RNA-Seq data was evaluated by FastQC (v0.10.1) and a series of Perl (v5.16.1) and R (v3.0.1) scripts. Normalized gene expression values expressed as fragments per kilobase of exon per million fragments mapped (FPKM) were determined using Cufflinks 2.0.2 with default settings, which reports the mean of the maximum likelihood estimates from each of three replicates processed independently.

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-p21 (Sc-756, Santa Cruz, 1:1000), anti-p53 (Sc-6243, Santa Cruz, 1:1000), anti-MDM2 (Sc-965, Santa Cruz 1:1000) and anti-Hsc70 (Sc-1059, Santa Cruz, 1:5,000).

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from PVCs 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 KAPA SYBR Green Master Mix

(KAPA). qRT-PCR was performed in a 96-well plate using BioRad (CFX96) instrument. The PCR program consisted of 20 sec at 95 ºC, followed by 40 cycles of 95 ºC for 15s and 60 ºC for 20s. Primer quality was analyzed by dissociation curves. The expression of miR-34abc and *Pdgfr*α*, Pdgfr*β *and* α*SMA* was normalized to *U6* and *Gapdh*, respectively.

Results

Currently, there is very little known about the cells that normally express *Arf,* motivating me to generate a cell culture model that accurately reflects the unique environment in which *Arf* is expressed developmentally. I decided to pursue this by taking advantage of *Arf* $^{Gfp/Gfp}$ mice in which Gfp replaces exon 1β of the endogenous *Arf* locus, rendering the mice *Arf* null. In this context, a retrolental mass persists in the primary vitreous space in which Gfp (+), *Arf*-expressing cells, in addition to other cell types including endothelial cells, are present (Figure 3.1A). It is important to note that the retrolental mass is not evident in WT or Gfp/+ animals postnatally, making it unfeasible to derive these cells under "wild type" conditions. I isolated the retrolental tissue from *Arf* $^{Gfp/Gfp}$ mice and retrieved a total of 38,000 Gfp-positive cells (averaging 633 cells/eye), which represented 2-3% of the total population (Figure 3.1B). I collected the *Arf* $^{Gfp/Gfp}$ Primary Vitreous Cells (PVCs) for *in vitro* culture and expansion. At

confluence, the PVCs form a monolayer and adopt a fibroblast-like morphology with some variation in Gfp expression (Figure 3.1C). I continued to expand the cells in culture and observed that Gfp expression persists through at least passage 15 (data not shown). At passage 5 and sub-confluence, the PVCs are elongated and spindle-like with long cytoplasmic processes. The cells continue to express Gfp while WT MEF cells cultured in tandem do not (Figure 3.1D). Although many laboratories, including our own, have successfully utilized classically immortalized fibroblasts and cancer cell models to gain valuable insight into some of the developmental and tumor suppressive functions of *Arf*, these systems are imperfect in recapitulating the non-pathological environment in which *Arf* is expressed. The PVCs represent, for the first time, a cell culture model in which the *Arf* promoter is endogenously turned on during development allowing us and other researchers to explore the capacity of $p19^{Arf}$ to control vascular remodeling and mural cell proliferation in the context of a cell that normally expresses this promoter. These cells will also be useful in clarifying the complex regulation of the *Arf* promoter.

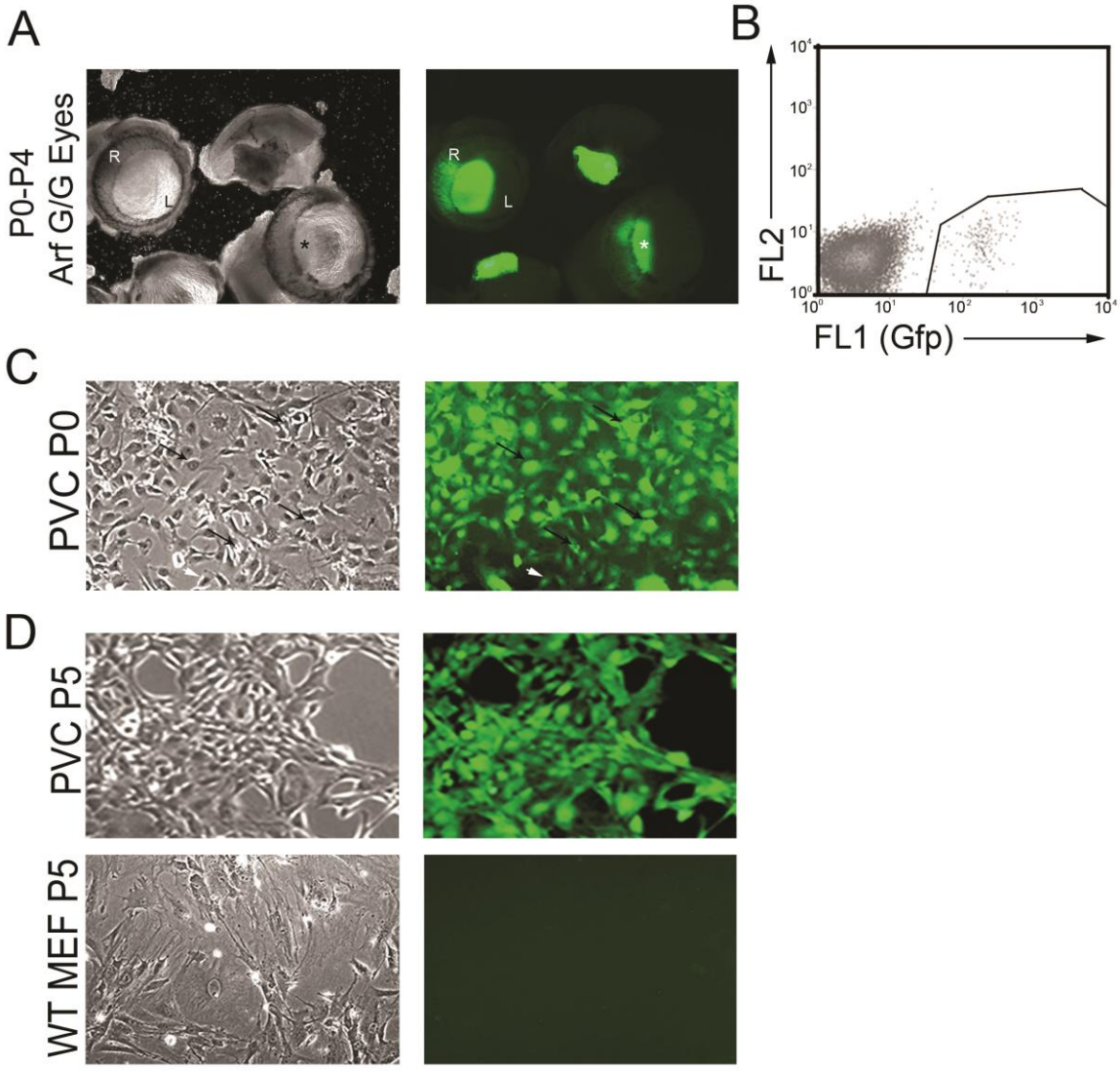

Figure 3.1: Isolation and expansion of *Arf* $^{Gfp/Gfp}$ PVCs (A) Representative photomicrograph of enucleated mouse eyes from P0-P4 *Arf*$^{Gfp/Gfp}$ mice. Phase contrast image (left), Gfp (right). The retrolental mass (*) is behind the lens (L) and expresses Gfp. (R= retina) (B) Purification of PVCs by fluorescence activated cell sorting (FACS) for Gfp. Representative images of cultured PVCs at passage 0 (C) and passage 5 (D). WT MEF (wild type mouse embryonic fibroblasts) cells at passage 5 (D). Phase contrast image (left), fluorescence image showing Gfp expression (right). Arrows indicate heterogeneous Gfp expression between high expressing (black) and low expressing (white) cells. (Performed with Caitlin Devitt)

Because we have not unequivocally established the identity of the *Arf*-expressing cells, I sought to capture the global gene expression profile of the *Arf* $^{Gfp/Gfp}$ PVCs in comparison to other cell culture models that have been previously used to study *Arf* biology. Like the PVCs, *Arf* $^{lacZ/lacZ}$ MEFs do not express a functional $p19^{Arf}$ protein, while 10T1/2 cells, a widely used pericyte model, carry a biallelic deletion of *Arf* (Freeman-Anderson et al., 2009; Zindy et al., 2003)(and unpublished data). When cultured in Pericyte Medium, all three cell lines resemble fibroblasts in their morphology (Figure 3.2A). To define these cells by gene expression, I cultured the *Arf* $^{Gfp/Gfp}$ PVCs, *Arf* $^{lacZ/lacZ}$ MEFs and 10T1/2 cells, extracted total RNA and performed high-throughput RNA-sequencing. There were 85.3, 86.7 and 83.8 million sequence reads for *Arf* $^{lacZ/lacZ}$ MEFs, 10T1/2 and PVCs, respectively; after applying a series of computational tools (see Methods), 71.6, 81.9 and 73.5 million reads were successfully mapped to the mouse reference genome. I examined how all 10,704 genes expressed in PVCs partitioned between *Arf* $^{lacZ/lacZ}$ MEFs and 10T1/2 cells. I found 970 expressed genes in PVCs that were also expressed in either *Arf* $^{lacZ/lacZ}$ MEFs or 10T1/2 cells; 769 genes were expressed in *Arf* $^{lacZ/lacZ}$ MEFs, while 201 were found in 10T1/2 cells (binomial test, $P = 2.8\times10^{-79}$) (Figure 3.2B). Based on this analysis, I conclude that on a genome-wide scale, the *Arf* $^{Gfp/Gfp}$ PVC*s* are more closely related to the *Arf* $^{lacZ/lacZ}$ MEFs than the 10T1/2 cells.

The fact that the MEFs and the PVCs share 86% similarity in gene expression reaffirmed previous studies utilizing *Arf* $^{lacZ/lacZ}$ MEFs to establish a pathway beginning from *Arf* induction to characterization of its downstream effects required for eye development. The Skapek laboratory has previously established that Tgfβ signaling drives *Arf* expression in *Arf* $^{lacZ/lacZ}$ MEFs and *in vivo* resulting in $p19^{Arf}$ mediated down-regulation of Pdgfrβ (Freeman-Anderson et al., 2009; Zheng et al., 2010). In order to understand if the *Arf* $^{Gfp/Gfp}$ PVCs were similar to the *Arf* $^{lacZ/lacZ}$ MEFs in this regard, I looked for the expression of all Tgfβ pathway genes as defined by KEGG and performed a hierarchical clustering (Kanehisa and Goto, 2000; Kanehisa et al., 2014). I found the components of the Tgfβ pathway that have so far been defined as important for *Arf* regulation, including *Smad2/3*, *Sp1* and *Cebpβ* to be expressed in all three cell lines (Figure 3.2B) (Zheng et al., 2013). Further, based on all Tgfβ pathway genes, I found that the PVCs clustered more closely to the *Arf* $^{lacZ/lacZ}$ MEFs than the 10T1/2 cells (data not shown).

To explicitly establish previous findings that the *Arf*-expressing cells of the primary vitreous are perivascular, I examined the expression of known markers that identify vascular/mural cells, as well as fibroblasts, endothelial cells and retinal cells. As has been previously shown, the *Arf* $^{Gfp/Gfp}$ PVCs express the transmembrane cell surface protein *Pdgfrβ* (Silva et al., 2005; Thornton et al.,

2007; Widau et al., 2012). I also observed expression of, *Angpt1*, which is critical for angiogenesis and vessel maturation as well as α*-SMA*, a bona fide perivascular cell marker (Suri et al., 1996a). *Vimentin*, a cytoskeletal component associated with mesenchymal cells, was also highly expressed in all three cell lines, further establishing that these cells are perivascular (Figure 3.2D) (Armulik et al., 2011). I found several markers of fibroblasts such as *S100a4, Col1a1 and Ph4b* to be expressed in all three cell types, while *Fap,* a marker of differentiated fibroblasts, was only present in the *Arf* $^{lacZ/lacZ}$ MEFs (Figure 3.2D) (Kalluri and Zeisberg, 2006). Further, I observed the lack of expression of endothelial specific genes, *Pecam, Cdh5 and vWf,* demonstrating that *Arf* is not expressed in the endothelial cell population that coexists with the PVCs in the developing eye (Figure 3.2D) (Garlanda and Dejana, 1997). Finally, to ensure that I did not contaminate the cell prep with neighboring retinal tissue, I checked for the expression of known retinal defining transcription factors: *Six3, Otx2, Nr2e3, Nrl* and *Crx* (Byerly and Blackshaw, 2009). None of these transcription factors were expressed in either cell line (Figure 3.2D). Based on this gene expression signature and the morphology of the cells, I assert that the *Arf* $^{Gfp/Gfp}$ PVCs are perivascular cells.

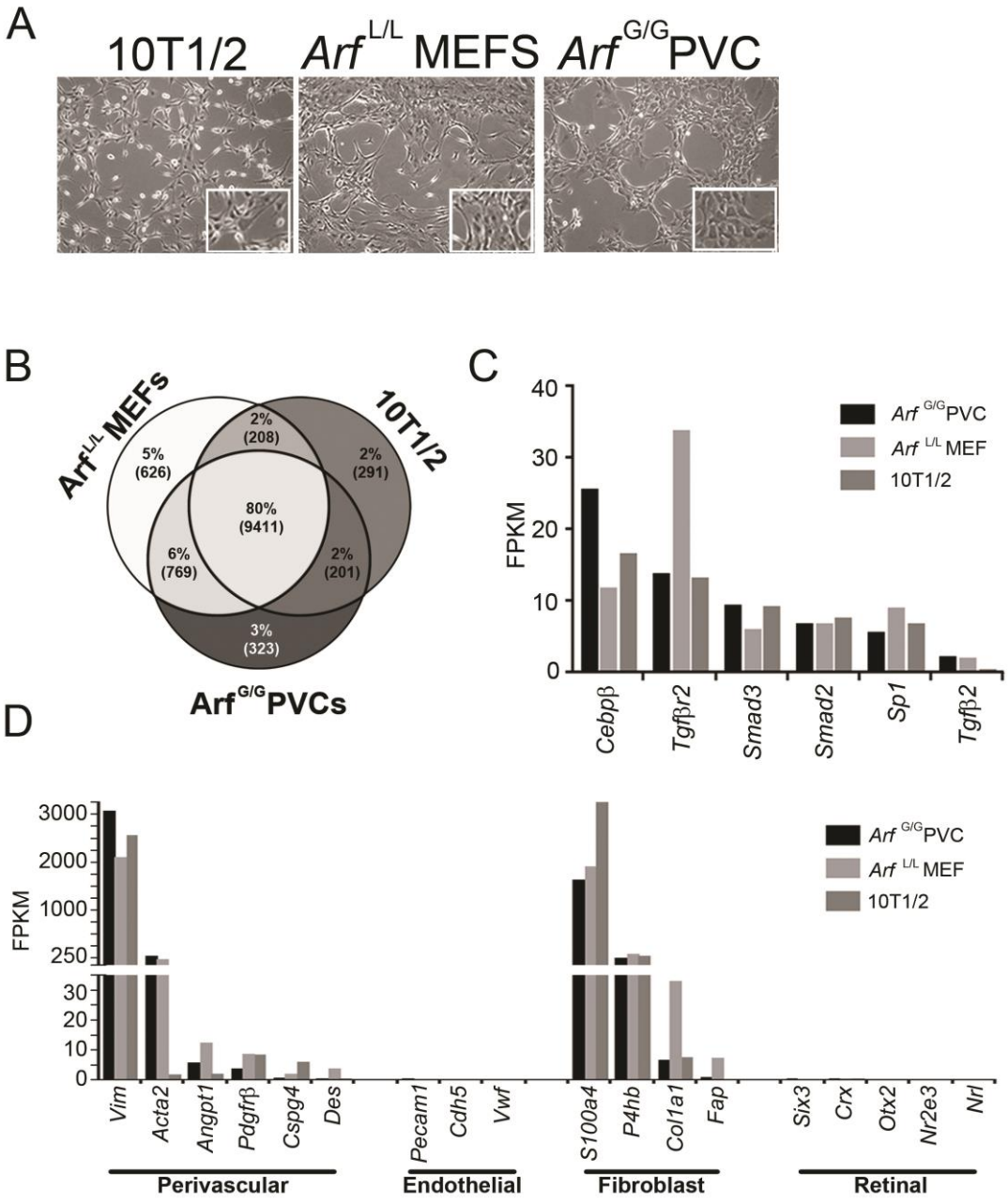

Figure 3.2: PVCs express perivascular genes (A) Representative phase contrast image of 10T1/2, *Arf*$^{lacZ/lacZ}$ MEFs and *Arf*$^{Gfp/Gfp}$ PVCs in culture. (B) Venn diagram comparison of all genes expressed in the three cell lines showing unique and overlapping transcripts. (C) Expression of a subset of Tgfβ pathway genes (FPKM). (D) Expression of cell type specific markers demonstrates that the PVCs express perivascular and fibroblast genes, not endothelial or retinal genes.

While 86% of all the genes expressed in the *Arf* $^{Gfp/Gfp}$ PVCs were also expressed in the *Arf* $^{lacZ/lacZ}$ MEFs, I found 323 genes representing 3% of the total genes expressed in PVCs to be mutually exclusive from those genes expressed in MEFs and 10T1/2 cells (Figure 3.2B). To understand how the PVCs are distinct from the other cultured cells, I subjected the dissimilar set of genes to Gene Ontology (GO) pathway analysis. KEGG pathway and GO terms were collected from the Molecular Signatures Database (Subramanian et al., 2005). I highlighted several pathways that were enriched in the *Arf* $^{Gfp/Gfp}$ PVCs ($p > 0.05$) (Figure 3.3A). Given their neural crest origin, it was not unexpected that I observed that the PVC only genes were enriched for the term Nervous System Development (Zheng et al., 2010). Of interest, I found that the term Anatomical Structure Morphogenesis was enriched with 9 genes expressed in the *Arf* $^{Gfp/Gfp}$ PVCs (-log p value = 2.84) including Pax6 and Eya2, both of which are important for eye development (Figure 3.3A) (Xu et al., 1997). Only the *Arf* $^{Gfp/Gfp}$ PVCs were significantly enriched for genes in the Tgfβ Receptor Signaling Pathway, including *Gdf15, Eng and Lefty1;* perhaps suggesting that the PVCs more aptly reflect a signaling environment to endogenously regulate *Arf (*Figure 3.3A). I also found the terms Cell Proliferation, Cellular Localization and Cell-Cell Signaling to be enriched in the PVC only gene set, reflecting the dynamic environment of the vitreous compartment as well as the requirement of $p19^{Arf}$ to blunt the proliferation of these cells.

I recently identified a previously unrecognized role for $p19^{Arf}$ during development in its capacity to regulate microRNA expression independently of p53 (Iqbal et al., 2014a). With this in mind, I sought to understand functional pathways targeted by microRNAs expressed in the PVCs. I found of all 10,704 genes expressed, 1.8% or 186 represented small non-coding RNA genes, 80 of which are defined as microRNAs based on annotation mm10 from the UCSC Genome Browser (data not shown) (Dreszer et al., 2012). In order to understand the function of the microRNAs, I analyzed all microRNAS expressed in the *Arf*$^{Gfp/Gfp}$ PVCs by DIANA-miRPath v2.0, a web-based server for microRNA target prediction and pathway analysis (Maragkakis et al., 2009). The most significantly enriched pathway targeted by microRNAs expressed in the *Arf* $^{Gfp/Gfp}$ PVCs was ECM-receptor interaction with 10 microRNAs expressed that target 21 different genes in this pathway (-log p value = 13.8) (Figure 3.3B). Of interest, Tgfβ signaling was also enriched in both the *Arf* $^{Gfp/Gfp}$ PVCs and *Arf* $^{lacZ/lacZ}$ MEFs (Figure 3.3B and data not shown). The *Arf* $^{Gfp/Gfp}$ PVCs expressed 12 microRNAs targeting 38 genes within this pathway (-log p value = 1.48) (Figure 3.3B). Tgfβ regulated microRNAs are known to target genes that promote angiogenesis and components of the epithelial to mesenchymal transition (EMT) program (Morrison et al., 2013). In this regard, I also found the terms Focal adhesion, Pathways in cancer and Transcriptional misregulation in cancer to

have significant enrichment of genes targeted by the repertoire of microRNAs expressed in the PVCs.

In line with the idea that these cells are derived from the neural crest, evidenced by lineage-tracing experiments using *Wnt1-Cre, Rosa26-LSL-tdTomato* and *Wnt1-Cre, Arf* $^{fl/fl}$ mouse models, and thus have undergone EMT, I was prompted to examine the expression of EMT associated genes in the PVCs (Figure 3.3C) (Zheng et al., 2010). I found high expression of known mesenchymal marker genes (*Cdh2)* as well as transcription factors such as *Twist1, Zeb1/2 and Snai1* that are required for EMT (Morrison et al., 2013). In contrast, the classical epithelial marker *Cdh1 (E-cadherin)*, was not expressed. Because $p19^{Arf}$ is turned on after the cells have migrated, an intriguing hypothesis posits that $p19^{Arf}$ expression in these cells negatively regulates the EMT program by inhibiting their proliferation and migration. This *ex vivo* model of the *Arf* $^{Gfp/Gfp}$ PVCs will be ideal for investigating how $p19^{Arf}$ controls aspects of the EMT program.

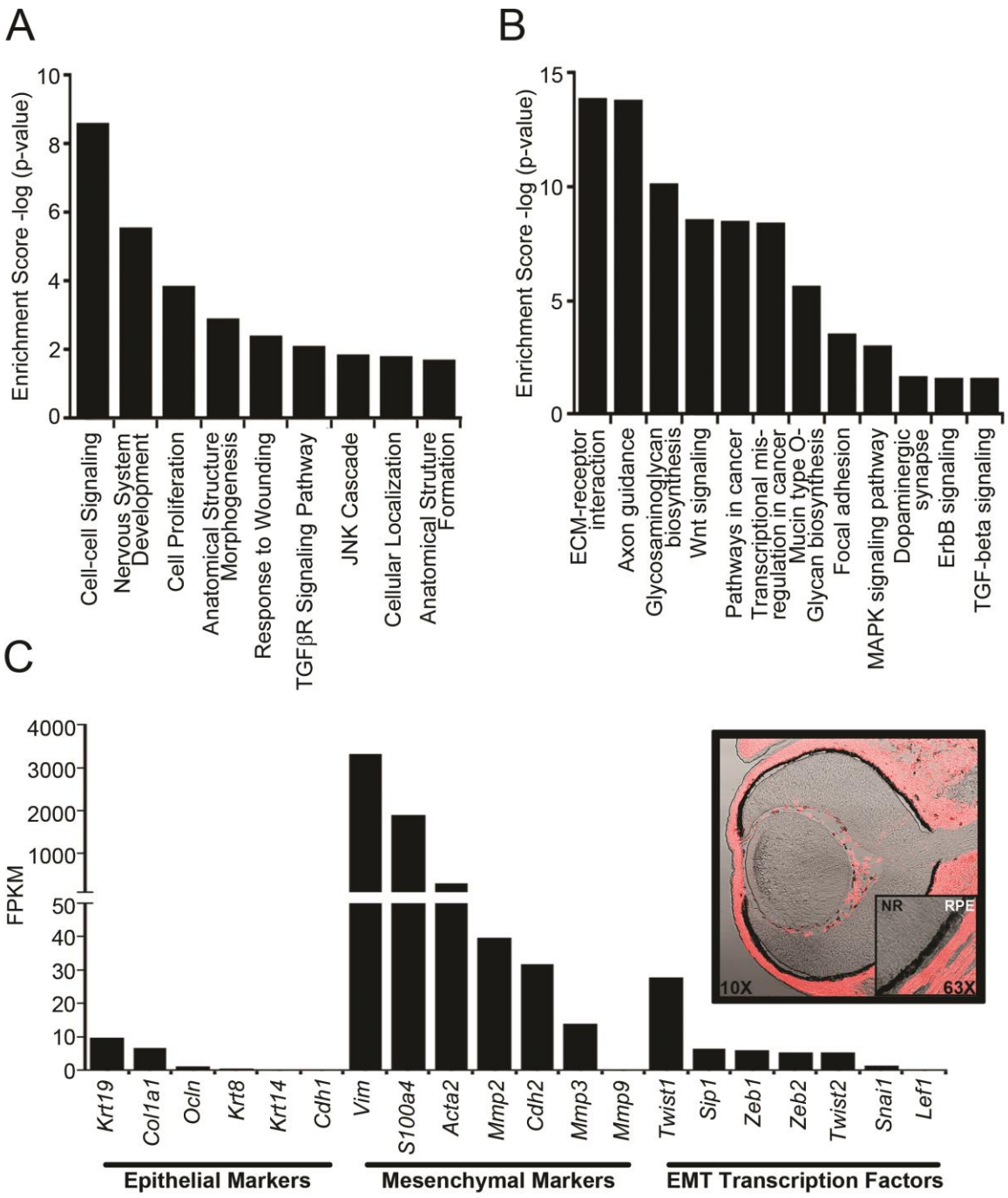

Figure 3.3: Gene set enrichment analysis of differentially expressed PVC genes identifies EMT related pathways (A) Gene Ontology (GO) term enrichment analysis for the PVC only genes. Indicated are a subset of enriched

pathways listed in order of significance (q>0.05). (B) KEGG Pathway analysis of microRNA target genes expressed in PVCs. microRNA target prediction was performed using Diana miRPath v2.0 Top ten pathways are listed in order of significance (q>0.05). (C) EMT marker gene expression in the PVCs (FPKM). Insert shows phase contrast and fluorescence overlay of Wnt1-cre, tdTomato E14.5 mouse eye. PVCs are derived from the neural crest (arrow) while retinal pigment epithelium (RPE) is not (arrowhead, inset). Image by Caitlin Devitt.

While characterizing these cells is critical, the real utility in this model stems from wanting to understand *Arf* activity in a cell that normally expresses the gene during development. To address this, I ectopically expressed *Arf-Rfp* by retroviral transduction in the *Arf* $^{Gfp/Gfp}$ PVCs (Figure 3.4A). Upon ectopic *Arf* expression, I observed activation of the p53 pathway as detected by expression of downstream target effectors that have been previously described, p53, MDM2 and p21 as compared to the Rfp control (Figure 3.4A) (Zhang et al., 1998). Moreover, I have recently shown that the expression of the miR-34 family is dependent upon *Arf* status in cultured cells and *in vivo.* In this analysis, I observed that ectopic *Arf* expression could upregulate all three members of the miR-34 family, miR-34abc in MEFs triple negative for *p53*, *MDM2* and *Arf* (TKO MEFs). Furthermore, shRNA knockdown of p19^{Arf} in *p53* $^{-/-}$ *MEFs*, reduced the expression of miR-34abc (Iqbal et al., 2014a). In the *Arf* $^{Gfp/Gfp}$ PVCs, I observed that ectopic *Arf* expression induced miR-34a and decreased the expression of miR-34b. miR-34c was not affected by p19^{Arf} in these cells (Figure 3.4C). Finally, because I observed that the PVCs had undergone EMT and expressed mesenchymal genes, I became interested in how re-expression of *Arf* affected vascular gene expression. As has been shown previously, Pdgfrβ is down-regulated in response to overexpression of *Arf* (Silva et al., 2005; Widau et al., 2012). Pdgfrα mRNA was not affected by *Arf,* while α*SMA* was significantly decreased upon reintroduction of *Arf* suggesting that it may play a role in

regulating vascular gene expression. These cells will be a useful tool in clarifying how $p19^{Arf}$ affects vascular smooth muscle biology.

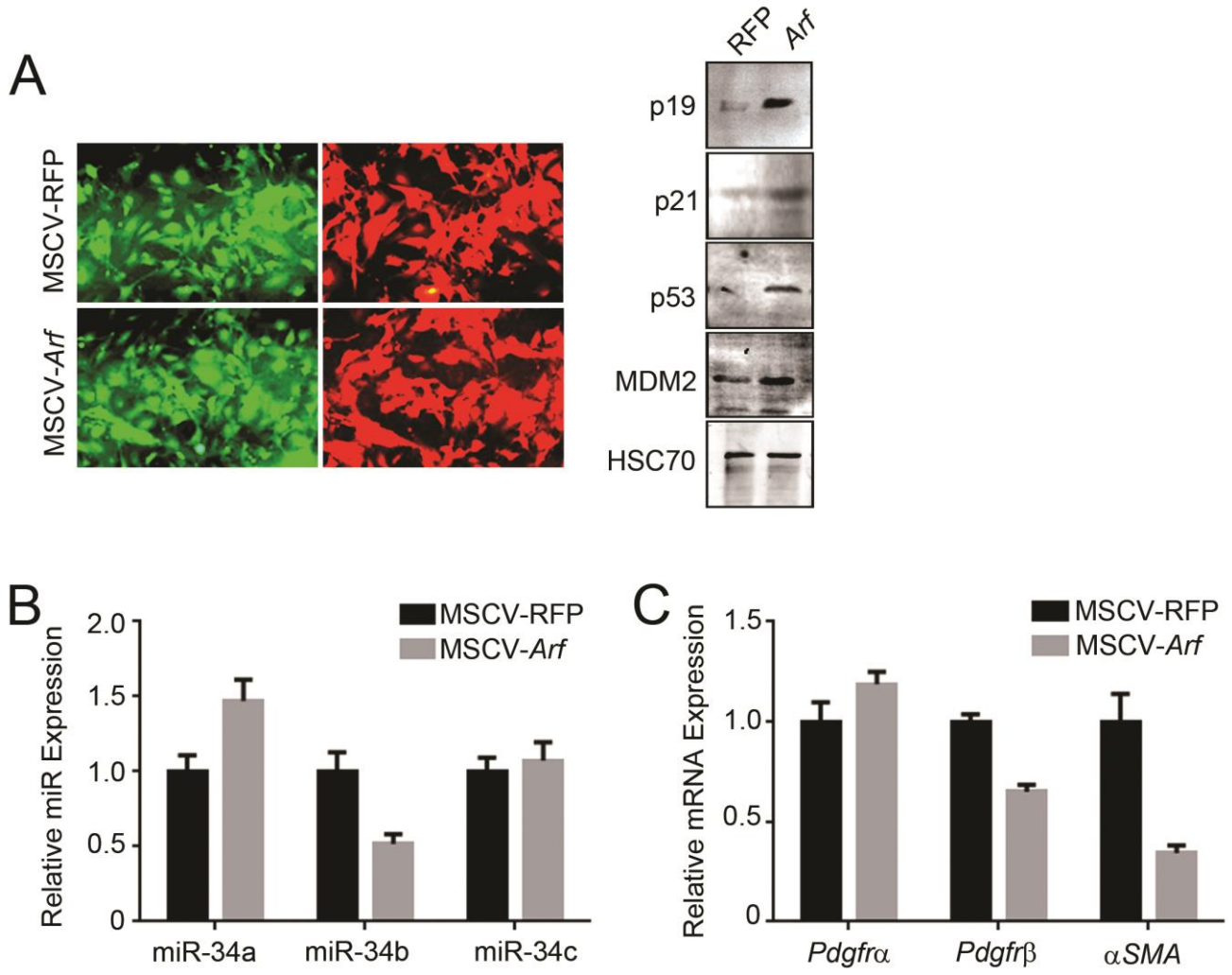

Figure 3.4: Ectopic p19^{Arf} expression in PVCs activates the p53 pathway (A) Retroviral transduction of PVCs with MSCV-RFP (top panel) or MSCV-Arf (bottom panel). (B) Representative western blot showing induction of p21, p53 and MDM2 upon ectopic p19^{Arf} expression (C) Quantitative analysis by qRT-PCR of miR-34abc family in PVCs upon retroviral transduction of *Arf*. microRNA expression is normalized to U6 and represented as relative to RFP control (D) Quantitative analysis by qRT-PCR of Pdgfrα, Pdgfrβ and αSMA in PVCs transduced with RFP or *Arf*. Expression is relative to RFP control and normalized to Gapdh.

Concluding Remarks

I believe that the PVCs will represent an important model for studying *Arf*, particularly for studies focused on how the *Arf* promoter is activated and the functional consequence of $p19^{Arf}$ expression in these cells. Furthermore, heterotypic interactions between perivascular and endothelial cells help to drive vascular stabilization and remodeling. Indeed, the molecular mechanism by which $p19^{Arf}$ regulates vascular/mural cell identity and proliferation, as well as the contribution of the human *CDKN2A* locus and intergenic 9p21 region to cardiovascular disease risk remain unclear. In the current model, loss of *Arf* in perivascular cells seems to derail the developmentally-timed regression of the underlying endothelial cells of the hyaloid vasculature. Given that *Arf* expression in normal cells is largely limited to perivascular cells embracing two vascular structures that become essentially functionless in the postnatal period, these cells could be particularly valuable tools to study perivascular-endothelial cell interactions. Additionally, by examining microRNA and protein changes that occur when *Arf* is expressed in these cells, we will be able to better understand the full repertoire of $p19^{Arf}$ dependent changes that drive vascular involution.

CHAPTER 4:

$p19^{Arf}$ limits primary vitreous cell proliferation driven by PDGF-B

Introduction

During mammalian eye development, the hyaloid vascular system (HVS) in the primary vitreous provides nutrients to foster lens and retina development. This vasculature consists of the hyaloid artery, which feeds the vasa hyaloidea propria (VHP) and tunica vasculosa lentis (TVL) (Goldberg, 1997; Ito and Yoshioka, 1999). To achieve optimal vision, this fully formed vasculature regresses in later stages of eye development, resulting in the avascular and largely acellular secondary vitreous. Defects in HVS regression and primary vitreous maturation lead to ocular disease in children. This disease, variously known as either persistent hyperplastic primary vitreous (PHPV) or persistent fetal vasculature (PFV) (Goldberg, 1997; Haddad et al., 1978) covers a wide spectrum of severity from mere remnants of the hyaloid artery stalk to cellular hyperplasia leading to erosion of the lens capsule posteriorly and dysplasia and tractional detachment of the retina, leading to blindness (Goldberg, 1997; Haddad et al., 1978). Currently, therapeutic interventions largely hinge on surgical interventions to try to preserve or restore vision or remove a severely diseased eye (Hunt et al., 2005; Pollard, 1997).

The underlying pathogenetic mechanisms are likely to vary with disease severity, and this notion is supported in pre-clinical mouse models of the disease.

These mechanisms can largely be divided into fundamental defects in pro-apoptotic processes needed to clear the cells from the vitreous and defects in processes that check hyperplastic expansion of primary vitreous cells – quite literally paralleling the two descriptors for the disease: PFV and PHPV. The former is reflected in mouse models with germ-line destruction of the p53 tumor suppressor gene (Ikeda et al., 1999) or angiopoietin-2 (Gale et al., 2002; Hackett et al., 2002) or in mice lacking macrophage-like hyalocytes (Lang and Bishop, 1993) or the WNT7B signals needed for them to exert cytotoxic effects on endothelial cells in the HVS (Lobov et al., 2005). In contrast, deregulated vascular endothelial growth factor (VEGF) (Mitchell et al., 1998; Rutland et al., 2007) or platelet-derived growth factor-B (PDGF-B) (Lei et al., 2010; Niklasson et al., 2010) support the hyperplastic expansion of primary vitreous cells and PHPV-like phenotype. Anti-proliferative factors have also been implicated. In particular, loss of the *Arf* tumor suppressor gene leads to hyperplasia of Pdgfrβ-expressing perivascular cells that normally flank the hyaloid artery (McKeller et al., 2002; Widau et al., 2012). The phenotype of *Arf* $^{-/-}$ mice mimics many aspects of PHPV, including microphthalmia; retrolental, fibrovascular mass that erodes the lens capsule and distorts the retina; and dense lens opacity resulting in blindness (Martin et al., 2004; McKeller et al., 2002). Although *Arf* $^{-/-}$ mice develop bilateral, severe eye disease, contrasting the typical clinical presentation, loss of *Arf* in just a subset of primary vitreous cells through somatic mosaic deletion leads to a

more variable phenotype with incomplete disease penetrance – better mimicking the clinical scenario (Mary-Sinclair et al., 2014).

Because *Arf* is normally expressed in perivascular cells of the primary vitreous, it represents an essential component of normal mouse eye development (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005). *Arf* encodes $p19^{Arf}$, a nuclear protein best known for its capacity to negatively regulate Mdm2, thereby stabilizing (and activating) p53 (Honda and Yasuda, 1999; Weber et al., 1999). A linear pathway from $p19^{Arf}$ to p53 cannot fully account for the developmental effects, though, because p53 deficient mice usually have normal eyes, whereas *Arf* loss leads to PHPV in a variety of pure and mixed mouse strains (Ikeda et al., 1999; McKeller et al., 2002; Reichel et al., 1998). Clues to the biochemical effects of $p19^{Arf}$ first came from genetic studies showing that the hyperplastic phenotype is ameliorated in *Arf* $^{-/-}$, *Pdgfrβ* $^{-/-}$ embryos (Widau et al., 2012), and $p19^{Arf}$ expression correlates with lower expression of Pdgfrβ in the developing eye, and in cultured mouse embryonic fibroblasts (MEFs) (Silva et al., 2005; Thornton et al., 2007; Widau et al., 2012). More detailed mechanistic studies – also conducted in MEFs and 10T1/2 fibroblasts – demonstrated that $p19^{Arf}$ expression can block *Pdgfrβ* mRNA transcription in a p53-dependent way and Pdgfrβ protein translation independently of p53 (Widau et al., 2012). Finally, miR-34a, a microRNA known

to be controlled by p53, is also induced by $p19^{Arf}$ and is required for p53-indendent translational repression of Pdgfrβ (Iqbal et al., 2014a). Though this detailed molecular mechanism explains the ocular phenotype in the absence of *Arf*, it is important to recognize that the experiments were conducted in mouse fibroblasts, whereas the cells that normally express $p19^{Arf}$ are pericyte-like cells derived from the neural crest (Silva et al., 2005; Zheng et al., 2010). In this report, I functionally characterize and confirm aspects of *Arf* biology previously defined in other cell types and *in vivo* in an *ex vivo* cell culture model that represents the first model in which the *Arf* promoter is normally active.

Methods

Cell isolation and culture

PVCs were isolated from early post-natal *Arf* $^{Gfp/Gfp}$ mice. Method for isolation is described in detail in (Iqbal et al., 2014b). PVCs were cultured in Pericyte Medium (PM) (ScienCell) and passaged using trypsin/EDTA. Tgfβ1 (R&D Systems), was added to cell culture medium at a dose of 5 ng/ml; an equivalent volume of vehicle (4mM HCl) was added into the medium as a control. PVCs were transduced with *MSCV-RFP* or *MSCV-Arf-RFP* retrovirus. 16 hours

post transduction, culture media was replaced. 50ng/mL PDGF-B was added to the cells for 16 hours prior to harvesting for cell cycle analysis.

RNA expression

Total RNA was extracted from PVCs with miRNeasy mini kit (Qiagen). For qRT-PCR, 1 μg of total RNA was reverse transcribed using Superscript III RT kit (Invitrogen) according to the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) was performed with KAPA SYBR Green Mastermix (KAPA) on BioRad (CFX96). The PCR program consisted of 20 sec at 95 ºC, followed by 40 cycles of 95 ºC for 15s and 60 ºC for 20s. Primer quality was analyzed by dissociation curves. The expression of *Pdgfrβ* was normalized to *Gapdh.*

Cell cycle Analysis

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 0.5mg/ml. After 1 hour of incubation at 37°C, PI was added to the suspension to a final concentration of 10μg/ml. PI-stained cells were analyzed for DNA content

with a BD Calibur flow cytometer and Watson Pragmatic Model to calculate the distribution of cells in G_1, S, and G_2 cell cycle phases. Results are averaged from 3 biological replicates.

Western blot

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, 1:5,000), anti-p53 (Sc-6243, Santa Cruz, 1:1000). Band intensity was quantified using ImageJ software. Results are averaged from 3 biological replicates

Results

To verify that the aforementioned molecular and genetic pathway is relevant to the cells normally expressing *Arf*, I took advantage of primary cultures of primary vitreous cells (PVCs) that I purified by flow cytometry based on expression of a Gfp reporter in *Arf* $^{Gfp/Gfp}$ (functionally, *Arf* $^{-/-}$) animals (Iqbal et al., 2014b). I previously characterized the PVCs by global gene expression analysis that highlighted, among other things, their pericyte-like nature (Iqbal et al.,

2014b). These cells are readily apparent as early as embryonic day (E) 11.5 (Martin et al., 2004; McKeller et al., 2002; Silva et al., 2005) and dramatically increase in number in the primary vitreous space by E13.5 (Silva et al., 2005) and Figure 4.1A). Several pieces of data indicate that Tgfβ2 is required for *Arf* induction in the primary vitreous. First, *Tgfβ2* $^{-/-}$ mice have a variety of developmental defects, including primary vitreous hyperplasia (Saika et al., 2001; Sanford et al., 1997), and this correlates with decreased expression of $p19^{Arf}$ (Freeman-Anderson et al., 2009). Second, transgenic expression of Tgfβ1 driven from the α-crystallin promoter can correct the primary vitreous hyperplasia in *Tgfβ2* $^{-/-}$ mouse embryos, but not in *Arf* $^{-/-}$ embryos, which indicates that $p19^{Arf}$ is needed for the anti-proliferative effects of Tgfβ in the eye (Zheng et al., 2010). Dual immunofluorescence staining shows $p19^{Arf}$ and the Tgfβ receptor TbrII to be coexpressed, suggesting that this protein signals to induce *Arf* directly (Freeman-Anderson et al., 2009). This was confirmed to be true in MEFs and 10T1/2 cells: exogenous Tgfβ1, 2, or 3 increases Smad2/3 binding to the *Arf* gene, recruits RNA polymerase II, and then increases *Arf* mRNA and protein expression (Freeman-Anderson et al., 2009; Zheng et al., 2010). Interestingly, even though I isolated the PVCs by virtue of Tgfβ2-driven *Arf* promoter activation and Gfp expression, addition of Tgfβ1 (5ng/ml) further increased *Gfp* mRNA expression in these cells *ex vivo* (Figure 4.1B). Hence, the developmental signaling pathway that is critical for eye development is maintained in cultured PVCs. This finding is

also consistent with previous global gene expression analyses showing that components of the Tgfβ pathway defined as being important for *Arf* induction, such as *Smad2/3*, *Sp1* and *Cebpβ*, are expressed in PVCs (Iqbal et al., 2014b).

As highlighted above, $p19^{Arf}$ inhibits cell proliferation and, in the context of eye development, it specifically blunts mitogenic effects of PDGF-B by down-regulating the expression of Pdgfrβ (Silva et al., 2005). To test whether $p19^{Arf}$ similarly arrests cultured PVCs, I utilized propidium iodide staining followed by flow cytometry and quantification using the Watson Pragmatic Model Watson 1987 (Watson et al., 1987). I generated MSCV-based retroviral vectors containing *Arf* cDNA upstream of an IRES element driving expression of Rfp (Iqbal et al., 2014b). Transducing PVCs with the *Arf-IRES-Rfp* vector significantly decreased the fraction of cells in S-phase with an accumulation of cells in G_1 and G_2 phases as compared to the Rfp control (Figure 4.1C). Of note, it is not currently possible to study the effect of <u>endogenous</u> *Arf* expression in PVCs for several reasons. First, $p19^{Arf}$ expression from the wild type allele in an $Arf^{Gfp/+}$ mouse severely restricts cell accumulation *in vitro*, and would likely do so *ex vivo*. Indeed, earlier work with $Arf^{+/-}$ MEFs demonstrated that serial expansion (which would be required if attempting to propagate PVCs from the very small numbers in a phenotypically normal $Arf^{Gfp/+}$ eye) usually results in cells that have lost the remaining wild type allele (Zindy et al., 1998). Second, it

is not yet possible to purify *Arf* $^{Gfp/+}$ PVCs before *Arf* is expressed as I depend on Gfp expression for the flow cytometry sorting, and the *Arf* promoter drives the Gfp reporter. Nonetheless, the capacity for ectopic expression of $p19^{Arf}$ to mimic the *in vivo* arrest provides a new opportunity for structure-function analyses of $p19^{Arf}$ and identification of downstream effectors in one of the very few cell types known to normally express this protein in the developing mouse.

As mentioned above, it is known that primary vitreous hyperplasia in the *Arf* $^{-/-}$ mouse is driven by Pdgfrβ (Silva et al., 2005; Widau et al., 2012). Hence, I evaluated how PDGF-B, the ligand for this receptor, influenced PVC proliferation and the capacity for $p19^{Arf}$ to block the effects using the explanted cells. First, exposure of serum-starved PVCs to PDGF-B (50ng/ml) for 16 hours increased the S-phase fraction by over two fold, but ectopic expression of $p19^{Arf}$ completely abrogated this effect (Figure 4.1D). I correlated this blockade with the ability for $p19^{Arf}$ to significantly repress *Pdgfrβ* mRNA and protein (Figures 4.1E and F). I conclude that *ex vivo* studies of PVCs faithfully reflect the *in vivo* biology: PDGF-B drives PVC proliferation and *Arf* expression blocks it by a mechanism that leads to decreased Pdgfrβ expression.

Although $p19^{Arf}$ is most well-known for its capacity to sequester Mdm2 and thereby stabilize p53 (Honda and Yasuda, 1999; Weber et al., 1999), the protein

also acts independently of p53 to inhibit ribosomal RNA processing (Sugimoto et al., 2003), associate with E2F1 and c-Myc to inhibit their trans-activating potential (Datta et al., 2004; Eymin et al., 2001; Qi et al., 2004), promote sumoylation of Mdm2, nucleophosmin and other proteins (Rizos et al., 2005; Tago et al., 2005) and more recently, mediate post-transcriptional repression of Drosha, a microRNA processing enzyme (Kuchenreuther and Weber, 2013). Genetic evidence from mouse studies suggests that $p19^{Arf}$ likely controls primary vitreous expansion in a manner that does not strictly depend on p53. Ocular development is normal in most strains of mice lacking p53; however, certain pure BALB/c and pure C57BL/6 lines of *p53* $^{-/-}$ mice have a PHPV/PFV-like phenotype with variable penetrance (Ikeda et al., 1999). Further, the developmental defect in C57BL/6 mice is abrogated when the animals are bred into a mixed C57BL/6 x 129/Sv background (Reichel et al., 1998). In contrast, PHPV consistently develops in *Arf* $^{-/-}$ mice in pure C57BL/6 and pure 129/Sv lines as well as mixed C57BL/6 x 129/Sv animals (McKeller et al., 2002). I have taken this to mean that while p53 may play a role in transcriptional repression of *Pdgfrβ* mRNA, *Arf* expression represses the protein through p53 independent mechanisms (Widau et al., 2012).

I utilized the PVC model to evaluate the role that p53 plays in *Arf*-dependent repression of Pdgfrβ mRNA and protein. Because the PVCs retain

the p53 gene, I developed PVC sub-lines in which LMP-based retroviral vectors (obtained from S. Lowe, Memorial Sloan Kettering Cancer Center) delivered either control or p53-specific shRNA, and puromycin was used to select pools of PVCs retaining p53 (shCTL) and those with p53 knockdown (shp53). Although not complete, the knockdown is functionally significant: ectopic expression of *Arf* in the shCTL cells robustly increases p53 (Figure 4.2E, lanes 1 and 2), but similar transduction of *Arf* into shp53 cells only increases p53 to a level that is still lower than baseline in the control cells (Figure 4.2E, compare lanes 2 and 4). As another functional measure, doxorubicin (Dox) augments the expression of p21Cip1, a well-known p53 target (Harper et al., 1993), in a dose-dependent manner; this effect is also dramatically decreased in the p53 knockdown PVCs (Figure 4.2A). I also observed that the baseline S-phase fraction was slightly increased in the shp53 PVCs at baseline (Figure 4.2B, lanes 1 vs 5), but in both cases exogenous PDGF-B drove additional cells into S phase (Figure 4.2B, lanes 1 vs 2 and 5 vs 6). Importantly, ectopic *Arf* expression blunted the mitogenic effects of PDGF-B in the presence and in the absence of p53 knockdown (Figure 4.2B, lanes 2 vs 4 and 6 vs 8, and Figure 4.2C).

I used this system to address the importance of p53 in two Arf-dependent responses: Pdgfrβ mRNA and protein repression and miR-34a induction. First, as with the parental PVCs (Figure 4.1E), ectopic *Arf* expression significantly

decreased *Pdgfrβ* mRNA but this effect was completely negated when p53 was knocked down (Figure 4.2D). In contrast, *Arf* expression still retained the capacity to repress Pdgfrβ protein in shp53 PVCs, though the effect was somewhat moderated (Figures 4.2E). In a similar way, *Arf* can increase the expression of primary miR-34a, but only in the presence of p53 (Figure 4.2F), but p53 is at least partly dispensable for induction of mature miR-34a – indicating a p53-independent role for $p19^{Arf}$ in processing of this microRNA (Figure 4.2G). All of these molecular effects are similar to those that observed *in vivo* during primary vitreous development and maturation (Martin et al., 2004; Silva et al., 2005; Widau et al., 2012).

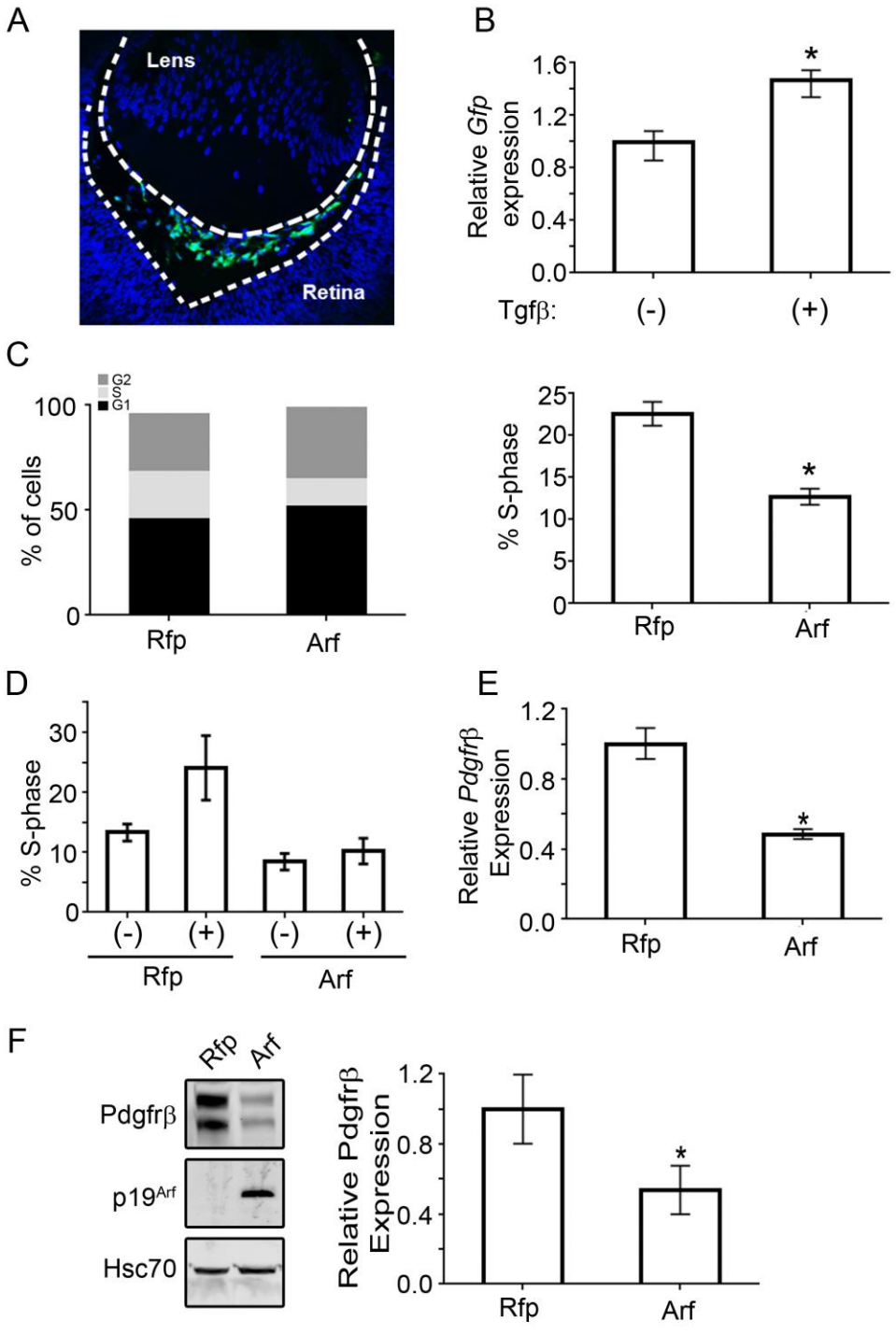

Figure 4.1: p19^{Arf} responds to exogenous Tgfβ and inhibits PDGF-B driven proliferation. A) Representative photomicrograph image of developing vitreous

at E13.5 from *Arf* $^{Gfp/Gfp}$ mouse. B) Relative expression of *Gfp* as a surrogate marker for exon1β measured by qRT-PCR. *Gfp* expression is normalized to *Gapdh* control. C) Quantification of DNA content of PVCs as detected by propidium iodide and flow cytometry. Quantification of S-phase fraction is shown on right. D) $p19^{Arf}$ inhibits PDGF-B driven proliferation. Cells were stimulated with 50ng/mL PDGF-B for 16 hours and cell cycle was analyzed as above. E) Relative expression of *Pdgfrβ.* F) Representative western blot showing Pdgfrβ, $p19^{Arf}$ and Hsc70 protein expression in lysates prepared from PVCs. Western signal (on right) is quantified using the Odyssey Image Studio Lite system and normalized to Hsc70.

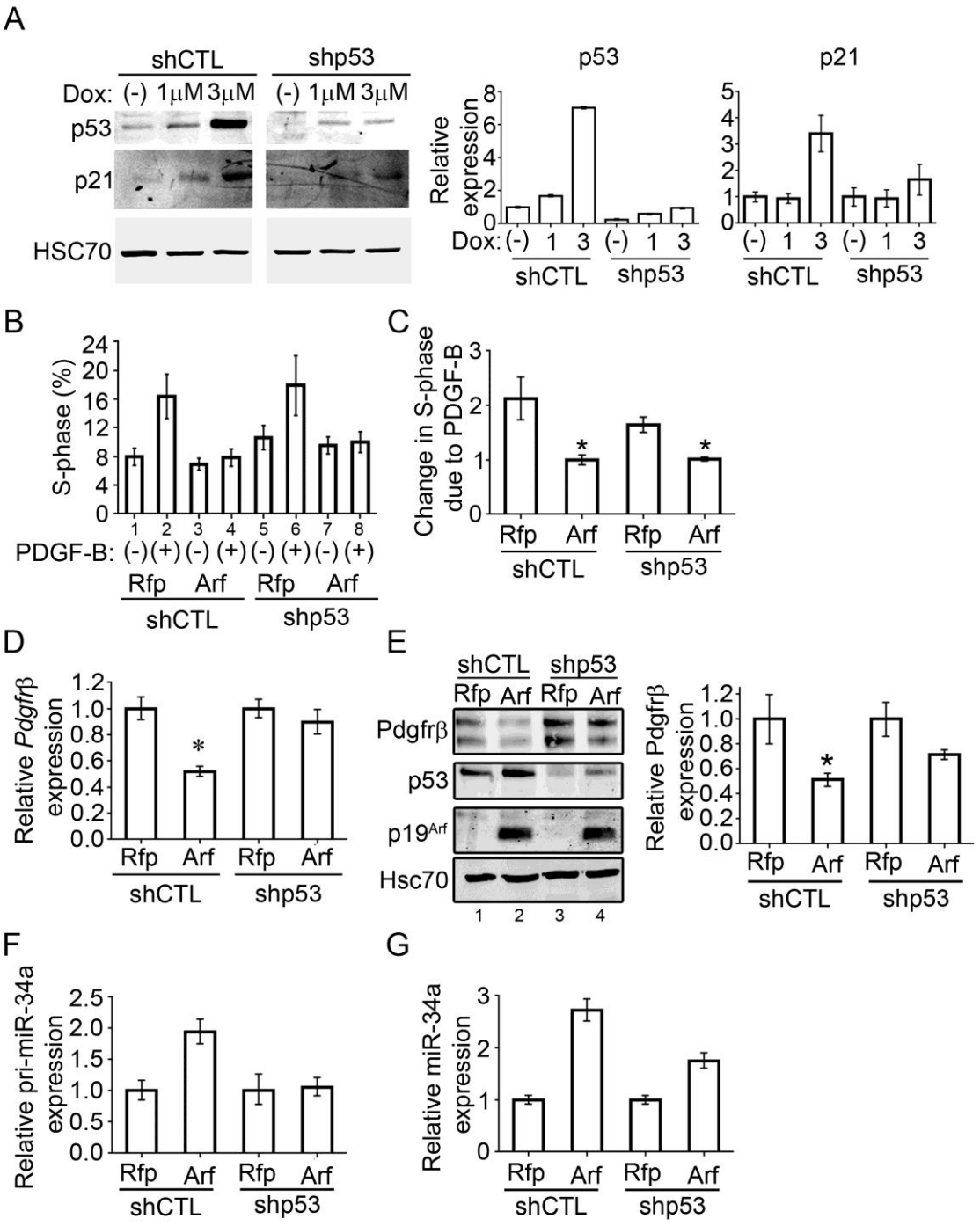

Figure 4.2: p19^{Arf} inhibits PDGF-B driven proliferation independently of p53.
A) Representative western blot showing p53 induction in response to doxorubicin

(Dox) treatment. Quantification, normalized to Hsc70, is on right. B) Quantification of percent of cells in S-phase as measured by propidium iodide and flow cytometry. C) Fold change of S-phase fraction of cells in response to PDGF-B. D) qRT-PCR showing relative expression of *Pdgfrβ* mRNA. E) Representative western blot showing Pdgfrβ, p53, $p19^{Arf}$ and Hsc70 protein expression in lysates prepared from shCTL or shp53 cells. Western signal (on right) is quantified using the Odyssey Image Studio Lite system and normalized to Hsc70. F) Relative expression of primary(pri)-miR-34a transcript. G) Relative expression of mature miR-34a.

Concluding Remarks

In the mouse, the mature testis, the developing eye and umbilical arteries are the only sites where numerous *Arf* expressing cells are normally found (Freeman-Anderson et al., 2009; Silva et al., 2005; Zindy et al., 2003). *Arf* expressing cells in the eye include those perivascular cells embracing the hyaloid artery and also cells scattered in the cornea (Silva et al., 2005; Thornton et al., 2007; Zindy et al., 2003). Primary vitreous hyperplasia is the only recognized developmental defect due to *Arf* gene inactivation (Martin et al., 2004; McKeller et al., 2002), and this ocular disease is due to Pdgfrβ-driven proliferation in the aforementioned perivascular cells (Silva et al., 2005; Widau et al., 2012). Because the PVCs are unique as the only cell type known to be altered without *Arf*, it was important to validate the functional and biochemical effects of $p19^{Arf}$ in these cells. Indeed, using this new *ex vivo* model, I have established the following: 1) The *Arf* promoter can be engaged by Tgfβ to induce *Arf* expression in PVCs *ex vivo*; 2) Ectopic *Arf* expression arrests PVC proliferation, including proliferation driven by PDGF-B; 3) Cell proliferation arrest by $p19^{Arf}$ correlates with repression of Pdgfrβ; 4) and *Arf* expression in PVCs checks proliferation, decreases Pdgfrβ protein, and induces mature miR-34a independently of p53. All of these new findings accurately reflect the *in vivo* effects of $p19^{Arf}$ during eye development.

CHAPTER 5:

Discussion and Future Directions

Although initially described as a tumor suppressor that acts through p53, $p19^{Arf}$ clearly has functional capabilities that do not depend on this downstream effector (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998). Such activities include its capacity to interfere with ribosomal RNA processing, perhaps by translational repression of Drosha; inhibition of signaling to NFκB; blockade of Myc-driven transcriptional activation; and fostering sumoylation of Mdm2 and other nuclear proteins (Datta et al., 2004; Kuchenreuther and Weber, 2013; Qi et al., 2004; Rizos et al., 2005; Rocha et al., 2003; Sugimoto et al., 2003; Tago et al., 2005). 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* $^{-/-}$*, Pdgfrβ* $^{-/-}$ animals demonstrates that this biochemical pathway is crucial for a normal developmental process (Widau et al., 2012).

The data in this report allow me to posit a model by which $p19^{Arf}$ controls Pdgfrβ expression by two mechanisms: p53-dependent transcriptional repression and post-transcriptional repression via miR-34a, a process that can be separated from p53 (Figure 5.1). 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 (Ikeda et al., 1999; McKeller et al., 2002; Reichel et al., 1998). 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* (Ikeda et al., 1999; McKeller et al., 2002; Reichel et al., 1998). However, in a mixed C57BL/6 x 129/Sv lineage, the eyes are usually normal (McKeller et al., 2002). It was previously speculated that this might be due to a p53-independent capacity for $p19^{Arf}$ to block Pdgfrβ translation, and its ability to do so might vary with genetic background (Widau et al., 2012). I can now attribute this behavior to *Arf*-dependent regulation of miR-34a and related microRNAs in the eye.

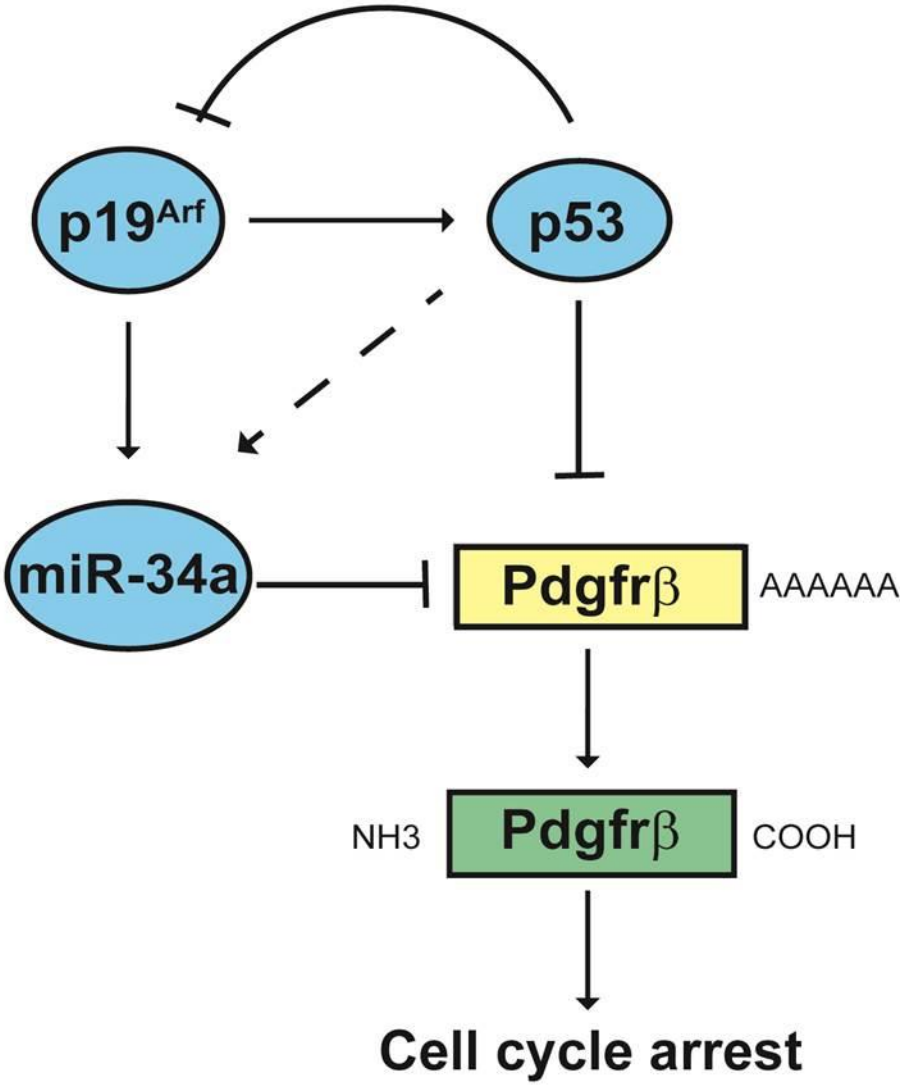

Figure 5.1: Model for Arf-mediated regulation of Pdgfrβ. $p19^{Arf}$ mediates transcriptional repression of *Pdgfrβ* by stabilizing p53 and post-transcriptional repression of Pdgfrβ protein levels via induction of miR-34a and potentially other microRNAs. Although *Arf* expression can induce miR-34a and other microRNAs independently of p53 in cultured MEFs (solid arrow), p53 may contribute to miR-34a expression in the developing mouse eye (dashed arrow).

This model helps to resolve another surprising paradox. Although miR-34a is well-known to be a transcriptional target of p53 and it exerts anti-proliferative effects in a number of tumor models, mice lacking all three members of the miR-34 microRNA family are seemingly normal with no overt developmental defects or cancer susceptibility (Chang et al., 2007; Concepcion et al., 2012; He et al., 2007; Tarasov et al., 2007). 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. However, loss of miR-34 family members might unmask an eye development defect in certain lines of *p53*-deficient mice. This can be evaluated by examining the eyes of mice null for the miR-34 family and p53.

These findings further 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 (Jacks et al., 1994; Kamijo et al., 1999). Tumorigenesis studies in the *RIP-Tag2* mouse model, as well as earlier studies of intestinal adenomas arising in *Arf, Ink4a* double knock-out mice bred into the *Min* mouse, indicate that $p19^{Arf}$ might influence tumor vascular biology (Gibson et al., 2003; Ulanet and Hanahan, 2010). In neither case is a molecular explanation clear. Indeed, Ulanet and Hanahan carried out a broad

array of molecular analyses, including RNA-based analysis of a panel of angiogenesis-related genes, and they found no significant differences (Ulanet and Hanahan, 2010). Given the 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 $p19^{Arf}$, protein-based assays might be more revealing as one explores possible anti-angiogenic effects and p53-independent tumor suppression by $p19^{Arf}$ (Kawagishi et al., 2010).

Since the *Arf* cDNA was first cloned, most of the cell-based functional studies have been carried out in MEFs, a wide range of fibroblast cell lines, and cancer cell lines – none of which reflect a "normal" cellular context. Despite that inherent limitation, much has been gleaned from such studies. Indeed, our own laboratory has defined most of the aforementioned molecular and cell biological effects of $p19^{Arf}$ in MEFs and 10T1/2 fibroblasts (Silva et al., 2005; Weber et al., 2000). However, a more granular understanding of *Arf* biology is likely to depend on context – expressing the protein in the right cell type. Gaining a better understanding of the nature of the perivascular cells and how *Arf* influences their biology represents one such area. These particular PVCs are nearly unique for two reasons. First, they embrace blood vessels that undergo dramatic regression in the immediate postnatal period. Second, they are the only cells in

the eye that express $p19^{Arf}$. They are “nearly” unique because the umbilical arteries branching from the internal iliac vessels represent another vascular system that becomes superfluous in the immediate postnatal period, and this is the only other vasculature enveloped by *Arf* expressing perivascular cells (Freeman-Anderson et al., 2009). Of note, we have not yet uncovered a defect in the umbilical vessels when $p19^{Arf}$ is absent. Nearly all work on *Arf* in the eye has focused on how it controls the number of these PVCs – essentially, how it blocks primary vitreous hyperplasia. Yet, the fact that *Arf* is expressed in the cells from E11.5, just as the hyaloid vessels are forming (Silva et al., 2005), and the expression is extinguished at P5, just as the vessels begin to regress (Mitchell et al., 1998), suggests another role for $p19^{Arf}$: it might actually be required to temporarily stabilize the otherwise transient vessels. Perivascular cells, especially pericytes that represent a particular type of vascular mural cell, provide trophic signals to underlying endothelial cells (Hanahan and Folkman, 1996; Yancopoulos et al., 2000). Having developed an *ex vivo* culture model for those PVCs, we are only now in a position to establish more sophisticated systems to study these potential heterotypic interactions with endothelial cells and the role that $p19^{Arf}$ may play.

Similar comments can be made about the emerging role that $p19^{Arf}$ can play as a microRNA regulator. I have shown that this protein can increase the

expression of miR-34a independently of the p53 pathway, and miR-34a is needed for post-transcriptional Pdgfrβ repression and p53-independent cell proliferation control. Others have shown – using MEFs – that $p19^{Arf}$ physically interacts with Drosha, and this interaction both increases and decreases the expression of a wide range of microRNAs (Kuchenreuther and Weber, 2013). I do not yet understand the mechanism by which $p19^{Arf}$ can induce miR-34a independently of p53, nor do I know the full spectrum of microRNA changes that accompany *Arf* expression in PVCs that normally express the protein. Because both of these are likely to be cell context-dependent, conducting the work in PVCs may provide the correct perspective for such studies. Ultimately, these types of analyses may inform our understanding of PHPV/PFV pathogenesis.

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