Expression of the Arf tumor suppressor gene is controlled by Tgfβ2 during development

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The Arf tumor suppressor (also known as Cdkn2a) acts as an oncogene sensor induced by ‘abnormal’ mitogenic signals in incipient cancer cells. It also plays a crucial role in embryonic development: newborn mice lacking Arf are blind due to a pathological process resembling severe persistent hyperplastic primary vitreous (PHPV), a human eye disease. The cell-intrinsic mechanism implied in the oncogene sensor model seems unlikely to explain Arf regulation during embryo development. Instead, transforming growth factor β2 (Tgfβ2) might control Arf expression, as we show that mice lacking Tgfβ2 have primary vitreous hyperplasia similar to Arf+/− mice. Consistent with a potential linear pathway, Tgfβ2 induces Arf transcription and p19Arf expression in cultured mouse embryo fibroblasts (MEFs); and Tgfβ2-dependent cell cycle arrest in MEFs is maintained in an Arf-dependent manner. Using a new model in which Arf expression can be tracked by β-galactosidase activity in Arflocz+/− mice, we show that Tgfβ2 is required for Arf transcription in the developing vitreous as well as in the cornea and the umbilical arteries, two previously unrecognized sites of Arf expression. Chemical and genetic strategies show that Arf promoter induction depends on Tgfβ receptor activation of Smad proteins; the induction correlates with Smad2 phosphorylation in MEFs and Arf-expressing cells in vivo. Chromatin immunoprecipitation shows that Smads bind to genomic DNA proximal to Arf exon 1β. In summary, Tgfβ2 and p19Arf act in a linear pathway during embryonic development. We present the first evidence that p19Arf expression can be coupled to extracellular cues in normal cells and suggest a new mechanism for Arf control in tumor cells.

KEY WORDS: Arf tumor suppressor gene, Ocular development, Tgf beta, Mouse

INTRODUCTION

It is now well recognized that p19Arf, encoded by Arf (Cdkn2a – Mouse Genome Informatics) at the mammalian Arf/Ink4a locus (Kamijo et al., 1997), has a range of p53-dependent and -independent effects that contribute to its anti-cancer activity (Sherr, 2006). However, how the expression of p19Arf is regulated is much less well understood. It was first discovered to be induced in cultured mouse embryo fibroblasts (MEFs) by what has been termed ‘culture shock’ (Kamijo et al., 1997; Sherr and DePinho, 2000) and by the expression of certain oncogenes (de Stanchina et al., 1998; Palmero et al., 1999; Zindy et al., 1998). Implicit in the ‘oncogene sensor’ model for p19Arf is that Arf expression is controlled by cell-intrinsic mechanisms. However, this concept has not been rigorously tested. We recently discovered that Arf is also expressed in a subset of perivascular cells enveloping the hyaloid vasculature in the vitreous of the developing eye (Martin et al., 2004; McKeller et al., 2002). The hyaloid vessels are unusual because they abruptly involute in the postnatal period, and Arf is required for this process. It seemed unlikely that cell-intrinsic responses to oncogenic signals would explain this restricted expression pattern.

As we explored candidate factors that might control Arf, we focused on members of the Transforming growth factor β (Tgfβ) family. Tgfβ2s were initially identified in mammalian fibroblasts transformed with murine or feline sarcoma viruses and in cultured human melanoma cell lines as proteins that alter fibroblast morphology, and promote proliferation and growth in soft agar (De Larco and Todaro, 1978; Marquardt et al., 1983; Marquardt et al., 1984). The three mammalian Tgfβs transduce signals through a heteromeric complex containing type I and II Tgfβ receptor serine/threonine kinases (TβRI and TβRII; Tgfbr1 and Tgfbr2, respectively – Mouse Genome Informatics); binding of Tgfβ to the receptor complex activates a variety of pathways via both Smad-dependent and Smad-independent signals (Bierie and Moses, 2006; Derynck and Zhang, 2003; Massague et al., 2000). Among their effects is the capacity to arrest cell proliferation and block cancer progression, which appears to contrast the original observations. Of the three members in this family, Tgfβ2 was the most interesting because both Arf−/− (McKeller et al., 2002; Martin et al., 2004) and Tgfβ2−/− (Saika et al., 2001; Sanford et al., 1997) mice display primary vitreous hyperplasia during embryonic development. We investigated the possible relationship between Tgfβ2 and Arf using complementary cell culture-based models and in vivo models.

MATERIALS AND METHODS

Mice and cell lines

Mice in which Arf exon 1β was inactivated (Kamijo et al., 1997) or replaced by a reporter gene encoding green fluorescence protein (Gfp) (Zindy et al., 2003) or encoding β-galactosidase (made in essentially the same way, A.C.M. and S.X.S., see Fig. S5 in the supplementary material) were maintained in a mixed C57BL/6 × 129/Sv genetic background. Tgfβ2−/− mice (Sanford et al., 1997) were purchased from Jackson Laboratories. Primary MEFs from wild-type and Arf−/− mice were cultivated as previously described (Zindy et al., 1997). Animal studies were approved by the St Jude Children’s Research Hospital and the University of Chicago Animal Care and Use Committees.

Histology studies

For in vivo studies, tissue was harvested from euthanized mice and fixed for 4 hours in 1% formaldehyde, 0.2% glutaraldehyde, 0.2% NP-40 and 0.1% SDS; washed in PBS; and stained in 1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5

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RESULTS

Ocular defects in Tgfb2−/− embryo eyes

Currently, the only established developmental activity of p19Arf is to control the accumulation of cells within the vitreous (Silva et al., 2005). If it acted with Tgfβ2 during this process, we reasoned that the developmental defects in the absence of Arf should parallel those in Tgfb2−/− embryos. Without Arf, increased numbers of vitreous cells are detectable at embryonic day (E) 13.5, approximately 1 day after its expression is evident (Silva et al., 2005). At that point, there is excess proliferation in the vitreous, a defect that is intrinsic to the Arf-expressing cells (Thornton et al., 2007). Notably, Arf loss does not measurably change the small number of apoptotic cells (Silva et al., 2005). Like Arf−/− mice, Tgfb2−/− mice displayed obvious primary vitreous hyperplasia at E18.5 and at birth, whereas Tgfb2−/− and Tgfb2−/− eyes were normal (Fig. 1A; see Fig. S2 in the supplementary material). Quantitative analyses of younger embryos revealed increased cell density in the primary vitreous of Tgfb2−/− versus wild-type embryos as early as E13.5, and this was associated with increased cell proliferation (Fig. 1A,B; see Fig. S3 in the supplementary material). As in Arf−/− embryos (Silva et al., 2005), the average percentage of apoptotic cells (3.1±3.8% versus 2.5±2.1%, n=4~350 cells counted for each genotype, P=0.81) was similar in the presence or absence of Tgfβ2, respectively. Proliferation continued in the hyperplastic primary vitreous of Arf−/− and Tgfb2−/− embryos at E18.5 (Fig. 1C). Lastly, nearly all of the hyperplastic vitreous cells expressed Pdgfr in Tgfb2−/− embryos (Fig. 1D), again mimicking the findings in Arf−/− embryos (Silva et al., 2005). Hence, loss of Tgfβ2 causes hyperplastic expansion of Pdgfr-expressing cells from early stages of primary vitreous formation, as in Arf−/− embryos.

Relationship between Tgfβ2 and p19Arf in MEFs

To begin to address whether Tgfβ2 could lie ‘upstream’ of Arf in a linear pathway, we explored how the presence or absence of Arf influenced mitogenic or anti-mitogenic effects of Tgfβ2 in cultured MEFs. Exposure of wild-type MEFs to Tgfβ2 for 24 and 72 hours decreased the fraction of cells in S phase by 15-25% (Fig. 2A, lanes 5 and 7). This was balanced by increases in the G0/G1 fraction at

mM K2Fe(CN)6, 2 mM MgCl2, 0.2% NP-40 and 0.1% SDS overnight at 30°C. Tissue was then photographed or processed for either cryostat or microtome sections, used for hematoxylin and cosin (H&E) or immunofluorescence staining for specific proteins.

Gfp, p19Arf, Pdgfrβ and TβII were detected by immunostaining mouse tissue as previously described (Silva et al., 2005) using rabbit anti-Gfp (A6455, Molecular Probes); rat anti-p19Arf (Bertwistle et al., 2004) (provided by C. J. Sherr); goat anti-Pdgfrβ (AF1042, R&D Systems); goat anti-TβII (SC 33931, Santa Cruz); rabbit anti-phosphoserine 465/467 Smad2 (3849, Chemicon); and species-specific secondary antibodies.

Cell density in the primary vitreous was assessed using ImagePro Plus software as follows: digital photomicrographs of H&E-stained sections were used to select an area of interest (AOI) encompassing the entire posterior chamber (see Fig. S1 in the supplementary material). The number of pixels within this AOI was noted. Next, all cells (excluding erythrocytes) within the AOI were highlighted, and the number of highlighted pixels was calculated. The cellularity was then determined by dividing the cell pixel number by the total pixel number in the AOI, and it was presented as a percentage. Staining for Ki67 (Mk167 – Mouse Genome Informatics) and TUNEL labeling were performed and quantified such that the number of stained cells was normalized to the vitreous area, determined using ImageProPlus software as previously described (Silva et al., 2005). The average area of the vitreous in midline sections used in the quantitative studies was the same in wild-type and Tgfb2−/− eyes at this point: 1.48 × 105 versus 1.66 × 105 pixels, respectively (P=0.537).

Stained embryos or slides were photographed using an Olympus BX60 microscope equipped with a SPOT RT Slider camera (Diagnostic Instruments) or using a Zeiss 510 NLO multiphoton/confocal laser scanning microscope.

Cell culture studies

Wild-type and Arf−/− MEFs (passage 2) were treated in parallel 1 day following plating, with or without Tgfβ2 (0, 1, 5, 10 ng/ml), Tgfβ1 (5 ng/ml), Pdgfrβ (50 ng/ml) or an equivalent volume of the relevant vehicle for 24, 48 and 72 hours, at which times cells were harvested for protein or RNA extraction, or cell cycle analysis. Cyclineximide (100 μM, Sigma) and SB431542 (10 μM, Tocris Cookson) were used in some studies.

Protein extraction and immunoblotting were performed essentially as described (Zindy et al., 1998) using 4-12% gradient gels (XCell II – BioRad). Following fixation in 0.5% glutaraldehyde, or by measuring β-galactosidase activity in cell lysates prepared by sequential freeze/thaw in 0.25 M Tris pH 7.8, using Galacto-Light Plus (PE Biosystems).

For cell cycle analyses, cells were incubated with BrdU, harvested with trypsin/EDTA, fixed, permeabilized and stained using anti-BrdU antibody (Sigma) followed by FITC-coupled anti-mouse antibody (DAKO) and propidium iodide (Sigma; 10 μg/ml) and analyzed using either a FACScan or FACS calibur (BD Biosciences, San Jose, CA, USA). Relative cell cycle change represents the percent difference in the fraction of Tgfβ2-treated cells in a particular phase as compared with vehicle treated cells.

Chromatin immunoprecipitation

Arf−/−Mef (3 × 106/immunoprecipitation) treated with Tgfβ2 (5 ng/ml) or vehicle for 1.5 hours were crosslinked, sonicated and immunoprecipitated with anti-Smad2/3 antibody (sc6033, Santa Cruz) or IgG (AB–108-C, R&D Systems) as a control. Protein A/G sepharose beads (sc2003, Santa Cruz) were used to collect the protein-chromatin complexes. The beads were washed sequentially with low salt, high salt, LiCl and TE buffers (Upstate ChIP Kit, Millipore) and eluted in 0.1 M NaHCO3, 1% SDS. Crosslinking was reversed by incubation at 67°C overnight and the genomic DNA was extracted with a Qiagen PCR Purification Kit. A total of 3% of the precipitated DNA and 1% input DNA was amplified by PCR using primer sets for Ild1 and different regions of Arf (primer sequences are available upon request). The PCR products were resolved on 1.5 % agarose gels and stained with ethidium bromide.

Quantitative and non-quantitative studies

All quantitative studies were accomplished using three or more samples from separate animals, or three or more separate cell culture experiments. Non-quantitative results from histological studies are representative of findings using at least three embryos of the indicated genotypes, obtained from two or more separate experiments. Statistical significance of any quantitative differences was assessed by Student’s t-test.

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both times and in the G2/M fraction at 24 hours (Fig. 2A, lanes 1, 3 and 9). Tgfβ2-dependent changes in Arf−/− MEFs paralleled those in wild-type cells at 24 hours (Fig. 2A, compare lanes 1 and 2; 5 and 6; 9 and 10). However, the pronounced block of cells in S phase and the accumulation of cells in G1 at 72 hours were not maintained without p19Arf (Fig. 2A, compare lanes 3 and 4; 7 and 8; 11 and 12). The requirement for Arf correlated with p19Arf induction by Tgfβ2 at 48 and 72 hours (Fig. 2B, lanes 2 and 3 versus lane 1; see Fig. S4A in the supplementary material); p19Arf was not induced at 24 hours (see Fig. S4B in the supplementary material). The ability of p19Arf to maintain Tgfβ2-driven growth arrest may depend on p53 (Trp53 – Mouse Genome Informatics), which cooperates with Tgfβ1 to control proliferation in MEFs (Cordenonsi et al., 2003). However, we did not observe Arf-dependent induction of p21Waf1 (Cdkn1a – Mouse Genome Informatics), a well-described p53 target, or other Cdk inhibitors (see Fig. S4A in the supplementary material). Moreover, p21−/− mice did not display vitreous hyperplasia (see Fig. S4C in the supplementary material), and it is hence not the main Tgfβ2 target in the eye.

Mechanistic studies in MEFs
We investigated the mechanistic aspects of Tgfβ2-driven p19Arf induction. We first explored whether p19Arf induction correlated with increased Arf mRNA and promoter activity. The latter was addressed using a new reporter mouse, in which Arf exon 1β was replaced by lacZ CDNA encoding the β-galactosidase reporter; X-gal staining in the mouse recapitulated the previously described Arf expression in the eye and the testis (Fig. 2C; see Fig. S5 and Fig. S6A,B in the supplementary material). Like Gfp expression in the ArfβlacZ mouse (Martin et al., 2004; Zindy et al., 2003), β-galactosidase activity increased as ArfβlacZ MEFs were cultivated using a 3T9 protocol (see Fig. S6C in the supplementary material), and as they grew more confluent (Fig. 2D, lanes 2, 5, 8), paralleling previous findings with native p19Arf (Kamijo et al., 1997; Sharpless et al., 2004). We observed that Tgfβ2 increased Arf mRNA in wild-type MEFs and Arf promoter transcription in ArfβlacZ MEFs at 72 but not 24 hours, correlating with changes at the protein level (Fig. 2D, lanes 8 and 10 versus 2 and 4; see Fig. S7A in the supplementary material), although the reporter detected Arf induction earlier than the qRT-PCR assay. Lastly, p19Arf expression fell following cycloheximide exposure in Tgfβ2-treated MEFs at least as rapidly as in the control (see Fig. S7B in the supplementary material). Hence, Tgfβ2 controls p19Arf expression by inducing its promoter without measurably changing the stability of the protein.

Next we addressed whether Tgfβ1 or platelet derived growth factor B (Pdgfb) shared the capacity to induce Arf expression in the eye, and whether they act as a potent mitogen in MEFs (Silva et al., 2005). All the effects of Tgfβ1 on Arf expression paralleled those of Tgfβ2 (Fig. 2D, lanes 3, 6, 9 versus 4, 8, 10; see Fig. S7A in the supplementary material). By contrast, Pdgfb did not induce the reporter at 48 or 72 hours (Fig. 2D, lanes 12 and 13; negative data not shown). Therefore, Arf induction in this cell culture model does not simply represent a response to supraphysiological mitogens.

The role of Smad proteins
We also tested if the observed effects depended on Smad2 because this signaling protein was phosphorylated in response to Tgfβ2 in MEFs (Fig. 2E). To test this, we exposed the cells to SB431542, an inhibitor of Tβr1 (Seay et al., 2005). This chemical inhibited Smad2 phosphorylation at serine 465/467 and blocked both p19Arf and β-galactosidase induction in wild-type and ArfβlacZ MEFs, respectively (Fig. 2F,G). We also used a genetic approach to exclude
the possibility that this might represent an off-target effect of the chemical. The ectopic expression of the inhibitory Smad6 (Derynck and Zhang, 2003) impeded Arf promoter activation by Tgfβ2 (Fig. 2H), proving that Smad-dependent signals stemming from the Tgfβ receptor drive p19Arf expression.

Next, we considered whether Smads directly influence the Arf promoter because multiple potential Smad binding elements (SBEs) flank Arf exon 1β (Fig. 3C). Chromatin immunoprecipitation (ChIP) using an antibody recognizing Smad2 and Smad3 is described to show Smad binding at promoters like that driving mouse Id1 expression (Smith et al., 2009). In our experiments, Smad2/3 binding was evident at baseline levels and it increased 1.5 hours following exposure of ArflacZ/lacZ MEFs to Tgfβ1 (Fig. 3A). To interrogate the Arf gene, we designed 12 PCR primer sets spanning the –2.2 kb to +1.1 kb region flanking exon 1β (Fig. 3B,C; primer sequences are available upon request). ChIP assay shows that
Smad2/3 binding was observed at amplicons 1 and 3 proximal to the first exon in ArflacZ/lacZ MEFs 1.5 hours following the addition of Tgfβ1 (Fig. 3C,D). Binding at amplicons 2 and 4 was also observed, but the signal was weaker. Selective Smad2/3 binding to the chromatin was not convincing at other regions, including those with putative SBEs. Interestingly, the relatively small amount of binding observed at amplicon 2 suggests that Smad2/3 might bind to two distinct regions in amplicons 1 and 3. Thus, although increased Arf expression was not readily detected until 48 hours following the addition of either Tgfβ1 or 2, increased Smad2/3 binding is present in the Arf promoter shortly after Tgfβ1 exposure.

Tgfβ2 controls Arf expression in the mouse eye

We felt it was important to verify that our findings in MEFs were relevant to Arf regulation in the mouse eye. Immunofluorescence staining detects p19Arf in subnuclear foci in the mouse testis (Bertwistle et al., 2004). p19Arf was similarly detected in vitreous cells in Tgfb2+/− and Tgfb2−/− embryos at E13.5, but not in the Tgfb2−/− littersmates stained in parallel (Fig. 4A). To determine whether decreased p19Arf represented decreased transcription in vivo, we employed ArfGfp/Gfp and ArfGfp/Gfp Tgfb2−/− embryos to ask this question. As expected, vitreous hyperplasia developed at E13.5 in ArfGfp/Gfp embryos that were heterozygous for Tgfβ2, and nearly all of the vitreous cells expressed the Arf promoter. We determined whether decreased p19Arf was associated with decreased transcription in vivo, we employed ArfGfp/Gfp and ArfGfp/Gfp Tgfb2−/− embryos to ask this question. As expected, vitreous hyperplasia developed at E13.5 in ArfGfp/Gfp embryos that were heterozygous for Tgfβ2, and nearly all of the vitreous cells expressed the Arf promoter (Fig. 4B, panel a). However, this was not the case for Tgfβ2−/− embryos (Fig. 4B, panel b). Consistent with this, our observations in ArfGfp/Gfp embryos (Fig. 2C) showed that Tgfβ2−/− embryos do not provide essential mitogenic signals to drive vitreous hyperplasia, but Gfp staining showed Arf promoter activity to be markedly decreased (Fig. 4B, panel c). Similar observations using Tgfb2−/− ArfGfp/Gfp embryos at E13.5 (Fig. 4C) proved that the effects of Tgfβ2 on Arf expression were not specific to a single reporter and did not depend on an Arf-deficient state. Consistent with potentially direct signaling, immunofluorescence staining revealed that Gfp and TβRII expression overlapped in some of the vitreous cells of ArfGfp/Gfp embryos (Fig. 4D). Furthermore, phosphoSmad2 was present in nuclei in the primary vitreous in E13.5 wild-type embryos and throughout the retrolental mass in newborn Arf−/− mice (Fig. 4E). Because essentially all of the cells in the retrolental mass expressed the Arf gene in the embryo (Fig. 4B) and in the newborn period (Martin et al., 2004), we conclude that Tgfβ2 directly impacts cells expressing Arf.

Tgfβ2 controls Arf expression at other embryonic sites

Finally, a survey of the ArfGfp/Gfp embryos revealed two new sites where Arf is expressed during development: within the stroma of the developing cornea at E12.5 and E13.5 (Fig. 5Aa,b) and around the umbilical arteries within the abdominal/pelvic cavities between E13.5 through to the early postnatal period (Fig. 5C; see Fig. S8A in the supplementary material). Expression in the cornea stroma at this stage is interesting because Tgfβ2 induces extracellular matrix proteins such as collagen I and lumican, enhancing cornea thickness (Saika et al., 2001). However, there is no obvious thinning in the Arf−/− cornea (see Fig. S9 in the supplementary material). Arf expression in the umbilical arteries, which are essential only during embryo development (like the hyaloid vasculature), parallels the pattern in the vitreous in that the cells are perivascular in location and some coexpress Pdgfrβ (Fig. 5Cc; see Fig. S8B in the supplementary material). However, aspects of umbilical artery biology appeared unaffected by Arf loss. For example, the atresia that typically develops in the left umbilical artery between E13.5 and E14.5 (Warot et al., 1997) still occurs without p19Arf (Fig. 5Cb). In both the cornea and the umbilical arteries, absence of Tgfβ2 diminished Arf expression (Fig. 5Ac,d; Fig. 5B,D). Although the functional relevance of this
pathway in umbilical artery and cornea development is not yet clear, we can confidently conclude that Tgfβ2-mediated control of Arf promoter activity extends beyond the vitreous of the eye.

DISCUSSION

The clear role that Arf plays in mouse development implies that its control must extend beyond that provided by the deregulated signals accompanying the activation of certain oncogenes. Whereas this regulatory paradigm focuses on cell-intrinsic signaling, our findings provide the first evidence that Arf expression can be controlled by specific cell-extrinsic signals from Tgfβ2 during development and in MEFs. We can now begin to integrate our findings with emerging knowledge regarding the regulation of p19Arf (Gil and Peters, 2006; Kim and Sharpless, 2006).

First, although there is some evidence that its expression is controlled by post-transcriptional mechanisms (Colombo et al., 2005), our findings using two different reporter systems indicate that the Tgfβ3-dependent induction depends largely, and perhaps exclusively, on transcriptional activation. Unfortunately, regulatory mechanisms guiding transcription of Arf and the flanking Ink4a (Cdkn2a – Mouse Genome Informatics) and Ink4b (Cdkn2b – Mouse Genome Informatics) genes are complex and incompletely understood. The activity of certain Polycomb group proteins (notably Bmi1) leads to the methylation and silencing of Arf, Ink4a and to a lesser extent Ink4b in MEFs (Jacobs et al., 1999). In the developing mouse, the relative importance of Bmi1-mediated repression of specific genes at this locus is cell type-dependent (Bruggeman et al., 2005; Molofsky et al., 2005). The entire
Despite the fact that our ChIP studies show Smad2/3 binding to sites flanking Arf1999) positively or negatively regulate mouse Myc (Zindy et al., 1998) and Twist (Maestro et al., 1998), mechanisms that are not reflected in a transiently expressed reporter. For example, FoxO family members bind a regulatory element further removed from the promoter. They could also indicate that Smad-dependent changes near the promoter must cooperate with other cis regulatory elements that are blocked by chemical inhibition of TβrI and the ectopic expression of Smad6, its candidacy is strengthened by the fact that Smad2 phosphorylation and can be diminished in the absence of Tgfβ2 expressing cells in vivo indicates that Tgfβ2 signaling can directly impact the cells that express the Arf promoter; second, because Arf induction correlates with Smad2 phosphorylation and can be blocked by chemical inhibition of TβrI and the ectopic expression of Smad6, it seems safe to conclude that a Smad-dependent process is required in MEFs; lastly, our ChIP assay using a Smad2/3-specific antibody showed that Smad proteins directly bind to the Arf gene. That Smad2 phosphorylation is detected in Arf-expressing cells in the embryo suggests but does not prove it to be the crucial Smad in vivo. Its candidacy is strengthened by the fact that Smad3β+ mice, which are viable, appear to have normal eyes (Banh et al., 2006; Zhu et al., 1998). Formally evaluating the role of Smad2 may require its knock-out in cells destined to express the Arf promoter because Smad2β– mice suffer early embryonic lethality (Nomura and Li, 1998; Weinstein et al., 1998). The genetically engineered mice needed to accomplish this experiment are not currently available.

Certain mechanistic facts must still be elucidated. Like in other scenarios, we suspect that Smads cooperate with other transcription factors to enhance Arf transcription. The region bound by Smad2/3 in our experiments contains many putative transcription factor binding sites, including potential sites for C/EBPβ (also known as Cebpβ), a known Smad-interacting protein (Ross and Hill, 2008) (TFSEARCH result; http://www.cbrc.jp/hbin/nph-tfsearch). However, its role in Arf regulation is not evident from the literature. The rapid localization of Smad2/3 to the promoter, and the delayed increase in measurable Arf expression, challenges us to more broadly consider the role Smads might play in this process. For example, Smad binding might initiate a cascade of events that make the chromatin more accessible to other transcriptional activators.
even those like FoxO that act at a distance. As such, gaining insight into the Tgfβ-dependent changes in DNA methylation and histone status is likely to be informative. Lastly, although Smad2/3 binding to the promoter is detectable, given the fact that Smad6 preferentially inhibits BMP signaling (Goto et al., 2007; Kirkbride et al., 2008), it is possible that BMPs; Smads 1, 5 and 8; and TβRII might also contribute to Arf regulation.

Our findings shed light on the molecular basis for the crucial developmental process guiding the maturation of the primary vitreous into the avascular and largely acellular secondary vitreous, a developmental process that is essential for normal vision, and this might be relevant to human eye disease. We previously established that without Arf, vision is severely compromised because the vitreous hyperplasia obscures the optic lens and destroys the retina (Martin et al., 2004). This ocular defect resembles severe PHPV, a disease long known to be caused by failed maturation of the primary vitreous and persistence of the hyaloid vessels (Goldberg, 1997; Haddad et al., 1978). Tgfβ2 resides upstream of Arf in this developmental program. Interestingly, this observation is also consistent with a recent finding that selective inactivation of TβRII using Cre recombinase expressed from a neural crest-specific promoter appears to mimic the Arf<sup>−/−</sup> eye phenotype (Ittner et al., 2005). Based on these mouse models, we suggest that a search for the molecular basis for the human disease should include studies of human Arf, TGFβ2 and the yet-to-be-defined components of the signaling pathway.

Tgfβ2 loss leads to a complex developmental phenotype that includes multiple craniofacial, cardiac, pulmonary, urogenital and skeletal defects (in addition to the ocular abnormalities mentioned here) (Sanford et al., 1997). Molecular mechanisms underlying these other defects are still elusive; certainly, the absence of these other defects in Arf<sup>−/−</sup> mice indicates that, unlike the primary vitreous hyperplasia, they do not arise merely due to failed induction of Arf. This also implies that, although Tgfβ2 is required for Arf expression at certain sites, it is not sufficient at others. We are taking advantage of our complementary cell culture-based model and the ArflacZ<sup>+/−</sup> reporter mouse to identify putative cooperating signaling proteins. More refined molecular or physiological studies will be required to determine the functional consequences of loss of Arf in the cornea and umbilical artery where the Tgfβ2/p19<sup>Arf</sup> pathway is intact but anatomic abnormalities are not apparent in the absence of Arf.

Beyond development and eye disease, our finding of a linear pathway between Tgfβ and p19<sup>Arf</sup> has potential implications for the tumor suppressor actions of these proteins. It is interesting that both Tgfβ1 and p19<sup>Arf</sup> block early papilloma formation triggered by DMBA/TPA in the mouse (Cui et al., 1996; Kelly-Spratt et al., 2004); however, Tgfβ1 does not hinder progression of the papilloma to an invasive cancer (Cui et al., 1996). Conceptually, anti-cancer effects of Tgfβ in this model could be a result of p19<sup>Arf</sup> induction, and Arf deletion might coincide with loss of the tumor suppressive effects of Tgfβ. Dynamic regulation of p19<sup>Arf</sup> at different phases of cancer development, like the sequential induction of Arf during Myc-driven lymphomagenesis, has been attributed to the accumulation of additional oncogenic events (Bertwistle and Sherr, 2007). Our observations support an alternative hypothesis that extracellular signals from Tgfβ, derived from either tumor cells or stroma elements, might contribute to Arf regulation at different stages of tumor development and progression.

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**Author contributions**

All authors contributed to experimental design and implementation and assisted with data analysis and writing; N.E.A. carried out experiments showing Arf regulation by Tgfβ<sub>1</sub> in vivo, cell cycle analyses and some in vitro mechanistic studies, and wrote the first manuscript draft; Y.Z. carried out most of the in vitro mechanistic studies, including experiments proving Smad-dependence and ChIP assays; A.C.M. generated the Arf lacZ targeting construct, Arf<sup>lacZ+</sup> mouse ES cells, and much of the phenotype analysis; S.S. conceived of and guided the overall project direction, assisted with experimental conduct and data analysis, and wrote final drafts of the manuscript.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/12/2081/DC1

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Tgfβ2 controls Arf expression


