Expression of the Arf tumor suppressor gene is controlled by Tgfb2 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 (Tgfb2) might control Arf expression, as we show that mice lacking Tgfb2 have primary vitreous hyperplasia similar to Arf–/– mice. Consistent with a potential linear pathway, Tgfb2 induces Arf transcription and p19Arf expression in cultured mouse embryo fibroblasts (MEFs); and Tgfb2-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 Tgfb2 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 Tgfb 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, Tgfb2 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 (Tgfb) family. Tgfb3s 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 Tgfb3s transduce signals through a heteromeric complex containing type I and II Tgfb receptors serine/threonine kinases (TβRI and TβRII; Tgfr1 and Tgfr2, respectively – Mouse Genome Informatics); binding of Tgfb 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, Tgfb2 was the most interesting because both Arf–/– (McKeller et al., 2002; Martin et al., 2004) and Tgfb2–/– (Saika et al., 2001; Sanford et al., 1997) mice display primary vitreous hyperplasia during embryonic development. We investigated the possible relationship between Tgfb2 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. Tgfb2–/– 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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mM K$_4$Fe(CN)$_6$, 2 mM MgCl$_2$, 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, Pdgfb and TßRII 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-Pdgfb (AF1042, R&D Systems); goat anti-TßRII (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 (Mk67 – 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 ImagePro Plus 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: 1.48×10^5 versus 1.66×10^5 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 Tgfb2 (0, 1, 5, 10 ng/ml), Tgfb1 (5 ng/ml), Pdgfb (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. Cycloheximide (100 μg/ml) and analyzed using either a FACScan fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, USA) and gene-specific primers for Real-time PCR 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.

**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 Tgfb2 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=350 cells counted for each genotype, P=0.81) was similar in the presence or absence of Tgfb2, 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 Pdgfb in Tgfb2–/– embryos (Fig. 1D), again mimicking the findings in Arf–/– embryos (Silva et al., 2005). Hence, loss of Tgfb2 causes hyperplastic expansion of Pdgfb-expressing cells from early stages of primary vitreous formation, as in Arf–/– embryos.

**Relationship between Tgfb2 and p19Arf in MEFs**

To begin to address whether Tgfb2 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 Tgfb2 in cultured MEFs. Exposure of wild-type MEFs to Tgfb2 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...
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 ArfGfp+ mouse (Martin et al., 2004; Zindy et al., 2003), β-galactosidase activity increased as ArflacZ/lacZ 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 ArflacZ/lacZ 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 in MEFs. The former was tested because it can reverse primary vitreous hyperplasia (Zhao and Overbeek, 2001), whereas the latter acts as a potent mitogen in MEFs (Silva et al., 2005). All of 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, Tgfβ2 but not Tgfβ1 should be considered as a major player in the regulation of p19Arf expression in MEFs.

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 TβrI (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 ArflacZ/lacZ 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 Tgfβ (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 ArfloacZ/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 newborn period (Martin et al., 2004), we conclude that Tgfβ2 directly impacts cells expressing Arf.

**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/+ and ArfloacZ/+ reporter mice. As expected, vitreous hyperplasia developed at E15.5 in ArfGfp/+ embryos that were heterozygous for Tgfβ2, and nearly all of the vitreous cells expressed the Arf promoter (Fig. 4B, panels a and b), consistent with our observations in ArfloacZ/lacZ embryos (Fig. 2C). Hyperplasia was also evident in ArfloacZ/+ Tgfb2−/− embryos (Fig. 4B, panel c) (formally establishing that Tgfβ2 does not provide essential mitogenic signals to drive vitreous hyperplasia), but Gfp staining showed Arf promoter activity to be markedly decreased (Fig. 4B, panel d). Similar observations using Tgfb2−/− ArfloacZ/+ 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/+ 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; additional data not shown). 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 controls Arf expression at other embryonic sites.

**Tgfβ2 controls Arf expression at other embryonic sites**

Finally, a survey of the ArfloacZ/+ 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. 5A,a,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 at this stage is interesting because Tgfβ2 is expressed during development: within the stroma of the umbilical arteries, absence of Tgfβ2 expression at other embryonic sites (Martin et al., 2004). We identified the retroperitoneal mass in newborn Arf−/− mice (Fig. 5Cb) and 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 (1999) positively or negatively regulate mouse Arf (et al., 1998), Myc (Zindy et al., 1998) and Twist (Maestro et al., 2007). Formal evaluation of the role of Smad2 may require its knockout in cells destined to express 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 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.

Another important mechanistic issue relates to exactly how Tgfb2 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 TgfβR1 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 Smad3−/− 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 knockout in cells destined to express the Arf promoter because Smad2−/− 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,

\[ \text{Expression of Tgfb2 is required for Arf expression in the embryonic cornea and umbilical arteries. (A) Photomicrographs of sections through the cornea in E13.5 mouse embryos of the indicated genotypes and stained with X-gal with (a,b) or without (c,d) Eosin. (B) Quantitative analysis showing that the number of X-gal-positive cells across the cornea from midline sections of eyes is higher in phenotypically normal (wild-type) E13.5 ArflacZ/+ Tgfb2+/+ embryos, as compared with ArflacZ/+ Tgfb2−/− embryos (P=0.0004). (C) Photomicrographs of X-gal-stained umbilical artery (arrows) adjacent to urinary bladder (∗) in E14.5 mouse embryos of the indicated genotype shown as whole mount (a,b) and as cross-section through the umbilical artery (c). Short arrows (a,b) indicate the right umbilical artery, whereas the left umbilical artery is much smaller at this stage. Arf-expressing cells (blue) are perivascular in the umbilical artery and flank endothelial cells (long arrow, c), which are not X-gal-positive. (D) Photomicrographs show that X-gal staining of right and left umbilical arteries in E13.5 ArflacZ/+ Tgfb2−/+ embryos (arrows, a) is diminished in the absence of Tgfb2 (arrowheads, b).} \]
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 avascularity 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 obliterates 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−/− eye phenotype (Iitner 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−/− 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 ArfflacZ/+ 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/p19Arf 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 p19Arf has potential implications for the tumor suppressor actions of these proteins. It is interesting that both Tgfβ1 and p19Arf 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 p19Arf induction, and Arf deletion might coincide with loss of the tumor suppressive effects of Tgfβ. Dynamic regulation of p19Arf 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.F.A. carried out experiments showing Arf regulation by Tgfβ 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+ mice 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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