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Arf Suppresses Hepatic Vascular Neoplasia in a Carcinogen-Exposed Murine Model

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Abstract

Hepatic haemangiosarcoma is a deadly malignancy whose aetiology remains poorly understood. Inactivation of the *CDKN2A* locus, which houses the *ARF* and *p16^{INK4a}* tumour suppressor genes, is a common event in haemangiosarcoma patients, but the precise role of ARF in vascular tumorigenesis is unknown. To determine the extent to which ARF suppresses vascular neoplasia, we examined the incidence of hepatic vascular lesions in *Arf*-deficient mice exposed to the carcinogen urethane (i.p. 1 mg/g). Loss of *Arf* resulted in elevated morbidity and increased the incidence of both haemangiomas and incipient haemangiosarcomas. Suppression of vascular lesion development by ARF was heavily dependent on both *Arf* gene-dosage and the genetic strain of the mouse. *Trp53*-deficient mice also developed hepatic vascular lesions after exposure to urethane, suggesting that ARF signals through a p53-dependent pathway to inhibit the development of hepatic haemangiosarcoma. Our findings provide strong evidence that inactivation of *Arf* is a causative event in vascular neoplasia and suggest that the ARF pathway may be a novel molecular target for therapeutic intervention in haemangiosarcoma patients.

Keywords

p19Arf; p14ARF; ethyl carbamate; liver; endothelium; haemorrhage; tumour progression

Introduction

Haemangiosarcoma is a rare but deadly disease that accounts for approximately one percent of all sarcoma cases and two percent of all primary liver malignancies in people [1–3]. Hepatic haemangiosarcoma development has been associated with occupational exposure to a number of environmental toxins, and presentation of both cutaneous and visceral haemangiosarcomas after radiotherapy is also common [4–8]. Despite the aggressive nature

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Statement of Author Contributions

SEB and CJK designed the study and interpreted data. SEB conducted experiments, analysed data, generated figures, and wrote the manuscript. KEG conducted experiments. RDM conducted experiments and interpreted data. All authors participated in drafting the manuscript and had final approval of the submitted and published versions.

of this disease, there are few treatment options for patients presenting with haemangiosarcoma of the liver or other internal sites. Aggressive surgical resection is the standard form of treatment, but post-operative chemotherapy regimens are limited in both number and efficacy. Consequently, five-year survival rates for haemangiosarcoma patients are generally poor [1, 2]. The limited success of adjuvant therapy reflects a fundamental lack of insight into the aetiology of haemangiosarcoma. An enhanced understanding of the molecular mechanisms underlying this disease will identify novel targets for clinical intervention.

The *CDKN2A* locus, which houses the *ARF* (*p14^{ARF}* in humans, *p19^{Arf}* in mice) and *p16^{INK4a}* tumour suppressor genes [9], is frequently inactivated in hepatic haemangiosarcoma [3]. Epigenetic silencing of *ARF* occurs in 26% of cases [3], but a direct, causative role for *ARF* in haemangiosarcoma formation has yet to be established. *ARF* is best known for stabilizing p53 activity in oncogene-stressed cells [10–12]. In recent years, additional functions of *ARF* have also been described [13], such as controlling the regression of the hyaloid vasculature system in the developing mouse eye [14–18]. However, the extent to which *ARF* regulates the proliferation or transformation of endothelial cells in adult mice and humans remains poorly studied.

Urethane (ethyl carbamate) is a chemical carcinogen frequently utilized in mouse pulmonary tumourigenesis studies. Occurring naturally in many foods and beverages as a fermentation by-product [19], urethane is metabolized by cytochrome P450 2E1 to form vinyl carbamate epoxide, which in turn produces potentially mutagenic DNA adducts [20]. Notably, urethane's metabolites produce the same DNA adducts as vinyl chloride [21], a chemical known to induce angiosarcoma of the liver in humans [22]. When administered at high doses or with recurrent low dose application, urethane induces long-latency hepatic vascular lesions in wild-type mice [23–26]. One case of urethane-associated hepatic haemangiosarcoma in a human patient has also been reported [27].

Here we show that *Arf*-deficient mice exposed to urethane exhibited a significantly increased incidence of hepatic haemangiomas and incipient haemangiosarcomas. *Arf* loss contributed to multiple stages of vascular tumourigenesis, from early, preneoplastic angiectasis to the ultimate formation of haemangiomas with cellular atypia and haemangiosarcomatous differentiation. We further demonstrate that suppression of vascular neoplasia by *ARF* is heavily influenced by genetic strain, is *Arf* gene-dose-dependent, and likely proceeds through a p53-dependent pathway. Our findings present strong evidence that *ARF* suppresses the formation of vascular tumours in adult mice and, furthermore, provide a model in which to study the aetiology of vascular neoplasia.

Materials and Methods

Animal Model

All experiments were approved by the FHCRC Institutional Animal Care and Use Committee. *Arf*-deficient mice [28] were backcrossed separately onto C57BL/6 (The Jackson Laboratory, Bar Harbor, ME, USA) and NIH/Ola (Harlan Olac, Oxfordshire, UK) strains for 16 and 20 generations, respectively. *Arf*^{+/-} mice were intercrossed and carcinogenesis studies performed on resultant littermates of each genotype: *Arf*^{-/-} (n = 69 for C57BL/6; n = 59 for NIH/Ola), *Arf*^{+/-} (n = 42 C57BL/6; n = 33 NIH/Ola), and *Arf*^{+/+} (n = 30 C57BL/6; n = 56 NIH/Ola). Mice deficient for *Trp53* [29] were backcrossed onto the C57BL/6 strain for 20 generations, and carcinogenesis studies were performed on male mice of each genotype: *Trp53*^{-/-} (n = 11), and *Trp53*^{+/-} (n = 22). DNA for genotyping was isolated by digestion of ear tissue with proteinase K in InstaGene Matrix solution (Bio-Rad, Hercules, CA, USA). Each mouse was genotyped as described [28, 29].

Arf- and *Trp53*-deficient mice were subjected to a single injection of urethane (Sigma, St. Louis, MO, USA) in a PBS vehicle (i.p. 1 mg/g bodyweight) between 21 and 28 days of age. An additional cohort of *Trp53*^{+/-} mice (n = 12) was injected with urethane at 14 days of age. A cohort of unexposed C57BL/6 strain *Arf*^{-/-} (n = 25), *Arf*^{+/-} (n = 23), and *Arf*^{+/+} mice (n = 22) was included for control. Animals were euthanized by CO₂ asphyxiation at indicated time points or when moribund. All tissues were examined for the presence of lesions during necropsy. Normal and pathological tissues were frozen in liquid nitrogen and/or fixed for subsequent analysis.

Histopathology and Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin for at least 4 hrs, then processed and embedded in paraffin. 4-µm-thick tissue sections were cut, mounted on glass slides, and stained with H&E. Tissue samples were submitted to the FHCRC institutional histopathology core for von Willebrand Factor staining (#A0082; Dako, Glostrup, Denmark). A small set of tissue sections was also stained for phosphorylated p42/44 MAPK (pERK) (#4376; Cell Signaling Technology, Danvers, MA, USA). Controls included mouse kidney and rabbit IgG isotype control. All H&E slides were analysed by a veterinary pathologist blind to genotype. Diagnosis of angiectasis was based on dilatation of the liver sinusoids in the absence of proliferating endothelial cells. Diagnosis of haemangioma was determined by the presence of abnormal vascular spaces lined by proliferating endothelial cells. A subset of more aggressive haemangiomas presented with cellular atypia, regions of solid growth, and incipient haemangiosarcomatous differentiation [30]. In order to match the original stained slide, global adjustments to white balance, brightness, and/or colour saturation were made to copies of some photomicrographs using Adobe Photoshop®.

Statistical Methods

Time until development of hepatic vascular lesions causing death or requiring euthanasia was graphically summarized in a Kaplan-Meier plot, and survival differences were analysed for significance using the log-rank test. Animals euthanized for reasons other than the presentation of vascular lesions were considered censored observations in the second log-rank analysis. These animals were included in the analysis of overall mortality for *Arf* mice. The incidence of lesions by 25 weeks post urethane-injection was compared between strains for each genotype, and between *Arf*- and *Trp53*-deficient animals; differences were analysed for significance using the Fisher's exact test. Mice who died before 25 weeks and did not present with macroscopic hepatic vascular lesions were excluded from the Fisher's exact test analyses.

Results

Accelerated morbidity of *Arf*-deficient mice after urethane exposure

To investigate the tumour suppressive role of ARF in vascular neoplasia, we exposed *Arf*-deficient mice to the carcinogen urethane. Kaplan-Meier analysis demonstrated that C57BL/6 *Arf*^{-/-} mice injected with urethane suffered significantly shortened lifespans compared to their *Arf*^{+/+}, *Arf*^{+/-} and untreated *Arf*^{-/-} littermates (Fig. 1A). The median life expectancy of urethane-exposed *Arf*^{-/-} mice was reduced to 262 days from greater than 400 days for all other genotypes. Both *Arf*^{-/-} and *Arf*^{+/-} mice also developed lymphomas and sarcomas (data not shown) at a rate similar to that previously reported [31]. Moribund *Arf*^{-/-} mice frequently presented with lethargy, swollen abdomens, and symptoms of anaemia (e.g. pale ears and digits). During necropsy these animals were found to have grossly diseased livers bearing multifocal, massive thromboses (Fig. 1B). Rupture of these thromboses resulted in abdominal haemorrhaging that caused near-immediate death. In contrast to the marked lethality of urethane to C57BL/6 *Arf*^{-/-} mice, no *Arf*^{+/+} or *Arf*^{+/-} littermates or unexposed

Arf^{-/-} mice succumbed to hepatic vascular lesions by 50 weeks post-injection (Fig. 1C). Lesion incidence and associated lethality were statistically similar between genders (data not shown).

Susceptibility to vascular lesions is strain-specific

Tumour susceptibility in mice is strongly influenced by genetic background. To investigate the effect of genetic background on urethane-induced vascular lesions, we examined tumour incidence in NIH/Ola strain *Arf*-deficient mice (Fig. 1D). As in the C57BL/6 *Arf*^{+/+} mice, urethane injection did not yield gross hepatic lesions in NIH/Ola *Arf*^{+/+} mice. However, in contrast to the 40.9% (9 of 22) of C57BL/6 *Arf*^{+/-} mice that exhibited macroscopic hepatic vascular lesions by 25 weeks, no NIH/Ola *Arf*^{+/-} mice (0 of 32) bore lesions at this age. Furthermore, only 66.7% of NIH/Ola *Arf*^{-/-} mice (12 of 18) developed lesions by 25 weeks, compared to 91.7% of C57BL/6 *Arf*^{-/-} mice (44 of 48). Hepatic vascular lesions that did develop in NIH/Ola strain mice were typically smaller and less physically deleterious than lesions in C57BL/6 mice. Therefore, the susceptibility of *Arf*-deficient animals to vascular carcinogenesis is modified by genetic background, with the relative risk of C57BL/6 strain exceeding NIH/Ola.

Loss of *Arf* facilitates the malignant progression of vascular neoplasia

To determine the histopathological basis of the hepatic lesions, H&E stains for a subset of samples collected at 25 and 50 weeks post-exposure were examined by a veterinary pathologist. The analysis uncovered a tumour progression spectrum of vascular neoplasia in *Arf*-deficient mice, with lesions ranging from angiectasis to benign haemangioma to haemangioma with cellular atypia (Table 1). Low magnification views of representative wild-type and *Arf*-deficient liver lobes are shown in Figure 2 (A, B), and characteristic images of the tumour diagnostic criteria are provided (Fig. 2C–E). By 50 weeks post-exposure, 40% of *Arf*^{+/+} mice exhibited focal angiectasis but were otherwise unaffected compared to *Arf*-deficient mice. At 25 weeks, all *Arf*^{+/-} mice examined presented with angiectasis of the liver. By 50 weeks, 80% of *Arf*^{+/-} mice exhibited haemangioma lesions, with one mouse displaying multifocal cellular atypia and haemangiosarcomatous differentiation. A nearly identical rate of haemangioma incidence (75%) was observed in *Arf*^{-/-} mice, but at the earlier time point of 25 weeks post-urethane exposure. 38% and 40% of *Arf*^{-/-} mice presented with incipient haemangiosarcomas at the 25 and >25 week time points, respectively. One unexposed *Arf*^{-/-} mouse (n = 3) developed a small haemangioma by 50 weeks, and no vascular lesions were observed in unexposed *Arf*^{+/-} mice at the same time point (n = 5; data not shown).

The diagnosis of vascular neoplasia was substantiated by immunohistochemical analysis with von Willebrand Factor (vWF) antibody, an established marker of endothelial cells (Fig. 2F–H). While vWF staining was restricted to the endothelial cells lining hepatic blood vessels in unexposed mice (Supplementary Fig. 1A), abundant vWF positive cells were present in urethane-induced tumours (Fig. 2G, H), confirming the endothelial nature of the hepatic lesions. Vascular lesions that developed in urethane-exposed, *Arf*-deficient animals – including a cardiac haemangioma – were also focally positive for phosphorylated ERK, an indicator of RAS pathway activation (Supplementary Fig. 1A, B). Taken together, these findings suggest that suppression of vascular tumour development by ARF in this model is time- and *Arf* gene-dose-dependent, in that *Arf*^{+/-} mice demonstrated both delayed neoplastic formation and malignant progression compared to their *Arf*^{-/-} littermates.

Incidence of vascular lesions in *Trp53*-deficient mice

Many of the tumour suppressive activities previously attributed to ARF are related to its role in stabilizing cellular p53 levels [13], and *Trp53* loss has been implicated in

haemangiosarcoma development in mice and humans [32–36]. These facts prompted us to determine the extent to which suppression of vascular neoplasia by ARF is p53-dependent. We analysed the effect of urethane exposure on C57BL/6 *Trp53*-deficient male mice. All *Arf*^{+/+}; *Trp53*^{-/-} animals (n = 11) succumbed to thymic lymphomas by 15 weeks post-exposure, and none exhibited hepatic vascular lesions at necropsy. However, 9.5% of *Arf*^{+/+}; *Trp53*^{+/-} mice (2 of 21) developed hepatic vascular lesions by 25 weeks (Fig. 3A), and a small cohort of mixed-gender *Arf*^{+/+}; *Trp53*^{+/-} mice exposed to urethane at an earlier time point (14 days) and allowed to age up to 58 weeks post-injection presented with malignant hepatic vascular lesions (3 of 12; 25%; Fig. 3A, B). Although the 25-week lesion incidence rate for *Trp53*^{+/-} mice (9.5%) was lower than that observed in *Arf*^{+/+} mice (3 of 12 male mice; 25%), this difference was not statistically significant ($P = 0.3275$). The development of vascular tumours in urethane-exposed *Trp53*-deficient mice supports the argument that tumour suppression by ARF in this model is likely mediated by p53.

Discussion

The aetiology of hepatic haemangiosarcoma is poorly understood, and, consequently, patients diagnosed with the disease face extremely poor prognoses. Weihrauch and colleagues previously reported that the *p14^{ARF}* locus is frequently silenced in haemangiosarcomas of the liver [3]. Their work provided correlative but not causative evidence for tumour suppression by ARF in vascular neoplasia. Here we report evidence for a direct, causative role for ARF and its downstream signalling partner p53 in suppressing the formation and malignant progression of primary hepatic vascular tumours. Mice with homozygous deletion of *Arf* exhibited a significant predisposition for the development of haemangiomas and incipient haemangiosarcomas after carcinogen exposure, demonstrating that loss of *Arf* is sufficient to promote vascular neoplasia.

Many cancers are known to develop through a step-wise process, from hyperplastic to benign to malignant lesions. Evidence for this type of evolutionary progression, however, has not been established for haemangiosarcoma development [37]. Our study captured a tumour progression spectrum of hepatic vascular lesions. *Arf*-deficient mice developed lesions ranging from angiectasis to benign haemangiomas and incipient haemangiosarcomas in a time- and *Arf* gene-dose-dependent manner. In particular, *Arf*^{+/-} mice displayed a dramatic disease progression between the 25 and 50-week time points. These findings elucidate the evolution of vascular tumours and, moreover, support the potential use of this mouse model in future investigations into the aetiology of haemangiosarcoma.

An interesting finding from our study was that mouse genetic background strongly influenced susceptibility to the development of vascular tumours after urethane exposure. C57BL/6 *Arf*^{-/-} mice presented with hepatic vascular lesions at a significantly higher frequency than NIH/Ola *Arf*^{-/-} mice. The strain-specificity of this disease phenotype indicates the presence of genetic modifiers that differentially regulate vascular tumourigenesis between strains. Although a previous study of C57BL/6 *Trp53*^{+/-} mice chronically exposed to urethane found a high susceptibility to hepatic vascular carcinogenesis [35], another study observed protection against hepatic vascular lesions in C57BL/6 strain *Vhl*-mutant mice compared to A/J and BALB/c strain mutants [38]. Clearly, further investigation is warranted. Our model may lend itself to the future identification of additional genes involved in haemangiosarcoma susceptibility.

Vascular tumours are believed to result from the aberrant proliferation and transformation of endothelial cells [1]. ARF is required for the developmentally controlled regression of the hyaloid vasculature system in the mouse eye [14], but the question of whether ARF contributes to vasculogenesis or angiogenesis in adults remains largely unanswered. Our

finding that loss of *Arf* promotes the formation of vascular tumours indicates that ARF may, under defined circumstances, control endothelial cell proliferation in adult animals. However, an alternative hypothesis regarding haemangiosarcoma histogenesis posits that the cell of origin is a pluripotent mesenchymal stem cell [30]. This hypothesis holds that although haemangiosarcomas present with an endothelial cell-like appearance, the tumours may in fact not be derived from mature endothelium but rather from a more primitive, progenitor cell.

Arf-deficient mice are prone to spontaneous sarcomagenesis [31], and there is mounting evidence that ARF suppresses the development of multiple sarcoma types in humans [39–44]. This association between altered *ARF/INK4a* expression and the development of a wide range of sarcoma subtypes argues that the *CDKN2A* locus may play a critical role in arresting the growth of a transformed mesenchymal progenitor cell. Indeed, *CDKN2A* was found to be deleted in hTERT-transduced adult mesenchymal stem cells that exhibited anchorage independence and formed tumours when injected into mice [45, 46]. Taken together with these findings, our study supports the argument that the development of vascular tumours upon *Arf* loss reflects changes in mesenchymal stem cell differentiation and proliferation rather than a hyperproliferation of endothelial cells. In either case, the absence of vascular lesions in unexposed *Arf*-deficient mice indicates that for haemangiosarcoma development to occur, *Arf* loss must be accompanied by a cooperative “event” provided by urethane, presumably an oncogenic mutation.

The canonical function of ARF is to antagonize the MDM2 (HDM2 in humans) ubiquitin ligase in oncogene-stressed cells, thereby stabilizing cellular p53 levels and engaging an anti-proliferative response [12, 47]. Urethane is known to cause oncogenic *Ras* mutations in rodents [48–50], and mutant *RAS* has also been identified in human hepatic haemangiosarcoma [51, 52]. In this study, we observed evidence of RAS-ERK pathway activation in urethane-induced vascular lesions. Moreover, we have previously demonstrated that ARF suppresses the malignant progression of RAS-driven squamous cell carcinoma through a p53-dependent pathway [53], and other groups have shown that stabilization of p53 by ARF is required for cancer protection many weeks after exposure to genotoxins [54, 55]. Our findings here reveal a similar pattern. The ARF-p53 axis acts as an important, delayed barrier to carcinogen-induced haemangiosarcoma development.

Our study provides the first causative link between *Arf* loss and vascular neoplasia. Nevertheless, the specific contributions of *ARF* and *INK4a*, the two genes at the *CDKN2A* locus, to tumour suppression in humans remain controversial [13, 28, 56–60]. Deletion of the entire chromosomal region is frequent [61–63], but it is clear that selective inactivation of *ARF* does occur in human cancer [60]. Weihrauch et al have published on the alterations of *INK4a* and *ARF* in human hepatic angiosarcoma [3]. Their findings show that loss of *INK4a* almost certainly contributes to hepatic vascular neoplasia, as 63% of tumours exhibited hypermethylation of *INK4a*. However, *ARF* was also found to be hypermethylated in human hepatic angiosarcoma (26%), and in 11% of patients hypermethylation was specific to *ARF* while *INK4a* remained unmethylated. Furthermore, the strong evidence that p53 and *HDM2* are altered in human vascular neoplasia suggests that their functional partner ARF may also play a role in the disease [33, 34, 36, 64]. In summary, these findings argue that there is selective pressure to inactivate both the ARF-HDM2-p53 pathway and the *INK4a*-RB pathway in human hepatic vascular neoplasia, as is seen in many other human malignancies [65, 66].

The need for novel therapeutics to treat haemangiosarcoma patients is critical. Current standards of treatment are focused primarily on aggressive surgical resection, and the availability of adjuvant chemotherapies is limited, in part because few studies to date have

elucidated the molecular pathways underlying vascular neoplasia [1, 2]. Prognoses for patients diagnosed with haemangiosarcoma will likely not improve without significant advancement in our knowledge of the aetiology of the disease. The identification of ARF as a suppressor of hepatic vascular lesion development illuminates the process of haemangiosarcomagenesis and suggests potential targets for future therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Arf-deficient mice developed lethal vascular tumours after urethane exposure. **A:** The overall mortality of *Arf*^{-/-} mice was increased relative to *Arf*^{+/+} and *Arf*^{+/-} littermates and untreated *Arf*^{-/-} mice (log-rank test, two-tailed; $P < 0.0001$). **B:** Representative images of livers from urethane exposed *Arf*^{+/+} (top) and *Arf*^{-/-} (bottom) mice. Massive, multifocal, blood-filled lesions (arrows) covered the liver surface in *Arf*-deficient mice. **C:** The incidence of lethal hepatic vascular lesions increased in *Arf*^{-/-} mice compared to *Arf*^{+/+}, *Arf*^{+/-}, and untreated *Arf*^{-/-} littermates. Data were censored to include only animals whose deaths were attributed to vascular lesions (log-rank test, two-tailed; $P = 0.0005$). **D:**

Incidence of macroscopic hepatic vascular lesions observed 25 weeks post-urethane exposure in NIH/Ola and C57BL/6 strain $Arf^{+/+}$, $Arf^{+/-}$, and $Arf^{-/-}$ mice (Fisher's exact test, two-tailed; * $P = 0.02$, *** $P < 0.0001$).

Figure 2.

Pathology of urethane-induced vascular lesions. **A–B:** Representative images of liver lobes from urethane-exposed mice (40X magnification). The hepatic parenchyma in *Arf*^{+/+} mice (**A**) remained largely unaffected. Vascular lesions on the hepatic surface in *Arf*^{-/-} mice (**B**) disrupted the hepatic architecture and caused substantial secondary change, including thrombosis and associated fibrosis (asterisk). Regions of dark background shading in the upper corners of (**A**) and (**B**) were removed using Adobe Photoshop®. **C:** Angiectasis. A single layer of endothelial cells lined the enlarged sinusoids (100X). **D:** Uniform, proliferating endothelial cells (arrowheads) formed irregular vascular channels characteristic of haemangioma (400X). **E:** Multifocally, some haemangiomas exhibited cellular atypia and incipient haemangiosarcomatous differentiation. Plump-to-elongated endothelial cells formed small vascular channels (arrows) and appeared clustered and multilayered (400X). **F–H:** vWF staining (400X) highlights the normal sinusoidal endothelial cells in angiectasis

(arrowheads, **F**), shows proliferating endothelial cells lining abnormal vascular spaces in haemangioma (**G**), and reveals atypical endothelial cells aligning in sheets in an incipient haemangiosarcoma (**H**).

Figure 3.

Hepatic vascular neoplasia in *Arf*-deficient mice resembles lesions observed in urethane-exposed *Trp53*-deficient mice. **A:** Incidence of hepatic vascular lesions observed 25 weeks post-urethane exposure in male C57BL/6 *Arf*^{+/-} (3 of 12; 25.0%) and *Trp53*^{+/-} (2 of 21; 9.5%) mice (Fisher's exact test, two-tailed; $P = 0.3275$). Incidence of lesions in *Trp53*^{+/-} mice rose at later time points, as 3 of 12 (25.0%) mice sacrificed between 30 and 58 weeks post-urethane exposure developed lesions. **B:** Haemangiosarcoma from a *Trp53*^{+/-} mouse (40X). Multiple large, blood-filled lakes are present, bordered by solid sheets of plump-to-spindle shaped endothelial cells (inset, 400X).

Vascular tumour progression is *Arf* gene-dosage and time-dependent

Table 1

Lesion grade	<i>Arf</i> ^{+/+}		<i>Arf</i> ^{+/-}		<i>Arf</i> ^{-/-}	
	25wk	50wk	25wk	50wk	25wk	>25wk ^a
	Lesion incidence % (number of cases/total # tissues examined ^b)					
Angiectasis	0 (0/5)	40 (2/5)	100 (4/4)	80 (4/5)	88 (7/8)	80 (4/5)
Haemangioma	0 (0/5)	0 (0/5)	0 (0/4)	80 (4/5)	75 (6/8)	80 (4/5)
Haemangioma w/atypia ^c	0 (0/5)	0 (0/5)	0 (0/4)	20 (1/5)	38 (3/8)	40 (2/5)

^a An insufficient number of C57BL/6 *Arf*^{-/-} mice survived to 50 weeks post-urethane exposure, necessitating the inclusion of samples collected at any time after 25 weeks in the analysis.

^b A subset of liver samples from urethane-exposed, female mice of each genotype was selected at random for scoring. H&E stained sections were diagnosed blind to genotype by a veterinary pathologist.

^c Diagnosis indicates presence of haemangioma with cellular atypia and haemangiosarcomatous differentiation.