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Development of mouse models of angiosarcoma driven by p53

Donald M. Salter1, Meredith Griffin2, Morwenna Muir2, Jayne Teo2, Jayne Culley2, James R. Smith3, Laura Gomez-Cuadrado2, Kylie Matchett2, Andrew H. Sims2, Larry Hayward2, Neil C. Henderson3 and Valerie G. Brunton2,*

INTRODUCTION

Angiosarcomas are a rare group of tumours which have poor prognosis and limited treatment options. The development of new therapies has been hampered by a lack of good preclinical models. Here, we describe the development of an autochthonous mouse model of angiosarcoma driven by loss of p53 in VE-cadherin-expressing endothelial cells. Using Cdh5-Cre to drive recombination in adult endothelial cells, mice developed angiosarcomas with 100% penetrance upon homozygous deletion of Trp53 with a median lifespan of 325 days. In contrast, expression of the R172H mutant p53 resulted in formation of thymic lymphomas with a more rapid onset (median lifespan 151 days). We also used Pdgfrb-Cre-expressing mice, allowing us to target predominantly pericytes, as these have been reported as the cell of origin for a number of soft tissue sarcomas. Pdgfrb-Cre also results in low levels of recombination in venous blood endothelial cells in multiple tissues during development. Upon deletion of Trp53 in Pdgfrb-Cre-expressing mice (Pdgfrb-Cre, Trp53+/− mice), 65% developed lymphomas and 21% developed pleomorphic undifferentiated soft tissue sarcomas. None developed angiosarcomas. In contrast, 75% of Pdgfrb-Cre, Trp53+/− mice developed angiosarcomas, with 60% of these mice also developing lymphomas. The median lifespan of the Pdgfrb-Cre, Trp53+/− mice was 151 days. Re-implantation of angiosarcoma tumour fragments from Cdh5-Cre, Trp53+/− mice provided a more consistent and rapid model of angiosarcoma than the two spontaneous models. The ability to passage tumour fragments through the mouse provides a novel model which is amenable to preclinical studies and will help the development of potential new therapies for angiosarcoma.

KEY WORDS: Angiosarcoma, Trp53, Genetically engineered mouse model, Lymphomas, Tumour
development beyond that seen following loss of the wild-type p53 protein alone (Blagosklonny, 2000; Sigal and Rotter, 2000). Individuals with Li-Fraumeni syndrome carry inherited mutations in TP53 and are predisposed to tumour development, including sarcomas. In mouse models of Li-Fraumeni syndrome, expression of Trp53R172H, which corresponds to the TP53R175H hotspot mutation in human tumours, in mice leads to the development of predominantly lymphomas, but a small percentage of these mice also develop angiosarcomas (Lang et al., 2004; Olive et al., 2004). We therefore generated mice in which Trp53R172H was expressed under the control of Pdgfrb-Cre and Cdh5-CreER\(^{12}\), in addition to those carrying a floxed Trp53 allele.

**RESULTS**

**Tumour development in Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) and Pdgfrb-Cre, Trp53\(^{fl/fl}\) mice**

Experimental cohorts consisted of mice expressing either one (Pdgfrb-Cre, Trp53\(^{R172H/+}\) \((n=16)\)) or two (Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) \((n=28)\) mutant Trp53\(^{R172H}\) alleles, or loss of both Trp53 alleles (Pdgfrb-Cre, Trp53\(^{309/0}\) \((n=14)\)). The median lifespan of the Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) mice was 93 days compared to >365 days for the Pdgfrb-Cre, Trp53\(^{R172H/+}\) mice and 189.5 days for the Pdgfrb-Cre, Trp53\(^{309/0}\) mice (Fig. 1A). The deaths of all Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) mice were due to tumour formation, in contrast to the Pdgfrb-Cre, Trp53\(^{R172H/+}\) cohort in which only 2/16 mice were culled owing to tumour formation. In the Pdgfrb-Cre, Trp53\(^{309/0}\) cohort, 12/14 mice were culled owing to tumour formation. Mice that were asymptomatic at 1 year of age were culled.

**Autopsy findings and tumour histology**

In the Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) cohort, 75% \((n=21/28)\) of the mice developed angiosarcomas. Of these 21 mice, nine demonstrated only angiosarcomas, whereas in the other 12 mice lymphomas were also identified. The remaining Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) mice developed either lymphomas \((n=5/28)\) or teratomas \((n=2/28)\) (Fig. 1B). Thus, the predominant tumour type was angiosarcoma, with most mice developing multiple angiosarcomas in a number of different organs (Table 1) (Fig. 1B: median 3, range 1-6 tumours). No angiosarcomas were seen in the Pdgfrb-Cre, Trp53\(^{R172H/+}\) mice. The two Pdgfrb-Cre, Trp53\(^{R172H/+}\) mice culled owing to tumour formation had developed lymphomas. At autopsy, following culling of the asymptomatic Pdgfrb-Cre, Trp53\(^{R172H/+}\) mice at 1 year, three were found to have developed lymphomas and one was found to have adenocarcinoma in the lung. The remainder showed no gross or histological abnormality (Fig. 1B). Within the Pdgfrb-Cre, Trp53\(^{309/0}\) mice, upon histological examination it was found that nine developed lymphomas and three developed undifferentiated sarcomas; the remaining two had no detectable tumour upon sacrifice (Fig. 1B).

**Characterization of angiosarcomas in Pdgfrb-Cre, Trp53\(^{R172H/R172H}\) mice**

The morphological appearances of the angiosarcomas were similar within and between mice. The tumours consisted of lobules of pleomorphic cells showing varying degrees of vascular formation, which is typical of high-grade angiosarcomas (Fig. 2A,B). Immunohistochemistry supported the morphological assessment, with the tumour cells expressing CD31 and ERG (Fig. 2C,D). There was no expression of CD34 by the tumour cells (not shown). The tumours also showed strong expression of p53 (Fig. 2E) in keeping with the stabilization of mutant p53 that is often seen in human tumours expressing mutant p53. PDGFR-\(\beta\) was expressed by stromal cells within the tumour masses, but not reliably by the angiosarcomatous cells (Fig. 2F).

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**Fig. 1. Tumour development in Pdgfrb-Cre mice.**

(A) Kaplan–Meier curves showing significant difference in survival between Trp53\(^{R172H/R172H}\) \((n=28)\), Trp53\(^{R172H/+}\) \((n=16)\) and Trp53\(^{309/0}\) \((n=14)\) mice (log-rank \(P<0.0001\)).

(B) Tumour incidence and type in the different mouse cohorts.
Development of undifferentiated sarcomas in Pdgfrb-Cre, Trp53fl/fl mice

Three of the 14 (21%) Pdgfrb-Cre, Trp53fl/fl mice developed tumours with the morphological features of high-grade spindle cell and pleomorphic undifferentiated soft tissue sarcoma (Fig. S1A). Immunohistochemistry showed no expression of p53, confirming the homozygous deletion of p53 in the Pdgfrb-Cre, Trp53fl/fl mice (Fig. S1B), and strong expression of PDGFR-β by the tumour cells (Fig. S1C). Less than 10% of the cells expressed SMA (Acta2) in two of the cases, whereas the other was completely negative for SMA. None of the tumour cells expressed CD31 (Fig. S1D) or ERG (not shown).

Pdgfrb-Cre-mediated recombination does not occur in adult CD31 endothelial cells

As the angiosarcomas that developed expressed CD31 but did not express PDGFR-β, we asked whether there was any Cre-mediated recombination in CD31-positive endothelial cells in adult mice. Using Ai14 reporter mice (single-fluorescent reporter mice that express tdTomato after Cre-mediated recombination) (Madisen et al., 2010), we found that Pdgfrb-Cre induced highly efficient recombination in mouse skin (Fig. 3A), a tissue in which a number of angiosarcomas arose in the Pdgfrb-Cre, Trp53R172H/R172H mice. To evaluate the specificity of recombination, we stained skin from Ai14;Pdgfrb-Cre mice for PDGFR-β and confirmed appropriate reporter expression using Pdgfrb-Cre (Fig. 3B). Staining of Ai14;Pdgfrb-Cre mice skin for CD31 expression showed that recombination did not target adult endothelial cells and, in some cases, PDGFR-β-expressing cells were seen surrounding small CD31-positive endothelial cells (Fig. 3C). This suggests that the angiosarcomas have arisen either from endothelial cell lineages that transiently express PDGFR-β during development (Stanczuk et al., 2015; Ulvmar et al., 2016) or from other PDGFR-β-expressing

Table 1. Incidence of angiosarcoma formation in Pdgfrb-Cre, p53R172H/R172H mice

| Mouse ID | Number | Position                                      |
|---------|--------|----------------------------------------------|
| 24      | 3      | Hip, shoulder, leg                           |
| 152     | 5      | Abdomen, flank, knee, hip, shoulder          |
| 156     | 2      | Mammary fat pad, leg                        |
| 187     | 3      | Heart, intramuscular back, fat shoulder      |
| 191     | 5      | Shoulder, flank, mammary fat pad, abdomen, back |
| 193     | 4      | Back, neck, flank, abdomen                   |
| 194     | 1      | Mammary fat pad                              |
| 195     | 3      | Heart, intramuscular leg, armpit             |
| 196     | 3      | Shoulder, back, neck                        |
| 197     | 1      | Flank                                        |
| 215     | 6      | Shoulder, intramuscular abdomen, subcutaneous back (×2), preputial, peritoneum |
| 249     | 2      | Subcutaneous back, soft tissue chest         |
| 251     | 2      | Muzzle, leg                                  |
| 259     | 2      | Leg, diaphragm                               |
| 265     | 3      | Diaphragm, heart, subcutaneous back          |
| 283     | 2      | Shoulder, mammary fat pad                    |
| 289     | 2      | Leg, chest                                   |
| 291     | 6      | Leg, mid abdomen, lower abdomen, back, neck, mammary fat pad |
| 298     | 2      | Mammary fat pad (×2)                         |
| 301     | 3      | Peritoneum, chest, shoulder                  |
| 330     | 2      | Stomach, heart                               |

Table 1. Incidence of angiosarcoma formation in Pdgfrb-Cre, p53R172H/R172H mice

Fig. 2. Histology and immunohistochemistry of mouse angiosarcomas in Pdgfrb-Cre, Trp53R172H/R172H mice. (A,B) H&E staining showing vascular lobulated tumour masses. These were composed of pleomorphic tumour cells showing variable vasoformative capability. (C-F) Immunohistochemical analysis with antibodies to CD31 (C), ERG (D), p53 (E) and PDGFR-β (F). Scale bars: 500 µm in A; 50 µm in B,C,F; 250 µm in D,E.
pericyte lineages. Interestingly the undifferentiated sarcomas that developed in the Pdgfrb-Cre, Trp53fl/fl mice had retained expression of PDGFR-β, suggesting a different cell of origin.

**Tumour development in Cdh5-CreERT2, Trp53R172H/R172H and Cdh5-CreERT2, Trp53fl/fl mice**

To determine whether we could promote more efficient generation of angiosarcomas, we directly induced expression of mutant p53 or loss of p53 in adult endothelial cells using Cdh5-CreERT2 mice, in which Cre recombinase is driven by Cdh5. Using Ai14;Cdh5-CreERT2 reporter mice we found that Cdh5-Cre induced recombination in CD31-positive endothelial cells (Fig. S2). Experimental cohorts consisted of mice expressing either one (Cdh5-CreERT2, Trp53R172H/+ (n=15)) or two (Cdh5-CreERT2, Trp53R172H/R172H (n=8)) mutant Trp53R172H alleles, or loss of one (Cdh5-CreERT2, Trp53fl/+ (n=9)) or both (Cdh5-CreERT2, Trp53fl/fl (n=13) Trp53 alleles. A control cohort of Cdh5-CreERT2 mice were also treated with tamoxifen (n=9). The median lifespan of Cdh5-CreERT2, Trp53R172H/R172H mice was 151 days (range 109-198) (Fig. 4A). In the Cdh5-CreERT2, Trp53R172H/+ mice, 2/16 mice developed tumours; all other mice were asymptomatic and sacrificed at 1 year (Fig. 4A,B). In the Cdh5-CreERT2, Trp53fl/+ mice, two developed tumours: one a thymic lymphoma and the other a hepatocellular carcinoma. At autopsy, following culling of the asymptomatic Cdh5-CreERT2, Trp53fl/+ mice at 1 year, the remainder showed no gross or histological abnormality. Thus, the predominant tumour type driven by Trp53R172H in the Cdh5-CreERT2 mice was lymphoma, in contrast to the angiosarcomas that developed in the Pdgfrb-Cre, Trp53R172H/R172H mice. Within the Cdh5-CreERT2, Trp53fl/fl cohort all mice developed angiosarcomas (13/13), many with multiple tumours that developed in a range of anatomical locations (Table 2) (Fig. 4B). The Cdh5-CreERT2, Trp53fl/+ mice had no detectable tumours upon sacrifice. When we looked at the latency of the angiosarcomas and the lymphomas in all experimental mice we found that the lymphomas developed more rapidly than the angiosarcomas, with a median survival of 163 days (range 109-198) days and 325 days (range 224-407), respectively (Fig. 4C).

**Autopsy findings and tumour histology**

In the Cdh5-CreERT2, Trp53R172H/R172H cohort, 7/8 mice developed thymic lymphomas, with evidence of thymic hyperplasia in the remaining mouse, but none developed angiosarcomas (Fig. 4B). Two of the mice that developed lymphomas also developed additional tumours: one an undifferentiated sarcoma and the other a hepatocellular carcinoma. In addition, one of the Cdh5-CreERT2 mice showed evidence of thymic hyperplasia, with all the others showing no evidence of tumour formation upon autopsy (Fig. 4B). In the Cdh5-CreERT2, Trp53R172H/+ mice, two developed tumours: one a thymic lymphoma and the other an angiosarcoma (Fig. 4B). At autopsy, following culling of the asymptomatic Cdh5-CreERT2, Trp53R172H/+ mice at 1 year, the remainder showed no gross or histological abnormality. Thus, the predominant tumour type driven by Trp53R172H in the Cdh5-CreERT2 mice was lymphoma, in contrast to the angiosarcomas that developed in the Pdgfrb-Cre, Trp53R172H/R172H mice. Within the Cdh5-CreERT2, Trp53R172H/+ cohort all mice developed angiosarcomas (13/13), many with multiple tumours that developed in a range of anatomical locations (Table 2) (Fig. 4B). The Cdh5-CreERT2, Trp53fl/+ mice had no detectable tumours upon sacrifice. When we looked at the latency of the angiosarcomas and the lymphomas in all experimental mice we found that the lymphomas developed more rapidly than the angiosarcomas, with a median survival of 163 days (range 109-198) days and 325 days (range 224-407), respectively (Fig. 4C).
Characterization of angiosarcomas in Cdh5-CreERT2, Trp53fl/fl mice

Many of the tumours were similar to those seen in the Pdgfrb-Cre, Trp53R172H/R172H mice, comprising lobules of pleomorphic cells showing varying degrees of vascular formation (Fig. 5A). However, many of the tumours showed extensive haemorrhage and necrosis. In some tumours, a cavernous/telangiectatic pattern was evident, with enlarged blood-filled spaces being lined by atypical endothelial cells. Immunohistochemistry supported the morphological assessment, with the tumour cells expressing ERG, CD31 and VE-cadherin, but not PDGFR-β (Fig. 5). Twelve of the 13 tumours that developed in the Cdh5-CreERT2, Trp53fl/fl mice did not express p53 (Fig. 5F). The reason for p53 expression in the remaining angiosarcoma is not known.

Comparison of gene expression profiles of Pdgfrb-Cre, Trp53R172H/R172H and Cdh5-CreERT2, Trp53fl/fl tumours

To understand the differences between the angiosarcomas that developed in the Pdgfrb-Cre, Trp53R172H/R172H and the Cdh5-CreERT2, Trp53fl/fl mice, we carried out gene expression analysis using the NanoString PanCancer Pathways panel. Tumour type-specific gene expression profiles were determined using unsupervised hierarchical clustering, with both tumour types also being significantly more different than normal VE-cadherin-derived endothelial cells (Fig. 6A). Gene ontology analysis of the genes that were significantly differentially expressed between the two different angiosarcoma subsets showed that pathways linked to p53 and angiogenesis, including FGF and VEGF signalling pathways, were over-represented (Fig. 6B). This indicates that the gain-of-function Trp53R172H mutant drives expression of a different set of genes to those seen in the Cdh5-CreERT2, Trp53fl/fl mice, to initiate angiosarcoma development.

To determine whether the changes seen in the differentially expressed genes in the mouse angiosarcomas reflect changes seen in human angiosarcomas, we carried out gene set enrichment analysis of the differentially expressed genes in the mouse angiosarcomas compared to normal endothelial cells and compared this with published human data from a set of human angiosarcomas and normal endothelial cells (GSE44115: Andersen et al., 2013). This showed a significant enrichment of genes associated with human angiosarcomas in the mouse tumours compared to those expressed in the normal endothelial cells (Fig. 6C), indicating that the mouse angiosarcomas represent a sub-population of human angiosarcomas.

Interestingly, analysis of genes that are associated with endothelial cell function showed that a number were significantly increased in angiosarcomas in both the human and mouse datasets (VEGFC, EPHA2), whereas others were differentially regulated in the human and mouse angiosarcomas (VEGFA, VEGFB, KDR, MYC) (Fig. 6D).

Generation of cell lines and transplantation model

We generated cell lines from four angiosarcomas that developed in the Pdgfrb-Cre, Trp53R172H/R172H mice. As with the spontaneous
tumours, the cell lines all expressed p53 (Fig. 7A) and genotyping showed that each cell line was homozygous for the R172H allele. Upon re-implantation of the cell lines into the flanks of mice, two of the lines from the *Pdgfrb-Cre, Trp53R172H/R172H* mice developed tumours, with morphological features of undifferentiated pleomorphic sarcomas (Fig. 7Bi). Neither the generated cell lines nor the tumours retained expression of CD31 (Fig. 7Bii). We also generated cell lines from four angiosarcomas that developed in the *Cdh5-CreERT2, Trp53fl/fl* mice. Genotyping confirmed that each cell line was homozygous for the floxed *Trp53* allele. However, none of the cell lines formed tumours when injected into the flanks of recipient mice. In an attempt to overcome the loss of endothelial markers upon culture of the angiosarcomas we implanted tumour fragments from three spontaneous angiosarcomas that developed in the *Cdh5-CreERT2, Trp53 fl/fl* mice. All formed tumours and histological examination confirmed that these were angiosarcomas expressing both CD31 and ERG (Fig. 7C,D). Furthermore, secondary implantation of frozen tumour fragments resulted in successful outgrowth of angiosarcomas in recipient wild-type mice (Fig. 7C,D).

**DISCUSSION**

We have generated two mouse models of angiosarcoma driven by deregulation of p53. We used the *Pdgfrb-Cre* mouse that is known to target both pericytes and endothelial cells during development. This resulted in 75% of *Pdgfrb-Cre, Trp53R172H/R172H* mice developing angiosarcomas, which is higher than the 62% reported when *Tie2-Cre* mice were crossed to *Trp53* floxed mice (Farhang Ghahremani et al., 2014). In this model p53 is deleted in both endothelium and the haematopoietic lineages. Interestingly, we saw no angiosarcomas in the *Pdgfrb-Cre, p53fl/fl* mice suggesting that the *Trp53R172H* mutant is exerting a gain-of-function activity that is required for angiosarcoma development when *Pdgfrb* cells are targeted. The development of angiosarcomas in a small percentage of mice in a model of Li-Fraumeni syndrome that expresses *Trp53R172H* supports the specific involvement of mutant p53 in the development of angiosarcoma (Lang et al., 2004; Olive et al., 2004). Analysis of *Pdgfrb-Cre* mice has shown that recombination occurs in a number of cell types during development, including endothelial and mural cells (Stanczuk et al., 2015; Ulvmar et al., 2016), so it is not possible to define the cell of origin in the angiosarcomas that developed in the *Pdgfrb-Cre, Trp53R172H/R172H* mice.

The cell of origin of sarcomas remains unclear, although mounting evidence suggests that they are derived from mesenchymal cells (Yang et al., 2014). NG2 is a cell-surface proteoglycan expressed by pericytes, which are mesenchymal cells that surround blood vessels (Sá da Bandeira et al., 2017). A recent study has shown that targeting p53 loss in adult mice using NG2-driven Cre recombination leads to the formation of bone and soft tissue sarcomas, which supports the mesenchymal origin of these tumours (Sato et al., 2016). In this study only one angiosarcoma developed, indicating that they can arise from mesenchymal precursors but that the efficiency is much lower than that of other sarcoma types such as osteosarcomas and undifferentiated pleomorphic sarcomas, which were the most frequent tumour types seen. Interestingly, we found that loss of p53 in the *Pdgfrb-Cre* mice gave rise to undifferentiated sarcomas that expressed PDGFR-β, albeit with a reduced efficiency and increased latency compared to that observed by Sato and colleagues (Sato et al., 2016). This disparity may reflect differences in the efficiency of recombination
Fig. 6. See next page for legend.
Fig. 6. Gene expression analysis demonstrates mouse angiosarcomas have common and distinct features, but largely resemble human angiosarcomas. (A) Unsupervised hierarchical clustering of the NanoString PanCancer Pathways panel demonstrated some variation between the mouse angiosarcomas, but these are significantly more different than normal VE-cadherin (Cdh5)-derived endothelial cells. (B) Analysis of PANTHER signalling pathways that were significantly differentially expressed between the two mouse angiosarcomas. (C) The 299 significantly differently expressed genes between the mouse angiosarcomas and normal VE-cadherin-derived endothelial cells were significantly enriched in human angiosarcomas compared to normal human endothelial cells. (D) Genes associated with endothelial cell function were significantly increased in angiosarcomas in both the human and mouse datasets (VEGFA, VEGFB, KDR and MYC). Bars represent median, boxes show upper to lower quartiles, whiskers show the range excluding outliers. Grey, normal human and mouse endothelial cells; green, human and mouse angiosarcomas.

In the Pdgfrb-Cre and Ng2-Cre mice or may be due to distinct pericyte subpopulations that are marked by PDGFR-β and NG2 during development (Birbrair et al., 2013). In the future, use of a conditional Pdgfrb-CreERT2 mouse (Claxton et al., 2008) that allows specific deletion of Trp53 in the adult, in which Pdgfrb expression is restricted to pericytes, will allow further evaluation of the role of distinct mesenchymal cell lineages to the development of different sarcoma subtypes.

To address whether direct targeting of adult endothelial cells would result in the development of angiosarcomas with higher efficiency we used the Cdh5-CreERT2 mouse. Surprisingly, all Cdh5-CreERT2, Trp53R172H/R172H mice developed lymphomas. Studies in Cdh5-CreERT2 mice have reported recombination of a small (0.3%) subpopulation of bone marrow cells that are haematopoietic in nature (Monvoisin et al., 2006). Combined with the propensity of mutant p53 to drive lymphomagenesis, this appears to be sufficient to drive development of lymphomas in the Cdh5-CreERT2, Trp53R172H/R172H mice. However, all of the Cdh5-CreERT2, Trp53R172H mice developed angiosarcomas, with no lymphomas detected. The enrichment of genes associated with p53 in the differentially expressed genes between the tumours that develop in the two models indicates that, as expected, the gain-of-function Trp53R172H mutant drives expression of a different set of genes to those seen in the Cdh5-CreERT2, Trp53R172H mice to initiate angiosarcoma development. Other mouse models targeting specific endothelial cell populations have also been reported. mTORC1 activation in endothelial cells following conditional deletion of its upstream inhibitor Tsc1 resulted in 100% of mice developing hepatic haemangiomatis, and ~80% developing cutaneous lymphangiosarcomas (Sun et al., 2015). This is in contrast to the aggressive angiosarcomas that developed upon direct targeting of Trp53 in endothelial cell lineages in the Cdh5-CreERT2, Trp53R172H mice. mTORC1 pathway activation has also been reported in human angiosarcomas (Sun et al., 2015; Chadwick et al., 2018) and has been linked to Myc-mediated transcriptional regulation of VEGFA (Sun et al., 2015); increased expression of both Myc and Vegfa in the angiosarcomas from the Cdh5-CreERT2, Trp53R172H mice suggest that a similar autocrine stimulation loop may be present in these tumours, although the involvement of mTORC signalling in these tumours is not known. Combined loss of Trp53, Pten and Ptpn12 also leads to the development of aggressive vascular lesions, which was associated with both mTORC and MEK pathway activation, suggesting that mTORC activation alone is not sufficient to drive aggressive angiosarcoma development (Chadwick et al., 2018). In this study, loss of Trp53 alone did not result in the development of angiosarcomas. However, the tumours arose from more restricted recombination in a subset of endothelial cells. Taken together with the distinctive spectrum of tumours seen upon deletion of Tsc1, this suggests that targeting distinct populations of endothelial cells in mice in combination with differential pathway activation impacts on the type and site of vascular tumour development.

Increased Myc expression downstream of the forkhead boxO (FoxO) pathway has been linked to endothelial cell proliferation and angiosarcoma development (Riddell et al., 2018; Wilhelm et al., 2016). Interestingly, conditional triple knockout of FOXO1/3/4 in mice results in development of thymic lymphomas and vascular lesions, predominantly haemangiomatis, in a number of tissues, with only 9% progressing to angiosarcomas, although there is no reported role for Myc in this model (Paik et al., 2007). This is in contrast to the Cdh5-CreERT2, Trp53R172H mice, in which angiosarcomas arise predominantly in soft tissues, as the vascular lesions that develop in the FOXO triple knockout mice are predominantly benign and arise in relation to the uterus and a number of other tissues (Paik et al., 2007). FOXOs and p53 share many common target genes and may act in a cooperative manner in order to regulate gene transcription (Fu and Tindall, 2008; Renault et al., 2011). It will be interesting to establish whether the deregulation of such pathways is required for driving the transition from benign to malignant disease in the mouse models.

Angiosarcomas in humans may be divided into several clinical groups. The majority of cases (∼50%) are cutaneous, breast parenchymal angiosarcomas account for ∼14%, soft tissue 11%, heart 7% and bone 4%, with a range of other sites accounting for the remainder (Laht et al., 2010). Little is known of the factors that predispose to angiosarcoma development in the clinical setting, and it may be that angiosarcomas constitute a range of interrelated clinical diseases with common endothelial features but different phenotypes and aetiologies. Comparison of gene expression profiles between the two mouse models and human angiosarcomas showed significant enrichment of angiosarcoma-associated genes in the two models, suggesting that the mouse models do represent some of the phenotypes present in the human disease. Use of these and other mouse models will help to unravel some of the targetable pathways in angiosarcoma.

Although genetically engineered models are used in preclinical studies, the long latency of tumour formation in the Cdh5-CreERT2, Trp53R172H mice (median lifespan 325 days) and the development of multiple tumours would make therapeutic efficacy studies costly and challenging. Knockout of p53 in lymphoepithelial Rag2−/−;Il2rg−− mice leads to a high frequency of angiosarcomas (over 65%), whereas only sporadic formation of lymphomas. With a mean latency of 18 weeks, this provides an alternative model that would be more amenable to therapeutic studies (Landuzzi et al., 2014). However, the lack of immune cell populations in these mice restricts their use for assessing immune modulators, which are showing promise in sarcoma. A recent report has shown that use of a lentiviral vector-based system to introduce oncogenic HrasG12V in combination with loss of Cdkn2a via intravenous injection into immune competent mice resulted in the formation of angiosarcomas. These develop very rapidly and in multiple sites, which is most likely influenced by the intravenous route of injection (Yang et al., 2014). This provides a useful model for preclinical studies, but the rapid development of multiple tumours will make these studies challenging.

To overcome these issues, we generated cell lines from angiosarcomas that developed in the Pdgfrb-Cre, Trp53R172H/R172H and Cdh5-CreERT2, Trp53R172H mice, in an attempt to generate syngeneic mouse models of angiosarcoma. However, as has been
reported previously (Farhang Ghahremani et al., 2014), endothelial markers were rapidly lost when the tumours were established in culture, even when grown with endothelial cell-specific supplements. In contrast, direct implantation of tumour fragments allowed us to establish angiosarcomas which could be frozen and passaged serially through wild-type recipient mice. This had the benefit of reducing the latency of tumour formation and restricting the number of tumours per mouse. Furthermore, the subcutaneous localization of the tumours allowed easy monitoring of tumour growth. We have previously used this transplantation approach to model HER2 breast cancer and have demonstrated its utility in determining drug efficacy and establishing models of drug resistance (Creedon et al., 2016).

Taken together, our data indicate that deleting p53 in endothelial cells in the adult mouse is the most effective way to generate angiosarcomas. This resulted in 100% penetrance with no formation of lymphomas. The development of lymphomas in the Cdh5-CreER{T2}, Trp53{R172H/R172H} mice supports a strong selection for angiosarcoma formation following loss of p53 in contrast to expression of the gain-of-function mutant p53. This is supported by angiosarcoma formation in mice in which loss of Trp53 is combined with loss of Ptpn12 and Pten (Sá da Bandeira et al., 2017). The further establishment of a transplantation model of angiosarcoma provides a novel approach for testing potential new therapeutics in this disease setting.

MATERIALS AND METHODS

Animals

Mice expressing Cre under the control of the Pdgfrb promoter (Pdgfrb-Cre) (Foo et al., 2006) or in the inducible control of the Cdh5 promoter.
procedures were approved by the University of Edinburgh Ethical review. Tumours and major organs were removed and fixed in 10% neutral buffered formalin in a maximum size of 1.5 cm or the mouse became sickly, as defined by UK Home Office guidelines. Following sacrifice, macroscopically identified tumours and major organs were removed and fixed in 10% neutral buffered formalin. In some instances, fresh samples were taken for generation of cell lines or tumour fragments taken for re-implantation. Animal studies and procedures were approved by the University of Edinburgh Ethical review committee (Application #PL01-16) and conducted in accordance with UK Home Office regulations.

Histology and immunohistochemistry
Formalin-fixed tissues were routinely processed into paraffin wax blocks and sections cut for Haematoxylin and Eosin (H&E) staining and immunohistochemistry. Immunohistochemistry was carried out as described previously (Creedon et al., 2016). Primary antibodies used were CD31 at 1:800 (Abcam, ab28364), ERK (Oko, I659, ready to use, 200 µl per slide), p53 at 1:2000 (Leica BioSystems, NCL-P53-CM5p), PDGFR-β at 1:100 (CST, 3169S), and VE-cadherin at 1:4000 (Abcam, ab33168).

Immunofluorescence
Shaved dorsal skin was mounted on 3 mm blotting paper and placed in 4% methanol-free formaldehyde (Thermo Fisher Scientific, 28906) at 4°C for 1 hour. The skin was then washed with PBS and transferred to 18% sucrose overnight for cryoprotection before embedding in OCT embedding matrix (CelHisto, KMA-0100-00A) and frozen on dry ice. Then 7µm cryosections were cut at room temperature in the dark for 30 min and washed in PBS containing 0.05% Tween 20 (Sigma-Aldrich, P2287). To image endogenous tdTomato fluorescence, sections were incubated with 1 µM DAPI for 10 min, washed twice with PBS and mounted with Prolong Gold Antifade reagent (Invitrogen). Images were acquired using a Zeiss LSM780 confocal microscope.

Cell culture
Tumours from Pdgfrb-Cre, p53<sup>R172H</sup>Trp<sup>S539F</sup> mice were freshly processed by rinsing in PBS and then mincing to ~1 mm<sup>2</sup> pieces using two scalpels. After transferring to a 15 ml Falcon tube containing 10 ml digestion media, sections were incubated with blocking buffer (PBS containing 5% goat serum and 0.3% Triton X-100) for 30 min, then incubated with primary antibodies PDGFR-β (1:25, Abcam, Ab32570) or CD31 (1:50, BD Pharmingen, 550274) for 2 h. Sections were then washed twice and incubated for 30 min with Alexa fluor 488 conjugated secondary antibodies [Molecular Probes; goat anti-rabbit antibody 488 (1:1000, A11034) or goat anti-rat antibody 488 (1:1000, A11006)], washed twice, incubated with DAPI and mounted as described above. Images were obtained using a Zeiss LSM780 confocal microscope.

Gene expression profiling
RNA prepared from angiosarcomas that developed in the Pdgfrb-Cre, p53<sup>R172H</sup>Trp<sup>S539F</sup> and Cdh5-CreERT<sup>2</sup>, Trp<sup>S539F</sup> mice was analyzed using the NanoString PanCancer Pathways panel (reproduces 750 cancer associated genes) on the NanoString nCounter DX platform as per the manufacturer’s instructions. For comparison, VE-cadherin-positive liver endothelial cells were isolated from Ai14;Cdh5-CreERT<sup>2</sup> mice 3 weeks after tamoxifen treatment (Lynch et al., 2018). A VE-cadherin (tdTomato)-positive population of endothelial cells was collected using a BD FACSAria II. Following standard nCounter normalisation, differentially expressed genes were identified using Student’s t-tests (P<0.05) between the angiosarcomas derived from the Pdgfrb-Cre, p53<sup>R172H</sup>Trp<sup>S539F</sup> and Cdh5-CreERT<sup>2</sup>, Trp<sup>S539F</sup> mice and relative to the normal endothelial cells. Hierarchical cluster analysis was performed with the Cluster and Treeview programs (Eisen et al., 1998). Gene set enrichment analysis (Subramanian et al., 2005) was performed using the Phenestin R package. Gene ontology analysis was performed using the PANTHER classification system (Paik et al., 2007).

Sub-cutaneous tumour growth
Cell lines and tumour fragments derived from the mouse angiosarcomas were injected into both flanks of 6- to 8-week-old female CD-1 nude mice (Charles River) and tumour growth measured twice weekly using calipers. Tumour volumes were calculated in Excel using the formula v=4/3πr<sup>3</sup>. Animals were sacrified when tumours reached the maximum size allowed, and collected and fixed in 10% neutral buffered formalin.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.G.B., D.M.S., L.H., N.C.H.; Methodology: V.G.B., D.M.S.; Validation: V.G.B., M.G., M.M., K.T., J.C., J.R.S., L.G.-C.; Formal analysis: D.M.S., A.H.S.; Investigation: D.M.S., M.G., M.M., K.T., J.C., J.R.S., K.M., L.G.-C.; Resources: N.C.H.; Writing - original draft: V.G.B.; Writing - review & editing: V.G.B., D.M.S., L.H., N.C.H.; Visualization: V.G.B., D.M.S., M.G., J.R.S., K.M., A.H.S.; Supervision: V.G.B., D.M.S.; Project administration: V.G.B.; Funding acquisition: V.G.B., D.M.S., L.H.

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