Generation of a murine hepatic angiosarcoma cell line and reproducible mouse tumor model

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Hepatic angiosarcoma (AS) is a rare and highly aggressive tumor of endothelial origin with dismal prognosis. Studies of the molecular biology of AS and treatment options are limited as animal models are rare. We have previously shown that inducible knockout of Notch1 in mice leads to spontaneous formation of hepatic AS. The aims of this study were to: (1) establish and characterize a cell line derived from this murine AS, (2) identify molecular pathways involved in the pathogenesis and potential therapeutic targets, and (3) generate a tumor transplantation model. AS cells retained specific endothelial properties such as tube formation activity, as well as expression of CD31 and Von Willebrand factor. However, electron microscopy analysis revealed signs of dedifferentiation with loss of fenestrae and loss of contact inhibition. Microarray and pathway analysis showed substantial changes in gene expression and revealed activation of the Myc pathway. Exposing the AS cells to sorafenib reduced migration, filopodia dynamics, and cell proliferation but did not induce apoptosis. In addition, sorafenib suppressed ERK phosphorylation and expression of cyclin D2. Injection of AS cells into NOD/SCID mice resulted in formation of undifferentiated tumors, confirming the tumorigenic potential of these cells. In summary, we established and characterized a murine model of spontaneous AS formation and hepatic AS cell lines as a useful *in vitro* tool. Our data demonstrate antitumor activity of sorafenib in AS cells with potent inhibition of migration, filopodia formation, and cell proliferation, supporting further evaluation of sorafenib as a novel treatment strategy. In addition, AS cell transplantation provides a subcutaneous tumor model useful for *in vivo* preclinical drug testing.

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Angiosarcoma (AS), a malignant neoplasm arising from endothelial cells, is an uncommon malignancy accounting for only 1% of all sarcomas.¹ Hepatic AS is a rare (2% of primary liver tumors), but aggressive cancer.^{2,3} Previous studies established an association between hepatic AS and exposure to environmental toxins such as thorotrast, vinyl chloride, inorganic arsenic, and others.^{2,4} Patient survival is very poor with a median life expectancy of 6 months; only 3% of affected patients live longer than 2 years.²

Currently, the conventional therapy of localized AS is surgical excision with the goal of complete resection.^{5–7} However, in the case of hepatic AS, this therapeutical option is applicable only in few cases, and liver transplantation is contraindicated. Because of high recurrence rates after local therapy, postoperative radiotherapy is recommended. Although up to 50% of the AS will generate metastases, there is no evidence

supporting survival benefit of adjuvant chemotherapy.^{5,6,8} The standard treatment option for metastatic AS is chemotherapy mainly based on anthracyclines, doxorubicin, and taxanes.^{6,9} However, chemotherapeutic agents are only transiently effective in disease control and new therapeutic approaches are urgently needed.¹⁰ For targeted therapy, very few randomized trials were conducted in patients with AS. For the multi-tyrosine kinase inhibitor sorafenib, a phase II study has been published by Maki *et al*¹¹ reporting antitumor activity in patients with AS. Nevertheless, contradictory findings have been published by the French Sarcoma Group stating that sorafenib has only limited activity against AS.¹² Sorafenib is an anti-angiogenic drug that targets Raf and vascular endothelial growth factors 2 and 3, and shows inhibitory activity against platelet-derived growth factor receptor- β . To promote targeted therapies and explore new

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therapeutic approaches for AS, it is essential to better understand its tumor biology.

We and others have recently shown that Notch1 is a tumor suppressor gene for endothelial cells and that inhibition of the Notch1/Dll4 pathway leads to formation of spontaneous vascular neoplasms and AS.^{13–15} Present literature only describes the occurrence of AS but detailed molecular characterization of this tumor is lacking. A major drawback of the AS models (inducible Notch1 deletion/loss of Notch1 heterozygosity/chronic DLL4 blockade) is the difficulty to predict tumor presence in individual animals. Therefore, a reproducible model, that is, by injecting isolated AS cells, is needed to study therapeutic strategies *in vivo*. Here, we describe the first

successful establishment of Notch1-deficient AS cell lines with molecular characterization and *in vitro* testing of the tyrosine kinase inhibitor sorafenib. Subcutaneous transplantation of AS cells into NOD/SCID mice gave rise to tumor formation, thus representing an AS tumor model that allows monitoring of tumor progression.

MATERIALS AND METHODS

Animals, Induction of Notch1 Deletion, Genotyping, and Sampling

MxCre Notch1 lox/lox mice or Notch 1 lox/lox wild-type (WT) mice on a C57Bl/6 background were injected with 300 μ g of

Figure 1 Global loss of Notch1 causes spontaneous angiosarcoma in liver. (a) Representative images of a tumor-bearing liver (left) taken from a Notch1 lox/lox MxCre +/+ mouse (KO) 52 weeks after recombination and a normal liver (right) taken from a Notch1 lox/lox MxCre -/- mouse (WT) 52 weeks after plpC injection. (b) H&E staining of liver AS from a Notch1 KO mouse 72 weeks after Notch1 deletion. The AS lesion shows focal disruption of the liver parenchyma by malignant and infiltrating endothelial cells. Low-power magnification ($\times 10$) depicts numerous variable-sized cavernous spaces filled with red blood cells. High-power magnification ($\times 20$ and $\times 60$) reveals dilated sinusoids that are lined by plump pleomorphic malignant endothelial cells with severe cytological atypia. (c) CD31 immunoreactivity in liver angiosarcoma derived from Notch1 KO mice confirmed the endothelial differentiation of the tumor cells. (d) SEM analysis of corrosion cast in a Notch1 KO mouse 43 weeks after plpC injection showed dilated sinusoids, characteristic for hepatic AS. (e) Vascular cast of a mouse liver with AS illustrating intussusceptive remodeling; typical rows of transcapillary pillars are indicated by arrowheads.

Figure 1 Continued.

polyinosinic–polycytidylic acid (pIpC) (InvivoGen, San Diego, CA, USA) at the age of 4 weeks at days 0, 3, and 6 to induce Cre expression for deletion of the loxP-flanked exon encoding the signal peptide of Notch 1 as previously described.^{13,16}

Animals were maintained in the animal facility of the Department of Biomedicine, University Hospital Basel, in a specific-pathogen free environment on a 12 h light and 12 h dark schedule. Food and drinking water were provided *ad libitum*. All procedures were approved by the veterinary office of the Canton Basel-Stadt, Switzerland.

Vascular casting

Vascular casts of the mouse liver were prepared similar to the previously described method.¹³

Isolation and generation of AS cell lines

Hepatic endothelial cells were isolated from Notch1 knockout (KO) mice harboring a visible AS as previously described with modifications.¹³ Long-term culturing of the primary cells selected malignant AS cells based on their atypical growth capacity and eventually led to a uniform cell population.

Vascular tube formation assay

Tube formation assay was performed as previously described, using various concentrations of sorafenib (1, 3, and 5 μM) or temsirolimus (1, 10, and 100 ng/ml) in a total dimethyl sulfoxide (DMSO) concentration of 0.1%. Controls were treated with 0.1% DMSO.¹³

RNA extraction and microarray analysis

Affymetrix Mouse Gene ST 1.0 was used for microarray analysis of AS cells.

Detailed information is included in the Supplementary Materials and Methods section. All original array data are deposited at the National Center for Biotechnology Information Gene Expression Omnibus database (GEO accession number: GSE57655).

Time-lapse microscopy and filopodia quantification

AS cells were pretreated for 5 h with 3 μM sorafenib or were untreated before being subjected to the tube formation assay. For time-lapse imaging, AS cells were seeded on matrigel-coated glass bottom dishes and grown in FBS-free ECM in the

presence or absence of sorafenib ($3 \mu\text{M}$). The average number of filopodia per cell was determined on still images at 0/2/4/6/8 h time points obtained from time-lapse series.

Cell proliferation assay

AS cell proliferation with/without sorafenib was measured using the xCELLigence System RTCA DP analyzer (ACEA Biosciences) as described in the Supplementary Materials and Methods.

Tumorigenicity assay in vivo

Tumorigenicity studies were performed for one of the three AS cell lines. HepAS-1 cells were suspended in $100 \mu\text{l}$ matrigel at a concentration of 2×10^6 cells and injected subcutaneously into the flank of NOD/SCID mice. Tumor size was measured *in vivo* by external caliper at weekly intervals. The mice were killed when the tumor size exceeded 1500 mm^3 .

Statistical analysis

Comparisons were performed using Student's *t*-test (unless otherwise specified) in GraphPad Prism software version 6.0 (in the figures statistical significance is designated as $*P < 0.05$, $**P < 0.005$, and $***P < 0.001$).

See Supplementary Materials and Methods for additional and detailed experimental procedures.

RESULTS

Disruption of Notch1 Leads to Spontaneous AS in Liver

Notch1 conditional KO mice spontaneously develop hepatic AS over time with a penetrance of 86% at 50 weeks after Notch1 deletion.¹³ On gross examination, hepatic AS presented as a diffuse liver tumor showing abnormally widened blood vessels on the liver surface (Figure 1a). Histologically, the neoplastic nodules were characterized by a proliferation of plump, oval, and fusiform hyperchromatic endothelial cells multilayered along dilated sinusoids, often forming a pelioid or cavernous growth pattern, typical of AS (Figure 1b). Tumors presented in a diffuse pattern of proliferating anastomosing blood-filled channels with focal solid patterns. Strong CD31 immunoreactivity (known as the best diagnostic AS marker) was detected in malignant endothelial cells lining the vascular channels (Figure 1c).¹⁷ Vascular casting for three-dimensional morphological analysis of the liver vasculature with AS was performed (Figure 1d): corresponding to the H&E sections displaying dilated sinusoids and a cavernous growth pattern, the casts showed very wide blood vessels primarily in subcapsular location. Furthermore, classical sprouting angiogenesis as well as intussusceptive angiogenesis were identified (Figure 1e). The latter angiogenic process is a unique form of vascular neoformation that has been recently described in tumor angiogenesis of solid malignancies.¹⁸

Isolation of AS cells and establishment of cell lines

Hepatic endothelial cells were isolated from livers of Notch1 KO mice bearing a hepatic AS by collagenase perfusion,

Figure 2 Scheme of the isolation process and the subsequent steps to establish the angiosarcoma cell line. LSECs were isolated from livers of Notch1 KO mice harboring a hepatic AS by collagenase perfusion, density centrifugation over a two-step Percoll gradient, followed by selective adherence. Long-term culturing of isolated AS cells in endothelial cell medium led to removal of contaminating fibroblasts, resulting in a homogenous AS cell line.

density centrifugation over a two-step Percoll gradient, followed by selective adherence. Long-term culturing in endothelial cell medium and passaging of isolated AS cells led to the establishment of an AS cell line (Figure 2). In this way we established three AS cell lines from three individual mice, named HepAS-1, HepAS-2, and HepAS-3. The cells show no senescence, have undergone more than 40 passages, and can easily be recovered from cryopreservation.

Morphological Characterization

The AS cells of all three established cell lines shared the same morphologic features. In culture, the cells tended to form coherent patches that extended to confluency displaying the characteristic endothelial cobblestone pattern (Figure 3a). Unlike liver sinusoidal endothelial cells (LSECs), cell growth was undiminished on uncoated plastic culture dishes. Notably, proliferation was not inhibited after establishment of a confluent monolayer, instead cells formed multiple cell layers as seen by phase contrast microscopy. The ultra-structure was further explored by scanning electron microscopy demonstrating few filopodial extensions, whereas fenestrae and contact inhibition (both characteristic features of sinusoidal endothelial cells) were lost (Figure 3b).

Using TEM, endothelial differentiation was further supported by identification of rod-shaped microtubulated bodies corresponding to Weibel–Palade bodies, the endothelial-specific storage organelle for regulated Von Willebrand factor secretion (Figure 3c).^{19,20} Finally, HepAS cells were found to express the endothelial markers CD31 and Von Willebrand factor by immunocytofluorescence (IF) staining (Figure 3d).

Transcriptional Profiling Identifies Myc Pathway Alterations in Malignant AS Cell Lines

To further characterize the new AS mouse cell lines, we performed gene expression analysis using whole transcriptome

Affymetrix microarrays. We profiled three samples each from WT LSECs, Notch1 KO LSECs, and the three established AS cell lines. Expression levels of selected genes were validated by qPCR analysis of the same samples (Supplementary Figure 1). Only 17 genes were significantly (mean fold change >2, adjusted *P*-value <0.05) up- or downregulated in KO LSECs compared with WT LSECs (Figure 4a). Using the same significance cutoff, a large number of genes (1733 upregulated and 1654 downregulated) were altered between KO LSECs and the AS cell lines (Figure 4a). To gain more insight into this profound dysregulation, we analyzed pathway activation in the AS cell lines using Gene Set Enrichment

Figure 3 Morphological and phenotypic characteristics of angiosarcoma cells. (a) Low-power ($\times 50$) and high-power ($\times 100$) phase contrast microscopy images of AS cell cultures (cell passage 5) display a cobblestone morphology at confluence, typical for endothelial cells. (b) Scanning electron micrograph of AS cells passage 4 cultured for 1 and 4 days. Cells retain endothelial characteristic filopodia, but contact inhibition is lost (left: bar = 10 μ m; right: bar = 30 μ m). (c) Electron micrograph of AS showing an endothelial cell with a markedly irregular nucleus, several invaginations, and prominent nucleoli and filopodia. The cytoplasm contains numerous micropinocytotic vesicles and rare rod-shaped microtubulated bodies, the so-called 'Weibel–Palade bodies' (arrowheads). On the right, a high-power view of a rod-shaped microtubulated body. The interior of the body shows the characteristic striated appearance. (d) AS cells maintain expression of the endothelial markers CD31 ($\times 200$ original magnification) and Von Willebrand factor ($\times 400$ original magnification) assessed by IF staining. Nuclei were stained with DAPI.

Figure 3 Continued.

Analysis (GSEA) with the Chemical and Genetic Perturbations database.²¹ To this end we compared the AS cell lines with all LSEC samples (WT and KO grouped together). In all, 20 gene sets were upregulated and 227 downregulated with false discovery rate (FDR) of <0.05 . Many of the downregulated gene sets were related to immune response and cytokine signaling (data not shown). We also observed that a number of high-scoring gene sets were related to the Myc pathway—out of the top 20 upregulated sets, 6 defined Myc targets (Figure 4b). The highest-ranked Myc-related gene set contained 18 genes including key drivers of cell proliferation such as cyclin D2 and cyclin-dependent kinase 4 (Figure 4c).

Sorafenib Shows Growth Inhibitory Effects *In Vitro*

The antiangiogenic agent sorafenib is a multikinase inhibitor that targets vascular endothelial growth factor receptors 2

and 3 (VEGFR-2 and -3), platelet-derived growth factor receptor (PDGFR), as well as the Raf kinases B-Raf and C-Raf, whose activation induces a downstream signaling transduction cascade eventually activating an array of transcription factors including c-Myc. Given the enrichment of the Myc pathway in AS cells, we sought to address the question of whether AS cells are sensitive to sorafenib.

In an initial experiment we tested the impact of various sorafenib concentrations from 1 to 5 μM on tube formation of AS cells that were found to still possess this unique endothelial cell (EC)-specific angiogenic feature. Indeed, the results showed a dose-dependent inhibition of tube formation with a significant decrease in the mean tube length already at 3 μM ($P = 0.0038$; Figure 5a). In contrast, tube formation of AS cells exposed to the mTOR inhibitor temsirolimus was not impaired (data not shown).

Figure 4 Angiosarcoma cell lines show massive alterations in gene expression with a pronounced dysregulation of the Myc pathway. **(a)** Volcano plots comparing the gene expression between the wild-type and Notch1 KO LSECs (left panel) and between Notch1 KO LSECs and AS cell lines (right panel). The x axis unit is the log2 fold change between the mean expression in the two biological groups, with the red dashed line indicating the cutoff value of twofold. The y axis unit is the false discovery rate from the moderated *t*-test, with the red dashed line indicating the cutoff value of 0.05. Every dot represents one gene. **(b)** Table showing the top 20 enriched categories according to the normalized enrichment score from the Gene Set Enrichment Analysis (GSEA). The columns indicate the gene set name according to GSEA (NAME), the number of genes participating in the gene set (SIZE), the absolute enrichment score (ES), the normalized enrichment score (NES), the empirical *P*-value obtained by resampling of the data (*P*-val), and the false discovery rate (FDR). The gene sets related to Myc are highlighted in red. **(c)** Gene set enrichment plot (left panel) of Zeller_Myc_up showing Myc-specific genes upregulated in AS compared with LSEC. The heatmap (right panel) shows the scaled expression values of the genes that belong to the Zeller_Myc_up set in the nine analyzed samples.

To visualize the inhibitory effect of sorafenib on the ability to form vascular tubes, time-lapse microscopy was performed. Tube formation is a highly dynamic process, com-

prising cell migration and adhesion (filopodia and lamello-podia), coalescence of cells, reorganization, and arrangement into tubules (Supplementary Movie 1). We observed that

sorafenib-treated AS cells displayed significantly reduced migration (Supplementary Movie 2). Development of filopodia was quantitatively assessed at various time points and was significantly reduced in the presence of 3 μM sorafenib (Figure 5b).

Next, we explored the effect of sorafenib on AS cell proliferation and adhesion using the xCELLigence System. Sorafenib significantly reduced the cell index in comparison with controls (DMSO) at 24 and 48 h after drug addition (Figure 5c). We then aimed to assess the induction of apoptosis by sorafenib using flow cytometry and immunoblotting for activated caspase 3. No elevation of activated caspase 3 levels was observed with sorafenib concentrations up to 10 μM (Figure 5d).

The pERK protein is one of the best-known signaling mediators downstream of the RAF/MEK/ERK pathway that is activated by growth factors including VEGF and PDGF. As VEGFR-1 and -2 are downregulated to almost undetectable levels in AS cells (Supplementary Figure 1) and expression of the PDGFR is dramatically reduced (data not shown), we hypothesized that the noted *in vitro* effects of sorafenib result from inhibition of the Raf kinase that phosphorylates ERK. Correspondingly, western blot analysis revealed reduced phosphorylation levels of ERK upon sorafenib treatment in a dose-dependent manner (Figure 5e). Given that activation of the RAF/MEK/ERK signaling cascade induces cyclin D expression via transcriptional activation of Myc,²² we assumed that in our AS cells sorafenib exerts its growth-inhibiting effects through suppressing cyclin D expression. Protein levels of cyclin D1 (Figure 5e) were decreased dose-dependently by sorafenib. Lower cyclin D1 protein levels in sorafenib-treated AS cells were confirmed by RT-PCR; cyclin E1 mRNA levels were also reduced (Supplementary Figure 2). These results indicate that the growth-suppressive effects of sorafenib result from inhibition of the RAF/MEK/ERK pathway with a consequent reduction of cyclin D1 expression levels.

Tumorigenicity of Established Angiosarcoma Cell Line

To investigate the tumor formation potential of the established AS cell line, we subcutaneously injected AS cells into NOD/SCID mice ($n=3$). After 3 months, the tumor started to grow, and within 2 weeks the tumor volume increased exponentially to a diameter of >15 mm, confirming the tumorigenic potential of these cells (Figures 6a and b). No metastasis formation occurred. Histological assessment revealed a poorly differentiated sarcoma-like tumor with a solid growth pattern and epitheloid appearance of the cells. The tumor cells showed a high mitotic activity, displayed an eosinophilic cytoplasm (Figure 6c), and were positive for Factor VIII, indicating its endothelial differentiation (Figure 6d).

DISCUSSION

In this study, we have established three murine AS cell lines from Notch1-deficient mice harboring liver AS that developed spontaneously over time. We provide analysis of molecular changes that occur during transformation from nonmalignant Notch1 KO LSECs to a malignant AS phenotype. We further used the generated AS cell lines to study molecular pathways driving tumorigenesis. Myc-related genes were found upregulated, suggesting a potential role for sorafenib in AS treatment. Consistently, we demonstrated growth inhibitory effects in AS cells treated with sorafenib. When we injected AS cells into immunodeficient mice, the cells formed tumors confirming their tumorigenic potential.

The Notch pathway plays a pivotal role during development and adult tissue homeostasis and regulates various fundamental processes, including cell identity, differentiation, proliferation, and apoptosis. Mutations of Notch signaling members cause several diseases such as Alagille syndrome, T-cell leukemia, and CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), and is implicated in cancer biology.²³ Considering its highly complex effects that are cell type,

Figure 5 Sorafenib shows inhibitory efficacy in angiosarcoma cells in a dose-dependent manner. **(a)** Tube formation of AS cells is inhibited by sorafenib in a dose-dependent manner. AS cells (5×10^4) were seeded in 4-well chamber slides coated with matrigel and treated with 1, 3, and 5 μM sorafenib or DMSO (vehicle). After 46 h, total tube length was quantified using CellP software. Results are representative of three independent experiments. **(b, left)** Representative still images of AS cells grown on matrigel in the presence or absence (DMSO) of sorafenib, 3 μM (left) at 0 and 6 h. **(b, right)** Quantification of the average number of filopodia per cell from time-lapse sequences captured at 0/2/4/6/8 h. The reduced number of filopodia at time point 0 is explained by the exposition to sorafenib 5 h before subjecting the cells to the TFA assay. In sorafenib-treated cells, the number of filopodial extensions was significant decreased (paired *t*-test) after 6 h. **(c)** Real-time monitoring using the xCELLigence system (ACEA) of sorafenib-induced growth inhibition of AS cells after 24 and 48 h treatment duration. AS cells were seeded in 16-well E-plates at 5×10^3 cells/well in complete endothelial cell medium. Baseline levels were assessed after 24 h followed by sorafenib or vehicle (DMSO 0.1%) addition. Results were from two independent experiments with at least 6 data points per condition. **(d, left)** Sorafenib did not induce apoptosis in AS cells after 24 h of treatment determined by propidium iodide staining and flow cytometry. Only at the highest concentration an increase in apoptotic cells was noted. Percentage of apoptotic cells was normalized to DMSO controls ($n=5$). **(d, right)** Total lysates were subjected to western blot analysis to measure caspase 3 activity. **(e)** Sorafenib inhibits Erk phosphorylation and cyclin D1 expression in AS cells. AS cells were incubated with various concentrations of sorafenib or DMSO (vehicle) for 8 h followed by serum starvation in the presence of sorafenib/vehicle overnight. After 24 h of treatment, cells were exposed to phorbol myristate acetate (PMA; 100 ng/ml) for 15 min. Phosphorylated and total protein levels were evaluated by western blotting (left panel). Protein levels of cyclin D1 after 24 h of treatment with sorafenib at the indicated doses were assessed by immunoblotting (right panel). Means $\pm$ s.e.m.

* $P<0.05$, ** $P<0.005$, and *** $P<0.001$.

Figure 6 Tumor growth *in vivo* of subcutaneously injected angiosarcoma cells in NOD/SCID mice. (a) Photograph of a tumor (arrow) grown in a NOD/SCID mouse 3 months after subcutaneous injection of angiosarcoma cells (2×10^6). (b) Dissected tumor (left) showing induction of angiogenesis (arrows) in the surrounding subcutaneous tissue. (c) Histology of tumor tissue derived from injected angiosarcoma cells. Hematoxylin/eosin staining demonstrated a poorly differentiated sarcoma-like tumor ($\times 10$ and $\times 20$ original magnification). (d) Immunohistochemical staining for factor VIII showed a strong signal in cells lining vascular-like structures as well as in cells composing the solid tumor ($\times 10$ and $\times 20$ original magnification).

time, and context dependent, it is not surprising that Notch signaling exerts a bifunctional role in cancer showing oncogenic features or acting as a tumor suppressor.²⁴

Especially in endothelial cells the Notch pathway has been associated with tumor suppressor functions.^{13–15} Chronic DLL4 blockade as well as loss of Notch1 heterozygosity induce vascular tumor formation.^{14,15} In line with these findings, we have recently reported that Notch1 acts as a tumor suppressor gene in LSECs.¹³ In our Notch1 KO mouse model, AS development occurs spontaneously over time. Of note, these tumors were exclusively found in the liver. Liu *et al*¹⁵ reported similar findings in their Notch1 loss of hetero-

zygosity mice with vascular tumor formation in different organs, but most prevalently in the liver. In this approach, Notch1 heterozygosity is lost upon Notch1 receptor activation. Thus, cells with frequent Notch1 activation are more likely to become Notch1 null. Using such a sophisticated genetic model, the authors demonstrated that liver ECs are most affected by Notch1 loss of heterozygosity. At the same time, they could show that Notch1 activation occurs more often in hepatic ECs than in ECs from other organs.¹⁵ Restriction of vascular tumor formation to the liver in our animal model mirrors this indispensable role of Notch1 in liver endothelium.

Myc dysregulation has been described in human AS where Myc amplification was implicated in tumorigenesis in radiation-induced AS as well as in a subset of primary AS, and may be associated with the angiogenic phenotype.²⁵ Molecular analysis of our AS cells showed enrichment of the Myc pathway. In our model, we assumed that enhanced Myc expression is caused by increased activity of upstream signaling cascades such as the RAF/MEK/ERK pathway. Based on this hypothesis, tyrosine kinase inhibitors like sorafenib were tested *in vitro* for antitumor activity.

Evidence-based treatment for AS is missing for two reasons. First, the rarity of this tumor, accounting for only 2% of soft tissue sarcoma²⁶ that in turn constitutes only 1% of all cancers, makes it impossible to conduct large clinical trials with a sufficient number of patients to test efficacy of new treatment strategies. Second, experimental *in vitro* and *in vivo* models are limited, and thus AS pathomechanisms are incompletely understood and therapeutic experiments remain challenging.

A number of angiosarcoma cell lines have been established from tumor tissue arising in different primary sites and originating from different species. Two human AS cell lines (ISO-HAS and AS-M) were established from human scalp AS.^{27,28} The AS cell line ISOS-1 was established from human scalp AS transplanted into immunodeficient mice.²⁹ Furthermore, a total of seven canine AS cell lines were established from three different xenograft canine tumors.³⁰ Although there are a number of AS cell lines derived from cutaneous tumors, so far only one cell line has been generated from hepatic AS.³¹ This cell line named HAEND was established from a post-mortem liver biopsy of a patient diagnosed with hepatic AS induced by vinyl chloride, a well-known causal agent.³² *In vivo* models of AS are limited. Two reported AS models in the literature are based on AS cell implantation into immunodeficient mice.^{33,34} One drawback of our Notch1 KO mouse model to study AS biology and treatment strategies is the fact that the time point of tumor occurrence is not predictable and varies from animal to animal. Using our generated AS cell lines, we have established a tumor implantation model leading to subcutaneous AS formation, allowing visual control of tumor development and providing an attractive model to determine the *in vivo* efficacy of anticancer drugs.

In conclusion, the establishment of these novel AS cell lines enable the exploration of AS biology. Our *in vitro* studies identified sorafenib as a potential candidate for AS therapy and the successful AS cell transplantation provides a subcutaneous tumor model useful for *in vivo* preclinical drug testing.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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