Vascular normalization by loss of Siah2−/− results in increased chemotherapeutic efficacy

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Abstract:
Tumor hypoxia is associated with resistance to anti-angiogenic therapy and poor prognosis. The Siah E3 ubiquitin ligases regulate the hypoxic response pathway by modulating the turnover of the master pro-angiogenic transcription factor Hif-1α. In this study, we demonstrate that genetic deficiency in the Siah family member Siah2 results in vascular normalization and delayed tumor growth in an established transgenic model of aggressive breast cancer. Tumors arising in a Siah2−/− genetic background showed increased perfusion and pericyte-associated vasculature, similar to that occurring with anti-angiogenic therapy. In support of the role of Siah2 in regulating levels of Hif-1α, expression of angiogenic factors was decreased in Siah2−/− tumors. Blood vessel normalization in Siah2−/− tumors resulted in an increased response to chemotherapy and prolonged survival. Together, our findings offer a preclinical proof of concept that targeting Siah2 is sufficient to attenuate HIF1α-mediated angiogenesis and hypoxia signaling, thereby improving responses to chemotherapy.
Introduction:

Angiogenesis in solid tumors is the result of the complex interplay between several signaling pathways in the epithelial and stromal fractions of tumors, which is triggered by an increased demand for oxygen in the growing tumor. Given the importance of the expansion of the vasculature in tumor growth and metastasis, anti-angiogenic therapy is regarded to be a key approach to molecularly-based cancer therapy (1). Early work demonstrated that VEGF-specific antibodies reduced tumor growth and angiogenesis in three cancer models (2). Bevacizumab (Avastin), a neutralizing humanized monoclonal antibody against VEGF, and VEGF receptor (VEGFR) inhibitors including sunitinib (Sutent) and sorafenib (Nexavar), are approved treatments for several cancers (3). VEGF and VEGFR inhibition transforms the morphology of tumor blood vessel from tortuous and leaky to pericyte-covered and elongated (1). However, resistance to anti-angiogenic treatment frequently occurs, with tumors resuming growth despite VEGF or VEGFR inhibition (4). The proposed reasons for tumor escape from treatment include up-regulation of alternate angiogenic molecules (either acquired or pre-existing), recruitment of pro-angiogenic cells from the bone marrow or tumor cell invasion along existing vascular networks (5). An important experimental insight into escape from anti-angiogenic therapy comes from a mouse model of pancreatic tumors treated with anti-VEGF antibodies (6). The tumors that evade the blockade of the VEGF pathway to induce angiogenesis up-regulate several other pro-angiogenic growth factors, including FGF, PDGF and Angiopoetins (Ang) (6). The induction of these pro-angiogenic factors has been attributed to the cellular response to hypoxia (6).

Cells adapt to a hypoxic environment through up-regulation of Hif-1α, which together with the constitutively expressed Hif-1β protein, forms the Hif-1 transcription factor (7). Hif-1α protein turnover is controlled largely through covalent protein modification, including hydroxylation by prolyl-hydroxylases (PHD-1, -2 & -3) on Pro402 and 564 (8). The hydroxylated Hif-1α has increased affinity for the E3 ubiquitin-protein ligase complex which is composed of the von Hippel-Lindau tumor suppressor protein VHL, elongin B & C and cullin. After poly-ubiquitination, Hif-1α
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is degraded by the 26S proteasome (8). Under hypoxia, Hif-1α hydroxylation decreases due to reduced PHD abundance and activity (8). We and others have shown that Siah proteins polyubiquitinate PHDs under hypoxic conditions (9, 10), thereby targeting them for proteasomal degradation and allowing unmodified Hif-1α protein to dimerise with Hif-1β. The role of Siah proteins in tumor progression is becoming clearer (11-13). Previously, we described that the loss of Siah2 reduces the occurrence of aggressive neuroendocrine tumors and metastasis of prostate adenocarcinoma (14) and Siah2 is required for melanoma metastasis through Hif-1 (15). Furthermore, a dominant negative version of Siah blocks oncogenic Ras signaling, thereby reducing pancreatic and lung tumor growth (16, 17). We have previously described that inhibition of Siah2 by using a high affinity blocking peptide that binds to its substrate recognition domain can reduce growth and vascular development of tumors in a syngeneic and orthotopic breast cancer mouse model (18). Importantly, we have shown that clinically, Siah2 protein expression is specifically increased in triple negative, basal-like breast cancer (19). Here, we explore the growth and vascularization of breast cancer in Siah2-/- mice in a PyMT transgenic background. We show that tumor growth is delayed in PyMT-Siah2-/- animals. End-stage tumors show reduced angiogenic factor expression, as well as a vascular morphology resembling that seen following anti-VEGF treatment. We observe higher perfusion of PyMT-Siah2-/- tumors which are more sensitive to doxorubicin-based chemotherapy. Our findings are the first to explore the effect of targeting the upstream regulator of the hypoxic response, Siah2, as an anti-tumoral strategy to achieve increased efficacy of conventional chemotherapy in an immune competent mouse model of cancer. This work has important implications for the utility of targeting this upstream regulator of hypoxia, angiogenesis and tumor cell survival.
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Material and Methods:

Animals: PyMT-Siah2−/− and PyMT-WT mice were generated by crossing C57Bl/6 Siah2−/− mice (20) with C57Bl/6 PyMT-transgenic mice (kindly provided by Carol MacLeod and Lionel Hebbard (UCSD, USA).

All procedures involving mice were conducted in accordance with NHMRC regulations on the use and care of experimental animals, and were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee.

Measurement of tumor size. Mammary glands were palpated every other day, and once apparent, the size of individual tumors measured with digital calipers. Mice were culled when the tumor volume for all the tumors in a mouse reached a total of 525 mm³ (endstage). Tumor volume was calculated as (π*length*width²/6).

Dissection and tissue extraction. Mice were culled and weighed, then the tumor dissected and fractioned for further extraction of mRNA and protein, as well as for histology (fresh frozen and 10% NBF fixation). Whole tissue samples of the mammary gland, lung, liver, spleen and spine were prepared in 10% NBF.

Primary cell isolation, cell culture. PyMT positive cell lines were generated as published (21), then further purified for the tumor epithelial population by FACS sorting using EpCAM antibodies (eBioscience). The epithelial lineage of established lines was confirmed by FACS with EpCAM antibodies. Cell culture was done as previously described (18).

Transplantable tumor model. 5 x 10⁵ PyMT positive primary or established cultured cell lines were injected into the 4th left mammary fatpad of each recipient. The mice were then monitored at least twice weekly and tumor progression recorded as above.

Mammary fat pad staining. 3rd and 4th left and right mammary glands were carefully removed en bloc, laid flat, fixed, stained with Carmine Red, and cleared in xylene.

Immunohistochemistry and immunofluorescence. Serial sections were obtained from FFPE or OCT (fresh frozen) imbedded tissues. Staining was done with antibodies for CD31 (BD Biosciences
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Pharmingen: 550274), NG2 (Millipore; AB5320) and SMA (Dako; M0851) according to manufacturer’s instructions and as described (18). Texas-red dextran injections were done as described (22). Pimonidazole (PIM) staining was carried out as described (23). Determination of CD31/NG2 co-localization and CD31/PIM minimum distances was done using Metamorph software in a semi-automated manner. 3-4 random fields of view per tumor were analyzed. Proliferation and apoptosis were assessed by PCNA (BD Biosciences; 610665) and TUNEL (Chemicon) according to manufacturers’ instructions. The positively stained cells were counted in 5 fields/mammary gland and expressed as percentage of total cells present.

Flow cytometry analysis. 3rd and 4th left and right mammary glands or endstage tumors were dissected, minced and then subjected to collagenase digestion before staining with APC-CD45, PE-F4/80 and PE-Cy7-CD11b antibodies prior to resuspension in FACS buffer containing 2% FBS and 1/100 dilution of 7AAD. The samples were analyzed on a BD FACSCanto II Loader machine.

Angiogenesis arrays, TIF. Tumor interstitial fluid (TIF) was prepared as described (24). TIFs were then probed with mouse angiogenesis antibody proteome profiler array from R&D Systems according to manufacturer’s instructions.

Western blot. Cells were treated as indicated, lysed and analyzed by Western Blotting as previously described (18).

mRNA extraction and qPCR. mRNA from cells or tissues was isolated by QiaShredder (Qiagen) and RNeasy (Qiagen) kits according to the manufacturers’ instructions.

Small animal PET and Ultrasound. 18FAZA tracer was synthesized and labeled in a dedicated in-house radiochemistry facility and injected into mice with 65 mm³ tumors in their third mammary gland. 3 hours post injection mice were imaged on a prototype A-PET (Phillips Mosaic) small animal PET scanner as described (25). A high-resolution small animal ultrasound system (Visualsonics, Inc.) was used to examine tumor vasculature and perfusion. In the 3D power Doppler acquisition mode, 2D images were acquired in 100 μm increments across the tumor and images stacked to produce a 3D representation of the tumor. Percent vasculature was then calculated as the...
ratio of the number of colored voxels within the tumor volume and the total number of voxels within the same volume. Perfusion was evaluated in the centre slice of the tumor using a destruction/replenishment technique employing microbubbles as described previously (26).

**Clonogenic Survival Assay.** Cells were treated with a range of doxorubicin concentrations for the indicated times before counting and plating. Cells were fixed, stained and colonies of >50 cells counted.

**Determination of doxorubicin concentrations in tumors.** 8 PyMT-WT tumor-bearing WT mice and 8 PyMT-Siah2<sup>−/−</sup> tumor-bearing Siah2<sup>−/−</sup> mice with tumor volumes of 525 mm<sup>3</sup> were injected with 5 mg/kg doxorubicin 3 hours before sacrifice. Doxorubicin concentration in tumors was measured fluorimetrically using an adaptation of the method described previously (27). Frozen tumors were cut into three parts and each slice extracted in 3 ml 0.75 M HCl/iso-propanol per gram of frozen tissue, using metal beads and a FastPrep 24 homogenising unit (MP Biomedicals, 4 x 30 sec homogenisation). Samples were clarified by centrifugation at 14000g for 5 minutes and doxorubicin concentration in the supernatant measured using a POLARstar fluorimeter, with excitation at 480 nm and emission at 610 nm. Background fluorescence from tumors without doxorubicin was subtracted from all values. Doxorubicin concentrations were converted to ng doxorubicin per gram of frozen tissue.

**Doxorubicin treatment of mice.** Mice were tail vein injected twice weekly for 4 weeks with the indicated doses when tumors reached 65 mm<sup>3</sup>. Animal weight and tumor dimensions were assessed as describe above. Animals were culled when tumors reached 525 mm<sup>3</sup>. 

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Results:

Loss of Siah2 delays tumor onset

We investigated the impact of Siah2 in breast cancer progression by generating MMTV-Polyoma Middle T (PyMT) transgenic mice on a Siah2<sup>−/−</sup> background. Loss of Siah2 resulted in a significantly increased time to tumor onset compared to PyMT-wild type animals (PyMT-WT; 81 days of age; PyMT-Siah2<sup>−/−</sup>; 121 days of age; p<0.001; Figure 1A). To determine whether this delay can also be seen at earlier stages, we next examined the mammary glands of mice before the onset of palpable tumors. Mammary gland whole mounts (Figure 1B and Supplementary Figure 1A, C, E) and histological analyses (Figure 1C and Supplementary Figure 1B, D) of 30, 50 and 70 day old mice revealed that the difference in tumor progression had already manifested at 50 days of age. The mammary glands of PyMT-Siah2<sup>−/−</sup> mice showed similar tumor progression to PyMT-WT animals but the effects were delayed by more than 3 weeks (Figure 1B; graph). Therefore, PyMT-Siah2<sup>−/−</sup> mice at 90 days of age resembled PyMT-WT mice of 70 days of age with regard to tumor burden and histological staging (Supplementary Figure 1E compared to Supplementary Figure 1C-left). To determine whether the differences in tumor onset could be associated with altered mammary development in a Siah2<sup>−/−</sup> background, we examined mammary fatpad morphology in young PyMT-negative animals from both Siah2 genotypes. The timing of formation, number and morphology of the ductal tree was indistinguishable between Siah2<sup>−/−</sup> and wild type animals (Supplementary Figure 2), suggesting that the delayed onset of tumor formation in mice was not a consequence of altered mammary development.

While there was a trend towards longer time to ethically allowed endpoint (525 mm<sup>3</sup>) in Siah2<sup>−/−</sup> mice, this was not statistically significant (p=0.36; Figure 1D). Although initially delayed, once tumors became palpable in Siah2<sup>−/−</sup> animals, they grew rapidly (Figure 1E). This data suggest...
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that the initial delay of tumor growth initiation in PyMT-Siah2−/− mice is associated with early events in tumor development but compensatory changes allow accelerated growth of the tumor.

Reduced stromal infiltration in Siah2−/− breast tumors:

Tumors in PyMT-WT mice were typically fluid or blood-filled, had a high degree of stromal infiltration and large areas of necrosis (Figure 2A). Tumors from PyMT-Siah2−/− mice were strikingly different, consisting of large epithelial sheets, very little fibroblast infiltration, and minimal necrosis (Figure 2A). Smooth muscle actin (SMA), a marker of fibroblasts and pericytes (28, 29), stained large areas of stromal tissue in PyMT-WT tumors, whereas PyMT-Siah2−/− tumors displayed greatly reduced staining (Figure 2B and Supplementary Figure 3).

To investigate the basis of the difference in tumor development in the PyMT-Siah2−/− animals, we first assessed PyMT-derived oncogenic signaling in cells isolated from mammary glands of 30 day old PyMT-WT and PyMT-Siah2−/− mice. There was a similar level of PyMT gene expression in both genotypes (Supplementary Fig 4A). PyMT expression induces, among other responses, the phosphorylation of Erk (30). The levels of phosphorylated Erk varied between mammary glands isolated from different 30 day old PyMT-WT and PyMT-Siah2−/− animals but there was no obvious difference between the two genotypes (Supplementary Fig 4B). We also observed no difference in the rates of apoptosis or proliferation in mammary glands from 50 and 70 day old mice as well as in endstage tumors (Supplementary Fig 4C-F). Siah2 is a member of the Siah ubiquitin ligase family, which also includes Siah1A and Siah1B in mice. To assess whether the loss of Siah2 function in the PyMT-Siah2−/− mice was compensated for by increased expression of either Siah1A or Siah1B, thus enabling tumor progression, the gene expression of these genes was examined in early mammary lesions and endstage tumors. We observed no difference in the expression of these two genes in primary epithelial cells of 30 day old PyMT-Siah2−/− and PyMT-WT mice or endpoint tumors (Supplementary Fig 4G and 4H).
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Normalized tumor vasculature in PyMT-Siah2−/− mice

We previously observed changes in the vasculature of syngeneic tumors in the presence of Siah substrate binding inhibitors (18). We therefore analyzed the vasculature in endstage tumors from both genotypes. While we observed no difference in the overall amount of CD31-positive vessels in the tumors (Figure 3A, Supplementary Figure 5A, data not shown) the vessels of PyMT-Siah2−/− tumors appeared less tortuous and possess slimmer lumen (Figure 3A, Supplementary Figure 5A). We therefore investigated the coverage of the vessels by pericytes. Consistent with previous reports (1, 31), we observed modest co-localisation of pericytes with endothelial cells in PyMT-WT tumors (Figure 3B and Supplementary Fig 5B). In contrast, PyMT-Siah2−/− tumors showed significantly more pericyte coverage of endothelial cells (Figure 3B and Supplementary Fig 5B), which is characteristic for more mature vessels (1).

To examine if these different vessel phenotypes are functionally similar, we injected Texas Red Dextran into the blood stream of tumor-bearing mice. By this method only blood-carrying vessels are labeled (22). PyMT-Siah2−/− tumors contain significantly more blood-carrying vessels compared with PyMT-WT tumors in both spontaneous and transplanted tumor models (Figure 3C and data not shown). To further corroborate this observation, blood flow and perfusion was assessed by high resolution 3D power Doppler ultrasound (32). For this experiment, single large tumors are required, rather than multiple foci occurring in the spontaneous model. Therefore, tumor cells derived from PyMT-WT and PyMT-Siah2−/− mice were injected into the 4th mammary fatpad of syngeneic mice (named PyMT-WT/WT and PyMT-Siah2−/−/KO, respectively). Tumors generated this way recapitulated the angiogenic, pericyte and stromal infiltration phenotypes observed in the spontaneous model (data not shown). In tumors of similar size (approximately 150 mm³, Figure 3D), PyMT-Siah2−/−/KO tumors had a significantly increased percentage of functional, blood-containing vasculature compared to those in PyMT-WT/WT tumors (Figure 3E). Furthermore,
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using replenishment kinetics following an ultrasound-induced destruction of microbubbles, we observed significantly elevated perfusion of the PyMT-Siah2+/KO tumors (Figure 3F).

To assess if increased tumor perfusion impacted on tumor-associated hypoxia, PyMT-WT and PyMT-Siah2+/ tumors were investigated by small animal PET using the hypoxia tracer 18F-AZA. PyMT-Siah2+/ tumors showed a trend towards reduced hypoxia (Figure 4A, p=0.09). The tissue localization of hypoxic areas was visualized using the hypoxia marker pimonidizole (PIM), which accumulates preferentially in areas of limited perfusion (25, 33). Co-staining with PIM and the vascular marker CD31 showed that in PyMT-Siah2+/ tumors, hypoxia was restricted to regions that were predominantly distant from blood vessels (Figure 4B and Supplementary Figure 6A). In contrast, significantly more regions of hypoxia associated with blood vessels in PyMT-WT tumors, consistent with the poor functional properties and transient occlusion (Figure 4B, Supplementary Figure 6) (34, 35) associated with the vasculature of many solid cancers.

Therefore, while the overall number of CD31-positive endothelial cells is similar in PyMT-Siah2+/ and PyMT-WT tumors, tumors developing in PyMT-Siah2+/ mice contain more functional vessels, resulting in increased perfusion in both early and endstage tumors.

Reduced pro-angiogenic growth factor production by PyMT-Siah2+/ tumors

We next aimed to determine the causes for the difference in neo-angiogenesis in PyMT-Siah2+/ tumors. Hif-1-dependent angiogenesis results in the recruitment of blood vessels from existing vasculature (22), thus allowing rapid tumor growth. Siah2 controls the abundance of the Hif-1α transcription factor (9). Accordingly, Hif-1α-positive cells in endstage tumors were lower in PyMT-Siah2+/ mice compared to PyMT-WT mice (Figure 4C). Hif-1α is responsible for the induction of a wide range of pro-angiogenic factors, including VEGF and angiopoetins (36) and the phenotypes observed above suggest that the loss of Siah2 in tumor cells causes an angiogenic normalization phenotype similar to VEGF inhibition. We therefore assessed the levels of VEGF and
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the VEGF receptors, Kdr (VEGFR2) and Flt1 (VEGFR1), as well as several FGFs and angiopoetin expression in the two tumor genotypes by qPCR. Expression of these pro-angiogenic factors was either similar in both tumor genotypes or significantly reduced in PyMT-Siah2−/− tumors (Figure 4D). These results demonstrated that the previously described hypoxia response-dependent pro-angiogenic pathway is largely suppressed in PyMT-Siah2−/− tumors. To uncover the mechanism and factors utilized by PyMT-Siah2−/− tumors to induce vessel formation, we used a proteomic approach to screen for angiogenic factors secreted at elevated levels into the tumor interstitial fluid (TIF) by PyMT-Siah2−/− tumors. We found variations between amounts and composition of angiogenic factors secreted from tumors of the same genotype attributable to the heterogeneity of this model (37). However, we found that PyMT-WT TIFs derived from both small (65mm³ volume) and endstage tumors consistently contained elevated levels of Angiopoietin 1 (A6), CXCL16 (A9), CCN1 (B2), FGF-1 (B9), CCN3 (D11) and PTX3 (E6); (Figure 4E, Supplementary Figure 5C and Supplementary Table 1). We furthermore confirmed that several factors, including FGF1 (B9) and Angiopoetin 1 (A6), which we found to be down-regulated on a gene expression level in PyMT-Siah2−/− tumors (Figure 4D), were also secreted at reduced levels by PyMT-Siah2−/− tumors (Figure 4E). Together, this data shows that PyMT-Siah2−/− tumors produce reduced amounts of pro-angiogenic growth factors compared to controls.

Tumors from PyMT-WT and PyMT-Siah2−/− mice are different with regard to their stromal content (Figure 2) and stromal lineages, especially macrophages. Using flow cytometric analysis, we found a slight increase of CD11b+/F4/80+ cells, representing macrophages, in PyMT-Siah2−/− lesions at 50 days compared to PyMT-WT mammary gland lesions. However, no difference of CD45+ cells and CD45+/CD11b+/F4/80+ cells was observed in normal mammary glands or endstage tumors (Supplementary Figure 6B). This data suggests that the difference in angiogenesis in the PyMT-Siah2−/− tumors is not due to reduced macrophage infiltration causing changes to the angiogenic switch.
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Loss of Siah2 results in increased sensitivity to standard chemotherapy

To test if the increased perfusion in the PyMT-Siah2<sup>−/−</sup> tumors is associated with enhanced sensitivity to chemotherapy, we first assessed the sensitivity of PyMT-WT and PyMT-Siah2<sup>−/−</sup> cells <i>in vitro</i> towards doxorubicin, a common first line treatment of breast cancer. The clonogenic survival of PyMT-WT and PyMT-Siah2<sup>−/−</sup> cells in the presence of various doxorubicin concentrations is similar (113.9 nM for PyMT-WT cells compared to 107.2 nM for PyMT-Siah2<sup>−/−</sup> cells) (Figure 5A), demonstrating that cells with both genotypes have similar sensitivity to doxorubicin <i>in vitro</i>. To measure if the vascular normalization described above results in elevated tumor concentrations of chemotherapeutics <i>in vivo</i>, doxorubicin was intravenously injected into tumor-bearing mice 3 hours prior to determining drug concentrations. We found that doxorubicin concentrations in PyMT-Siah2-/- tumors are approximately 120% higher than in PyMT-WT tumors (Figure 5B). To assess if the different drug concentrations results in changed efficacy of doxorubicin on established orthotopic tumors in the 4<sup>th</sup> mammary glands, PyMT-WT and PyMT-Siah2<sup>−/−</sup> tumors were grown to 65 mm<sup>3</sup> then twice weekly treatment of doxorubicin was commenced. PyMT-WT/WT tumors grew steadily, reaching 250 mm<sup>3</sup> within 30 days (Figure 5C). The treatment of PyMT-Siah2<sup>−/−</sup>/KO tumor-bearing animals with doxorubicin resulted in stable tumor sizes which are significantly smaller than in the PyMT-WT/WT group (Figure 5C). After cessation of the treatment, we followed the tumor growth until endstage (525 mm<sup>3</sup>) and observed significantly prolonged survival of PyMT-Siah2<sup>−/−</sup>/KO mice after treatment with doxorubicin compared to PyMT-WT/WT mice (Figure 5D; p<0.05). Collectively, this data demonstrate that the increased perfusion of PyMT-Siah2<sup>−/−</sup> tumors results in a significantly elevated efficacy of doxorubicin despite similar resistance of PyMT-Siah2<sup>−/−</sup> tumor cells <i>in vitro</i>. 

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Discussion:

Malignant tumor progression past an initial “avascular phase” requires new blood vessel formation once the tumor grows beyond 500 μm in diameter (38). Hypoxia is a key driver in the neo-angiogenic process required to form a new but inefficient tumor-associated vasculature network (39). While no single protein seems to be the master regulator of neo-angiogenesis, the loss or inhibition of VEGF or its kinase receptors (VEGF-Rs) results in a normalization of the tumor vasculature. As a normalized vasculature would lead to more efficient delivery of blood through tumor-associated vessels, anti-angiogenic therapy might result in higher sensitivity of tumors to standard chemotherapy (40). This prediction has since been verified in animal models in which VEGF or VEGF-R function has been attenuated. More recently, anti-VEGF treatment of glioblastoma patients has been associated with improved tumor perfusion and enhanced survival been proven in glioblastoma patients (41). Anti-angiogenic therapy through EGFR inhibition with erlotinib was also shown to result in increased sensitivity towards chemo- and radiotherapy (42). Similarly, we find in this study that the loss of Siah2 results in vascular normalization, increased tumor perfusion and elevated sensitivity to doxorubicin-based chemotherapy (Summarized in the schematic in Figure 5E).

Although blocking proteins downstream of Hif-1α, such as VEGF, is an attractive therapeutic approach, the clinical success of anti-angiogenic therapies has been limited. Hif-1 regulates a wider range of responses that allow cancer cells to survive in hypoxic environments and it appears that targeting just a subset of these allows the emergence of bypass mechanisms and restoration of tumor growth (6). In addition, inhibition of single Hif-1 targets may leave other hypoxic responses, such as metastatic spread, uncontrolled or enhanced, with unintended consequences for patients (43, 44). For these reasons, targeting the Hif-1α protein directly represents a promising approach that should be explored in more detail.

As a transcription factor, targeting Hif-1 activity directly is difficult and most drugs that inhibit Hif-1α activity do so by altering other signaling pathway components that influence Hif-1α
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stability (8). In addition, some chemotherapeutic drugs, including doxorubicin, have been shown to block Hif-1 DNA binding and thereby act as Hif-1α inhibitors (8). In this study, we have explored an alternative pathway of inhibiting hypoxic-response signaling by genetic targeting of the Siah2 ubiquitin ligase, which regulates the abundance of PHD proteins under hypoxia and therefore the stabilization of Hif-1α (9). In contrast to the inhibition of downstream proteins of the hypoxic-response pathway, we do not observe the up-regulation of alternate pro-angiogenic molecules or the recruitment of pro-angiogenic cell lineages. Despite this, Siah2−/− tumors start growing after an initial delay. It is possible that this initial delay in tumor growth represents a delayed induction of the angiogenic switch in these tumors or that the resulting vascular normalization phenotype in Siah2−/− tumors represents a stage similar to that of normal angiogenesis of organs during development. Alternatively, other pro-angiogenic factors, which we did not detect in our approaches, might be in part responsible for the differential timing of angiogenesis in Siah2−/− tumors. Importantly, macrophages have previously been shown in the PyMT-driven mouse model to be essential contributors of pro-angiogenic proteins to the tumor microenvironment and the ablation of macrophages results in a delayed angiogenic switch (22). While we find no differences in macrophage infiltration into the Siah2−/− tumors at early or late stages, further work will define whether Siah2 plays a role in angiogenic factor production in macrophages. With the close resemblance to that seen in human disease, including molecular pathway activation (i.e. Ras, PI3K and Src signaling);(45-47) and biomarker expression (37), the PyMT-driven breast cancer model can assist in the future to further define the various roles of Siah2 in breast cancer progression.

Our findings, that the loss of Siah2 results in increased tumor perfusion through vascular normalization, are in accordance with previous work from our group and others showing that blockade of Siah substrate binding (14, 15, 18) and dominant negative Siah proteins (16, 17) have tumor inhibiting properties in various cancer models. Siah inhibitors can be expected to inhibit several pro-tumoral responses, in addition to the hypoxic-response pathway (48), including Ras (16, 17, 49), estrogen receptors (50, 51) and DNA damage signaling networks (13, 52). Inhibition of...
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Siah has been linked to Hif-1α dependent and independent mechanisms (15, 17). However, untangling the relative contribution of inhibition to each of these pathways to attenuation of tumor growth is challenging (12).

We have previously generated the peptide-based Siah inhibitor PHYL (18, 53) which required stable expression in tumor cells. Due to its peptide nature, it is not suitable for studies assessing the systemic effect of Siah blockade on tumor progression. Recently, Vitamin K3 has been reported as an inhibitor of Siah ubiquitin ligases, although with reasonably low affinity (54). Improving this Siah inhibitor further might provide a novel anti-angiogenic treatment option for solid cancers in the future. In a recent study, we have demonstrated that Siah2 expression is correlated with triple negative, basal-like breast cancer in patients (19). This type of breast cancer is characterized by a strong hypoxic phenotype and inhibition of Siah and therefore hypoxic response signaling, in combination with standard chemotherapy, might provide a novel and promising treatment approach in patients with these treatment-refractory tumors. As Siah inhibitory drugs would result in vascular normalization and elevated tumor perfusion, their administration should ideally occur before application of chemotherapeutic drugs. Vascular normalization has been shown to generally improve oxygenation of tumors, providing a healthier environment for tumor growth (1). It is thought that alleviation of hypoxia not only abrogates continuous hypoxic signaling and the promotion of pro-survival mechanisms, but also reduces their drug resistance and the selective pressure on tumor cells to metastasize (1, 3, 55). It will be interesting to determine in the future if the reduction of hypoxia signaling in Siah-inhibited tumors also results in a less aggressive, metastatic and more drug-sensitive phenotype. Thus, our experiments identify Siah as both a valuable target for molecular therapy as well as an adjunct to standard chemotherapy in breast cancer.
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References:

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Figure Legends:

Figure 1. PyMT-Siah2⁺/⁻ mice show delayed onset in a PyMT model of breast cancer

A) Mammary tumor incidence in PyMT-Siah2⁺/⁻ (black) and PyMT-WT (red) mice (n = 24 (WT) and 27 (Siah2⁺/⁻) mice/group, respectively) is depicted as percentage of tumor-free mice in a Kaplan-Meier graph. Mice were considered tumor-free until a palpable mass (~ 0.3 mm x 0.3 mm) was detected and persisted for more than 6 days. (p<0.001).

B) Representative fourth mammary gland whole mounts of PyMT-WT (left) and PyMT-Siah2⁺/⁻ (right) mice at 50 days of age. Primary tumor can be observed at the nipple region (NR) at this stage and the difference in primary tumor size can already be distinguished. Lymph nodes (LN) are indicated. Size bar: 1 cm. Bar chart summarizing data from 4th mammary gland whole mounts of mice at 50 and 70 days based on primary tumor size.

C) Representative FFPE sections of the nipple region of fourth mammary glands of PyMT-WT (left) and PyMT-Siah2⁺/⁻ (right) mice at 50 days of age following hematoxylin and eosin (H&E) staining (Size bar: 200 μm). Bar chart summarizing data from H&E sections from 4th mammary glands of mice at 50 and 70 days based on tumor stage.

D) Kaplan-Meier analysis of overall survival of PyMT-WT (red) and PyMT-Siah2⁺/⁻ (black) mice (n = 24 WT and 27 Siah2⁺/⁻ mice/group, respectively; p = 0.36).

E) Kaplan-Meier analysis of survival of PyMT-WT (red) and PyMT-Siah2⁺/⁻ (black) mice after tumor onset (n = 24 WT and 27 Siah2⁺/⁻ mice/group, respectively; p = 0.14). Survival endpoint for (D) and (E) is determined by the total area of all the mammary tumors in the mouse reaching 525 mm³.

Figure 2. PyMT-WT endstage tumors show more stromal infiltration than PyMT-Siah2⁺/⁻ endstage tumors.

A) Representative FFPE sections of endstage tumors from PyMT-WT (left) and PyMT-Siah2⁺/⁻ (right) mice following hematoxylin and eosin (H&E) staining. Endstage is determined by the total volume of all the mammary tumors in the mouse reaching 525 mm³. Size bar: 1mm.
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The extent of stromal infiltration in PyMT-WT (left) and PyMT-Siah2−/− (right) endstage tumors was assessed by immunohistochemical staining using anti-smooth muscle actin (SMA) antibody against fibroblasts on FFPE sections of tumor. Representative 10x images of the tumors depict stromal infiltration stained brown by anti-SMA. Size bar: 200 μm.

Figure 3. Delayed angiogenic switch and normalized vasculature in Siah2−/− tumors.
(A) Slimmer, elongated and more normalized blood vessel morphology in PyMT-Siah2−/− compared to PyMT-WT endstage tumors. Fresh frozen sections were stained with a marker for endothelial cells, anti-CD31, to visualize blood vessel morphology. DAPI was used as a nuclear stain. Representative images of blood vessel morphology in tumors of the respective genotypes. Size bar: 100 μm.
(B) Increased pericycle coverage of tumor blood vessels in PyMT-Siah2−/− tumors. Immunofluorescence staining of PyMT-WT and PyMT-Siah2−/− tumors with anti-CD31 (endothelial) and anti-NG2 (pericycle) antibodies. Tissue sections were counterstained with DAPI. Merged representative pictures are shown. Size bar: 10 μm. Metamorph-based quantification of co-localization of CD31-positive endothelial cells and NG2-positive pericytes. n=10 for PyMT-WT, n=11 for PyMT-Siah2−/−, 3 to 4 random fields of view were analyzed per tumor, ** denotes p<0.01.
(C) Texas red dextran-injected PyMT-WT and PyMT-Siah2−/− tumors were prepared and counterstained with DAPI. Size bar: 100 μm. Quantification of 5 PyMT-WT and 6 PyMT-Siah2−/− tumors is shown on the right (p<0.05).
(D) Ultrasound analyses of tumor volumes show no differences between PyMT-WT/WT and PyMT-Siah2−/−/KO tumors (n=5 for PyMT-WT/WT and n=6 for PyMT-Siah2−/−/KO).
(E) PyMT-Siah2−/− tumors have a significantly higher blood carrying vessel density (expressed as % vasculature) compared to wildtype tumor (p<0.05, n=5 and 6 for PyMT-WT/WT and PyMT-Siah2−/−/KO, respectively)
(F) PyMT-Siah2−/− tumors have a significantly increased perfused area compared to PyMT-WT tumors (p<0.0001; n=5 (PyMT-WT/WT) n=6 (PyMT-Siah2−/−/KO), measured in triplicates). Size bar: 100 μm.

Figure 4. Angiogenic mechanism in Siah2−/− tumors.
(A) Small animal FAZA PET scan of PyMT-WT/WT (n=11) and PyMT-Siah2−/−/KO (n=14) mice bearing tumors in the 3rd or 4th mammary glands. Tumors (left, middle image; 3rd mammary glands) are indicated with arrows. The other strong signal is from the gut. Quantification of the tumor to background (taken at neck region) ratio of each mouse/tumor is shown in graph on right, p=0.09. Tumor volumes are similar between PyMT-WT/WT and PyMT-Siah2−/−/KO mice.
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(B) Pimonidazole (PIM, green) staining of tumors from PyMT-WT/WT and PyMT-Siah2−/−/KO tumors co-stained with CD31 (red) and DAPI (blue). Size bar: 100 μm. Minimum vessel distance to hypoxic areas was quantified in PyMT-WT (n=4) and PyMT-Siah2−/− (n=4) tumors. The percentage of vessels in close proximity (1-10 μm), mid range (11 – 100 μm) and distant (101-200 μm) to the nearest hypoxic area is depicted on the right. * denotes p<0.05.

(C) Hif-1α staining in PyMT-WT and PyMT-Siah2−/− endstage tumors. Quantification of the Hif-1α signal is shown on the right (n=12 for PyMT-WT and n=11 for PyMT-Siah2−/−; p=0.06).

(D) Quantitative RT-PCR analysis of gene expression levels of pro-angiogenic growth factors in PyMT-WT and PyMT-Siah2−/− at endstage tumors. The expression of the different growth factors was normalized to a house keeping gene and then the levels in PyMT-WT tumors set as 1. Fold change in PyMT-Siah2−/− tumors is represented for PyMT-WT (n=8) and PyMT-Siah2−/− (n=9), values are means +/- SEM.

(E) Angiogenesis profiler array analysis of tumor interstitial fluid (TIF) of PyMT-WT and PyMT-Siah2−/− tumors at endstage (525 mm3).

Figure 5. Loss of Siah2 increases tumor sensitivity to chemotherapeutic treatment with Doxorubicin

(A) Dose-response curve showing number of colonies formed in a clonogenic survival assay after 1 hr treatment of PyMT-WT and PyMT-Siah2−/− cells with 5 doxorubicin concentrations. Experiment was repeated three times for each cell line at each doxorubicin concentration.

(B) Tumor doxorubicin concentration 3 hours after injection of 5 mg/kg doxorubicin in PyMT-WT/WT (n=8) and PyMT-Siah2−/−/KO (n=8) animals. Each tumor was divided into three pieces and each extracted independently. Each extraction was measured in duplicate and values are shown as means. Mean +/- SEM for each genotype are shown, p<0.001.

(C) Graph showing tumor volume in mice injected intravenously with 5 mg/kg doxorubicin or PBS twice weekly for 4 weeks. The dotted line indicates starting tumor size of 65 mm3 (n=7, n=7, n=3 and n-3 for PyMT-Siah2−/−/KO, PyMT-WT/WT, PBS control PyMT-Siah2−/−/KO and PBS control PyMT-WT/WT, respectively. p<0.01 between doxorubicin treated PyMT-Siah2−/−/KO and PyMT-WT/WT groups).

(D) Kaplan-Meier curve of survival of PyMT-Siah2−/−/KO and PyMT-WT/WT after end of 5 mg/kg doxorubicin treatment (n=7 and n=7 for PyMT-Siah2−/−/KO and PyMT-WT/WT respectively, p<0.05).

(E) Summary showing that in response to tumor hypoxia, loss of Siah2 results in increased perfusion, a reduction of pro-angiogenic protein production and increased sensitivity to doxorubicin-based chemotherapy.
A

H&E PyMT-WT

H&E PyMT-Siah2-/−

B

αSMA PyMT-WT

αSMA PyMT-Siah2−/−
A

Number of colonies

Log Concentration of Dox [log(μM)]

B

Doxorubicin (ng/g tumor tissue)

C

Tumor volume (mm³)

Days after 65 mm³

D

Percent survival

Days after end of Dox treatment

E

Wildtype

Siah2⁻/⁻

Tumor Hypoxia

Angiogenic factors

Abnormal vasculature

Poor perfusion

CTx resistance

Normalized vasculature

Improved perfusion

Increased CTx efficacy

Wong et al., Figure 5
Vascular normalization by loss of Siah2−/− results in increased chemotherapeutic efficacy
Christina SF Wong, Jaclyn E Sceneay, Colin M House, et al.
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