Targeting the angiogenic 'splice' switch; inhibition of SRPK1 as a novel therapeutic approach in prostate cancer

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Pathological angiogenesis occurs when the balance between pro-angiogenic and anti-angiogenic factors is tipped enabling an angiogenic switch. VEGF-A is the primary inducer of angiogenesis but is alternatively spliced at the pre-mRNA level to produce pro-angiogenic and anti-angiogenic isoforms. In previous work we showed that the balance of VEGF-A isoforms is regulated by SRSF1, which is regulated by phosphorylation from SRPK1 in renal epithelial cells, colon cancer cells, retinal epithelial cells and melanoma cells. SRPK1 and SRSF1 promote expression of pro-angiogenic VEGF-A165a and knockdown or inhibition of SRPK1 switches this balance towards anti-angiogenic VEGF-A165b, which inhibits angiogenesis and tumor growth in colon cancer and melanoma cells. In our recent study, Mavrou et al., 2014, we report that SRPK1 expression is upregulated in both prostatic intraepithelial neoplasia and malignant tissue sections from patients with radical prostatectomy. Knockdown or inhibition of SRPK1 with small molecule inhibitors leads to a splicing switch towards anti-angiogenic VEGF-A in PC-3 cells, a reduction in angiogenesis and inhibition of tumor growth in xenograft models. Specifically inhibiting pro-angiogenic VEGF-A splice isoforms through targeted inhibition of the splicing kinase SRPK1 is a novel approach which could enable development of therapies that are safer and more efficacious than current drugs. This study provides proof-of-concept for modulation of SRPK1 to normalise the endogenous VEGF-A splicing balance, enabling inhibition of angiogenesis and tumor growth in prostate cancer. Here, we discuss our recent findings and ongoing work to develop these findings into a novel therapeutic approach.

Keywords: Angiogenesis; SRPK1; alternative splicing; VEGF-A; prostate cancer

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Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, is a hallmark of cancer, required to supply oxygen and nutrients and remove waste products to sustain tumor proliferation. Angiogenesis can also facilitate metastatic spread by providing escape routes for disseminating tumor cells [1-3]. During tumor progression the balance of pro-angiogenic and anti-angiogenic factors, which maintains normal quiescent vasculature, is altered during an 'angiogenic switch', leading to aberrant neovascularization [4, 5]. The best described inducers and inhibitors of angiogenesis are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) respectively [6]. The balance of angiogenic regulators can be shifted by altered gene transcription, for example VEGF-A gene expression is upregulated in response to hypoxia and oncogene signaling [7, 8]. VEGF-A can also be released from the extracellular
matrix by the actions of proteases \(^9\). An additional mechanism for upregulation of pro-angiogenic VEGF-A involves a switch in alternative splicing towards pro-angiogenic VEGF-A isoforms \(^{10-12}\). This ‘angiogenic splice switch’ was discovered in our laboratory more than 10 years ago, with the finding of 3’ alternative splicing in the terminal exon of VEGF-A pre-mRNA \(^{10}\). VEGF-A pre-mRNA can be alternatively spliced to produce multiple distinct isoforms \(^{13}\). Proximal splice site selection in exon 8 leads to production of pro-angiogenic VEGF-A\(_{165\text{a}}\) isoforms, whereas distal splice site selection leads to the generation of anti-angiogenic, cytoprotective VEGF-A\(_{165\text{b}}\) isoforms, predominantly VEGF-A\(_{165\text{b}}\) \(^{10}\). Angiogenesis occurs when the balance between these pro- and anti-angiogenic forms of VEGF-A is switched by mechanisms of differential splicing of the VEGF-A mRNA to the pro-angiogenic form \(^{10, 12-16}\). Recent work in our laboratory showed that the balance of endogenous pro/anti-angiogenic VEGF-A splice isoforms is regulated by SRPK1 signalling via the splicing factor SRSF1 \(^{17, 18}\). Following phosphorylation by SRPK1, SRSF1 binds to the VEGF mRNA and promotes VEGF-A\(_{165\text{a}}\) production. Knockdown or inhibition of SRPK1 in colon cancer or melanoma changes splicing towards the anti-angiogenic VEGF\(_{165\text{b}}\) form, leading to inhibition of new blood vessel growth and suppression of tumour growth \(^{16-18}\). In addition, the anti-angiogenic VEGF\(_{165\text{b}}\) splice isoform is downregulated in malignant human prostate tissue compared with benign tissue and in malignant tissue the pro-angiogenic isoform dominates \(^{15}\), suggesting that a switch in VEGF-A mRNA splicing might also be important during prostate cancer progression. In our recent paper (Mavrou et al., 2014), we extend these findings and show that SRPK1 regulates angiogenesis and tumor growth in prostate cancer by controlling the balance of VEGF-A alternative splicing.

**Results and Discussion**

Mavrou et al., 2014 show that SRPK1 immunohistochemistry staining is significantly increased in both prostatic intraepithelial neoplasia (PIN) and malignant regions of tissue and SRSF1 is increased in malignant lesions from 17 patients with radical prostatectomy. SRPK1 mRNA and protein levels were higher in prostate cancer cell lines compared to normal prostate epithelial cells. Consistent with these findings, SRSF1 has been described as an oncogene and is up-regulated or amplified in several cancers \(^{19, 20}\) and overexpression of SRPK1 has been described in pancreatic, breast and colon tumors, where expression is correlated with tumor grade \(^{17, 21, 22}\). SRPK1 has also been suggested to be involved in ovarian cancer, hepatocellular carcinoma, non-small cell lung carcinoma and glioma \(^{23-27}\). Furthermore, immunohistochemistry staining of breast cancer tissue microarrays show that SRPK1 expression is linked to patient prognosis (our unpublished work \(^{22}\)) and SRPK1 has recently been implicated in the regulation of breast cancer cell apoptosis, migration and metastasis \(^{28}\). Further analysis of large tissue patient datasets with follow up clinical information would enhance our understanding of the implications of SRPK1 and SRSF1 expression in prostate cancer.

Mavrou et al., 2014 hypothesized that high SRPK1 expression might account for the predominantly pro-angiogenic VEGF-A isoform expression previously observed in malignant prostate cancer \(^{15}\). Consistently, knockdown of SRPK1 using lentiviral shRNA switched splicing of VEGF-A mRNA from predominantly pro-angiogenic VEGF-A\(_{165\text{a}}\) in control cells to increased anti-angiogenic VEGF-A\(_{165\text{b}}\) in SRPK1 knockdown PC-3 cells. This switch in VEGF-A splice isoforms led to a decrease in microvessel density and tumor growth in subcutaneous xenograft models with SRPK1-KD PC-3 cells and in a model with control cells injected with anti-angiogenic VEGF-A\(_{165\text{b}}\). Furthermore, SRPK1 copy number correlated with tumor volume.

The roles of alternative splicing in multiple aspects of tumor progression continue to be elucidated \(^{29}\), further suggesting that SRPK1 might be a key regulator of tumor progression. Alternative splicing is increasingly recognized as an important process expanding proteomic and functional complexity in physiological processes and in disease, particularly in cancer \(^{30, 31}\). Recent high-throughput sequencing studies indicate that more than 90% of human genes are alternatively spliced \(^{32}\). In the last few years a multitude of alternative isoforms have been described that are specific to and regulate hallmark stages of cancer \(^{29}\). SRPK1-SRSF1 signaling has the potential to regulate splicing of multiple genes involved in tumor progression. However, recent studies indicate that the effect of SRPK1 inhibition might lead to specific alterations in splicing outcome depending on the context \(^{33}\). Mavrou et al., 2014 show that the effect of SRPK1 knockdown or inhibition in prostate cancer growth is mediated through alterations in angiogenic VEGF-A splice isoform production, as changes in VEGF-A splice isoforms and levels of angiogenesis in tumor xenografts were demonstrated and no effects were observed on cell growth, proliferation, invasion or migration. This conclusion is strengthened by a rescue experiment in which overexpression of VEGF-A\(_{165}\) insensitive to splicing control rescued the tumor growth in SRPK1 knockdown xenografts, and additional publication\(^{16}\), that demonstrates SRPK1 inhibition can prevent melanoma growth in mice.

The effect of SRPK1 inhibition on different potential targets downstream is likely to be complex,
context-dependent and subtle. There are a number of known SRPK1 targets, not all of which are inhibited by treatment with SRPK1 inhibitors at concentrations that inhibit pro-angiogenic VEGF-A splicing. It appears that the VEGF-A system is exquisitely sensitive to SRPK1 activity and subsequent SRSF1 phosphorylation – other targets such as Rac1B, hnRNPA2/B1 and MKNK2 [19, 20, 34], have differing sensitivities – MKNK2 and hnRNPA2/B1 are both altered in highly expressing SRPK1 cell types [17] whereas Rac1B is not and neither hnRNPA2/B1 nor Rac1B are affected in other cell types such as RPE cells (unpublished data). Future assessments of the role of additional splicing proteins and interplay with other signaling pathways would lead to a comprehensive understanding of the role of the SRPK1-SRSF1 axis in prostate cancer. Both increased and decreased levels of SRPK1 have been found to promote cancer by interfering with the phosphorylation status of Akt signaling. SRSF1 is regulated through tightly controlled phosphorylation of the RS domain by SRPK1 in a processive manner such that excessive or insufficient phosphorylation inhibits SRSF1-mediated splicing [35]. It would therefore be interesting to test the phosphorylation status of SRPK1-SRSF1 signaling in different stages of tumors from patients in future work, and identify splice changes that are linked with this.

These findings from our laboratory and others suggest that SRPK1 might be a good target for developing novel therapies in prostate cancer. Angiogenesis has been considered an attractive therapeutic target for decades since the discovery of its importance in tumor progression in multiple types of cancer [3, 36]. Generation of anti-angiogenic drugs has been an area of intense research and as a result anti-angiogenic therapies have been approved for some cancers and for neovascular diseases of the eye. Most anti-angiogenic therapies target VEGF or its receptors. In 2004 the FDA approved the monoclonal antibody, bevacizumab (Avastin), for the treatment of several cancers. Avastin binds to VEGF and inhibits signaling, and was a landmark breakthrough in anti-angiogenic therapy. In clinical trials, Avastin slightly, but statistically significantly, increased response rates and/or survival among patients with colorectal, lung, renal and certain types of brain cancer. Since the approval of Avastin, several inhibitors have been developed that target VEGF-A-driven angiogenesis and VEGF-A is a validated target [2]. However, current anti-angiogenic therapies are associated with limited efficacy, non-specific effects and toxicity. Several phase III studies of anti-angiogenic therapies have not matched expectation. For example, trials of Avastin, sunitinib or aflibercept in patients with prostate cancer failed to show improvement in overall survival [37, 39]. In addition, several studies have raised concerns about the impact of anti-angiogenic therapy on tumor progression, metastasis and drug resistance, leading to the hypothesis that vascular normalization may be a better approach to improve patient outcome [36, 39]. All current anti-VEGF-A therapies are non-specific, working via broad mechanisms that do not discriminate between the pro- and anti-angiogenic forms of VEGF-A. Avastin binds both pro-angiogenic tumour-supporting VEGF-A165a and anti-angiogenic tumour-inhibitory VEGF-A165b, with roughly equal affinity [14]. Likewise, agents that inhibit the VEGF receptor prevent both pro- and anti-angiogenic signalling from occurring. These broad anti-VEGF-A activities have the potential to generate significant cardiovascular and other off-target toxicities. Development of specific SRPK1 inhibitors would be expected to have more targeted effects and less toxic side effects due to selective inhibition of pro-angiogenic VEGF-A165a without inhibition of anti-angiogenic, cytoprotective VEGF-A165b. Furthermore, normalising the balance of endogenous pro/anti-angiogenic VEGF-A splice isoforms could counteract the abnormal blood vessel growth observed during tumour angiogenesis. Improved targeted small molecule kinase inhibitors of angiogenesis would be particularly appreciated in prostate cancer, where there is a need for improved therapeutic approaches but where previous clinical trials with other anti-angiogenic agents have not been successful [37, 38].

Previous and ongoing work in our laboratory shows that several SRPK1 inhibitors can switch VEGF-A splicing towards anti-angiogenic isoforms in vitro and have anti-angiogenic effects in vivo, in colon, breast and prostate cancer, in renal disease and in neovascular diseases of the eye including wet age-related macular degeneration [16, 17, 40, 41]. Mavrou et al., 2014 demonstrate that two inhibitors, SPHINX and SRPIN340 [41, 42] inhibit EGF-induced SRSF1 phosphorylation. SPHINX treatment decreased pro-angiogenic VEGF165a and increased protein levels of VEGF-A165b and decreased tumour growth in vivo following intraperitoneal administration to orthotopic PC-3 xenografts. These studies provide proof-of-concept that small molecule inhibitors of SRPK1 can inhibit tumour growth in vivo via regulation of VEGF-A alternative splicing and inhibition of angiogenesis.

Since SRPK1 inhibition switches the balance from pro-angiogenic to anti-angiogenic VEGF-A, decreases vessel density and tumour growth, our findings support the idea that angiogenesis is a causal driver of prostate cancer progression rather than a consequence of hypoxia in the growing tumour microenvironment. It has been suggested that once the angiogenic switch has been activated, the extent of neovascularisation can vary between tumours and remains under the control of the balance of angiogenic signalling.
between cancer cells and their microenvironment. [5, 6] Similarly, the balance of VEGF isoforms is likely continually regulated in response to SRPK1 signalling. Further work involving splicing reporters will provide insight into the control of alternative splicing during tumour progression [43, 44].

Our recent work provides proof-of-concept for the use of SRPK1 inhibitors to treat colon and prostate cancer and as topical eye-drop formulations to treat neovascular eye diseases such as wet age-related macular degeneration (wAMD), with potential for significant advantages over existing therapies. In current and future work we are continuing to investigate the underlying biology of SRPK1 in cancer progression, VEGF-A splicing and regulation of angiogenesis and developing novel inhibitors to translate this work into clinical therapeutics. Anti-angiogenic therapies have been the subject of intense research and anticipation with some notable successes, for example Sunitinib is a small molecule inhibitor approved to treat kidney cancer and the VEGF-A antibody fragment Lucentis is the standard of care for treatment of wAMD. However, there is an unmet need for novel approaches due to limited efficacy, off-target effects and toxicities associated with current therapies. These problems and the disappointing results from recent trials with novel anti-angiogenic therapies may be explained, at least partly, through incomplete understanding of the complexity of VEGF-A regulation and biology of angiogenesis. By specifically inhibiting pro-angiogenic VEGF-A_{ext} isoforms through modulation of SRPK1-mediated alternative splicing, we aim to develop more efficacious and safer therapeutics.

**Conflicting interests**

DO Bates is an inventor on patents related to control of splicing of VEGF, founder, shareholder and CSO of Exonate Ltd, a spin-out company from the University of Nottingham. J Batson is an employee of Exonate Ltd.

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