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14. ABSTRACT The VEGF receptor Flt-1 mediates VEGF survival signaling in cancer cells and has been correlated with a high risk metastasis and relapse in breast cancer. However, the function of Flt-1 in breast cancer is not yet known. Here we report an increase in Flt-1 expression in breast tumor cells exposed to a hypoxic environment. Interestingly, no increase in Flt-1 expression was observed in pre-malignant breast epithelial cells. Furthermore, Flt-1 was identified as a target of miR-10b, which is deregulated in breast cancer. We find that expression of mature miR-10b in breast cancer cells results in significant down-regulation of Flt-1 expression. These findings will serve as a basis for functional studies of the role of Flt-1 and miR-10b in the etiology of breast cancer.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusion.....	5
References.....	5

Introduction

Vascular endothelial growth factor (VEGF) is an essential factor in breast cancer progression that signals to cancer cells in a way that facilitates their survival^{1,2}. The survival of these cells is dependent on the interaction between VEGF and its receptor, Flt-1^{2,3}. Recent clinical studies have correlated high levels of Flt-1 expression in breast cancer with a high risk of metastasis and relapse^{4,5}, indicating that the interaction between VEGF and Flt-1 promotes aggressive behavior in breast cancer cells. However, the exact function of Flt-1 in breast cancer is not yet known. My overall goal is to understand the function of Flt-1 in breast cancer, with a specific focus on the role of VEGF and Flt-1 in breast cancer growth and metastasis. Specifically, I hypothesize that Flt-1 mediates VEGF survival signals and contributes to aggressive behavior in breast cancer. The scope of this project includes understanding the function of Flt-1 in breast cancer cells during tumor growth and metastasis, identifying the signaling pathways which are stimulated by Flt-1 activation, and the regulation of Flt-1 expression. This investigation into the function and signaling pathways of Flt-1 in human breast cancer will enable us to define the contribution of Flt-1 to breast cancer progression and to determine how Flt-1 signaling contributes to enhanced cancer progression. Furthermore, this work will provide the groundwork for refining current VEGF-targeted therapies, such as Bevacizumab, to target breast tumor cells directly.

Body

To understand the function of Flt-1 in breast cancer growth and metastasis, I have been focusing on knockdown of Flt-1 expression in the SUM-159 human breast cancer cell line using RNAi. I have had limited success with consistently repressing expression of Flt-1 in this cell line, as well as others, using RNAi directed against Flt-1. Commercially available reagents have failed to inhibit Flt-1 expression using both si- and shRNA. Flt-1 knockdown using siRNA has been reported in the literature, however, I failed to reproduce the published results in my cell lines using the same siRNAs and transfection reagents. I recently entered into collaboration with Phillip Zamore, who specializes in small RNAs. Together, we have designed an siRNA which targets the 3' untranslated region of Flt-1, as opposed to the coding sequence. Preliminary studies show that this sequence is successful in knocking down Flt-1 protein expression by western immunoblot, as compared to a non-targeting siRNA (Figure 1).

To gain further insight into Flt-1 function, I have begun to examine the impact of hypoxia on Flt-1 expression in the MCF-10A series of breast cancer cells. In all four stages, ranging from normal breast epithelial cells to metastatic breast cancer cells, Flt-1 expression was assessed by western immunoblot following exposure to a severely hypoxic environment (0.1% O₂) for 24 hours. Flt-1 levels were enhanced in the tumorigenic cell lines MCF-10CA1h and MCF-10CA1a, but did not change in normal MCF-10A or pre-malignant MCF-10AT cells, as compared to control cells maintained in normoxia for the same period of time (Figure 2).

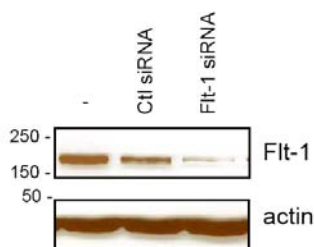


Figure 1. Flt-1 siRNA decreases Flt-1 protein expression in SUM-159 cells.

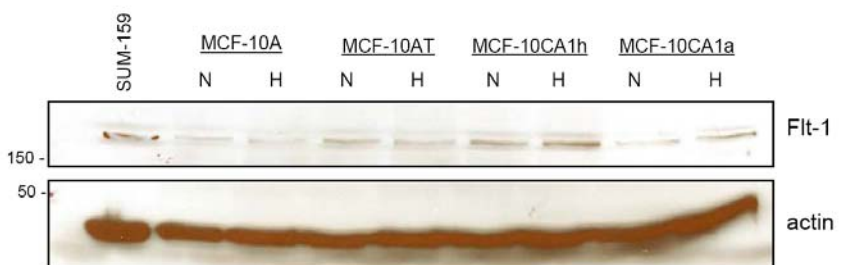


Figure 2. Expression of Flt-1 protein increases following 24 hours in a hypoxic environment (0.1% O₂) in tumorigenic variants of MCF-10A cells, but not in non-tumorigenic or premalignant variants.

To address Flt-1 signaling in breast cancer, I proposed to use an Flt-1/CSF-1 chimeric receptor composed of the Flt-1 intracellular and transmembrane domains fused to the CSF-1 extra-cellular domain⁶. However, CSF-1R was recently found to be commonly expressed in both normal breast epithelium and breast tumors. Additionally, CSF-1R is reported to play a significant role in normal breast development, as well as in mammary tumor progression to metastasis^{7,8}. I examined several human breast cancer cell lines using reverse-transcriptase RT-PCR and found them all to express CSF-1R and/or its ligand CSF-1 (Figure 3). As a result, this

chimeric receptor is not suitable for use in these cells, as basal stimulation by endogenous ligand would present a confounding factor. I am currently investigating other options for examining Flt-1 signaling in breast cancer.

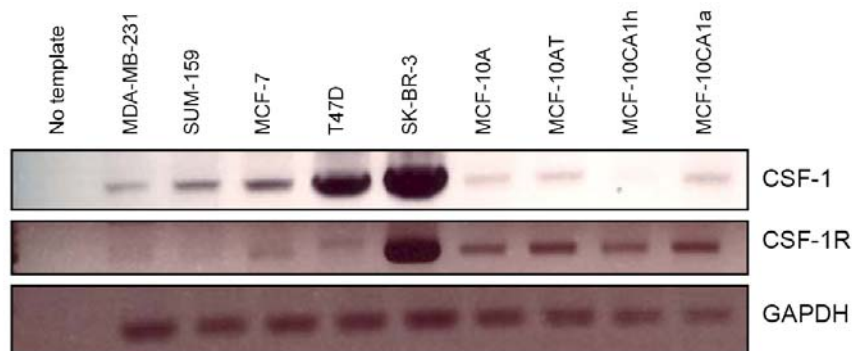


Figure 3. Expression of a CSF-1 and CSF-1 receptor in human breast cancer cell lines by RT-PCR.

Finally, I have begun to examine the regulation of Flt-1 expression in breast cancer to determine what factor or factors are causing Flt-1 levels to increase in cancer cells. Recent publications have implicated miR-10b as one of several microRNAs deregulated in breast cancer^{9,10}. Flt-1 is one of many genes predicted by computer algorithms to be a target of miR-10b, therefore I hypothesized that miR-10b regulates the expression of Flt-1 in breast carcinoma. Utilizing a miR-10b mimic and a mutant miR-10b containing a single base-pair substitution in the seed region as a control, I found that expression of miR-10b results in a downregulation of Flt-1 protein by western immunoblot in SUM-159 breast cancer cells lacking endogenous miR-10b. Expression of the miR-10b seed mutant or a non-targeting control mimic had no effect on Flt-1 levels as compared to untreated cells (Figure 4). I plan to continue these studies, using ectopic expression of miR-10b in breast cancer cells as well as antisense for miR-10b silencing, to examine the function of miR-10b in breast cancer and its regulation of Flt-1 expression. Furthermore, I will assess the regulation of Flt-1 by several other miRNAs that are predicted to target the Flt-1 3' UTR.

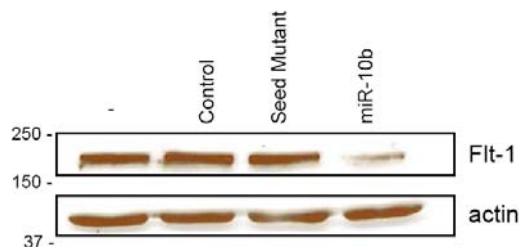


Figure 4. Expression of a miR-10b mimic results in downregulation of Flt-1 protein expression.

Key Research Accomplishments

- Hypoxia increases expression of Flt-1 in tumorigenic breast cell lines, but not in pre-malignant breast epithelial cells
- Flt-1 expression in breast cancer is regulated, at least in part, by miR-10b
- Presentation of work at lab meetings and departmental data club
- Attendance at UMass Memorial's monthly Breast Cancer Conference
- Attendance at the AACR Special Conference: The Role of Non-Coding RNAs in Cancer

Reportable Outcomes

None

Conclusion

In conclusion, hypoxia was found to regulate expression of Flt-1 in breast cancer and Flt-1 was validated as a target of miR-10b, a microRNA which is deregulated in breast cancer. Future work will address the significance of these findings with specific regard to Flt-1 function, as well as to the function of miR-10b, in breast cancer growth and metastasis. The mechanisms of Flt-1 regulation and Flt-1 signaling in breast cancer will also be addressed. An understanding of how Flt-1 contributes to growth and progression in breast cancer will have broad implications for other common epithelial cancers and will provide a foundation of knowledge that will potentially enable the development of therapeutic strategies aimed at targeting this important pathway in breast cancer.

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