THE EFFECTS OF CAVIN-2 ON ANGIOGENESIS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effects of Cavin-2 on Angiogenesis

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Caveolae are bulb-shaped invaginations about 50-60nm in size on the plasma membrane of mammalian cells that play a key role in cell signaling, endocytosis, plasma membrane adaptations, and lipid homeostasis. Caveolae are known to be important in angiogenesis and are found in the microvasculature of the skeletal muscle, heart and lung, making up to 60% of the plasma membrane. While the mechanisms of caveolae regulation are not completely understood, Cavin family proteins appear to be critical regulators of caveolae. Cavin proteins form subcomplexes through homo- and hetero-oligomerization which regulate caveolae dynamics. Cavin-2, also known as serum deprivation-response protein, or SDPR, is a key regulator of caveolae dynamics by binding to Cavin-1 and introducing membrane tubules. To discover genes important for the transition of endothelial cells from a quiescent to an activated state, a switch that is critical for initiation of angiogenesis, we performed RNA sequencing analysis comparing unstimulated to activated, invading endothelial cells using a three-dimensional assay that mimics angiogenesis. We found expression of Cavin-2 was downregulated 73% in invading cells compared to non-invading cells. This study tested whether Cavin-2 might suppress endothelial sprouting responses. We found that overexpression of Cavin-2 had no statistically significant effect on cell invasion using this three-dimensional model of angiogenesis, indicating that Cavin-2 does not have a suppressive effect on endothelial sprout initiation.

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SECTION I

INTRODUCTION

Background

The Bayless lab investigates angiogenesis, a process that occurs normally during wound healing and various steps of the female reproductive cycle; however, angiogenesis can also be excessive in pathological situations such as tumor growth, or insufficient following a heart attack or stroke, which prevents healing within those infarcted tissues. Thus, a need exists clinically to be able to inhibit or enhance angiogenesis, depending on the patient's needs. Understanding the molecular basis of angiogenesis is vital for manipulating blood vessel growth clinically. As a result, much research has been conducted in an effort to understand the roles of various proteins and mechanisms involved in angiogenesis. One such angiogenic pathway is the regulation and formation of caveolae.

Caveolae are bulb-shaped invaginations of 50-60nm on the plasma membrane of cells and can be found in many types of mammalian cells, including adipocytes, muscles cells and endothelial cells. Caveolae play a key role in cell signaling, endocytosis, plasma membrane adaptations, and lipid homeostasis. Although the mechanisms of caveolae regulation are not completely understood, Cavin family proteins appear to be critical regulators of caveolae. Three members have been identified to date, including Cavin-1, Cavin-2, and Cavin-3. Cavin proteins form subcomplexes through homo- and hetero-oligomerization which regulate caveolae dynamics. Cavin-2 is also known as serum deprivation-response protein, or SDPR, and is upregulated following serum deprivation. Cavin-2 can also regulate caveolae dynamics by binding to Cavin-1

and introducing membrane tubules. Recent research has shown that Cavin-1 expression and caveolae formation are limited after silencing Cavin-2, which suggests that Cavin-2 is a vital contributor to caveolae morphology and dynamics. While not completely understood, caveolae appear to play a role in protein-protein and protein-lipid signaling, mechanosensing, organization of the plasma membrane and possibly function to suppress tumor formation (Bai et al. 2012, Echarri and Del Pozo 2015, Kovtun et al. 2015). Cavin proteins have also been linked as potential therapeutic targets and possible prognostic indicators of certain types of cancer. In a recent study of breast cancer, Cavin family proteins were down-regulated in cancerous tissue compared to healthy adjacent tissue, possibly indicating that Cavin proteins can function as tumor suppressors (Bai et al. 2012). Other research has shown that Cavin proteins play a role in controlling stress fibers, such as actin filaments, to control plasma membrane dynamics (Echarri and Del Pozo 2015). In addition to research findings by other labs, Cavin-2 was identified as a potential target of interest by the Bayless lab due to recent data collected through RNA sequencing and qPCR. These preliminary data show that Cavin-2 was downregulated after endothelial cell activation and invasion, corresponding to findings from other labs where Cavin-2 expression was decreased in tumors compared to tumor adjacent normal tissue (Bai 2012). These findings, along with data from literature searches, suggest that Cavin-2 might provide an inhibitory signal during angiogenesis by potentially regulating caveolae dynamics or other important cellular processes, such as signaling, plasma membrane organization and lipid homeostasis within endothelial cells.

Objective

The primary objective of this project is to better understand the role of Cavin-2 during endothelial cell sprouting using a 3D model of angiogenesis and test whether Cavin-2 might suppress sprout initiation.

SECTION II

METHODS

RNA sequencing

Human umbilical vein endothelial cells (HUVECs) were analyzed using RNA sequencing to identify targets to further study. The genes, such as Cavin-2, were considered to be targets of interest if the gene met certain strict criteria. These criteria included: greater than two-fold difference in expression in invading compared to quiescent endothelial cells, the protein's effects on the regulation of angiogenesis is not currently understood, a high level of mRNA expression is seen in a microarray analysis previously performed, and the endothelial cell expression pattern is closely correlated with cancer progression.

Cell culture

HUVECs were maintained from passages 3-6 in endothelial growth medium, as previously described (Bayless, 2009). 293FT cells (Invitrogen) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and used from passages 2-12.

Cloning Cavin-2

Cavin-2 was amplified from HUVEC cDNA using primers: 5' AGTCGCGGCCGCGATGGGAGAGGACGCTGCACAG 3' and 5' CTAGGATATCTCAGGAGGTCTGGTGCACCTG 3' and inserted into the pFlag-CMV2 vector using *NotI* and *EcoRV* restriction sites, adding an N-terminal flag tag. Positive clones were identified through test transfection and sequencing. Flag-Cavin-2 was then amplified from the

pFlag-CMV2 vector using primers: 5’ GATCGTCGACGCCACCATGGACTACAAAGACGATG 3’ and 5’ CTAGGATATCTCAGGAGGTCTGGTGCACCTG 3’ and subcloned into the pENTR4 vector using the *SalI* and *EcoRV* restriction sites. Positive clones were verified through sequencing. Flag-Cavin-2 was then recombined with the pLenti5/V6 lentiviral vector. Positive clones were verified through test transfection and sequencing.

Overexpression of Flag-Cavin-2 in HUVECs

To generate GFP (generated as previously described in Lee et al., 2009) and Flag-Cavin-2 lentiviruses, 2.5 µg of GFP or Flag-Cavin-2 backbone was added to 7.5 µg of VIRAPOWER helper plasmids and 5µg/µL polyethyleneimine (PEI) and incubated with confluent 293FT cells in T25 flasks for 60 hours. Lentiviral particles were harvested by centrifuging for 10 minutes at 1000xg. Lentiviruses were concentrated overnight with Lenti-X-Concentrator (Clontech) following manufacturer’s instructions. HUVECs were transduced with lentiviral particles in the presence of 12 µg/mL polybrene (Sigma) for 4 days. After this time, the culture medium was replaced, and cells were used for invasion assays on day 5.

Invasion assay

Invasion assay protocol was followed as previously described by Bayless et al., 2009. The HUVECs used in this study were purchased from Lonza. HUVECs were seeded as a monolayer at 30,000cells/50µL on the collagen matrix on a half-area 96 well plate. Each collagen matrix consisted of 28µL of collagen at 2.5mg/mL containing 1µM sphingosine 1-phosphate (Sigma) that was allowed to polymerize for 45 minutes at 37°C, 5% CO_2. 50µL of cell suspension was

added to each collagen matrix and allowed to incubate 30 minutes at 37°C, 5% CO_2. Growth factors (RSII (1x reduced serum II (500 µg/mL BSA, 5 µg/mL insulin, 5 µg/mL human holo-transferrin, 4.28 µg/mL oleic acid, 5 ng/mL sodium selenite; Sigma, USA), ascorbic acid 50µg/mL, VEGF 40ng/mL, basic FGF-2 40ng/µL) were then added in 50 µL M199 before incubating for 22 hours at 37°C, 5% CO_2. Extracts were taken at the start, 1hr, 5 hr and 22 hr post-invasion for western blot analysis to examine Cavin-2 expression. Cells were fixed using 3% glutaraldehyde after 22 hours. Assays were stained with toluidine blue and counted manually on a 10x10 ocular grid using a 20X objective. HUVECs were considered to be invading if any part of the cell had left the monolayer of seeded cells and invaded into the collagen matrix.

Western blot analysis

Cell protein extracts were prepared by removing collagen gels from wells at indicated time points and then added to boiling 1.5x Laemmli sample buffer containing 2% 2-mercaptoethanol and incubating at 100°C for 10 minutes before storing at -20°C. Proteins were separated using 8.5% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes purchased from Millipore and blocked with 5% milk in 1% TBST at room temperature for 1 hr. Membranes were then incubated overnight at 4°C with the primary antibody, rabbit anti-Flag at a 1:1000 dilution. After washing with Tween-20 Saline solution, the secondary antibodies, at a 1:5000 dilution, were added and incubated at room temperature for 1 hr. Secondary antibodies were washed off and membranes were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and HyBlot CL autoradiography film (Denville Scientific, South Plainfield, NJ). The following antibodies were used in this study: rabbit anti-flag (F7425, Sigma), mouse anti-actin (CP01, Millipore), and HRP-conjugated secondary antibodies (Dako, Carpinteria, CA).

SECTION III

RESULTS

RNA sequencing of HUVECs was used to determine genes important for regulation of angiogenesis by comparing invading to non-invading cells. Cavin-2 was considered to be a target of interest after being defined as having a high fold expression change in invading compared to non-invading cells, a high absolute level of mRNA expression in a microarray analysis, an endothelial cell expression pattern that correlates with cancer progression and its effects on angiogenesis are currently not understood. After being identified as a potential regulator of angiogenesis, experiments were designed to study the effects of Cavin-2 on angiogenesis.

The RNA sequencing data results in Figure 1 show fold changes of Cavin-2 mRNA expression. Fold changes on the left are shown comparing HUVECs at 1 hour that were either given proangiogenic stimuli (+/+) or not (-/-). Fold changes from RNA sequencing are also shown comparing HUVECs at 6 hours that were either given proangiogenic stimuli (+/+) or not (-/-). After 6 hours of treatment, the invading cells (+/+) have a 73% lower expression of Cavin-2 compared to non-invading cells (-/-). Therefore, Cavin-2 expression decreases during invasion. Figure 1 also shows qPCR analysis performed to confirm the RNA sequencing results. Independent experiments were used to generate cDNA to use in qPCR experiments with primers complementary to Cavin-2 to confirm RNA sequencing data. Cavin-2 expression was normalized to GAPDH expression. The qPCR results confirm the results seen in the treatment groups analyzed using RNA sequencing. Additionally, a third treatment group was analyzed with qPCR, comparing the fold difference between 1 and 6 hours of activation with S1P and growth factors

(VEGF and bFGF). These results show approximately a 60% decrease in Cavin-2 expression during invasion or after S1P and GF treatment. This fold difference indicates that longer exposure to the factors or the invasion process itself is downregulating the expression of Cavin-2.

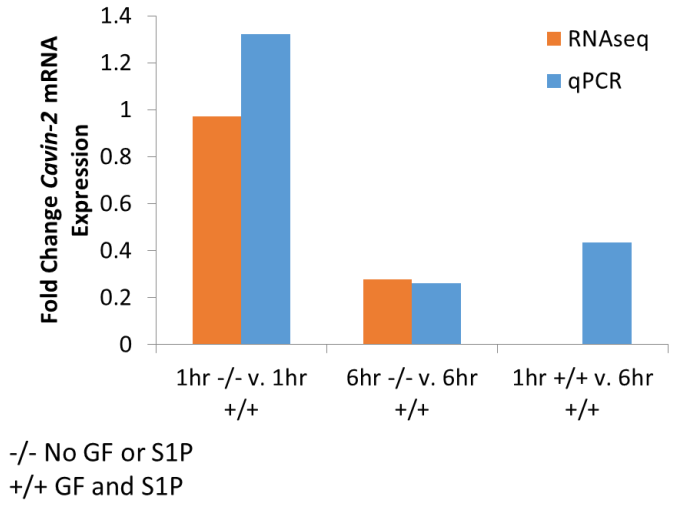

Figure 1. Cavin-2 expression was downregulated in response to proangiogenic stimuli. The graph shows expression changes between 1 hr and 6 hr experiments that were either with or without growth factors (GF, 40 ng/mL bFGF and VEGF) and S1P (1 µM). The -/- treated endothelial cells are not invading or activated, while the +/+ treated endothelial cells have been activated by GF and S1P and are invading.

The next step was to determine the source of the downregulation in the expression of Cavin-2. This was an important step since Cavin-2 is also known as serum deprivation response protein (SDPR) because it was previously found to be upregulated in response to the removal of serum. In the RNA sequencing experiment, the cells in all treatment groups were placed under serum starvation conditions during invasion. However, the (+/+) treatment group have been treated with the most active component found in serum, S1P. Thus, the (-/-) group could be considered serum starved, which could lead to an increase in Cavin-2 expression. The serum starvation during invasion could be mitigated in the (+/+) group from the addition of S1P. This difference

in treatment (+/+ vs -/-) could obscure differences in Cavin-2 expression in invading versus quiescent endothelial cells caused by the process of invasion. Determining the source of the downregulation of Cavin-2 in the RNA sequencing experiment (Figure 1) was done through analyzing mRNA expression in HUVECs that were treated with endothelial growth media containing serum (supermedia), serum starved or serum starved and then treated with 1µM S1P. Figure 2 shows the fold changes in mRNA expression observed from these treatments. If the downregulation of Cavin-2 expression in invading (+/+) treated cells seen in the qPCR and RNA sequencing experiments is caused by S1P treatment, and not due to invasion, Cavin-2 expression should be decreased in the +S1P treated cells compared to the serum starved cells. As expected from previous studies, serum starvation increased the expression of Cavin-2 compared to cells in supermedia. Surprisingly, treatment of endothelial cells with S1P after serum starvation further increased the expression of Cavin-2 compared to serum starved cells (Figure 2). This indicates that although S1P is an active component of serum, it does not rescue the effects of serum starvation on Cavin-2 expression. Further, this suggests that the downregulation of Cavin-2 expression in invading (+/+) S1P and GF treated cells (Figure 1) is not caused by the addition of S1P, but rather by the process of invasion.

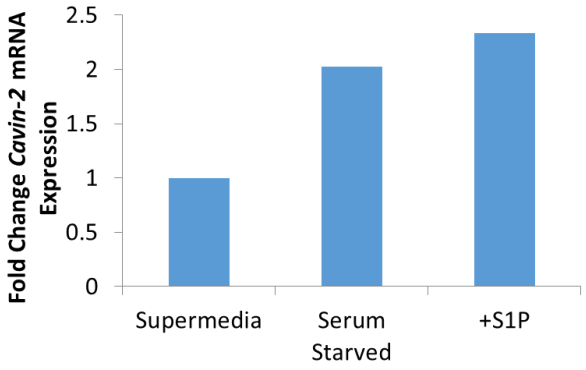

Figure 2. S1P increased Cavin-2 mRNA expression. The graph shows fold changes of Cavin-2 mRNA expression during serum treatments. The results indicate that prolonged serum exposure is not the primary contributing factor for Cavin-2 expression changes in the 1 hr vs 6 hr HUVECs that were treated with S1P and growth factors.

After identifying Cavin-2 as a potential regulator of angiogenesis and determining the general cause of mRNA expression change, this study examined the effect of Cavin-2 on angiogenesis, using a three-dimensional (3D) model of endothelial sprouting with human umbilical vein endothelial cells (HUVECs). To generate expression constructs, Cavin-2 was amplified from HUVEC cDNA and then inserted into the pFlag-CMV2 vector using *NotI* and *EcoRV* restriction sites. Positive clones were identified using test transfection and sequencing. Flag-Cavin-2 was amplified and then subcloned into the pENTR4 vector. The positive clones were verified through restriction digest and sequencing. Flag-Cavin-2 was recombined with pLenti5/V6 vector and positive clones were sequenced and used to transduce HUVECs. Whole cell lysates from this transduction were used for Western blotting, shown in Figure 3. This Western blot shows Flag-Cavin-2 expression in all of the clones, and the clone with the highest expression, Cavin-2 C,

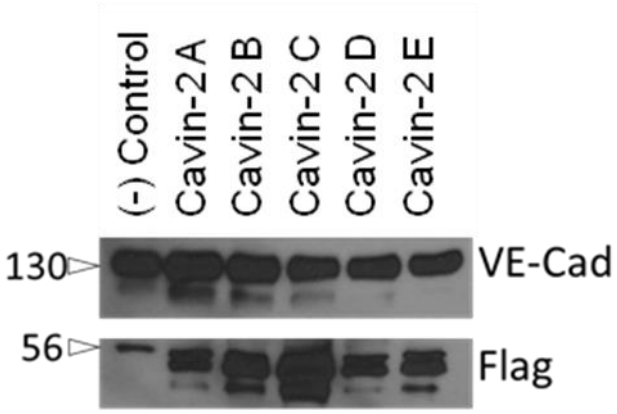

Figure 3. Cavin-2C clone displayed the most robust HUVEC transduction. A small scale test with Flag-Cavin-2 lentiviruses were prepared using 5 clones of Cavin-2. Cell lysates were probed with antibodies directed to VE-cad and Flag using western blotting. The highest expression clone, Cavin-2 C, was then used to continue.

was used for the following steps of the study. Once the highest expression clone was identified, the HUVECs were then transduced using lentiviruses to overexpress Flag-Cavin-2 or GFP as a control. After 5 days of transduction HUVECs that were not transduced, or transduced to

overexpress Cavin-2 or GFP were seeded onto 3D collagen matrices containing 1 µM S1P. Cells were supplemented with bFGF, VEGF, ascorbic acid and RSII in the media. Overexpression of Flag-Cavin-2 in the HUVECs was determined by taking extracts before and 18 hours post-invasion. GFP expression was confirmed visually, under UV light (data not shown). Results from the western blot are shown in Figure 4. Overexpression of Cavin-2 is clearly shown at 18 hours post-invasion. This indicates that the overexpression was successful and the invasion assay results should demonstrate the effect of increased Cavin-2 expression on this 3D model of angiogenesis.

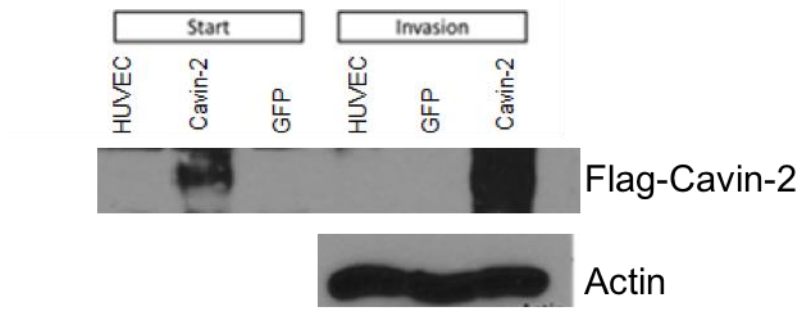

Figure 4. Flag-Cavin-2 was expressed in HUVECs used for invasion assasys. Invasion extracts using pre-invasion and 18 hour post-invasion extracts were used for a Western blot. Clear overexpression is shown in the Cavin-2 18 hour post-invasion. This overexpression indicates that the lentivirus successfully transduced and the Cavin-2 expression is significantly different from the HUVEC control.

After the assay was fixed with 3% glutaraldehyde at 22 hours post-invasion, the assay was stained and manually counted. The number of HUVECs that invaded into the collagen matrix was recorded. Results of the invasion counts are shown in Figure 5. No statistically significant differences were found across the three groups. The number of invading cells for HUVECs and the GFP control were within normal ranges for this assay at this concentration based on previous results in the Bayless lab. The lack of a statistical difference between the Cavin-2 overexpressed

group and the HUVEC and GFP control groups indicate that Cavin-2 overexpression has no effect on HUVEC invasion in this 3D model of angiogenesis.

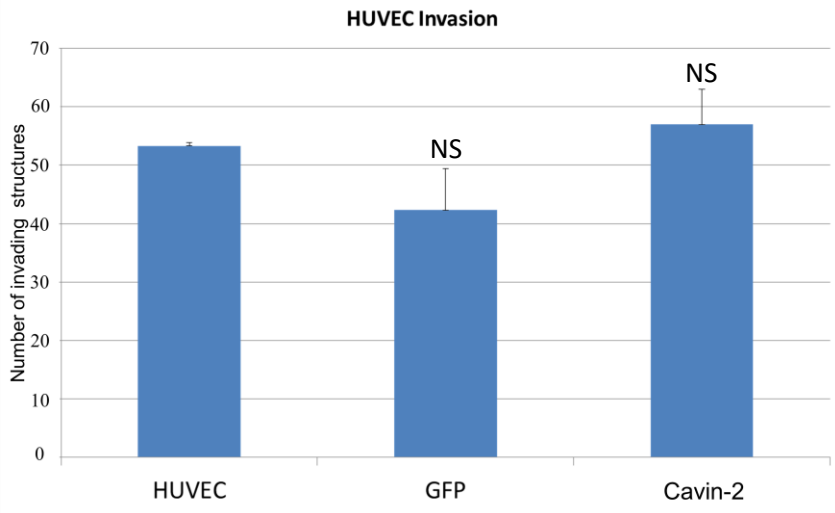

Figure 5. Cavin-2 overexpression did not affect endothelial sprouting. This graph shows the average number of invading structures at 20x with standard deviation bars. N=3 for each of the groups. NS, no significant differences were observed between groups.

SECTION IV

DISCUSSION

Based on the RNA sequencing data, Cavin-2 showed to be a promising protein to study due to its unknown influence on the regulation of angiogenesis and the clear fold change difference between invading and non-invading cells. The cloning experiments of Cavin-2 yielded positive clones at the expected molecular weight of 47kDa for all 5 clones. However, the highest expression clone, Cavin-2 C, was the only clone used to continue the study. Next, the lentiviral transduction was successful based on the overexpression seen in the western blot for the Cavin-2 transduced HUVECs (Figure 4). The actin loading control was consistent across the three groups and the Flag-tag added to Cavin-2 was clearly expressed. Thus, the invasion assay should show the influence of Cavin-2 overexpression. Nonetheless, after performing three invasion assays, no statistical differences were seen between the non-transduced HUVECs, GFP-expressing cells and the Cavin-2 expressing cells. The average HUVEC invasion responses were similar to the average counts previously obtained from endothelial cell invasion using this three-dimensional model. These results indicate that Cavin-2 does not limit human endothelial cell invasion when overexpressed. If overexpression of Cavin-2 had affected endothelial sprouting, the next step would have been to study Cavin-2 in an *in vivo* model such as wound healing. This involves taking tissue samples in the actively repairing tissues adjacent to the wound site in order to examine differences in sprouting. However, the lack of a significant response in the *in vitro* model indicates that an intensive *in vivo* approach is not the best use of time and resources. As a result, screening other proteins of interest from the RNA sequencing data is likely a better option

for finding key proteins that influence angiogenesis based on the current understanding of Cavin-2.

While Cavin family proteins have been identified as structural components of caveolae, their functional role is still not understood. Also, all Cavin family proteins appear to undergo extensive post-translations modifications (Kovtun, 2015). This could complicate studies and possibly effect results of *in vitro* experiments. Nonetheless, we still would expect to see a difference in Cavin-2 transduced HUVECs from this study. Thus, the results from this study indicate that Cavin-2 does not limit invasion when overexpressed.

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