

Recombinant Production of Vitronectin and Insights into its Structure and Role in Fibrinolysis

Cameron T. Landers
The University of Tennessee – Knoxville
Chancellor's Honors Program

Senior Thesis
May 2011

Mentor: Dr. Cynthia B. Peterson

Recombinant Production of Vitronectin and Insights into its Structure and Role in Fibrinolysis

Cameron T. Landers, Tihami Qureshi, Cynthia B. Peterson

Abstract

Vitronectin is an abundant glycoprotein located in both the circulation and the extra cellular matrix that surrounds tissues. Vitronectin has a myriad of roles pertaining to cells, including implications in cell-cell adhesion, tumor malignancy, cell migration, and fibrinolysis. To date, obtaining vitronectin requires extensive preparation steps beginning with human plasma or the use of virally-infected cells. Attempts at expression in a prokaryotic vector have been largely unsuccessful. However, given that each of these methods has limitations and risks, a eukaryotic expression system in Schneider 2 *Drosophila* cells was used to allow for harvesting a large quantity of vitronectin in a properly glycosylated form comparable to that produced in the human body. This method allows for the production of a considerable amount of protein (approximately 10-20 mg in a 450 mL cell culture) in as little as one to two weeks from a maintained cell stock, and should be further investigated as a possible replacement for the human plasma purification methods currently in use.

In addition to the purification strategies and the methods and results of the project, this thesis will discuss the specifics of how vitronectin influences the fibrinolytic cascade of plasminogen activation by interacting with and stabilizing plasminogen activator inhibitor 1 or PAI-1. This interaction makes vitronectin an indirect, non-catalytic inhibitor of plasminogen proteolysis to active plasmin, an enzyme that breaks down fibrin clots. By this cascade of interactions, vitronectin is a contributor to the buildup of blood clots. Therefore, further understanding of the vitronectin-PAI-1 complex, as well as possible interactions with metal ions, will allow for considerable insight into conditions such as strokes, heart disease, and blood clotting disorders. It is hoped that the availability of a recombinant source of vitronectin will serve to expedite future studies into these diseases.

Introduction

Vitronectin is known to have a multitude of roles in the human body that are the direct result of the diverse variety of ligands with which it interacts. A glycoprotein of 459 amino acid residues, it occurs in two forms in the blood: a 75 kDa single peptide form and a disulfide-linked dimer of 65 kDa and 10 kDa fragments (Tomasini and Mosher, 1986). The vast majority of vitronectin is produced by liver hepatocytes, after which it is exported to the blood stream along with many other platelet components where it circulates at concentrations of around 0.2 mg/mL (Sano et al 2010). Prior to its transport into

the blood, it is at times cleaved by an undetermined protease at arginine 379 toward its C-terminus to produce the 65 kDa form, which is often visible as a separate species from the 75 kDa vitronectin during electrophoresis (Podor et al 2002).

While a crystal structure for vitronectin has yet to be produced, homology modeling has been used to characterize its four different domains. Xu et al, modeled the domains in 2001 by comparing them to other known structures with similar sequences and motifs and using docking experiments. Vitronectin consists of a 44-residue N-terminal domain known as the somatomedin B domain (SMB), an intrinsically disordered region (residues 48-130), a central domain (residues 131-323) with four beta-propeller domains, and finally a C-terminal heparin-binding domain (residues 354-456). Between the SMB domain and the disordered regions are the sites for the RGD integrin binding.

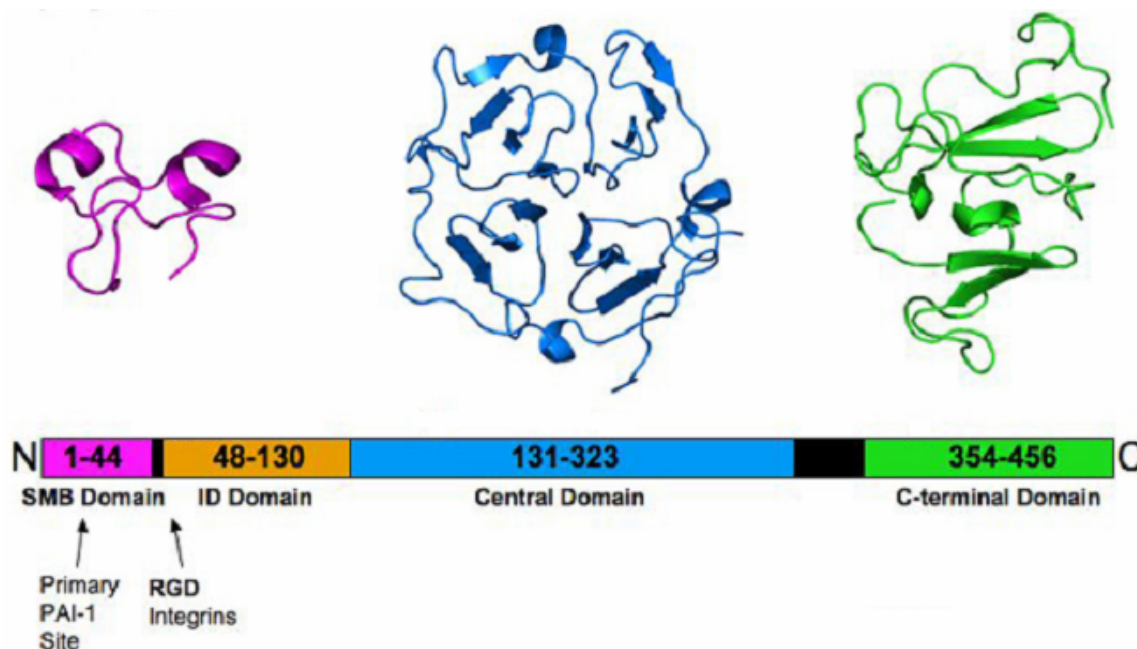


Figure 2: The four domains of vitronectin. These structures are homology models based on computational methods and direct experiments. Not shown is the heparin binding site on the C-terminus, as well as the glycosylation sites near the SMB domain. Image adapted from work by Xu et al, 2001.

Perhaps the most critical domain of vitronectin is the somatomedin B domain (SMB). This domain contains the binding sites for PAI-1, uPAR, and integrins (Mayasundari et al 2004). In fact, the crystal structure for PAI-1 in complex with only the SMB domain has been solved, and is shown below. In that circumstance, SMB was expressed as an isolated domain in *E. coli*.

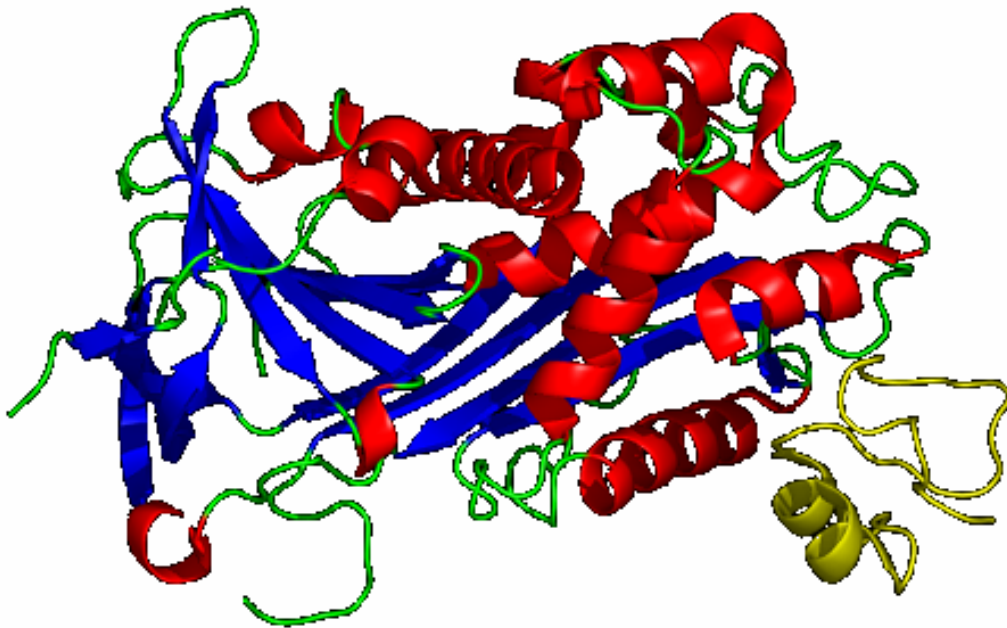


Figure 3: PAI-1 is shown here in complex with the SMB region of vitronectin. SMB is shown in yellow. This crystal structure was produced by Zhou, et al in 2003.

Vitronectin was initially identified as a protein involved cell adhesion, growth, and spreading in the extra cellular matrix (Holmes, 1967). Since then it has been shown to be involved with cell adhesion via the Arg-Gly-Asp (RGD) integrin binding motif (Gladson and Cheresh, 1991) as well as tumor adherence (Carreiras et al 1999) and malignancy (Sidenius and Blasi, 2003) due to its interactions with the urokinase plasminogen activator receptor (uPAR).

Another major area of interactions for vitronectin is its contributions to maintaining hemostasis. Vitronectin binds heparin, an anticoagulant, and impairs its ability to interact with thrombin and antithrombin (Preissner and Berghaus 1986). This helps to promote the coagulation cascade, leading to the conversion of fibrinogen to fibrin and the formation of clots. Vitronectin is involved not only in helping to lead to the formation of fibrin clots, but also in preventing the clots from breaking broken down, a process called fibrinolysis. It does this by prolonging the active conformation of plasminogen activator inhibitor 1, or PAI-1 (Schar et al 2008).

PAI-1 is a serine protease inhibitor, or serpin, which directly binds to and inhibits tissue plasminogen activator and urokinase plasminogen activator, both of which are serine proteases. In the absence of PAI-1, these two proteins would normally cleave plasminogen to plasmin. Plasmin is a serine protease itself, and

it dissolves the fibrin clots, effectively ending the coagulation event (Wiman and Collen, 1978). Therefore, PAI-1 can be viewed as a factor that promotes the longevity of fibrin clots. An interesting caveat of PAI-1 is its inherent thermodynamic instability. With a half-life of approximately 60 to 70 minutes in the blood, PAI-1 freely converts from its active conformation to an inactive form (Chorostowska-Wynimko, Skrzypczak-Jankun, and Jankun, 2004). However, vitronectin serves to stabilize the active conformation, thus allowing PAI-1 to continue binding and inhibiting tissue plasminogen activator and urokinase plasminogen activator. This interaction means that vitronectin prolongs fibrin clots and coagulation events, and therefore any insights that can be gained into the PAI-1 – Vitronectin interaction could be useful for understanding the process of fibrinolysis as well as providing later targets for future drugs to treat conditions such as heart disease and strokes.

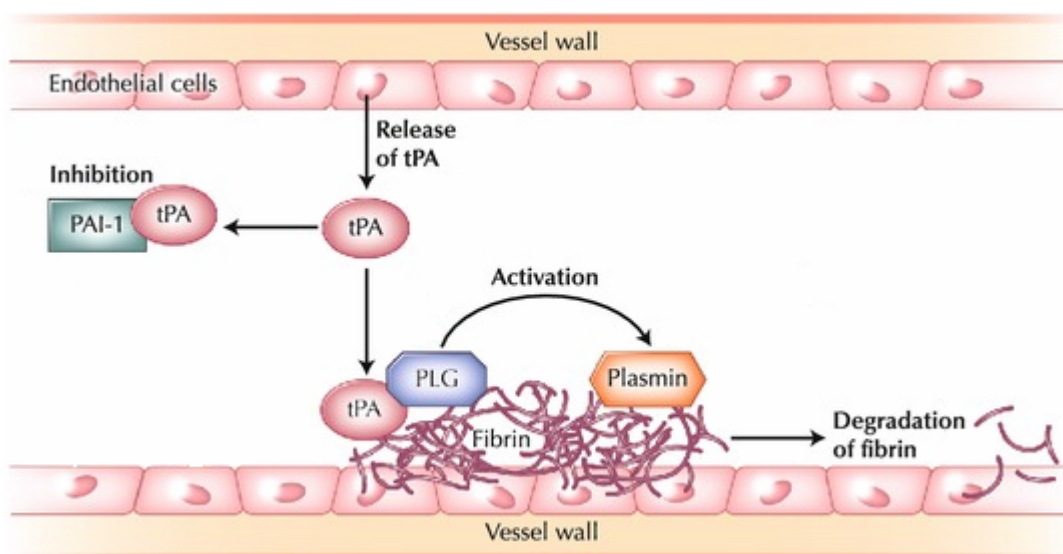


Figure 1: PAI-1 is shown inhibiting tissue plasminogen activator (tPA). tPA converts plasminogen (PLG) to active plasmin, which degrades fibrin clots. Vitronectin stabilizes PAI-1 in its active conformation, improving its ability to inhibit tPA and uPA. (*Image adapted from Krone, Allen, and McCrae, 2010.*)

Given the great importance of vitronectin to human health and its variety of roles in the body, the availability of a practical recombinant source of the protein would be extremely beneficial. However, several features of vitronectin make this somewhat difficult. First, vitronectin has over 400 amino acid residues, which makes it difficult to produce in a prokaryotic vector compared to the relative ease with which many recombinant proteins (PAI-1 for example) can be produced. Furthermore, since vitronectin contains three glycosylation sites, which account for as much as 30% of its mass (Schvartz, Seger, and Shaltiel, 1999), an expression system with proper eukaryotic glycosylation is preferred. Another alternative is to use virally infected Sf9 or Hi5 cells (Schar et al 2008). This method indeed produces functional protein; however, this does not represent a stable cell line, and the yields of such a production system are relatively low.

The current method used to produce native vitronectin is to purify it from human blood plasma. The process itself is time consuming (typically 3-5 weeks of work) and expensive, and it has the inherent risks of manipulating pooled human plasma from donors. Furthermore, while this method yields very large

amounts of protein, it does not allow for any mutagenesis to be performed. As will be explained later in this thesis, this proposed system of expressing vitronectin in a stable line of Schneider 2 *Drosophila* cells produces a large amount of protein, in a form that appears to be comparable to native human, in a time-span that is significantly less.

	Post Translational Modification	Time Required	Amount Generated
Purification from human blood plasma	Yes	3-5 weeks	10-50 mg from 10 plasma bags
Viral Infection	Yes	1-2 weeks	~ 500 ug
Recombinant Prokaryotic Expression (<i>E. coli</i>)	No, protein too large	1 week	None Usable
Recombinant Eukaryotic Expression (S2 <i>Drosophila</i>)	Yes	5-10 days from maintained cell line	5-25 mg from a 450 mL S2 Culture at 2×10^7 cells/mL.

Table 1: Comparison of methods to acquire vitronectin. An added benefit of a recombinant system is the ability to perform mutagenesis experiments ranging from single amino-acid modifications to full domain deletions. An outline of the scheme used to produce the results in the last row is proposed in the next section.

A recent attempt in the Peterson laboratory at creating a recombinant source of vitronectin has been met with some success. Dr. Lawrence Thompson

helped to develop the method described here, providing the foundation for this work with some important distinctions. One concern was whether or not vitronectin would form higher order complexes within or immediately after excretion from the cell. For these reasons, Dr. Thompson chose to add a Kringle domain to the protein, with the expectation that after purification, the domain could be cleaved away using proteases. These domains are common in blood coagulation factors (plasminogen, prothombin, and apolipoprotein, for example), and are believed to regulate proteolytic interactions (Patthy et al, 1984). An added benefit is that Kringle domains bind lysine and therefore lysine columns with a high affinity, which facilitate purification (Chang et al, 1998).

In this method, stable cell lines of S2 *Drosophila* were developed that produced both vitronectin and a mutant form lacking the somatomedin B domain. These proteins were purified using a lysine column, and 15 mg of protein was generated from five liters of cells at a density of $\sim 10^6$ cells/mL ^{Note}. However, despite successful purification of the proteins, removal of the kringle domain was recalcitrant to all efforts. Some proteases cleaved away the kringle domain but also cleaved sites in vitronectin, while others did not cleave the domain at all. It was decided that a new strategy forgoing the kringle domain and using the poly-histidine tag for purification should be attempted, and the procedure in this thesis was the result.

^{Note} The concentration of S2 cells here is approximately 20-fold less than that used in the expression described later. This was due to poor cell growth in large four liter containers used in this particular expression. This may have been due to the cells being too dilute when passaged into the large containers, which could have starved them of necessary growth factors. Based on that, smaller cultures at higher concentration (see methods section) are recommended for any future work, as the larger cultures do not seem ideal for S2 growth.

Materials, Methods, and Results

Materials – *Drosophila* Schneider 2 Cells were obtained from Invitrogen, as was the pMT/BiP/V5-His C vector chosen for the transfection of the S2 cells. Transfection of the S2 line was completed using Invitrogen's calcium phosphate transfection kit and protocol. Additionally, pCoBlast selection vector from Invitrogen was also used to generate the stable cell line and select for transfected cells. All S2 cultures were grown in Hyclone SFX Insect Media from Thermo Scientific. Depending on the circumstance (see next section), some cultures were supplemented with fetal bovine serum from Gibco, Penicillin-Streptomycin from Gibco, and/or Blastidicin S from Invitrogen.

The S2 cell line is derived from embryonic *Drosophila* and exhibit characteristics that suggest they are derived from a macrophage lineage (Schneider, 1976). The pMT vector itself contains several useful features designed to work with S2 cells that are ideal for vitronectin expression. First, it contains restriction sites that are distinct from any sequences in vitronectin itself. Secondly, it codes for a BiP (Binding Immunoglobulin Protein) signal sequence, which targets the protein for excretion via the endoplasmic reticulum (Walter and Johnson, 1994). This excretion is also useful since much of the posttranslational modification vitronectin experiences will occur in the Golgi apparatus and ER. Furthermore, this plasmid also codes for a poly-histidine C terminal tag. This tag will allow for purification later on a Nickel-chelating

Sepharose - Fast Flow resin column from Amersham Biosciences. Finally, the plasmid contains a *Drosophila* metallothionein (MT) promoter site and a polyadenylation signal, which will allow for efficient transcription and translation of vitronectin, respectfully.

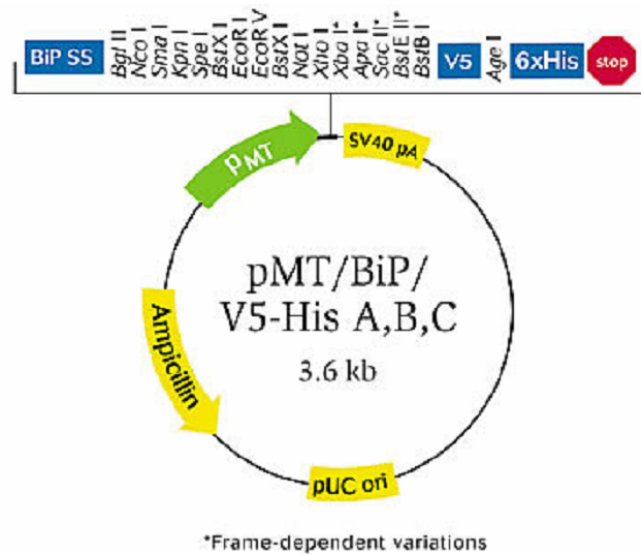


Figure 4: The pMT vector from Invitrogen. This vector has several features that make it useful for vitronectin expression. For ligation into this plasmid, the Sma I and Age I restriction sites were used.

For all preparations of DNA plasmids, Wizard Plus SV Miniprep DNA Purification kits and protocol were used from Promega. Initial screening of vitronectin sequences was completed in TOP10 *E. Coli* from Invitrogen using the PCR 2.1 vector and its accompanying kit.

Methods – Using the vitronectin template from Dr. Thompson’s earlier endeavors, a polymerase chain reaction was performed to generate DNA for both vitronectin (VN) and a mutant form lacking the Somatomedin B domain (Δ SMBVN). This was accomplished by using the primers shown below:

	VN	Δ SMBVN
Forward Primer 5' to 3'	CCCGGGACCAAGAGTCATGCAAGGG	CCCCGGGGTGTTCACCTATGCCGGAG
Reverse Primer 5' to 3'	ACCGGTCAGATGGCCAGGAGCTGGGC	ACCGGTGGGCTGAGGTCTCCCTGGAT

Table 2: Both forward primers include recognition sites for the Age I (ACCGGT) restriction endonuclease, while the reverse primers have a site for the Sma I (CCCGGG) restriction endonucleases. For Δ SMBVN, after the restriction site, the primer begins at the intrinsically disordered domain (IDD).

Following the PCR reaction, VN and Δ SMBVN DNA were ligated into respective PCR 2.1 vectors and used to transform TOP10 *E. Coli*. Transformed cells were then plated on plates containing ampicillin and kanamycin.^{Note 2} The PCR 2.1 vector enables a blue/white screen in which any insert into the plasmid disrupts the beta-galactosidase gene. Upon treatment with X-gal, transformed

^{Note 2} Unlike other cell lines, including TOP10F' (Invitrogen), TOP 10 do not require IPTG or any other reagent to induce expression of galactosidase since it lacks the lac repressor.

colonies lacking an insert will appear blue as X-gal is degraded by galactosidase to form a colored product, while colonies with an insert and the plasmid will be both antibiotic resistant and white from a lack of X-gal processing. Individual white colonies were selected, grown in 15 mL Terrific Broth cultures, and their plasmids isolated using a Wizard Plus SV Miniprep kit. The plasmids were subject to DNA sequencing until plasmids without any mistakes in the sequences for both VN and Δ SMBVN were obtained.

Next, the plasmids and pMT vectors were digested using Sma I and Age I, gel purified to ensure only digested plasmid and sequences remained, and then ligated together to form two pMT vectors, one containing the vitronectin sequence and one the Δ SMB vitronectin sequence. These were again screened and sequenced. Following each confirmed sequence, 25% glycerol stocks were made and stored at -80° C.

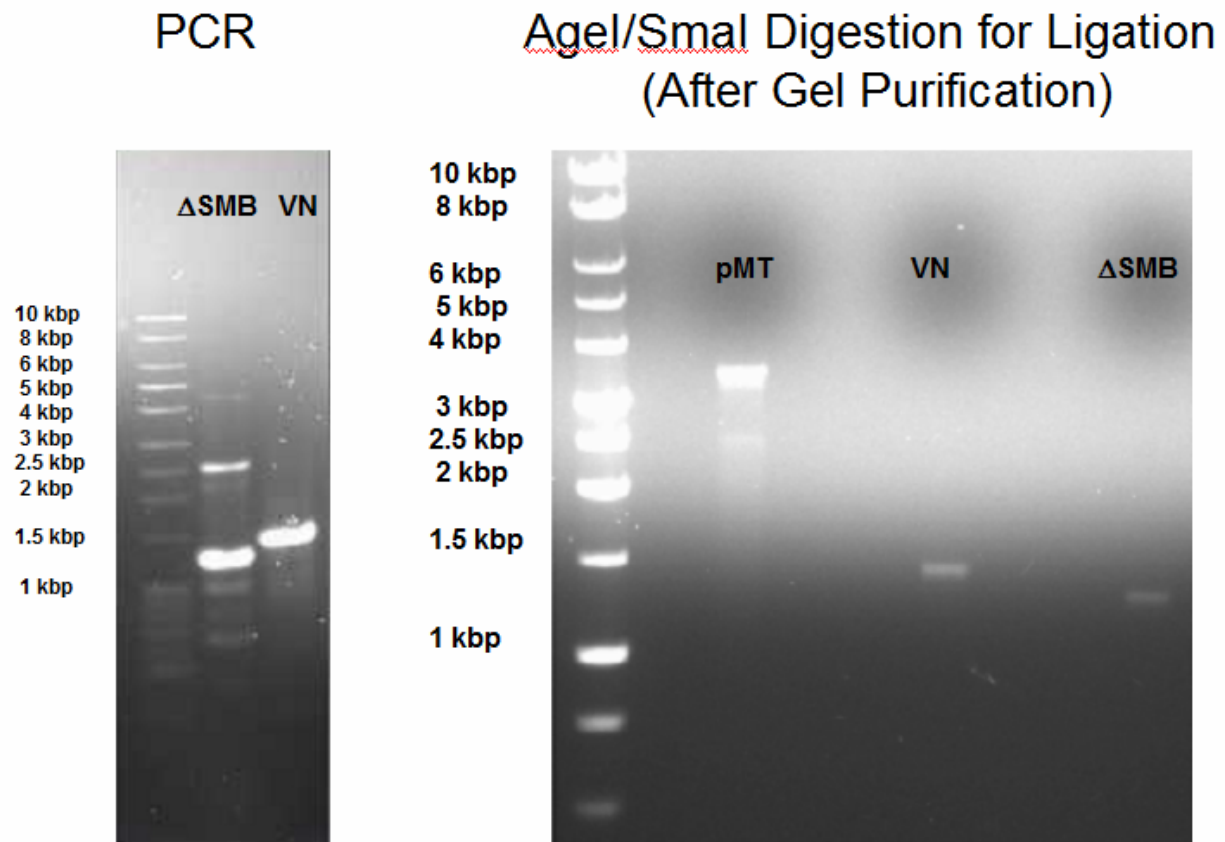


Figure 5: 0.8% Agarose electrophoresis gels. The gel on the right was used for gel purification by physically excising the bands from the gel and purifying them to yield pure, digested DNA with overhangs for ligation.

Schneider 2 *Drosophila* 1 mL cultures were thawed from liquid nitrogen storage and added to 25 mm² flat bottom flasks along with 4 mL of Hyclone SFC

Insect Media + 50 units/mL Penstrep + 10% FBS (v/v).^{Note 3} These cultures are stored in 10% DMSO, which needed to be removed to promote cell growth. The cells were allowed to adhere to the surface of the flask for around one hour, and then by tilting the flask, removing the media by pipetting, and quickly adding fresh media (again, with FBS), the majority of the DMSO is removed. These cultures were allowed to grow and were gradually passaged until the transfection was performed. Cells were seeded at 10^6 cell/mL concentration in 35mm well plates, and a calcium phosphate transfection was performed according to the protocol in the Invitrogen S2 Drosophila and pMT/Bip/V5-His A,B,C kit.

Initially, only a transient transfection of the S2 cells was completed to quickly screen the effectiveness of the vector in the cells. However, no significant protein could be detected on SDS-Page Gels, nor could it be detected using a GFP-pMT positive control that would fluoresce if GFP was being produced. Assuming that this meant transfection efficiency was low, a stable cell line transfection was begun to select for only cells that were co-transfected with the pCOBlast plasmid. Cells were again seeded on 35 mm well plates, and the transfection was performed. Three days post transfection, Blastidicin containing media (25 ug/mL) was added to the wells in place of the original media. This media still contained FBS, but now had a selection agent to

^{Note 3} Whether or not FBS is added to the media is dependent on the next step in the process. Preparing cells for transfection or selection requires the use of the FBS, since without it cell growth is initially slow and they may lack proper nutrients to grow well under selection pressure. However, when cells stabling expressing protein are thawed, one option is to forgo FBS, since cells will need to be weaned off it prior to inducing expression. This does result in slower initial growth, but it also saves the time of weaning the cells.

kill any cells that were not resistant to blasticidin. The concentration of cells in all of the wells was routinely measured, and a control, a line that would eventually produce vitronectin, and a line that would eventually produce deltaSMB vitronectin are shown below graphically. Every three to five days the media was removed without disturbing the cell layer, and fresh media was added. After almost 4 weeks, larger cultures of the successfully transfected cell lines were started.

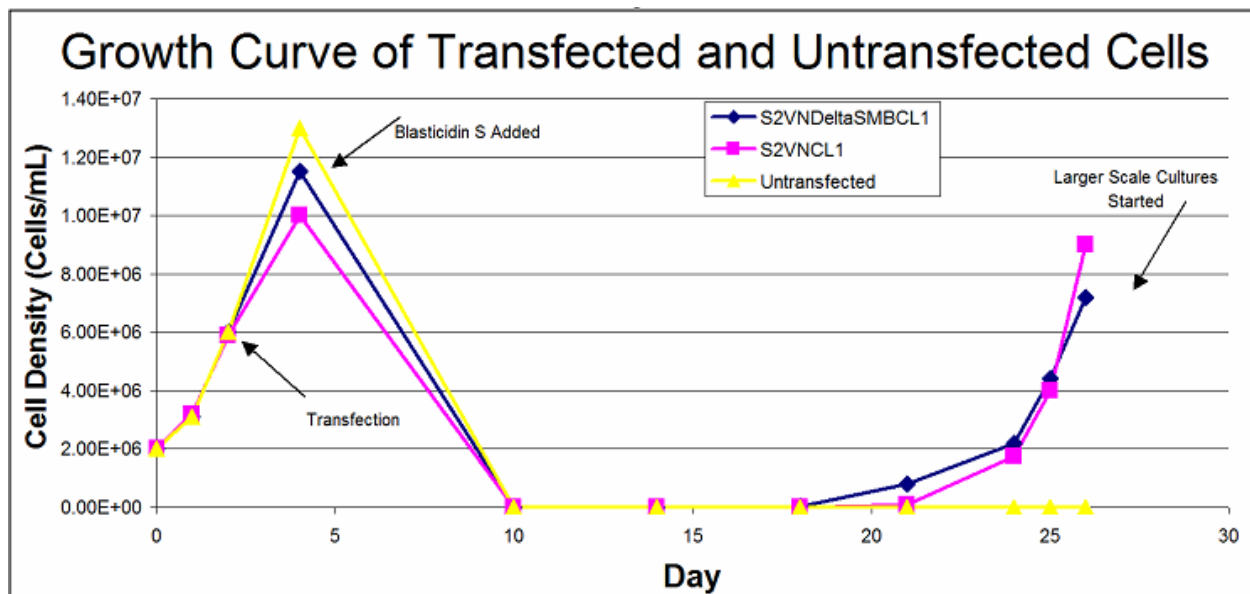
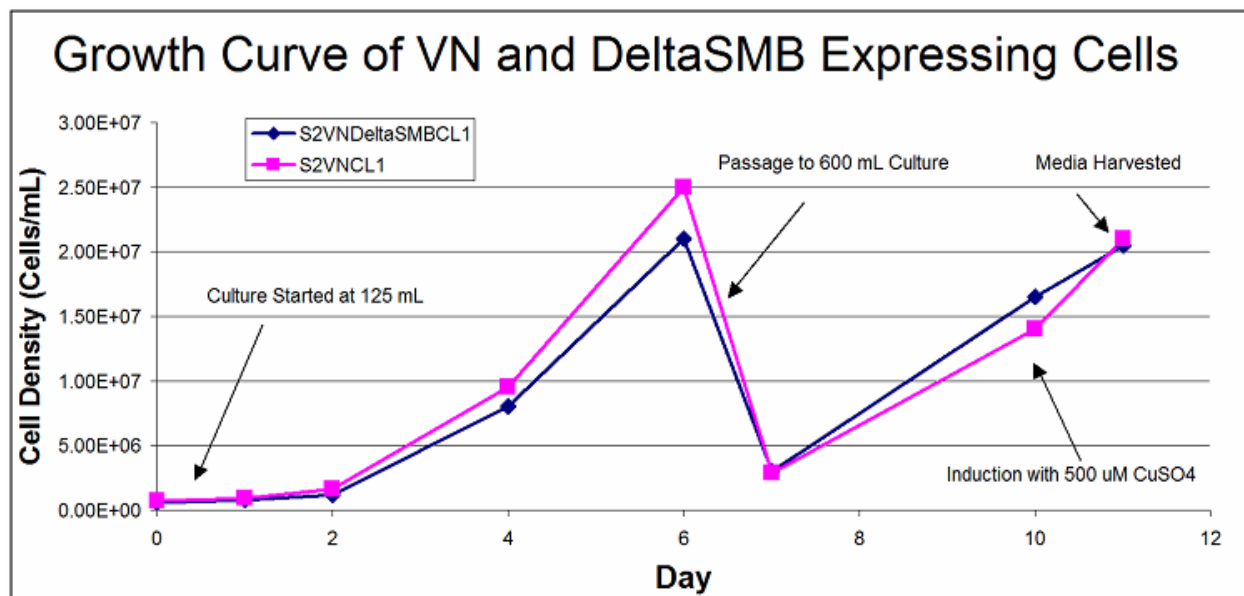


Figure 6: Cells without Blasticidin S resistance (Untransfected) are quickly killed following the addition of blasticidin. Even in wells where transfection occurred, the vast majority of cells appeared to not be resistant. However, after many days at a concentration so low that only a marginal percentage of cells were alive, Δ SMBVN transfected cells and VN transfected cells resistant to blasticidin began to proliferate.

The new cell lines were renamed S2VN Δ SMBCL1 and S2VNCL1 to distinguish them from previous kringle-VN producing cell lines, and larger flat bottom flask cultures and eventually larger scale shaker flask cultures were grown. As the cultures were scaled up, Hyclone SFX Media was added without FBS (though Blasticidin S and Penstrep were maintained^{Note 4}), a process that weaned the cells so that the proteins in FBS would not be present in any future analyses. After the cells reached a high concentration in 125 mL cultures, some initial screening for protein production was completed, while larger cultures of 450 mL to 600 mL were also made. In either case, both were induced with 500 μ M Copper Sulfate (making use of the metallothionein promoter in the pMT vector).



^{Note 4} Typically, Penstrep was left in all cultures no matter the size. However, due to its cost, blasticidin was only used for small-scale cultures. Despite the selection processing being complete, blasticidin is also potentially antimycotic (Izumi et al 1991). Its use in small-scale cultures prevents contamination. In addition to using blasticidin, all work with S2 cells should always be performed in a laminar flow hood.

Figure 7: For both S2VN Δ SMBCL1 and S2VNCL1 cell lines, the growth of the cells were comparable to the 24 hour doubling rate reported by Invitrogen.

Approximately thirty hours after induction, cell cultures were centrifuged at 1000 X g to pellet the cells, and then the media was decanted and filtered using a .22uM Corning Vacuum Filter Flask. An initial sample of the media was collected to see if the cell lines expressed vitronectin and Δ SMBVN as hoped, and to see if they secreted the product. BCA assays were also performed on the media at this point to assess total protein, determining there to be 83 mg of protein in one particular 450 mL culture. While the majority of this is likely not vitronectin, that vitronectin is the dominant species (as seems to be the case in **Figure 8**) bodes well for the method. After the proteins were determined to be vitronectin and Δ SMBVN, permanent cell stocks able to express each were frozen in liquid nitrogen according to Invitrogen's protocol for freezing permanent Schneider 2 *Drosophila* cell cultures. To be certain the cells were still viable after freezing, an aliquot of both S2-VN and S2- Δ SMBVN cells were thawed several weeks later and again tested for growth and expression. The thawed cells showed growth and expression virtually identical to those described throughout the remainder of this method.

SDS Page Gel of Media Containing Excreted VN and VN Δ SMB Before Purification

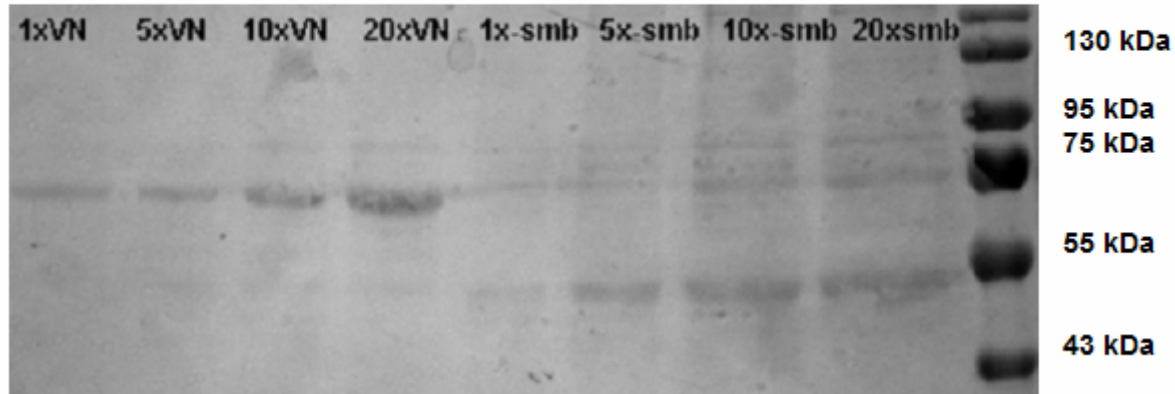


Figure 8: SDS-Page Acrylamide Reducing gel. The two S2 cell lines were shown to be expressing vitronectin and Δ SMB as hoped. The concentrations shown are relative to the media, and both appear to be the dominant protein species in the media.

After the protein SDS-Page gel confirmed that the cells secreted the desired proteins, the media containing the expressed full-length vitronectin was dialyzed for 48 hours into 20 mM Imidazole, 50 mM NaH₂PO₄, and 500 mM NaCl at pH 7, the buffer to be used for purification on a nickel column. Initially, a gradient of 50 mM imidazole to 500 mM imidazole was used to purify the protein; however, the gradient produced a gradual elution without any true peaks. Instead, a multi-wash system was used. After the pooled protein from the previous gradient was loaded on to the Nickel-chelating Sepharose Fast Flow column, a 30 mM imidazole, 50 mM NaH₂PO₄, and 500 mM NaCl, pH 7 wash was

run, followed by a 60 mM imidazole, 50 mM NaH_2PO_4 , and 500 mM NaCl, pH 7 elution, followed by a 500 mM Imidazole, 50 mM NaH_2PO_4 , and 500 mM NaCl pH 7 elution.

A reducing gel showed that the composition of the 60 mM imidazole and 500 mM imidazole elutions were indistinguishable, so they were pooled together and concentrated. It should be noted that during this purification there was a substantial amount of vitronectin that did not bind the column and came out in the flow through. To help mitigate this, the flow through was run over the column again and the elutions repeated. Another BSA assay was performed, which indicated 3.6 mg of protein had been purified. This amount seems low relative to the high amount measured in the original media, but it is suspected that much of it was lost in the gradient attempt and in the lack of binding in the flow through.

Vitronectin Purification on the Nickel Chelating Sepharose Column

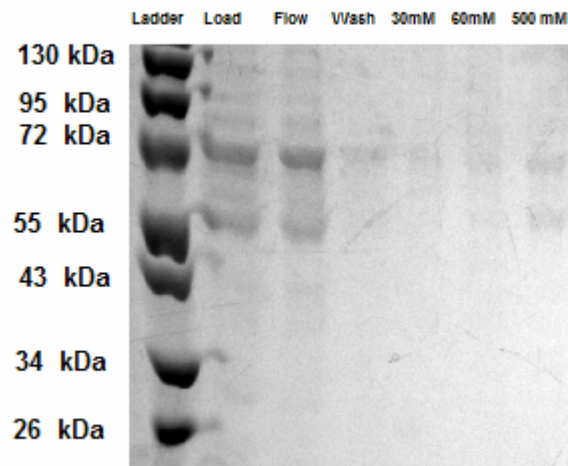


Figure 9: Elutions of Vitronectin in three concentrations of Imidazole buffer as described above. It is worth noting that considerable amounts of the protein failed to bind to the nickel column. This could be due to a chelating agent in the media that was not dialyzed out.

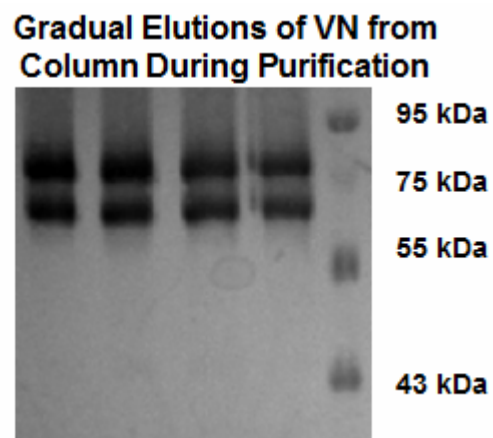


Figure 10: Western blot made using Rabbit Anti-VN antibodies conjugated to Peroxidase labeled anti-rabbit antibodies. The double banding pattern is

typical of human purified vitronectin as described in the introduction, indicating that the proteolysis that occurs could be common to many animal cells.

Despite the relative success of the vitronectin purification scheme, a sample of unpurified Δ SMBVN was run over both DEAE Sepharose column and a SP column. Under neither circumstance was there significant binding of the protein to the column.

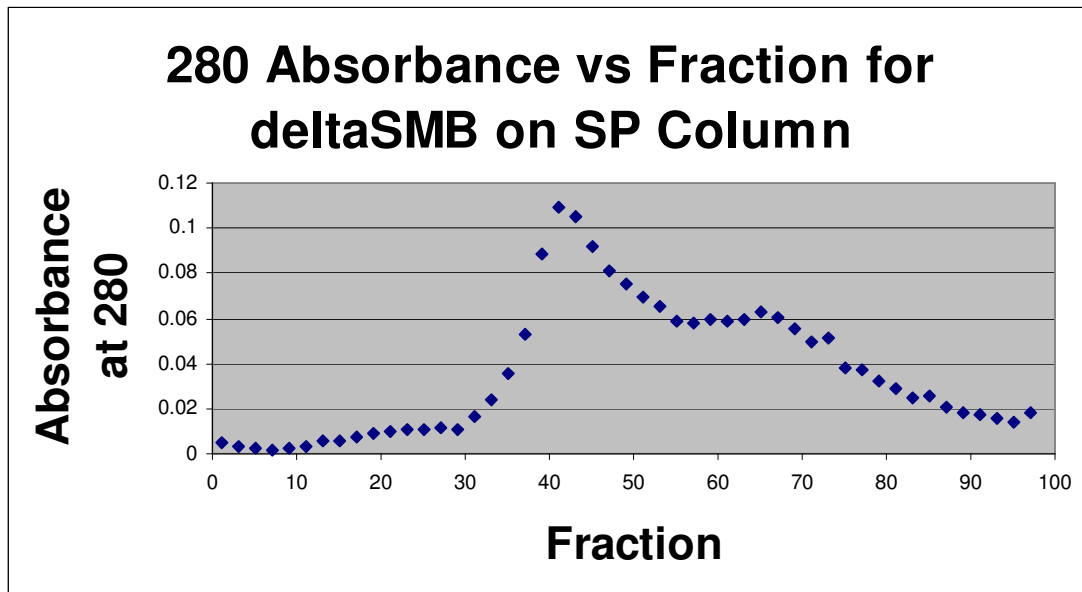


Figure 11: While slight peaks were observed on the SP column, the low value of the absorbance indicates that little bound. To be certain, a reducing and non-reducing SDS Page Gel was made to see what protein species were eluted.

Reducing and Non Reducing Dye SDS Gels of Delta SMB after SP Column

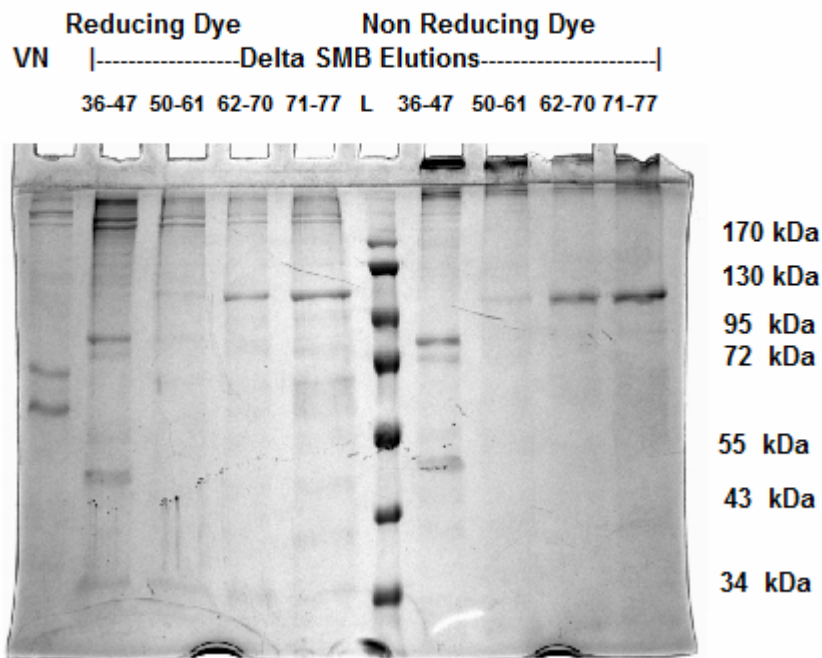


Figure 12: After concentration of the pooled fractions listed above from the SP column, a reducing and non-reducing SDS Page Gel was run to further examine higher order complexes. It would appear that in the case of Δ SMBVN very little of the protein existed outside of the complexes.

Additionally, Δ SMBVN purified from the nickel column did not show reactivity with anti-VN antibodies during western blots. Furthermore, it appeared to have very large amounts of higher order complexes. Purified vitronectin also exhibited large complexes in addition to the 65 kDa and 75 kDa bands, and it is unclear if those are another protein in complex with vitronectin or larger aggregates of vitronectin and Δ SMBVN themselves. An attempt was made to

purify vitronectin from the larger complexes using a S-200 size exclusion column. However, the larger complex and vitronectin appeared to elute at the same time. This would seem to indicate that at least some of the purified vitronectin is in complex with another molecule or with large complexes of itself, and that the two may not be able to be separated at this stage in the purification.



Figure 13: After the use of a size exclusion column, the larger higher-order bands are still clearly visible on this SDS Page Gel of Vitronectin. This indicates that there is likely a large complex being formed that causes the two to elute together.

Discussion and Future Directions

These results indicate that a large amount of vitronectin and the Δ SMB mutant can be produced in S2 *Drosophila* cells. Based on the findings, it would seem that the best course of action would be to immediately dialyze all unpurified samples after a 24 to 48 hour induction and cell removal, followed by nickel column purification and 3 elutions of 30 mM imidazole, 50 mM NaH_2PO_4 , and 500 mM NaCl, pH 7; 60 mM imidazole, 50 mM NaH_2PO_4 , and 500 mM NaCl, pH 7; and 500 mM Imidazole, 50 mM NaH_2PO_4 , and 500 mM NaCl pH 7. In light of the lack of binding of the protein to the DEAE column and SP column, it is not recommended that ion exchange be used without a new rational not known at the time of this writing. One option may be to use a vitronectin-specific antibody column immediately, followed by, or in place of, the nickel chelating Sepharose column. The key will be finding a way to prevent both aggregation and complex formation with other proteins in the media.

It should be noted that several other samples of unpurified protein, even when left at 4°C for a few days, almost universally were problematic for later purification attempts. For example, one sample appeared to have been proteolyzed during the wait, so from then on a protease inhibitor cocktail was added to all samples. This practice should be continued for all future purification strategies. Additionally, purification of the protein should occur as quickly as possible to prevent aggregation, if that is indeed the problem. Based on the 83 mg of protein measured by the BCA assay in the unpurified media, this

method appears to have great promise as a source of recombinant vitronectin and Δ SMBVN so long as a method for removing or preventing the high order complexes can be developed and the purification process can be streamlined.

In addition to improving the strategy for purifying these recombinant proteins, surface plasmon resonance (SPR) studies of recombinant vitronectin and Δ SMB vitronectin are recommended. SPR analysis will allow for the binding constants between vitronectin and PAI-1 to be measured (Pattnaik 2005), and compared to those of human purified vitronectin. An added benefit of SPR is that the protein does not need to be completely pure, which may be useful given the difficulty of removing the large molecular weight complexes.

Additionally, assays assessing the effects of recombinant vitronectin on PAI-1 stability could help to confirm that the interaction between the two is consistent with what is observed in human purified vitronectin. If the constants for full-length recombinant vitronectin is comparable to those of human purified vitronectin, and if the purification strategy for the recombinant form can be improved, then a genuine recombinant source of vitronectin will have been created that is both practical, reliable, and considerably more efficient than current methods. This would open the door to future mutagenesis studies, as well as allowing for more frequent analyses of full-length vitronectin due to it being more easily produced. Hopefully, by developing this recombinant source that makes vitronectin easier to obtain, future insights into strokes, heart disease,

tumor malignancy, and many other diseases may be expedited, paving the way for new drugs to treat these conditions.

Acknowledgements:

The author would like to sincerely thank each and every member of the Peterson lab. I can say with absolute honesty that I consider each of you a friend and a valued part of my time at Tennessee.

Dr. Cynthia Peterson has been a truly wonderful mentor and has shown a constant interest in my success for which I am extremely grateful. She has pushed me to keep trying despite setbacks, celebrated my accomplishments, welcomed my input, and taught me important skills that will benefit me tremendously throughout my career.

Tihami Qureshi has been like a second mentor to me, who has helped me daily in the lab. She deserves much credit for the successes of this project, and has always been willing to lend me assistance on her own time. Her helpfulness is greatly appreciated, as is her patience and desire to help me succeed.

Nancy Horn, Joel Bucci, Letitia Olson, Dr. Sumit Goswami and Dr. Carlee McClintock have all helped me many times, either through tried and true advice, practical insight, showing me new techniques, or providing endless entertainment to break up the workday.

Dr. Lawrence Thompson provided much of the groundwork for this project and helped acquaint me to the lab during my first months there. Despite my likely silly questions and complete lack of lab knowledge at the start, he was patient and willing to help me learn what I needed to get this project started. I am certain that his influence is a major part of why I have enjoyed this experience from the very beginning.

References

Carreiras F., Cruet, S., Staedel, C., Sichel, F, and Gauduchon, P. (1999) "Human Ovarian Adenocarcinoma Cells Synthesize Vitronectin and Use It to Organize Their Adhesion." *Gynecologic Oncology*: 72, 312-322.

Chang, Y., Mochalkin, I., McCance, S. G., Cheng, B., Tulinsky, A., and Castellino, F. J. (1998) "Structure and Ligand Binding Determinants of the Recombinant Kringle 5 Domain of Human Plasminogen." *Biochemistry*: 37, 3258-3271.

Gladson, C. L. and Cheresh, D. A., (1991) "Glioblastoma Expression of Vitronectin and the Alpha V Beta 3 Integrin. Adhesion Mechanism for Transformed Glial Cells." *Journal of Clinical Investigation*: 88, 1924-1932.

Holmes, R. (1967) "Preparation from Human Serum of an Alpha-One Protein Which Induces the Immediate Growth of Unadapted Cells in Vitro." *Journal of Cell Biology*: 32, 297-308.

Horn, N. A., Hurst, G. B., Mayasundari, A., Whittmore, N. A., Serpersu, E. H., and Peterson, C. B. "Assignment of the Four Disulfides in the N-terminal Somatomedin B Domain of Native Vitronectin Isolated from Human Plasma." *Journal of Biological Chemistry*: 279, 35867-35878.

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991) "Blasticidin S-Resistance Gene (bsr): A Novel Selectable Marker for Mammalian Cells." *Experimental Cell Research*: 197, 229-233.

Krone, K. A., Allen, K. L., and McCrae, K. R. (2010) "Impaired Fibrinolysis in the Antiphospholipid Syndrome." *Current Rheumatology Reports*: 12, 53-57.

Mayasundari, A., Whittmore, N. A., Serpersu, E. H., and Peterson, C. B. (2004) "The Solution Structure of the N-terminal Domain of Human Vitronectin." *Journal of Biological Chemistry*: 279, 29359 - 29366.

Patthy, L., Trexler, M., Banyai, L., Varadi, A., and Vali, Z. (1984) "Kringle: Modules Specialized for Protein Binding. Homology of the Gelatin-Binding Region of Fibronectin with the Kringle Structures of Proteases." *FEBS Letters*: 171, 131-136.

Pattnaik, P. (2005). "Surface Plasmon Resonance: Applications in Understanding Receptor-Ligand Interaction." *Applied Biochemistry and Biotechnology*: 126, 79-92.

Podor, T. J., Campbell, S., Chindemi, P., Foulon, D. M., Farrell, D. H., Walton, P. D., Weitz, J. I., and Peterson, C. B. (2002) "Incorporation of Vitronectin into Fibrin Clots." *Journal of Biological Chemistry*: 277, 7520 - 7528.

Preissner, K. T. and Muller-Berghaus, G. (1986) "S Protein Modulates the Heparin-catalyzed Inhibition of Thrombin by Antithrombin III." *European Journal of Biochemistry*: 156, 645-650.

Sano, K., Miyamoto, Y., Kawasaki, N., Hashil, N., Itoh, S., Murase, M., Date, K., Yokoyama, M., Sato C., Kitajima, K., Ogawa, H. (2010). "Survival Signals of Hepatic Stellate Cells in Liver Regeneration are Regulated by Glycosylation Changes in Rat Vitronectin, Especially Decreased Sialylation." *Journal of Biological Chemistry*: 285, 17301-17309.

Schar, C. R., Blouse, G. E., Minor, K. H., and Peterson, C. B. (2008) "A Deletion Mutant of Vitronectin Lacking the Somatomedin B Domain Exhibits Residual Plasminogen Activator Inhibitor-1 Binding Activity." *Journal of Biological Chemistry*: 283, 10297-10309.

Schneider, I. (1976) "Cell Lines Derived from Late Embryonic Stages of *Drosophila Melanogaster*." *Journal of Embryological Experimental Morphology*: 27: 353-365.

Schvartz, I., Seger, D., and Shaltiel, S. (1999) "Vitronectin." *The International Journal of Biochemistry and Cell Biology*: 31, 539-544.

Sidenius, N. and Blasi, F. (2003) "The Urokinase Plasminogen Activator System in Cancer: Recent Advances and Implication for Prognosis and Therapy." *Cancer and Metastasis*: 22, 205-222.

Tomasini, B. R. and Mosher D. F. (1986). "On the Identity of Vitronectin and S-protein: Immunological Crossreactivity and Functional Studies." *Blood*: 68, 737-742.

Walter, P. and Johnson, A. E. (1994) "Signal Sequence Recognition and Protein Targeting to the Endoplasmic Reticulum Membrane." *Annual Review of Cell Biology*: 10, 87-119.

Wiman, B. and Collen, D. (1978) "Molecular Mechanism of Physiological Fibrinolysis." *Nature*: 272, 549-550.

Wynimko, J. C., Skrzypczak, E., and Jankun, J. (2004) "Plasminogen Activator Inhibitor Type 1: its Structure, Biological Activity, and Role in Tumourigenesis." *International Journal of Molecular Medicine*: 13.

Xu D., Baburaj, K., Peterson, C. B., and Xu, Y. (2001). "Model for the Three-Dimensional Structure of Vitronectin: Predictions for the Multi-domain Protein from Threading and Docking." *Proteins: Structure, Function, and Bioinformatics*: 44, 312-320.

Zhou, A., Huntington, J. A., Pannu, N. S., Carrell, R. W., and Read R. J. (2003) "How Vitronectin Binds PAI-1 to Modulate Fibrinolysis and Cell Migration." *Nature Structural Biology*: 10, 541-544.