The Creation of α2NC1 Transgenic Drosophila melanogaster

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THE CREATION OF α2NC1 TRANSGENIC DROSOPHILA MELANOGASTER

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By

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2013

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ABSTRACT

The α2 isoform of collagen IV’s noncollagenous 1 domain (α2NC1 or canstatin) has shown promise as an anti-cancer molecule, possessing both angiostatic and pro-apoptotic properties. This work aims to further the knowledge of α2NC1 by conducting a truly in vivo study in which the Gal4-induced endogenous overexpression of α2NC1 in Drosophila melanogaster will be used to assess the effects of α2NC1 upon normal development as well as tumorigenesis. To this end, transgenic fly lines capable of overexpressing α2NC1 were created. Initial overexpression studies indicated no developmental phenotype was caused by abundance of α2NC1. However, future overexpression studies in which α2NC1 is exported to its native extracellular location may reveal roles for α2NC1 in normal development and/or tumorigenesis.

Keywords: NC1, basement membrane, development, cancer, angiogenesis, Drosophila melanogaster
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ................................................................................................................................. iii</td>
</tr>
<tr>
<td>Acknowledgements ................................................................................................................... iv</td>
</tr>
<tr>
<td>Vita ........................................................................................................................................... v</td>
</tr>
<tr>
<td>List of Figures and Tables ...................................................................................................... viii</td>
</tr>
<tr>
<td>Chapters:</td>
</tr>
<tr>
<td>1. Introduction ....................................................................................................................... 1</td>
</tr>
<tr>
<td>2. Methods ............................................................................................................................... 13</td>
</tr>
<tr>
<td>3. Results ................................................................................................................................ 23</td>
</tr>
<tr>
<td>4. Discussion ........................................................................................................................... 49</td>
</tr>
<tr>
<td>Bibliography ............................................................................................................................ 54</td>
</tr>
<tr>
<td>Abbreviations Not Listed in Text ............................................................................................ 56</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Structure of Collagen IV</td>
<td>10</td>
</tr>
<tr>
<td>1.2 Angiogenesis and Metastasis</td>
<td>11</td>
</tr>
<tr>
<td>1.3 Binding of Gal4 to the Upstream Activation Sequence Causes Robust Expression of a Downstream Gene (NC1)</td>
<td>13</td>
</tr>
<tr>
<td>3.1 PCR Amplification of α2NC1 DNA</td>
<td>31</td>
</tr>
<tr>
<td>3.2 Primers Used to Amplify α2NC1 from Viking Incorporated Start/Stop Codons and Restriction Sites</td>
<td>32</td>
</tr>
<tr>
<td>3.3 Double-Digestion of BS and α2NC1</td>
<td>33</td>
</tr>
<tr>
<td>3.4 BS-α2NC1</td>
<td>34</td>
</tr>
<tr>
<td>3.5 Putative BS-α2NC1 Clones</td>
<td>35</td>
</tr>
<tr>
<td>3.6 Digestion of Putative BS-α2NC1 Clones</td>
<td>36</td>
</tr>
<tr>
<td>3.7 Double-Digestion of pUAST and BS-α2NC1</td>
<td>37</td>
</tr>
<tr>
<td>3.8 pUAST-α2NC1</td>
<td>38</td>
</tr>
<tr>
<td>3.9 Putative pUAST-α2NC1 Clones</td>
<td>39</td>
</tr>
<tr>
<td>3.10 PCR Amplification of α2NC1 from a Putative Clone Confirms Its Identity as pUAST-α2NC1</td>
<td>40</td>
</tr>
<tr>
<td>3.11 Sequencing of pUAST-α2NC1</td>
<td>41</td>
</tr>
<tr>
<td>3.12 Cleavage Prediction</td>
<td>42</td>
</tr>
</tbody>
</table>
3.13 Digestion of pIDTSmart-Lα2NC1 and pUAST........................................ 43
3.14 pUAST-Lα2NC1...................................................................................... 44
3.15 Digestion of Potential pUAST-Lα2NC1 Clones........................................ 45
3.16 Digestion with BglII Can Identify Orientation of LαNC1........................ 46
3.17 Sequencing of pUAST-Lα2NC1............................................................... 47

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Primers used for PCR and Sequencing Reactions................................. 21</td>
</tr>
<tr>
<td>2.2</td>
<td>Gal4 Driver Lines.................................................................................. 22</td>
</tr>
<tr>
<td>3.1</td>
<td>JO1 Transgenic Lines............................................................................ 48</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

One often hears someone mention finding “the cure” for cancer. However, no two cancers are exactly alike; rather, “cancer” refers to any unchecked proliferation of cells. This rapid growth causes tumors to form. There are several factors which confer on tumors the ability to continuously grow and ultimately metastasize. Such hallmarks of cancer include the evasion of apoptosis (programmed cell death), sustained angiogenesis (the formation of new blood vessels), self-sustaining growth signals, and insensitivity to anti-growth signals (1). Research focused on each of these cancer hallmarks is needed in order to combat the various subtypes that encompass this disease.

One such area of research focuses on basement membrane, whose components play numerous important roles in the formation, growth, and spreading of tumors. Basement membrane, or “BM”, is the 50-100nm-thick specialized extracellular matrix that lines both layers of epithelial cells and the endothelial cells that line blood vessels (2). BM plays an important structural role, separating monolayers of epithelia from stroma or neighboring tissue and providing support. It is also important in numerous biochemical capacities, as
components of BM have been shown to modulate signaling across the cell membrane (2,3). Such signals promote cell migration, differentiation, and growth and can vary depending on the unique microenvironment created by the BM components, many of which have tissue-specific isoforms (2,3,4).

One such component of BM is collagen IV (2,3,4). The genes for collagen IV are highly conserved across species (5) and code for six distinct isoforms of the α-chain, which differ primarily within their globular C-terminal noncollagenous (NC1) domains (2-6). α-chains self-assemble in the extracellular space to form the complex collagen IV suprastructure shown in Figure 1.1. In the first step of self-assembly, NC1 domains coordinate the association of three α-chains into trimers referred to as protomers. Several unique combinations of α-chain isoforms have been observed, with α1α1α2 being the most common (2,3,6). Two protomers associate into a hexamer of α-chains via thioester bonds between conserved methionine and lysine residues present in each NC1. Each methionine bonds with the lysine on the opposite NC1 residue, and vice-versa (2). Interactions between the N-terminal 7S domains mediate the tetrameric association of hexamers to form the “spider-like” shape characteristic of the collagen IV matrix (2,3).

There are several other components of BM, most of which are proteins and glycoproteins (2,3,5). The second-most abundant component is the glycoprotein laminin which, like collagen IV, self-assembles into a network composed of laminin trimers (in this case, heterotrimers, each consisting of an α,
β, and γ subunits). A variety of laminin isoforms contributes to the heterogeneity observed within individual BMs and between BMs of various tissues (2,3). Additionally, the presence of less abundant collagens (i.e. collagen XV and collagen XVIII) lends specificity to particular BMs. Other protein components include nidogen/entactin (which links the collagen and laminin scaffolds), heparan-sulphate proteoglycans (such as perlecan and agrin, which sequester stores of vascular endothelial growth factor), SPARC/BM-40/osteopontin, and fibulins (3). Not only are each of these proteins structurally important for BM, but nearly all of them have been shown to regulate angiogenesis during remodeling (2,3).

BM is anchored to the basolateral side of epithelium and endothelium primarily by interactions between the five G-like (LG) domains of laminin α-chains and cell-surface molecules including integrins and dystroglycans (2,3,6,7). Specifically, LG1-3 have been shown to bind integrins α3β1, α6β1, α7β1, & α6β4, while LG4-5 bind α-dystroglycan (7,8). However, the chemical natures of these interactions remain unknown (8). Additionally, interactions between the triple-helical region of collagen IV and integrins α1β1 & α2β1 further promote cellular adhesion (6).

BM components are continuously turned over (degraded and reformed) in a process called BM remodeling. While BM remodeling occurs at a normal basal level in all tissues, it plays a particularly important role in processes such as development and angiogenesis. For example, in Drosophila melanogaster each
adult tissue begins as an “imaginal disc” or precursor tissue, in the *Drosophila* larva. Each disc is surrounded by a basement membrane which is turned over during disc eversion, the process by which the imaginal discs develop into adult tissues (9). Due to this critical role of BM remodeling, *Drosophila* is an excellent model organism for studying the effects of BM components on development, a research topic with which the reagents created in this study will aid.

However, the relevance of BM remodeling on development extends well beyond the fly, as it is important for the development and partitioning of tissues in all multicellular animals (2,3,10,11). For example, in mice and other mammals, the specialized basement membrane of the kidney glomerulus (GBM) is formed during embryonic development from the remodeling and fusion of two distinct BMs – one synthesized by glomerular epithelium and the other by glomerular endothelium. Failure of the GBM to form properly will result in an incompletely vascularized glomerulus and a leaky blood-urine barrier (11).

BM remodeling is also required for the proliferation and migration of endothelial cells to form new blood vessels in the process of angiogenesis. Not only is BM degradation needed in a physical sense, to un-anchor endothelial cells and provide space for a new branch of the blood vessel, but many of the protein fragments generated during remodeling promote angiogenesis and endothelial cell migration (2,3). Furthermore, stores of vascular endothelial growth factor (VEGF) that were previously sequestered in the BM are released during remodeling (2,3).
As previously mentioned, angiogenesis is critical for tumor growth and metastasis, as new blood vessels are needed if the growing tumor is to receive the nutrients required for rapid and continued growth. Tumors often therefore upregulate factors, such as matrix metalloproteases (MMPs), which degrade BM and promote blood vessel formation (5). In addition to providing nutrients, these new vessels also provide access to the bloodstream – a single cancerous cell may break off from the primary tumor, enter the blood stream, and exit the blood stream in another tissue, forming secondary tumors (Figure 1.2). This is the process of metastasis and the most clinically devastating feature of cancer.

Given the numerous pro-angiogenic factors generated by BM remodeling, it makes sense that there would be a subsequent signal to “turn off” angiogenesis. This seems to be the role of collagen IV’s C-terminal non-collagenous 1 (NC1) domain. NC1 is one of the smallest and last products to be generated by MMPs in the degradation of BM, and several of its isoforms have been shown to inhibit angiogenesis (2-6,12-14). One particularly potent isoform is that of the α2 chain, or α2NC1 (also called canstatin in some literature). This 25KDa molecule has been shown to decrease tumor growth and metastasis by inhibiting two cancer strategies – sustained angiogenesis and evasion of apoptosis.

Several studies have demonstrated the angiostatic and pro-apoptotic properties of α2NC1 (and other NC1 isoforms) (4,6,12-14). Many of these studies were conducted in vitro, using HUVECs (human umbilical vein endothelial cells)
to demonstrate decreased growth, migration, & tubal formation as well as increased apoptosis of these cells when treated with α2NC1 (4,12). Others were conducted in xenograft models, in which human cancer cells were implanted subcutaneously in mice to mimic "natural" tumors. These studies showed decreased vascularization and growth of implanted tumors when treated with α2NC1, either by direct injection or injection of an adenovirus coding for α2NC1 (4,12,13). Extension of α2NC1’s half-life by fusing it to human serum albumin (HSA) resulted in a more dramatic anti-tumor effect and was even shown to decrease the levels of metastasis in mice (12,13). Whether or not α2NC1 promotes apoptosis in tumor cells as well as endothelial cells remains controversial, as α2NC1-HSA was shown to have a pro-apoptotic effect but unfused α2NC1 was not (4,12-14).

Several studies have investigated the mechanism by which α2NC1 elicits its effects. One possible anti-angiogenic mechanism of α2NC1 was proposed by Petitclerc et al., who suggested that soluble NC1 binds along the triple-helical region of collagen IV, thereby disrupting the lateral associations of collagen IV with other BM components (i.e. the laminin scaffold) and cell-surface anchors (specifically β1 integrins). As a result, the basement membrane assembly required for blood vessel formation is obstructed and angiogenesis is prevented (6).

More research has been done, however, based on the results of a study by Magnon et al. which found that α2NC1 binds to αv integrins including αvβ3
and αvβ5. Interactions between αv integrins and BM components have been shown to mediate migration of endothelial and tumor cells. It is therefore thought that the binding of α2NC1 to αv integrins blocks BM-integrin interaction and thereby inhibits the migration of endothelial and tumor cells seen in angiogenesis and metastasis (12). A later study by Magnon et al. demonstrated that due to these interactions with αv integrins, α2NC1 is even more effective at decreasing tumor angiogenesis and growth when paired with standard radiation therapy, as radiation increases expression of αvβ3 and αvβ5 integrins (13).

Additionally, α2NC1-integrin interactions have been shown to induce caspase-8 dependent apoptosis in endothelial cells by increasing Fas ligand expression and downregulating the expression of the anti-apoptotic protein FLIP via the disruption of FAK/PI3K/Akt signaling (12,14). Downregulation of FLIP decreases the mitochondrial membrane potential, causing the release of cytochrome c which results in caspase-9 activation. Caspase-9 dependent apoptosis was demonstrated in α2NC1-treated endothelial cells and in α2NC1-HSA-treated tumor cells (12,14). Furthermore, Petitclerc et al. imply that, like previously-studied αvβ3 antagonists, α2NC1 may elicit its pro-apoptotic effect by disrupting signal transduction pathways leading to altered expression/activity of Bcl-2, Bax, and p53 (6).

To further investigate the roles of α2NC1, we proposed a unique in vivo study in which α2NC1 would be endogenously overexpressed within a well-studied model organism, *Drosophila melanogaster* (the fruit fly). Endogenous
overexpression in otherwise wild-type flies will allow us to observe the effects of α2NC1 on normal organismal development. In subsequent experiments, α2NC1 will be overexpressed in flies with mutations in the genes Ras (a conserved and much-studied oncogene) and scribble (a conserved cell polarity protein whose inactivation disrupts cell polarity, inhibits apoptosis and induces dysplasia). This double mutation results in tumors which display features of human metastatic tumors. For example, these tumors result in the loss of cell-cell junctions and cell polarity (15). By comparing the size and number or tumors observed in α2NC1-overexpressing flies versus “normal” Ras/scribble flies, we will be able to assess α2NC1’s effect upon tumorigenesis.

To achieve the overexpression of α2NC1 in flies, we chose to utilize the powerful UAS-Gal4 binary genetic technique. The various elements of this powerful genetic system were originally identified in yeast and manipulated later for use in Drosophila. Gal4 is a transcriptional activator which binds to a specific sequence of DNA called an upstream activator sequence (UAS), resulting in robust transcription of any downstream gene (16) (Figure 1.3).

In this thesis, work related to cloning of α2NC1 in pUAST and subsequent generation of transgenic flies is presented. Two types of UAS-α2NC1 flies were created - one with the signal sequence (that aids in the secretion of the protein) and the other without this sequence resulting in one set of flies capable of secreting the protein and the other set where the secretion would be impaired. These two reagents will aid in better understanding of the consequences of
α2NC1 overexpression on normal development and tumor progression in a living organism.
Figure 1.1 Structure of Collagen IV. The collagen IV suprastructure is formed by the self-assembly of various α-chains (each ~400nm). In addition to a central collagenous domain characterized by a repeated Gly-X-Y motif in which X and Y are most often hydroxyproline and hydroxylysine, each α-chain contains an N-terminal 7S domain and a C-terminal noncollagenous domain. NC1 domains initiate the association of three α-chains into a trimer or "protomer". Two protomers interact via their NC1 domains, forming a hexamer. Hexamers interact via their 7S domains to form a spider-like tetramer. Bridging of multiple tetramers creates the fibrous collagen IV network that comprises more than 50% of the basement membrane (2,3). Source: Kalluri, R., et al. (3)
Figure 1.2. Angiogenesis and Metastasis. Tumors promote angiogenesis in order to receive the nutrients required for continued growth. As illustrated, the events of tumor angiogenesis and basement membrane degradation are closely linked. New blood vessels provide easy access to the blood stream, allowing tumors to metastasize. Source: Adapted from Zetter, B. R. (17)
Figure 1.3. Binding of Gal4 to the Upstream Activation Sequence Causes Robust Expression of a Downstream Gene (NC1). The UAS-Gal4 system has been manipulated for use in Drosophila in order to overexpress genes of interest. In this study, crossing transgenic UAS-α2NC1 males with Gal4 driver females results in progeny which overexpress NC1. Source: Adapted from Heberlein, U., et al. (18)
CHAPTER 2

METHODS

PCR Amplification of α2NC1 cDNA:

The DNA sequence for α2NC1 was amplified from Viking cDNA via PCR. 120ng Viking cDNA, 100ng each of primers JO1US and JO1DS (Table 2.1), 5μl of 5nM dNTPs (Invitrogen), 5μl of 10X Pfu Turbo Buffer (Agilent Technologies, Inc.), 1μl of Pfu (Agilent Technologies, Inc.), and nanopure water to 50μl were included in PCR reaction. Thermal cycler conditions were as follows: 5 minutes at 94°C, 35X (2 minutes at 94°C, 1 minute at 56°C, 1.5 minutes at 72°C), 10 minutes at 72°C. The PCR products were run on a 0.8% agarose gel with 0.6μg/mL ethidium bromide at 100V for 1hr, and the band corresponding to α2NC1 (~900bp) was excised and purified using QIAGEN’s Qiaex II Gel Extraction Kit, according to the manufacturer’s protocol.

Digestion of α2NC1:

Purified α2NC1 DNA and Bluescript II SK+ (BS) DNA were each double-digested with EcoRI and XbaI as follows: 1μg α2NC1 or 40μg BS DNA, 10μl 10X Buffer M (Invitrogen), 2.5μl EcoRI (~25U, Invitrogen), 2.5μl (~25U, Invitrogen),
and nanopure water to 100μl. Digestions took place in a thermal cycler at 37°C for 2 hours. The digests were run on a 0.8% agarose gel with 0.6μg/mL ethidium bromide at 100V for 1hr and the bands corresponding to α2NC1 (~900bp) and BS (~3000bp) were excised and the DNA purified using the QIAGEN Qiaex II Gel Extraction Kit according to the manufacturer’s protocol.

**Ligation of α2NC1 into Bluescript II SK+:**

113ng digested BS and 113ng digested α2NC1 (3:1 molar ratio) were ligated in a 10μl reaction which also contained 1μl of 10X T4 DNA Ligase Buffer (New England Biolabs), 0.5μl T4 DNA Ligase (New England Biolabs), and nanopure water. The ligation took place overnight (18 hours) at 10°C. The entire ligation (10μl) reaction was used to transform chemically-competent DH5α E. coli via heat shock. Heat shock was carried out by incubating the ligation reaction with 50μl cells for 20 minutes on ice, followed by 90 seconds on a 42°C heat block and another 3 minutes on ice. Cells were then given 300μl LB media and incubated at 37°C for 45 minutes. Transformants were selected by growing the cells overnight at 37°C on LB-agar with 75μg/mL ampicillin.

**Screening of Transformants for Recombinant BS-α2NC1:**

4mL liquid LB/ampicillin (75μg/mL) cultures were inoculated with each of the forty-two resulting transformants and grown overnight at 37°C/300rpm. Plasmid DNA was isolated from each of these liquid cultures using Qiagen’s
QIAprep Spin Miniprep Kit according to the manufacturer’s protocol. Isolated plasmid DNA was run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. DNA from putative positive clones was double-digested with EcoRI and XbaI for two hours at 37°C (200ng plasmid DNA, 0.5µl each enzyme [~5U, Invitrogen], 2µl 10X Buffer M [Invitrogen], nanopure water to 20µl) and run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. Miniprepped DNA from a confirmed BS-α2NC1 clone was used to transform chemically-competent DH5α E. coli cells via heat shock (as previously described). A resulting colony was used to inoculate a 4mL liquid LB/ampicillin (75µg/mL) culture, which was grown for 14 hours at 37°C/300rpm. 500µl of this starter culture was used to inoculate a 100mL liquid LB/ampicillin (75µg/mL) culture, which was grown up overnight, also at 37°C/300rpm. Plasmid DNA was isolated using Qiagen’s Spin Maxiprep Kit according to the manufacturer’s protocol.

**Subcloning of α2NC1 from BS-α2NC1 to pUAST:**

BS-α2NC1 and pUAST were double-digested with EcoRI and XbaI (47.3µg of BS-α2NC1 or 42.5µg pUAST, 2.5µl each enzyme [~25U, Invitrogen], 10µl 10X Buffer M [Invitrogen], nanopure water to 100µl). Digests were run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr, and the bands corresponding to α2NC1 (~900bp) and pUAST (~9000bp) were gel extracted using Qiagen’s Qiaex II Gel Extraction Kit according to the manufacturer’s protocol. The digested α2NC1 and pUAST were ligated in a 3:1
ratio (150ng digested pUAST, 50ng α2NC1, 0.5µl T4 DNA Ligase [200U, NEB], 1µl 10X Buffer [NEB], nanopure water to 10µl) overnight (14 hours) at 10°C using. The entire ligation reaction (10µl) was used to transform chemically-
competent DH5α E. coli using the previously-described heat shock protocol. Transformants were selected by growing the cells overnight at 37°C on LB-agar plates with 75µg/mL ampicillin.

**Screening of Transformants for Recombinant pUAST-α2NC1:**

4mL liquid LB/ampicillin (75µg/mL) cultures were inoculated with each of the eighteen resulting transformants and grown overnight at 37°C/300rpm. Plasmid DNA was isolated from each of these liquid cultures using Qiagen’s QIAprep Spin Miniprep Kit according to the manufacturer’s protocol. Isolated plasmid DNA was run on a 0.8% agarose gel with GelRed (Biotium) at 100V for 1hr. The identity of a putative positive clone was confirmed by amplifying α2NC1 from it via PCR. The PCR reaction included 1µg plasmid DNA, 100ng each of primers JO1US and JO1DS (Table 2.1), 45µl PCR Supermix (Invitrogen), and nanopure water to 50µl. Thermal cycler conditions were as follows: 5 minutes at 94°C, 35X(2 minutes at 94°C, 1 minute at 56°C, 1.5 minutes at 72°C), 10 minutes at 72°C. 20µl of the PCR products were run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. DNA from the positive clone was used to transform chemically-competent DH5α E. coli via heat shock (as...
previously described) and maxiprep pUAST-α2NC1 using the Qiagen Spin Maxiprep Kit according to the manufacturer’s protocol.

**Standard Dideoxy Sequencing of pUAST-α2NC1:**

The α2NC1 DNA sequence was PCR amplified using the following conditions: 10pmol of primer UAAS-2 or pUASTrev (Table 2.1), 12ng pUAST-α2NC1 (template DNA), 2µl 5X BigDye Sequencing Juice, and 2µl 5X buffer. Thermal cycler conditions were as follows: 3min at 96°C, 25X (30sec at 96°C, 15sec at 50°C, 4min at 60°C). Following PCR, amplification products were purified from the reaction mix using Qiagen’s DyeEx 2.0 Spin Kit. Samples were subsequently evaporated by centrifugation in a centrivap vacuum concentrator for 30 minutes at room temperature and resuspended in 20µl formamide buffer. Samples were then loaded onto the ABI 3130 Prism Sequencer.

**Microinjection of pUAST-α2NC1:**

75µg of pUAST-α2NC1 were sent to Genetic Services Inc. for microinjection into *Drosophila melanogaster* embryos.

**Genetic Crosses for Overexpression of α2NC1:**

Each of the five UAS-α2NC1 lines was crossed to each of the ten Gal4 lines shown previously in Table 2.1. Five transgenic males and five virgin Gal4 females were used in each cross, and crosses were kept at 25°C. Once larvae
were observed, the parental flies were transferred to new vials and kept at 29°C. After larvae appeared in the 29°C crosses, parental flies were discarded. F1 flies were observed using light microscopy and a CO₂ plate.

**Definition and Synthesis of Leader-α2NC1 (Lα2NC1):**

In order to create a leader-α2NC1 (Lα2NC1) construct, the online program “SignalIP-4.0 Prediction” (http://www.cbs.dtu.dk/services/SignalP/) was used to predict collagen IV’s leader sequence. The required Lα2NC1 nucleotide sequence was synthesized *in vitro* as part of a plasmid (pIDTSmart-Lα2NC1). 15ng of pIDTSmart-Lα2NC1 was used to transform chemically-competent DH5α E. coli via heat shock, as previously described. Transformants were selected by plating the transformation reaction on LB-agar with 75µg/mL ampicillin and growing the cells overnight at 37°C. A single resulting colony was picked and used to maxiprep large quantities of pIDTSmart-LαNC1 using the Qiagen Spin Maxiprep Kit according to the manufacturer’s protocol.

**Cloning of Lα2NC1 into pUAST:**

pIDTSmart-Lα2NC1 and pUAST were each digested with EcoRI (36µg pIDTSmart-Lα2NC1 or 21µg pUAST, 3µl EcoRI [~30U, Invitrogen], 8µl 10X Buffer H [Invitrogen], nanopure water to 80µl) and run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. The bands corresponding to Lα2NC1 (1005bp) and pUAST (~9000bp) were excised, and the DNA was
purified by gel extraction using Qiagen’s Qiaex II Gel Extraction Kit according to the manufacturer’s protocol. The digested insert and vector were ligated in a 12:1 ratio (43ng digested pUAST, 57ng digested Lα2NC1, 0.5µl T4 DNA Ligase [200U, NEB], 1µl 10X buffer [NEB], nanopure water to 10µl) overnight at 16°C. The entire ligation reaction (10µl) was used to transform chemically-competent DH5α E. coli via heat shock, as previously described. Transformants were selected by growing the cells overnight at 37°C on LB-agar with 75µg/mL ampicillin.

**Screening of Transformants for Recombinant pUAST-Lα2NC1:**

4mL liquid LB/ampicillin (75µg/mL) cultures were inoculated from each of the nineteen resulting transformants and grown overnight at 37°C/300rpm. Plasmid DNA was isolated from each of these liquid cultures using Qiagen’s QIAprep Spin Miniprep Kit according to the manufacturer’s protocol. Plasmid DNA was then digested with BglII (100ng plasmid DNA, 1µl BglII [~5U, Invitrogen], 2µl 10X Buffer M [Invitrogen], nanopure water to 20µl) for 2 hours at 37°C (in a thermal cycler) and run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. Positive clones were identified and large quantities of pUAST-Lα2NC1 were generated by maxiprep using Qiagen’s Spin Maxiprep Kit according to the manufacturer’s protocol.
Standard Dideoxy Sequencing of pUAST-Lα2NC1:

Sequencing was conducted following a similar protocol as that used for pUAST-α2NC1. ~50ng of pUAST-Lα2NC1 were used in each sequencing reaction, and cleanup was achieved by two subsequent isopropanol precipitations as follows. Four volumes (40µl) of 75% isopropanol were added to the sequencing reaction followed by centrifugation for 20 minutes in a table top centrifuge at 21,000Xg. The supernatant was removed and 250µl isopropanol added, followed by 10 minutes of centrifugation at 21,000Xg. Once again, the supernatant was removed, and residual liquid was evaporated in a centrivap vacuum concentrator for 10 minutes at room temperature. Samples were resuspended in 20µl of formamide buffer and loaded onto the ABI 3130 Prism Sequencer.

Microinjection of pUAST-Lα2NC1:

75µg of pUAST-Lα2NC1 were sent to Genetic Services Inc. for microinjection into Drosophila melanogaster embryos.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Use</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JO1US</td>
<td>Amplification of α2NC1</td>
<td>TGAATTCATGATTGGATTCCCAAGGACAG</td>
</tr>
<tr>
<td>JO1DS</td>
<td>Amplification of α2NC1</td>
<td>ATTCTAGATTACCTGGACTTTGCGAGTGCCG</td>
</tr>
<tr>
<td>UAAS-2</td>
<td>Sequencing</td>
<td>ACCAGCAACCAAGTAAAATCAACTGC</td>
</tr>
<tr>
<td>pUASTrev</td>
<td>Sequencing</td>
<td>GTCACACCACAGAAGTAAGG</td>
</tr>
</tbody>
</table>

**Table 2.1 Primers used for PCR and Sequencing Reactions.**
<table>
<thead>
<tr>
<th>Gal4 Line</th>
<th>Expresses in</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eyeless</em>-Gal4</td>
<td>Eye</td>
</tr>
<tr>
<td><em>Panier</em>-Gal4</td>
<td>Dorsal cells</td>
</tr>
<tr>
<td><em>Scalloped</em>-Gal4</td>
<td>Wing</td>
</tr>
<tr>
<td><em>Cg</em>-Gal4</td>
<td>Hemocytes</td>
</tr>
<tr>
<td><em>Vestigial</em>-Gal4</td>
<td>Wing</td>
</tr>
<tr>
<td><em>Ultra bithorax</em>-Gal4</td>
<td>Thorax, abdomen, wing, haltere</td>
</tr>
<tr>
<td><em>LSP2</em>-Gal4</td>
<td>Larval fat body/adult hemolymph</td>
</tr>
<tr>
<td><em>Patched</em>-Gal4</td>
<td>Antero-posterior compartment boundary</td>
</tr>
<tr>
<td><em>GMR</em>-Gal4</td>
<td>Eye</td>
</tr>
<tr>
<td><em>Tubulin</em>-Gal4</td>
<td>Whole body</td>
</tr>
</tbody>
</table>

**Table 2.2. Gal4 Driver Lines.** The various driver lines of *Drosophila melanogaster* each express Gal4 in a particular tissue/pattern. Breeding these lines with a UAS-transgene line (i.e. UAS-α2NC1), results in progeny that overexpress said transgene in the same tissue/pattern (16).
CHAPTER 3

RESULTS

Successful PCR Amplification of α2NC1 from Viking cDNA:

PCR using Viking cDNA as a template successfully amplified the DNA fragment corresponding to α2NC1, as shown by the appropriately-sized ~900bp band observed following gel electrophoresis of the PCR products (Figure 3.1). Furthermore, the primers used were designed such that they incorporated start (ATG) and stop (TAA) codons (allowing the α2NC1 gene fragment to be translated properly in vivo) as well as restriction sites (forward: EcoRI, reverse: XbaI) to be used in future restriction digests (Figure 3.2). The band corresponding to the amplified α2NC1 was gel purified for use in the subsequent cloning steps.

Cloning of α2NC1 into Bluescript II SK+:

Restriction digestion with EcoRI and XbaI resulted in linear Bluescript II SK+ (BS), as indicated by the appropriately-sized ~3000bp band observed following gel electrophoresis of the completed digest (Figure 3.3). Along with double-digested α2NC1 DNA, the linearized BS was used in the subsequent
ligation reaction to generate BS-α2NC1 (Figure 3.4). Heat shock transformation of *E. coli* cells using the ligation reaction resulted in several transformants, as indicated by the growth of colonies on selective LB-agar/ampicillin medium.

**Screening of Transformants Identified Recombinant BS-α2NC1:**

Electrophoretic analysis of undigested plasmid DNA isolated from each transformant suggested that there were several positive BS-α2NC1 clones, as indicated by the shift in molecular weight seen between empty BS and several of the samples (Figure 3.5). Digestion of four of these putative BS-α2NC1 samples with EcoRI and XbaI confirmed their identity, as gel electrophoresis of each digest resulted in two distinct bands, one corresponding to BS (~3000bp) and the other to α2NC1 (~900bp) (Figure 3.6).

**Subcloning of α2NC1 from BS-α2NC1 to pUAST:**

Digestion of BS-α2NC1 with EcoRI and XbaI successfully liberated the α2NC1 DNA fragment from the vector, as indicated by the two distinct bands observed following gel electrophoresis of the completed digest (~900bp and ~3000bp, corresponding to α2NC1 and BS, respectively) (Figure 3.7). Double-digestion of pUAST with the same two enzymes successfully linearized the vector, as indicated by the ~9000bp band observed following gel electrophoresis of the completed digest (Figure 3.7). The digested α2NC1 and pUAST fragments were purified from the gel, and used in the subsequent ligation reaction, which
resulted in the creation of recombinant pUAST-α2NC1 (Figure 3.8). Heat shock transformation of *E. coli* cells using the ligation reaction resulted in several transformants, as indicated by their growth on selective LB-agar/ampicillin medium.

**Screening of Transformants Identified Recombinant pUAST-α2NC1:**

Electrophoretic analysis of undigested plasmid DNA isolated from each transformant suggested that nearly all transformants were successful pUAST-α2NC1 clones, as indicated by the shift in molecular weight seen between empty pUAST and all but one of the samples (Figure 3.9). PCR amplification of α2NC1 from one of these putative pUAST-α2NC1 clones confirmed its identity, as gel electrophoresis of the PCR product yielded a single band at ~900bp (Figure 3.10).

**Sequencing of pUAST-α2NC1 Revealed that the α2NC1-Coding DNA in the Construct is Not Defective:**

PCR which incorporated fluorescent ddNTPs amplified α2NC1 DNA from pUAST-α2NC1, as described in the methods. Half of the reactions utilized primer UAAS-2, which anneals to the 5’ side of pUAST’s multiple cloning site, near the UAS. The other half of the sequencing reactions used the primer pUASTrev, which anneals to the opposite strand of pUAST, just to the 3’ side of the multiple cloning site. Sequencing analysis was performed by the ABI 3130 Prism
Sequencer. Reverse complementation was performed on results from pUASTrev reactions, as this primer amplifies from the nonsense strand. Results were compared to the nucleotide sequence “Drosophila melanogaster collagen type IV alpha 2 (DmColA2) mRNA, complete cds” using NCBI’s basic local alignment search tool (BLAST). Whenever discrepancies were observed between the results and accepted sequence, it was determined that the quality of sequencing reads for that region was poor and the sequence was confirmed by proper alignment in duplicate runs (see example, Figure 3.11). It was ultimately determined that no errors in cloning or mutations had occurred, and that the vector contained the proper coding sequence for α2NC1 as well as flanking start and stop codons.

**Generation of UAS-α2NC1 Flies:**

pUAST-α2NC1 was sent to Genetic Services Incorporated (GSI) where, along with a transposase element, it was microinjected into the germ line cells of *Drosophila* embryos. Because pUAST contains a P-element, microinjection resulted in the incorporation of the vector into the fly genome. Inbreeding of these initial transgenics resulted in animals with the insert present in the genomic DNA of every cell and tissue. Five viable transgenic UAS-α2NC1 lines (referred to as “JO1” lines) were received from GSI, of which two were homozygous for UAS-α2NC1 on the second chromosome and two were homozygous for UAS-α2NC1 on the third chromosome. An additional second chromosome line was homozygous lethal and therefore balanced over CyO on the second
chromosome (Table 3.1). Each of these transgenic lines should enable the overexpression of α2NC1, which for the purposes of these experiments was defined as amino acids 1450-1749 of the 1761 amino acid collagen IV α2 protein. These residues contain both α2NC1 and provide extra “contextual” amino acids that may help maintain α2NC1’s normal folding.

Overexpression of α2NC1 in Flies Produces No Developmental Phenotype:

Various Gal4 driver lines of Drosophila melanogaster are available which drive expression of Gal4 in a tissue-specific pattern. These Gal4 driver lines contain the gene for Gal4 under the control of various enhancers. Each driver line therefore expresses Gal4 in a specific pattern based upon its unique enhancer. For example, vestigial (vg) is a gene expressed primarily in the wing imaginal disc, along the ventral-dorsal compartment boundary (19). Vg-Gal4 flies therefore express Gal4 in a similar pattern within the wing. By crossing UAS-α2NC1 flies with various Gal4 driver lines, the expression of α2NC1 should follow the same pattern as the Gal4 expression. This allowed us to investigate any tissue specific effects of α2NC1’s expression. This is an important question to ask, given that BM composition differs greatly between various organs and tissues (2,3).

Crosses between the Gal4 drivers lines shown in Table 2.1 and the five JO1 lines produced no developmental phenotype at 25°C. Crosses conducted at 29°C similarly showed no outstanding characteristics, despite the fact that the
Gal4 system is temperature sensitive and overexpresses more at higher temperatures. This lack of effect is likely due to a requirement for extracellular localization of α2NC1 for its functionality. This possibility will be investigated in future crosses between UAS-Łα2NC1 (JO2) and Gal4 lines, as these progeny will overexpress a secreted version of α2NC1.

**Definition and Synthesis of Leader-α2NC1 (Łα2NC1):**

SignalIP-4.0 Prediction indicated collagen IV α2’s signal peptidase cleavage site as being between amino acids 28 and 29 (Figure 3.12). In order to conserve the context of the cleavage site, amino acids 1-35 were chosen to be joined to aa1450-1749 (α2NC1) of collagen IV. The Łα2NC1 DNA sequence was created *in vitro* by Integrated DNA Technologies (IDT) as part of a simple plasmid (pIDTSmart-Łα2NC1).

The fusion of the leader sequence to α2NC1 should result in the cotranslational localization of the peptide to the rough endoplasmic reticulum. Once fed into the ER lumen, α2NC1 will be cleaved from the membrane-bound leader sequence by signal peptidase. Like other soluble ER-localized peptides, α2NC1 will be directed to the Golgi apparatus and eventually to the plasma membrane for export from the cell to its native extracellular location.

**Cloning of Łα2NC1 into pUAST:**

Digestion with EcoRI liberated the Łα2NC1 DNA fragment from pIDTSmart-Łα2NC1, as indicated by the two distinct bands observed following
gel electrophoresis of the completed digest (~1005bp and ~2000bp, corresponding to Lα2NC1 and pIDTSmart, respectively) (Figure 3.13). Digestion of pUAST with EcoRI successfully linearized the vector, as indicated by the ~9000bp band observed following gel electrophoresis of the completed digest (Figure 3.13). These digested fragments were gel purified and used in the subsequent ligation reaction which resulted in the successful creation of recombinant pUAST-Lα2NC1 (Figure 3.14). Heat shock transformation of E. coli cells using the ligation reaction resulted in several transformants, as indicated by their growth on selective LB-agar/ampicillin medium.

**Screening of Transformants Identified Recombinant pUAST-Lα2NC1:**

Digestion of plasmid DNA isolated from each transformant identified one as containing recombinant pUAST-Lα2NC1, as digestion with BglII followed by gel electrophoresis resulted in two visible bands, one corresponding to pUAST (~9000bp) and the other to Lα2NC1 (1005bp) (Figure 3.15). This restriction pattern indicated that Lα2NC1 had inserted in the forward orientation relative to the UAS in pUAST (Figure 3.16).

**Sequencing of pUAST-Lα2NC1 Revealed that the Lα2NC1-Coding DNA in the Construct is Not Defective:**

Sequencing of pUAST-Lα2NC1 was performed in a manner similar to that used for pUAST-α2NC1. Results were compared using BLAST to the nucleotide
sequences “Drosophila melanogaster collagen type IV alpha 2 (DmColA2)” and “viking, transcript variant C” to verify the integrity of the α2NC1 and leader sequences within pUAST-Lα2NC1. Using the analysis strategy described for pUAST-α2NC1, it was determined that this construct contained the proper genetic sequences for both the leader sequence and α2NC1 (Figure 3.17).

**Generation of UAS-Lα2NC1 Flies:**

pUAST-Lα2NC1 DNA was sent to Genetic Services Incorporated (GSI) where, it was microinjected into *Drosophila* embryos, as described for the microinjection of pUAST-α2NC1. These flies will contain a leader-α2NC1 fusion sequence, again under the control of a UAS. The added leader sequence should direct the overexpressed α2NC1 peptide to the endoplasmic reticulum so that it can be exported from the cell to its native extracellular location.
Figure 3.1. PCR Amplification of α2NC1 DNA. PCR successfully amplified the genetic sequence for α2NC1. The PCR products were run on a 0.8% agarose gel with 0.6µg/mL at 100V for 1hr, which yielded a single band of the appropriate size (~900bp) (lanes 3 and 5). Lane 1 contains a standard 1kB+ DNA Ladder (Invitrogen). The PCR product was excised and subsequently purified using Qiagen’s Qiaex II Gel Extraction Kit.
Figure 3.2. Primers Used to Amplify α2NC1 from Viking Incorporated Start/Stop Codons and Restriction Sites. The primers shown above were designed to anneal to the forward and reverse strands of Viking cDNA, flanking the base pairs corresponding to amino acids 1450-1749 (NC1) of collagen IV α2. The forward primer incorporated a start codon (ATG) and EcoRI restriction site (GAATTC). The reverse primer incorporated a stop codon (TTA-reverse complement) and XbaI restriction site (TCTAGA). Figure not to scale.
Figure 3.3. Double-Digestion of BS and α2NC1. 40µg of BS and 1µg of PCR-amplified α2NC1 DNA were digested with EcoRI and XbaI. Digests were run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. Lanes 1-3 contain digested α2NC1. Lanes 4-6 contain digested (linear) BS. Digested BS and α2NC1 DNA were purified from the gels using Qiagen’s Qiaex II Gel Extraction Kit for use in the subsequent ligation reaction to create BS-α2NC1.
Figure 3.4. BS-α2NC1. Bluescript was used as an initial carrier for the α2NC1 genetic sequence, as it is much smaller than pUAST and therefore easier to use in the cloning reaction. The completed BS-α2NC1 plasmid contained an ampicillin resistance gene in addition to a bacterial origin of replication and the inserted α2NC1 genetic sequence, which was flanked by EcoRI and XbaI sites. Large quantities of BS-α2NC1 were generated and digested in order to produce the α2NC1 DNA needed for cloning into pUAST (20).
Figure 3.5. Putative BS-α2NC1 Clones. Plasmid DNA isolated from various clones via miniprep was run on a 0.8% agarose gel at 100V for 1hr. For comparison, lane 2 contains supercoiled BS DNA. The gel shift seen between the bands in lane 2 and those in lanes 3-9, 13, 15, and 16 indicates that these are most likely recombinant (BS-α2NC1) molecules. Lanes 10, 12, and 14 appear to contain empty vector, and the molecule represented in lane 11 is possibly a BS molecule that incorporated two copies of α2NC1. Lane 17 contains nanopure H₂O.
Figure 3.6. Digestion of Putative BS-α2NC1 Clones. Four putative BS-α2NC1 clones (see Figure 3.5) were digested with EcoRI and XbaI in order to liberate the incorporated α2NC1 fragment and confirm their identity. Digests were run on a 0.8% agarose gel with 0.6µg/mL at 100V for 1hr. For comparison, lane 2 contains digested BS (empty). Lanes 3-5 are clearly BS-α2NC1, as bands corresponding to BS (~3000bp) and α2NC1 (~900bp) are easily visible. The clone represented in lane 6 is also most likely BS-α2NC1, but the presence of an additional band just below 3000bp raises some question; the additional band is most likely a result of star activity, which was seen in some other digests due to the fact that EcoRI functions non-ideally in the medium-salt XbaI buffer that was used for the double-digestion. The clone represented in lane 4 was selected for future use.
Figure 3.7. Double-Digestion of pUAST and BS-α2NC1. 42.5μg of pUAST and 47.3μg of BS-α2NC1 were digested with EcoRI and XbaI. Each digest was run on a 0.8% agarose gel with 0.6μg/mL at 100V for 1hr. A) Lanes 3-5 contain digested (linear) pUAST. Lane 6 contains nanopure water. B) Lanes 2-5 contain digested BS-α2NC1 (upper band is linear BS ~3000bp and lower band is α2NC1 ~900bp). Lane 6 contains nanopure water. Digested pUAST and α2NC1 DNA was purified from the gels using Qiagen’s Qiaex II Gel Extraction Kit for use in the subsequent ligation reaction to create pUAST-α2NC1.
Figure 3.8. pUAST-α2NC1. This vector contained a gene for ampicillin resistance, a bacterial origin of replication, and the gene fragment for α2NC1 just downstream of the UAS ("5xGal4 DBD"). This vector was microinjected into Drosophila embryos, and due to its P element capability, it integrated into the genome, resulting in five lines of UAS-α2NC1 or “JO1” flies (20).
Figure 3.9. Putative pUAST-α2NC1 Clones. Plasmid DNA isolated from various clones via miniprep was run on a 0.8% agarose gel with 0.6µg/mL at 100V for 1hr. For comparison, lane 1 contains supercoiled pUAST DNA. The gel shift seen between the bands in lane 1 and those in lanes 2-8 and 10-19 indicates that these are most likely recombinant (pUAST-α2NC1) molecules. The identity of the band in lane 9 is unknown.
Figure 3.10. PCR Amplification of α2NC1 from a Putative Clone Confirms Its Identity as pUAST-α2NC1. PCR successfully amplified the α2NC1 genetic sequence from one of the putative pUAST-α2NC1 clones seen in Figure 3.6. The PCR products were run on a 0.8% agarose gel with GelRed (Biotium) at 100V for 1hr, and the ~900bp band corresponding to α2NC1 is easily visible in lane 2. Lane 1 contains a 1kB+ DNA Ladder (Invitrogen).
Figure 3.11. Sequencing of pUAST-α2NC1. Above are representative sequencing results for pUAST-α2NC1. Results were compared using BLASTn to “Drosophila melanogaster collagen type IV alpha 2 (DmColA2) mRNA, complete cds.” Mismatches typically corresponded to poor sequencing reads (see yellow highlighted reads above). Duplicate sequencing reactions were performed in order to obtain enough good reads to confirm that the entire α2NC1 sequence was correct.
Figure 3.12. Cleavage Prediction. The online “SignalIP-4.0 Prediction” software was used to predict which amino acids comprise the leader peptide that targets collagenIVα2 for translation at the rough ER and eventual secretion from the cell. The output above shows three scores. The C score is highest at the position predicted to be just after the cleavage site. The S score (green) is high for those residues thought to comprise the leader sequence. The Y score (blue) is a combination of the C and S scores and peaks at the most likely cleavage sites. Based on this output, the most likely cleavage site is between residues 27 and 28, with a second potential site between residues 30 and 31. As such, amino acids 1-35 were chosen for fusion with the C-terminal α2NC1 domain. When this fused protein (Lα2NC1) is expressed in vivo, the leader should be cleaved off by a signal peptidase in the ER, and α2NC1 will be exported to the extracellular space where it can enact its biological functions.
Figure 3.13. Digestion of pIDTSmart-Lα2NC1 and pUAST. pIDTSmart-Lα2NC1 and pUAST were digested with EcoRI. Each digest was run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. Lane 1 contains a standard 1kb+ DNA ladder (Invitrogen). Lanes 2 and 3 contain digested pIDTSmart-Lα2NC1 (upper band is linear pIDTSmart (~2000bp) and lower band is Lα2NC1 ~1000bp). Lanes 4 and 5 contain digested (linear) pUAST (~9000bp). Lane 6 contains nanopure water. Digested pUAST and Lα2NC1 DNA was purified from the gel using Qiagen’s Qiaex II Gel Extraction Kit for use in the subsequent ligation reaction to create pUAST-Lα2NC1.
Figure 3.14. pUAST-La2NC1. This vector contained a gene for ampicillin resistance, a bacterial origin of replication, and the genetic sequence coding for a leader peptide and α2NC1 (La2NC1) just downstream of the UAS ("5xGal4 DBD"). This vector was sent to Genetic Services Incorporated for microinjection into Drosophila embryos, and due to its P element capability, it will integrate into the genome, resulting in transgenic lines of UAS-La2NC1 or “JO2” flies (20).
Figure 3.15. Digestion of Potential pUAST-Lα2NC1 Clones. Eighteen putative BS-α2NC1 clones (only 6 shown) were digested with BglII in order to liberate the incorporated Lα2NC1 fragment and so identify any recombinant (pUAST-Lα2NC1) molecules. Digestion with BglII (as opposed to the enzyme used to clone [EcoRI]) allowed for directional screening, to ensure proper orientation of the inserted Lα2NC1 fragment (see Figure 2.2). Digests were run on a 0.8% agarose gel with 0.6µg/mL ethidium bromide at 100V for 1hr. Lane 1 contains a standard 1kB+ DNA ladder (Invitrogen). For comparison, lane 2 contains digested pUAST (empty). Lane 4 is clearly pUAST-Lα2NC1, as bands corresponding to pUAST (~9000bp) and Lα2NC1 (1005bp) are easily visible. Lanes 5-9 appear to be empty pUAST. Lane 10 is nanopure water.
Figure 3.16. Digestion with BglII Can Identify Orientation of LaNC1. The construct on the left shows La2NC1 in the proper orientation relative to the UAS (“5xGal4 DBD”). As shown by the marked restriction sites, digestion of this molecule with BglIII would result in three fragments, 80bp, 914bp, and 8921bp. If La2NC1 inserted in the reverse orientation (right), fragments 80bp, 29bp, and 9806bp long would be expected following BglIII digestion (20).
Drosophila melanogaster collagen type IV alpha 2 (DmColA2) mRNA, complete cds

Leader:  
Query  67  ATGTTACCAGAGACAAAGGGA  126
Sbjct  154  ATGTTACCAGAGACAGAGAGGTGCCGTCGGATATCGCGGTGACCAGGGCGAGGTGGGC  231

α2NC1:  
Query  172  ATTGGATTCCAAGGACAGAGAGGTGCCGTCGGATATCGCGGTGACCAGGGCGAGGTGGGC  231
Sbjct  4495  ATTGGATTCCAAGGACAGAGAGGTGCCGTCGGATATCGCGGTGACCAGGGCGAGGTGGGC  4554

Figure 3.17. Sequencing of pUAST-Lα2NC1. Above are representative sequencing results for pUAST-Lα2NC1. Results were compared using BLASTn to "Drosophila melanogaster collagen type IV alpha 2 (DmColA2) mRNA, complete cds." Mismatches typically corresponded to poor sequencing reads (see yellow highlighted reads above). Duplicate sequencing reactions were performed in order to obtain enough good reads to confirm that both parts of the Lα2NC1 sequence were correct.
Table 3.1. JO1 Transgenic Lines. Five lines of transgenic *Drosophila melanogaster* were received from Genetic Services Incorporated following microinjection of pUAST-α2NC1.
CHAPTER 4

DISCUSSION

Three recombinant vectors were generated as a result of this work. The first of these (BS-α2NC1) was generated by cloning of the genetic sequence for α2NC1 into the multiple cloning site of the common laboratory vector Bluescript II SK+. α2NC1 DNA was generated by PCR amplification of corresponding region from Viking cDNA (Viking being the Drosophila melanogaster gene for the full α2 chain of collagen IV). The primers used for PCR were designed to incorporate start and stop codons (allowing the α2NC1 gene fragment to be translated properly in vivo) as well as restriction sites (EcoRI and XbaI, to be used in cloning) on either side of the α2NC1 sequence. Double-digestion of the PCR product and Bluescript SK+ with said enzymes, followed by ligation using T4 DNA ligase, successfully produced the recombinant BS-α2NC1 molecule.

The second vector was pUAST-α2NC1, which was generated by subcloning of the α2NC1 genetic sequence from BS-α2NC1 to the Drosophila vector pUAST. Digestion of pUAST and excision of α2NC1 from BS-α2NC1 were carried out using the same enzymes as before (EcoRI and XbaI), ensuring that αNC1 was ligated into pUAST in the forward orientation relative to pUAST’s upstream activation sequence (UAS).
The third and final construct, pUAST-Lα2NC1, contained the genetic sequence for a leader-α2NC1 fusion construct under the control of pUAST’s UAS. The native α2-chain leader sequence was determined, and the Lα2NC1 fusion sequence (with flanking EcoRI sites) was synthesized *in vitro* as part of a simple bacterial plasmid. Restriction digestion with EcoRI excised the Lα2NC1 fragment from said plasmid, and ligation with EcoRI-digested pUAST resulted in recombinant pUAST-Lα2NC1. Due to the use of a single enzyme in this cloning procedure, several plasmid molecules resulting from the ligation were merely empty pUAST that had not incorporated an insert, and one was found to be pUAST that incorporated Lα2NC1 in the reverse orientation relative to the UAS. However, a second round of ligation resulted in a pUAST-Lα2NC1 molecule with Lα2NC1 in the proper orientation.

The two pUAST constructs were sent to Genetic Services Incorporated where they were microinjected into *Drosophila melanogaster* embryos, resulting in transgenic animals containing α2NC1 under the control of an upstream activation sequence (UAS). Five lines of UAS-α2NC1 (JO1) flies were received from GSI (see Table 3.1), and mapped UAS-Lα2NC1 (JO2) lines are expected to be received in the near future.

The creation of these transgenic lines is the first critical step in this study of α2NC1 in organismal development and tumorigenesis, as they have and will allow for the Gal4-dependent tissue-specific overexpression of α2NC1 in wild-type and tumor-bearing flies. Crosses between each of the five UAS-α2NC1 (JO1) lines and ten different Gal4 driver lines (see Tables 3.1 and 1.1) all
produced viable offspring with no observed phenotypic abnormalities. The absence of an effect was surprising; it was expected that, given the critical role of BM in *Drosophila* development, perturbing its composition would result in lethality or improper/incomplete development of various structures, depending on the expression pattern in question.

However, there are potential explanations for the lack of developmental phenotype. First, in order to rule out the possibility that α2NC1 was not being overexpressed as expected, reverse transcription PCR will be used to quantify α2NC1 RNA levels in overexpressing versus wild type flies. Assuming that α2NC1 levels are indeed significantly more robust in the overexpressing flies, one likely reason for the lack of phenotype could be the improper localization of α2NC1 from UAS-α2NC1. Because basement membrane is extracellular in nature and because α2NC1 is derived from the BM, it is logical to infer that α2NC1 should be localized to the outside of the cell. The extracellular localization of collagen IV is dependent on the N-terminal signal peptide. However, α2NC1 is a C-terminal domain of collagen IV, and as part of the UAS-α2NC1 construct does not contain the amino acid sequence that directs the α-chain to the endoplasmic reticulum during translation and thereby targets it for secretion from the cell. Therefore, it is very likely that the recombinant α2NC1 being overexpressed in the JO1 X Gal4 progeny is located in the cytoplasm where it is unable to exert its effects (such as binding to the extracellular domains of α3 and αv integrins). In the near future, crosses between UAS-Lα2NC1 (JO2) and Gal4
flies will test this hypothesis, as these progeny will overexpress a leader sequence-α2NC1 fusion molecule that will target α2NC1 for secretion.

In said JO2 X Gal4 crosses, it is expected that distinct developmental abnormalities will be observed as a result of abundant extracellular α2NC1 which, based upon previous work, is likely to disrupt formation of basement membrane and decrease branching of the tracheal system. Branching of the tracheal system (whose vessels deliver oxygen to various tissues) in Drosophila has been shown to be in many ways homologous to the mammalian process of angiogenesis (21). For this reason, the list of Gal4 drivers may be expanded to include a btl-Gal4 line which will drive expression of α2NC1 in the tracheal cells themselves. Furthermore, in order to test the hypothesis that α2NC1 overexpression (resulting from JO2 X Gal4 crosses) will diminish formation of tracheal vessels, studies may be conducted in which larvae are stained via immunohistochemistry with a labeled anti-2A12 antibody, a marker used in other studies to specifically identify tracheal cells (22).

In tumorigenesis studies, it is expected that α2NC1 overexpression will result in fewer and smaller tumors (again likely due to disrupted BM formation and decreased tracheal branching), and future experiments will work to investigate the mechanism of such results.

Other future studies may involve the creation of α2NC1 transgenics with a fluorescent or antigen tag, so that localization of α2NC1 can be seen within the fly and on a smaller scale, it’s (extra)cellular location. Furthermore, we would like to conduct further overexpression studies in order to investigate the
developmental and tumorigenic roles of other collagen IV fragments (such as the N-terminal 7S domain) as well as other basement membrane molecules; for example, collagens, laminin, SPARC, and perlecan, have all been shown in other studies to be bioactive and regulators of angiogenesis (2,3).

These investigations will shed light upon the numerous interactions that take place during basement membrane remodeling, both in normal developmental situations and during the invasive processes of tumor angiogenesis and metastasis, and thus will contribute toward the ultimate goal of fully understanding and combating tumor development and progression in patients diagnosed with cancer.


ABBREVIATIONS NOT LISTED IN TEXT

SPARC........................................... secreted protein acidic and rich in cysteine
FLIP.................................................. FLICE-like inhibitory protein
FAK.................................................... focal adhesion kinase
PI3K.................................................. phosphoinositide 3-kinase
Bcl-2.................................................. B-cell lymphoma 2 (a protein)
Bax.................................................... Bcl-2-associated X protein
PCR.................................................. polymerase chain reaction
dNTP............................................... deoxyribonucleotide triphosphate
LB.................................................... Luria-Bertani
ddNTP............................................. dideoxynucleotide triphosphate