Recombinant G3 Domain Protein of the Rat Laminin-5 α3 Chain Binds To Integrins on Tumorogenic Breast Cancer Cells To Induce Apoptosis

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RECOMBINANT G3 DOMAIN PROTEIN OF THE RAT LAMININ-5 α3 CHAIN BINDS TO INTEGRINS ON TUMOROGENIC BREAST CANCER CELLS TO INDUCE APOPTOSIS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

by
Brittany Paige Turner
August 2008

Accepted by:
Dr. Thomas R. Scott, Committee Chair
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Dr. Lyndon Larcom
Dr. Charlie Wei
ABSTRACT

The extracellular matrix (ECM) is a network of proteins for holding tissues and cells together. Among the constituents of the ECM is the large molecular weight glycoprotein laminin. Laminin is predominately found in the basement membrane of tissues. Laminins are composed of α, β, and γ chains with 5 globular (G) subdomains at the carboxyl (C) terminal end of the α chain. Of the 12 different laminin isoforms, laminin-5 has been targeted for cancer research. Specifically, the G3 domain has been studied as the specific binding domain. Cells are able to migrate through cell surface adhesion receptors known as integrins. These heterodimeric receptors are comprised of α and β subunits. Studies have demonstrated integrin α-3 and -6 preferentially bind to laminin-5 alpha3-chain G domains.

The G3 domain of the rat laminin-5 alpha3-chain was expressed in a prokaryotic system engineered with chaperone proteins that allow for soluble protein expression in a cold temperature (11.5°C) environment. The expressed recombinant G3 (rG3) has a predicted molecular weight (MW) of 27 kDa, which matches the approximated MW for the sequence including the histidine tag. Following expression and verification through Western Blotting, the protein was purified using chromatography.

Upon separation, adhesion and proliferation assays with rG3 were performed on three breast cancer cell lines. The breast cancer cells (MDA-MB-231, MDA-MB-435, and MCF-7) expressed various levels of the α-3, -6, and β-1 integrin, as determined by flow cytometry. For all three breast cancer cell lines, rG3 demonstrated dose-dependent adhesion but an inhibitory effect on proliferation. Each cell line demonstrated
morphological changes characteristic of apoptosis at 3, 6, and 12 hours of rG3 treatment. The inhibitory property of rG3 prompted determination of caspase activation. Following a 12-hour treatment with rG3, each cell line was assayed for activation of caspase-3/7, -8, and -9. In each cell line, all three caspases were activated over the levels observed in untreated breast cancer cells. Of particular interest are the elevated caspase-9 levels seen in all cell lines, resulting from altered cell signaling events post-rG3 treatment. Further signaling assays revealed altered levels of phosphorylation of the kinase Akt. Therefore, rG3 binding to cancer cells is specific for the α subunit, but fails to engage the β subunit necessary for induction of cell signaling events for cell survival. This ligand binding specificity leads to reduced cell viability through specific pro-apoptotic signaling events.
DEDICATION

I’d like to dedicate this dissertation to my advisor, Dr. Thomas R. Scott. The completion and success of this work is in large part due to his enthusiasm, tenacity, and encouragement. His support and patience through this personal accomplishment has inspired self confidence that has been instrumental in my growth as a scientist. As my mentor and friend, I share in the success of this work with you.
ACKNOWLEDGMENTS

In addition to the support of my major advisor, I am grateful to my committee members for opening their doors to this research and providing advice throughout the course of this work. To Dr. Charlie Rice, for sharing his passion of research and joyful, animated personality; Dr. Lyndon Larcom, for providing an excellent role model of a well-rounded scientist who is educated in numerous fields; and Dr. Charlie Wei, for opening his mind to this research, and providing fresh ideas for the future of this work.

I would also like to thank Dr. Marcy Owens for showing me how to function in a laboratory. Endless hours spent troubleshooting have shaped my outlook and approach to research. Her work ethic and persistence in the lab has helped form the scientist I am today.

To my best friend, Jonathan Ivey, I am deeply grateful for your immeasurable support, love, and faith in me. You’ve shared in all my joys and failures, and without you, this accomplishment would not be nearly as sweet. You represent everything good and beautiful in life, and I am a better person for knowing you.

I would also like to thank a few friends who have truly made this experience enjoyable. To Jason God, for his biting wit and steadfast friendship; Dr. Marlee Marsh, for her sound advice, friendship, and tasty dinners; and Holly Nance for her friendship, hilarious one-liners, and of course yummy cuisine.

Lastly, I am profoundly grateful to my family who always believed I was capable of great success. I am indebted to them for their unwavering love and support, and I hope I continue to make you proud in the future.
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1. LITERATURE REVIEW

Cell migration plays a chief role in a variety of biological processes, ranging from embryogenesis to wound healing, and unfortunately, tumor progression. A cell extends a protrusion at its leading edge to attach to the basement membrane (BM), eventually contracting to move forward, towards the protrusion (Lauffenburger and Horwitz, 1996). Invasion through the BM is a defining moment for carcinoma progression. Once completed, cancerous cells are granted access to the underlying tissues and lymphatics, and thus begins cancer metastasis (Liotta and Stevenson, 1991).

The BM is a specialized form of extracellular matrices (ECM). Sheet-like in structure, it covers the basal portion of all epithelia and endothelia, in addition to surrounding muscle, fat, and peripheral nerve cells (Sasaki et al., 2004). The BM is composed of the lamina lucida, densa, and fibroreticularsir (Merker, 1994). It is a vital component of tissue formation in all animals, as it separates the epithelium from underlying connective tissue. It also provides mechanical stability, and influences cell proliferation, adhesion, migration, differentiation, and gene expression (Katayama et al., 2004). The first BM protein to be identified and analyzed was collagen IV, followed by the discovery of the major non-collagenous glycoprotein laminin (Chung et al., 1979). Other constituents of the BM include nidogens, fibulins, and proteoglycans such as perlecan (Lohi, 2001; Sasaki et al., 2004). Because the BM denies movement of epithelial cells due to small pore size, only active degradation of the BM will allow passage of cells (Flug et al., 1995).
Laminins

As mentioned, the glycoprotein laminin is the predominant non-collagenous protein found in the BM. As a prerequisite for the formation of BM in developing tissues, laminin proteins hold dual roles in providing architectural support and cell-interactive properties (Colognato and Yercheno, 2000). The first laminin trimer discovered was named EHS-laminin, after isolation from the mouse tumor Engelbreth-Holm-Swarm. At the time, the individual proteins of the trimer were referred to as A, B, and B2 chains (Rouselle et al., 1991). Following the discovery of other laminin isoforms, EHS-laminin was renamed Laminin-1, and chains called α1, β1, and γ1 (Lohi, 2001).

Each laminin molecule forms the well-known cruciform structure with its α, β, and γ chains linked by disulfide bonds. Five α, three β, and three γ chains have been identified (Kariya et al., 2003). At nearly a million Daltons in molecular mass, laminins are large molecules (Colognato and Yercheno, 2000). Various combinations of the three chains yield at least 15 known laminin isoforms (Laminin 1-15; Kim et al., 2004). With the range of different laminin chains discovered, mathematically there are 45 potential combinations; however, the actual number of laminin isoforms is much smaller due to chain assembly restrictions. For example, the γ2 and β1 chains are never joined in forming a trimer (Colognato and Yercheno, 2000). Laminin isoforms display the most diversity in arm length and the number of domains in the short arm (Katayama and Sekiguchi, 2003). Full-length domains in the short arms are seen in Laminins -1-4 and 12, whereas extreme truncations, or shortened sequences, are seen in the α arm of Laminins -6-9. Due to a larger than normal α chain, Laminins -10 and -11 are much larger than typical full-sized laminin molecules. Laminin-5 currently remains the only
laminin isoform with truncations in all short arms. When present, the greatest sequence homology is seen in the N-terminal short arm domains (Colognato and Yercheno, 2000). Despite the unique features of each laminin isoform, they all share a common feature with the presence of larger globular (G) subdomains at the carboxyl (C) terminal end of the α chain and at the amino (N) terminal regions of the β and γ chains (Kariya et al., 2003).

Laminin-5, formerly known as kalinin, nicelin, epiligrin, or radsin, is comprised of α3Aβ3γ2 chains (Katayama and Sekiguchi, 2004). A variant form of Laminin-5 was recently discovered by Kariya et al. (2004). Named Laminin-5B for its full sized α chain, α3B, it has been associated with some immunological processes like inflammation (Kariya et al., 2004). Laminin-5 is found in the epidermis, oral mucosa, and bone, in addition to the BM of breast epithelium (Plopper et al., 1998). In the role of BM formation, Laminin-5 has been shown to bind to type VII collagen (Colognato and Yercheno, 2000). In vivo, it is a chief substrate for the adhesion of many types of epithelial cells (Kariya et al., 2003). In vitro experiments have shown Laminin-5 to promote adhesion and migration of cells much more strongly than Laminin-1, Laminin-2/4, fibronectin, and vitronectin (Miyazaki et al., 1993; Kikkawa et al., 1994). In normal breast epithelium, Laminin-5 plays a critical role in maintenance as demonstrated by the localization of Laminin-5 in normal rat breast gland (Plopper et al., 1998). Laminin-5 is also an elemental component of the dermal-epidermal junction. Should genetically induced defects modify Laminin-5 function, grave disorders in the basal membrane, such as skin blisters, may arise (Kunneken et al., 2003).
Structurally, Laminin-5 is characterized by a rod-like $\alpha$-helical coiled-coil domain of all three chains (Kunneken et al., 2003). The $\alpha3$ chain of Laminin-5 contains five homologous G subdomains, G1-G5, often called laminin G-like modules (Kariya et al., 2003). In 1988, Sasaki et al. were the first to gather evidence supporting the presence of globular structures at the C terminal end of the $\alpha$ chain. The migratory function of laminin through its G subdomains was first proposed by Engvall and Wewer in 1996. Initially secreted by epithelial cells as a 460 kDa heterotrimer, the $\alpha3A$ and $\gamma2$ chains undergo further proteolysis to yield a 400 kDa form of Laminin-5 (Colognato and Yercheno, 2000). This proteolytic processing generates the mature form of Laminin-5, which contains only G subdomains G1-3, as seen in Figure 1. A spacer sequence connecting the G3 and G4 domain, Gln(1337)-Asp(1338), has been identified as the cleavage site, releasing the G4-G5 fragment (Tsubato et al., 2000; Kunneken et al., 2004). The proteolytic processing of the $\alpha3$ chain in Laminin-5 has been reported to enhance cell migration in invasive carcinomas (Lohi, 2001; Pyke et al., 1995). Because of the migratory promoting activity in normal epithelial cells, it is very likely to provide the same role in carcinomas. Various immunohistochemical experiments have shown a strong correlation between Laminin-5 expression and tumor invasion. Interestingly, Laminin-5 expression is frequently located in invasive epithelial carcinomas, including thyroid tumors, ovarian carcinomas, and breast carcinoma, and is found concentrated at the invading fronts of malignant carcinomas (Katayama and Sekiguchi, 2004).
This processed form of Laminin-5 has been shown to alter the migratory behavior of cells through integrin-mediated binding (Kariya et al., 2003). Localization of Laminin-5 in the epithelial-stromal interface of numerous invasive carcinomas has led scientists to believe that Laminin-5 behaves as a ligand for migrating carcinoma cells. The integrins $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_3\beta_1$ have been identified as receptors for Laminin-5, advocating cell adhesion and migration resulting in the progression of epithelial carcinomas (Lohi, 2001).

**Integrins**

Integrins are cell surface adhesion receptors that are essential mediators and integrators of communication with components of the extracellular matrix (Alghisi and Ruegg, 2006). They are found expressed on many cells, and several integrins are usually expressed together on most cells (Hynes, 1992). Evolutionarily, their role is highly conserved from primitive metazoans like coral and sponges to mammals (Burke, 1999;
Kuphal et al., 2005). Research in varying fields, such as biomedical research and immunology, led to the discovery of integrins in the 1980s. The collective efforts of hematologists and immunologists soon unraveled the mechanisms of platelet aggregation, the characterization of cell surface molecules involved in cell-cell adhesion events, and identification of receptors on fibroblasts and epithelial cells responsible for fundamental biological functions. In 1986, the term “integrin” was coined by Tamkun and Hynes to signify the role of these proteins as a necessary membrane complex in the association linking the ECM and the cytoskeleton. Remarkably, integrins are capable of recognizing short amino acid sequences on exposed loops of their appropriate ligands, like the exemplar RGD sequence on fibronectin (Alghisi and Ruegg, 2006).

Integrin heterodimers are formed by the noncovalent association between α and β subunits. These adhesion molecules bind to proteins of the ECM, members of the immunoglobulin superfamily, and other proteins in a divalent-cation dependent manner. Currently, there are 18 α and 8 β subunits capable of producing 24 different integrins. Slightly larger, the α chain is approximately 140 kDa in size, while the β chain is somewhat smaller. The heterodimer composition largely defines ligand specificity. For example, a receptor for the ECM protein fibronectin is created by the combination of the α5 chain and β1 chains (Alghisi and Ruegg, 2006).

Integrins are categorized into three main subfamilies. The largest is the β1 family which is found on all cell types and mainly links cells to the ECM. Cells expressing members of the β2 and β7 family are primarily on bone-marrow derived circulating cells, and mediate associations with intercellular adhesion molecules (ICAMs), such as E-cadherin and fibrinogen (Kuphal, et al., 2005). Members of the αv family are important
in mammalian organogenesis and are seen expressed on adherent cells, and advocate cell migration and survival through various ligands (Aiyer and Varner, 2005; Kuphal et al., 2005). High expression of the β3 subfamily appears to predict tumorigenicity of malignant melanocytes. Following cell attachment to the appropriate ligand, modifications in cell migration and proliferation result (Kuphal et al., 2005).

The integrin subunits are composed of an extended extracellular domain, a single transmembrane segment, and a short cytoplasmic domain. Structurally, integrin molecules appear as if they are two legs with a head region at the N-terminus (Kuphal et al., 2005). As shown in Figure 2, the binding region is generated through the two extracellular head regions.

Figure 2. Integrin Structure (Aiyer and Varner, 2005).

The N-terminus of the α subunit contains several repeats of 60 amino acids that fold to create a β-propeller arrangement that rests on the divalent cation-binding region of the β subunit. An additional inserted domain, referred to as the I-domain, is found in some α chains and assists in ligand-binding. It is the α subunit that is largely responsible for
determining the ligand specificity of the integrin (Alghisi and Ruegg, 2006). Divalent cations, such as calcium and magnesium, are also found in each integrin α subunit and function to create salt-bridges with the ligand. The β subunit contains four EGF-like repeats, and ligand binding can take place through an I-like domain in the β subunit should the α chain lack an I-domain. Structural analyses have shown that when an integrin molecule is not bound to its ligand, the two extracellular head regions are bent toward the cell membrane in a low-affinity state, and the cytoplasmic tails tend to associate closely with one another. Once an integrin is engaged, the cytoplasmic tails shift from one another to allow association with other proteins, thereby triggering extension of the entire integrin molecule (Mizejewski, 1999). The cytoplasmic tails interact with proteins including vinculin, α-actin, talin, and kinases including focal adhesion kinase (FAK). Focal adhesion plaques are ultimately formed by the association of additional kinases and proteins that connect the integrin to the actin cytoskeleton. The cytoplasmic region of the β subunit has proven to be the principal link between the integrin and cytoplasmic molecules. It is this communication that leads to changes in cell migration, proliferation, adhesion, and survival (Aiyer and Varner, 2005).

Integrin signaling is known to be bi-directional, meaning signaling can occur from outside-to-inside and from inside-to-outside. Cells require sophisticated attachment-detachment signals, so that unwanted adhesion does not lead to inflammation, and appropriate detachment allows cells to undergo mitosis or migration when necessary (Mizjewski, 1999). Integrins are known to promote cell survival through activating assorted kinases including phosphatidylinositol 3-kinase (PI3K), integrin-linked kinase (ILK), extracellular regulated kinase (Erk), and c-Jun N-terminal kinase (JNK).
Activation of PI3K can ultimately lead to the phosphorylation of the serine/threonine kinase Akt (also referred to as protein kinase B, PKB; Alghisi and Ruegg, 2006). Biochemical support shows that ILK directly phosphorylates Akt, thereby fully activating the enzyme (Schatzmann et al., 2004). Signaling via Akt effects cellular processes including glucose metabolism, cell proliferation, migration, and survival. Akt has been shown to advocate tumor progression through the phosphorylation, and therefore inactivation, of proapoptotic factors BAD, BAX, and Caspase-9 (Alghisi and Ruegg, 2006). The transcription factor nuclear factor–kappa B (NFκB) is also positively influenced by Akt. Necessary survival genes are expressed following the phosphorylation of the NFκB inhibitor IκB (Brakebusch et al., 2002). The tumor suppressor protein p53 modulates integrin-mediated signaling through caspase-mediated inactivation of Akt (Mizjewski, 1999). Figure 3 demonstrates activation of the Akt pathway through integrin binding of Laminin-5.

Figure 3. Activation of Akt Pathway (Turner et al., 2008)
Cancer and Integrins

Integrin function is widespread, and because these molecules mediate the ECM influence on cell growth and differentiation as well as cell migration and extravasation, their role in tumor growth and metastasis is obvious (Mizejewski, 1999). Several studies have demonstrated that integrin expression is often modified during malignant transformation. Particularly, the upregulation of specific integrins is correlated with the acquisition of a more metastatic phenotype (Kuphal et al., 2005). Numerous studies have shown integrin expression and distribution in breast lesions and mammary adenocarcinomas to be greater compared to normal breast tissue (Mizejewski, 1999). For example, in 1995, Friedrichs et al. investigated the expression of human α6 integrin in invasive breast carcinomas of 119 women, and found that in 50% of the tumors, α6 was expressed on the majority of cells. This expression unfortunately correlated with reduced survival time. Interestingly, 24% of patients with breast tumors lacking α6 expression survived. Expression levels of α6 were determined in a variety of breast cancer cells by RT/PCR, immunoprecipitation, and flow cytometry. Cells with the highest levels of α6 expression grew the most aggressively and displayed metastatic characteristics upon injection in nude mice. Low levels of α6 expression demonstrated poor tumorigenicity and were non-metastatic in nude mice. Also, cells with high α6 expression produced significantly more lung metastases than the low α6 expressing cell lines (Mukhopadhyay et al., 1999).

Likewise, increased expression levels of α3 have correlated with metastatic melanoma, ovarian carcinomas of various pathological grades, as well as breast cancer (Ahmed et al., 2005). Studies report the integrin α3β1 to promote pulmonary metastasis
(Aiyer and Varner, 2005). Also, preferential adhesion through α3β1 is seen in the basement membrane of normal breast epithelium (Plopper, et al., 1998). The α6 subunit is expressed on all normal epithelia. This particular chain exists in two isoforms, A and B, by alternative splicing and is often found co-expressed on most cell lines. The α6 chain associates with the β1 or β4 subunit and both adhere to the ECM protein laminin (Friedrichs, et al., 1995).

Integrins are found on virtually every cell and tissue, and expression levels increase gradually during adulthood (Mizjewski, 1999). The β1 chain is mostly expressed on lymphocytes and leukocytes and levels typically increase upon antigen stimulation. Through attachment to ECM proteins, β1 is involved in extravasation and migration of activated lymphocytes (Hynes, 1992). The β1 chain can dimerize with ten different α subunits, including α1-α9, and αv. Members of the β1 family can bind numerous components of the ECM such as collagens, fibronectins, vitronectins, and laminins (Brakebusch, et al., 1997). The influence of an altered level of a given integrin on tumor progression has stirred much debate. For example, some studies link tumorigenicity to reduced β1 expression, and others to an elevated expression (Brakebusch et al., 1997). Controversial results have led many scientists to believe that tumorigenicity depends on several cell specific parameters, including the expression levels of different integrins. Also, the ECM component of each tissue is unique, thereby fostering tumor cells with dissimilar integrin expression patterns. Where certain integrins may play a defined role in one tumor, they may play no significant role in another tumor type when the appropriate ligands are not available in the tumor stroma (Kuphal et al., 2005).
Excluding skin cancer, breast cancer is the most common form of cancer among U.S. women, accounting for one in three cancers diagnosed (American Cancer Society, 2008). As already discussed, Laminin-5 and its specific integrin receptors have been shown in various carcinomas, including breast cancer. More specifically, studies concentrated on the adhesive (and migratory) properties of the G3 domain of the α chain of Laminin-5 have revealed a highly significant role for this domain (Kim et al., 2004). A groundbreaking study reported by Kariya et al. (2003) demonstrated that specific deletions or mutations within the G3 domain led to a marked loss of cell adhesion activity with the integrins α6β1, α6β4, and α3β1. The study further suggested that two different sites within G3 were responsible for cell adhesion and migration. In contrast, a study reported by Kim et al. (2004) designated the PPFLMLLKGR peptide sequence within the human G3 domain as the major site for cell adhesion. Substitution mutation experiments further suggested that an arginine (R) residue was critical for adherence of α3β1 expressing cells.

The present study is a continuation of work initiated in our laboratory with the G3 domain of the α3 chain of rat Laminin-5. Four expression vectors containing various lengths of the α3 chain of Laminin-5 were previously constructed, and the pH9 vector was chosen for expression because it contained the sequence for the G3 domain of the Laminin-5 α3 chain (Borick, 2004). The G3 domain was chosen to test for specific adherence to an α6-integrin expressing breast cancer cell line (MDA-MB-435). The recombinant rat Laminin-5 α3 G3 protein was expressed in the yeast system Pichia pastoris. Upon purification of the expressed histidine-tagged recombinant G3 protein, adhesion and proliferation assays were performed. Cellular activity of MDA-MB-435
cells decreased upon increasing concentration of recombinant rat Laminin-5 α3 G3, showing the first report of a negative effect of laminin on the proliferative activity of cancerous cells (Borick, 2004). The addition of an anti-α6 integrin antibody with the recombinant rat Laminin-5 α3 G3 protein exhibited an even further reduced activity of the cancerous cells. This suggested an additive effect of the anti-α6 integrin antibody and the recombinant rat Laminin-5 α3 G3.

The G3 domain in the present study was expressed as a recombinant protein in *E. coli* and used in subsequent biological assays. Although normally a highly glycosylated protein, G3 produced within a prokaryotic system has been shown to adhere to integrins to the same extent as the glycosylated form (Kunnkeken et al., 2004). With this knowledge of G3 activity associated with a prokaryotic protein product, the present study was designed to improve production and recovery of rG3 over that provided by the *Pichia* system. The *Pichia* system had the advantage of large-scale protein production in a batch fermenter, but the expressed His-tagged protein was difficult to recover and purify (Borick, 2004). The expression system used in the present study provided an intact, soluble rG3 that was efficiently recovered for bioassay tests.

Initially expressed as an insoluble protein in BL21 Star™ (DE3) *E. coli* cells, rG3 was solubilized with the addition of an 8 M urea solution (Turner, 2005). Subsequent biological assays were performed with MDA-MB-435 cells to assay for protein activity; however, cell lysis due to high urea concentration prohibited the production of reliable data. In an effort to overcome this problem, several steps were taken to remove the urea. Most published methods suggested eliminating the denaturing agent using dialysis, where a decreasing denaturant gradient was present. The difficulty with this approach occurred
when lowering the denaturant concentration to allow protein folding, while at the same time preventing aggregation. Because the protein is exposed for an extended period of time to an intermediate denaturant concentration, such as 2 to 4 M urea, where the protein is not yet folded but no longer denatured, it made the protein extremely prone to aggregation. Numerous other methods to remove the urea, like using the charged amino acids arginine and glutamic acid in dialysis, were attempted. Unfortunately, most efforts either failed or rendered themselves unreliable. Ultimately, the urea component was controlled for in biological assays to deduce protein activity. Although insoluble, rG3 nevertheless produced significant adhesion and reduced cell viability of MDA-MB-435 cells upon treatment. These results promoted the efforts reported herein to discover improved methods of protein production and solubility. With enhanced solubility, the potency of rG3 greatly surpassed that seen with an insoluble product.
2. MATERIALS AND METHODS

Recombinant G3 Expression

The G3 domain of the rat Laminin-5 $\alpha_3$ chain was generated through polymerase chain reaction (PCR) amplification from the plasmid pHB9 containing a length of the G3 domain previously shown through nucleotide-nucleotide alignments with the $\alpha_3$ chain of rat laminin-5 (Seen in Appendix, Figure A1; Borick, 2004). The G3 cDNA was cloned into the pET100 TOPO cloning vector provided with the Champion™ pET Directional TOPO® Expression kit (Invitrogen Life Technologies, Carlsbad, CA). The pET100 vector permitted high-level isopropylthio-β-galactoside (IPTG) induced gene expression in *E. coli* expressing a T7 RNA polymerase (Seen in Appendix, Figure A2). The vector also included an ampicillin resistance gene to allow for selection of the plasmid and an N-terminal 6X-histidine tag. The G3 cDNA sequence was verified using the ABI Big Dye® Terminator v3.1 Cycle Sequencing kit (Seen in Appendix, Figure A3; Applied Biosystems, Foster City, CA; Turner, 2005).

Instructions provided by the ArcticExpress™ Competent Cell manual (Stratagene, La Jolla, CA) were followed for expression of recombinant G3 (rG3). ArcticExpress™ cells co-express the cold-adapted chaperone proteins Cpn 10 and Cpn 60 from the psychrophilic bacterium, *Oleispira antarctica*. When expressed in ArcticExpress™ cells at lower temperatures of 4-12°C, these chaperonins confer improved protein processing, enhancing the production of active, soluble recombinant protein. First, a transformation reaction was performed by the addition of 10 ng of plasmid DNA into a tube of ArcticExpress™ competent (DE3) cells containing 2 µl of diluted β-mercaptoethanol. The reaction was incubated on ice for 30 min. The cells were then heat-shocked in a
42°C water bath for 20 sec, and then placed on ice for 2 min. Preheated SOC medium was added to the transformation reaction and placed in a shaker incubator at 37°C for 1 hr. The cells were transferred to LB agar plates containing ampicillin for selection of the expression plasmid. Following overnight incubation at 37°C, single colonies appeared, representing successfully transformed cells.

Following the transformation of competent cells, expression of rG3 began with plucking a single colony, and inoculating 1 ml aliquots of LB broth containing gentamycin and ampicillin. Because the ArcticExpress™ cells were genetically engineered to express the chaperone proteins Cpn 10 and Cpn 60 with an expression plasmid that is gentamycin-resistant, this antibiotic is required in the overnight culture. The cultures incubated overnight in a shaker incubator at 37°C. The next morning, 3 ml aliquots of LB broth, containing no antibiotics, were inoculated with 60 µl of the overnight cultures. The remaining 900 µl of overnight culture were stored at –80°C at a 1:1 dilution with 40% glycerol as stock for future inoculations. The 3-ml cultures were incubated at 30°C with shaking for 3 hr. Next, the temperature was lowered to 11.5°C, and the cells were allowed to equilibrate for 10 min before inducing expression of rG3 by the addition of IPTG at a final concentration of 1 mM. Only half of the cultures were induced to express rG3, leaving the other cultures uninduced to serve as negative controls. The cultures were allowed to shake at 11.5°C for 24 hr before the cells were harvested.

Once cells were induced for protein expression and harvested, they were placed on ice. To determine successful expression of soluble rG3, the cultures were prepared for separation by SDS-PAGE. First, 20 µl of both the induced and uninduced cultures were
added to 20 µl of 2x SDS gel sample buffer and heated to 95°C for 5 min to facilitate lysing of the cells. Protein samples for both cultures were loaded at a 40 µl total volume onto a 10-20% Ready Gel Precast Gel (Bio-Rad Laboratories, Inc., Hercules, CA) together with a Dual Color Precision Plus Protein Standard (Bio-Rad Laboratories, Inc., Hercules, CA). The gel was run in the Ready Gel System (Bio-Rad Laboratories, Inc., Hercules, CA) at 120 V for approximately 1 hr. Following a 30 min wash in dH$_2$O, the gel was stained with GelCode® Blue Stain Reagent (Pierce Biotechnology, Inc., Rockford, IL) to visualize protein bands.

Once soluble rG3 expression was confirmed, a 250 ml culture was prepared to yield a larger volume of the recombinant protein for purification. The initial ArcticExpress™ (DE3) transformation stored at -80°C was used to inoculate 10 ml of LB with ampicillin and gentamycin and placed in a shaker incubator at 37°C overnight. The following day, 250 ml of LB with no antibiotics was inoculated with the entire 10 ml of overnight culture and grown for 3 hr in a shaker incubator at 30°C. The temperature was then lowered to 11.5°C, and the cells were allowed to equilibrate for 10 min. IPTG was added at a final concentration of 1 mM to induce expression, and the culture was grown at 11.5°C in a shaker incubator for 24 hr before the cells were harvested by centrifugation at 4,600 x g for 10 min at 4°C.

To lyse the cell pellet, each component of the lysis buffer was added individually. First, 10 ml of dH$_2$O was added to re-suspend the cells, followed by 2 ml of 1 M Na$_2$HPO$_4$. Once the cells were fully re-suspended, 200 µl of Halt™ Protease Inhibitor Cocktail (Pierce, Rockford, IL) was added. To aid in cell lysis, 2 ml of 10% Triton X-100, 0.6 ml of 5 M NaCl, and 0.04 ml of 10 M NaOH were added and mixed thoroughly.
Lysis of the cells was facilitated by freezing the sample in liquid nitrogen followed by a short thaw at 42°C and repeated two times. To separate the insoluble protein fraction from the soluble, the cells were centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was harvested and a protein concentration was determined by performing a Bio-Rad Bradford Protein Assay.

**rG3 Purification**

Following cell lysis, the soluble protein fraction was applied to a G50 Sephadex column for separation by size exclusion. As previously demonstrated, the molecular weight of rG3 including the histidine tag is ~27 kDa (Turner, 2005), and with a fractionation range between 1,500 and 30,000 kDa, G50 fine Sephadex proved an appropriate separation medium. To begin, 10 g of G50 fine Sephadex was swelled overnight at 4°C in 200 ml of 0.5 M CH₃COONH₄, pH 7.0. The Sephadex and CH₃COONH₄ buffer were then de-gassed for at least 1 hr to remove air before the column was poured. Next, 50 ml of G50 were poured into a column and allowed to pack for an ultimate height of 20-25 cm. To determine the void volume, 3 mg of blue dextran was dissolved in 2 ml of a buffer containing 50 mM Tris-HCl, 100 mM KCl, pH 7.5. A drop of glycerol was added to the blue dextran and then pippetted slowly onto the column. The void volume was collected and measured in a graduated cylinder. The soluble protein sample containing rG3 was prepared and applied to the column, and after the appropriate volume was voided, 2 ml fractions were collected. To determine which fractions contained the purified protein, an OD reading was taken using a BiotTek plate reader at 280 nm. The rG3 containing fractions were stored at -20°C overnight, and then
freeze-dried to remove the ammonium acetate buffer. The fractions were then re-suspended in phosphate-buffered saline (PBS) and stored at -20°C for future use.

**Western Blotting**

To confirm the identity of the expressed soluble protein as rG3, a Western Immunoblot was performed using an anti-polyHistidine monoclonal antibody. First, both Arctic cell lysate and post-G50 purified protein samples were prepared for separation by SDS-PAGE. A 1:1 dilution of protein sample and 2x SDS gel sample buffer was performed and heated to 95°C for 5 min. Protein samples were loaded onto a 10-20% Ready Gel Precast Gel (Bio-Rad Laboratories, Inc., Hercules, CA) together with a Dual Color Precision Plus Protein Standard (Bio-Rad Laboratories, Inc., Hercules, CA). The gel was run in the Ready Gel System (Bio-Rad Laboratories, Inc., Hercules, CA) at 120 V for approximately 1 hr. Meanwhile, a sheet of Immuno-Blot™ PVDF Membrane and two pieces of 14 x 11 cm filter pads (Bio-Rad Laboratories, Inc., Hercules, CA) were soaked in chilled Western transfer buffer for at least 30 min. Following electrophoresis, the gel was also allowed to equilibrate in cold Western transfer buffer for 30 min. The filter pads, gel, and PVDF membrane were arranged in a sandwich-like fashion, and protein transfer was completed using a Trans-Blot Semi-Dry Transfer Cell. Non-specific proteins were blocked with a 30 min Blotto incubation at RT, followed by alternating washes with PBS and TBS. A 1 hr primary antibody incubation at RT was performed using a monoclonal anti-polyHistidine antibody (Sigma-Aldrich Chemical Co., St. Louis, MO) at a 1:4000 dilution in PBS with 5% goat serum. Following antibody incubation, several 10 min washes with PBS and TBS were performed to remove unbound antibody. Because the antibody was a direct alkaline phosphatase conjugate, the membrane was
immediately developed. The substrate solution was prepared following instructions provided by the BCIP/NBT substrate kit IV (Vector Laboratories, Inc., Burlingame, CA) and applied to the membrane for visualization of specific protein bands.

**Cell Culture**

MDA-MB-435, MDA-MB-231, and MCF-7 cells were used for biological assays. MDA-MB-435 cells were obtained through Dr. J. Price, MD from the Anderson Cancer Center (Borick, 2004). MDA-MB-435 cells are a metastatic human breast carcinoma cell line obtained from a 31-year-old female in 1976. It is a non-estrogen and -progesterone receptor expressing cell line that immediately forms tumors upon injection into mammary fat pads of mice (Chen et al., 2004). However, in 2000, a study published by Ross et al. suggested MDA-MB-435 cells to be of melanocyte origin, due to their similarity to melanoma cell lines. Since then, controversy has surrounded this cell line, as other reports confirm that these cells indeed originated from breast epithelium (Sellappan, et al., 2004). The cells were maintained in Dulbecco’s Modified Eagles’ Medium (DMEM) with 4.0 mM L-glutamine and 4500 mg/L glucose (HyClone, Logan, Utah). The culture medium was supplemented with 10% bovine growth serum, penicillin G (100 units/ml)/streptomycin sulfate (100 µg/ml) and sodium pyruvate (0.11 µg/ml). MDA-MB-231 cells are a metastatic breast cancer cell line obtained from a 51-year old female patient, derived from a pleural effusion removed on October 17, 1974. This invasive cell line displays an epithelial-like morphology and forms mammary fat pad tumors in nude mice. These cells have lost expression of both estrogen and progesterone receptors (Brinkley, BR et al., 1980). MDA-MB-231 cells were maintained in the same medium as MDA-MB-435 cells. MCF-7 cells, also a breast cancer cell line, originated from a 69-
year old female and were established from pleural effusion in 1970. This cell line is positive for the expression of both estrogen and progesterone receptors, and form tumors in nude mice (Dickson, et al., 1986). Not unlike MDA-MB-435 cells, this cell line has also become the topic of much debate. While the origin of these cells has not come into question, the metastatic characteristics are uncertain. Current opinion of this cell line is of weak malignance or complete non-invasiveness (Plopper et al., 1998; Cariati et al., 2008). MCF-7 cells were grown in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml)/streptomycin sulfate (100 µg/ml), and insulin (10 µg/ml). All cell lines were grown in 75 cm² tissue culture flasks (Corning inc., Corning, NY) and maintained at 37°C and 5% CO₂. To prevent overcrowding of cells, they were routinely passaged using 1X Trypsin/EDTA (HyClone, Logan, Utah) which removed the cells from the bottom of the flask. Before cells were transferred into a new culture flask, they were washed at least one time with fresh culture medium and centrifuged at 400 x g for 5 min at 23°C.

Flow Cytometry

To obtain a cell surface profile for target integrin molecules, flow cytometry was performed with the MDA-MB-435, MDA-MB-231, and MCF-7 cells. The following antibodies were used for labeling: rat anti-α6 integrin (GoH3, ICN Biomedicals, Inc.), mouse anti-α3 integrin (CD49, Chemicon International), and mouse anti-β1 integrin (CD29, Chemicon International), along with the proper isotype controls: rat IgG2a (Sigma Chemical Co.) and mouse IgG1 (Sigma Chemical Co.). The cells were collected from culture by a 5 min trypsinization and pelleted by centrifugation at 400 x g for 5 min at 23°C. The cells were washed twice with fresh culture medium and stained with trypan
blue before a viable cell count was performed on a hemacytometer. The cell concentration was adjusted to deliver a final cell concentration of $1 \times 10^6$ cells/tube (100 µl). Each tube then received 10 µg of either primary antibody or isotype control antibody, and was incubated on ice for 1 hr with frequent mixing. The cells were then pelleted by a short 30 sec spin at 14,000 x g in a bench-top micro-centrifuge at RT. Two 1 ml washes with sterile PBS were performed, and the cells were pelleted as stated above. Following the second wash, the cells were re-suspended in 100 µl of the appropriate secondary antibody; either goat anti-rat Ig Fab-specific FITC-conjugated antibody (Fab$_2$ flow cytometry grade antibody, Sigma Chemical Co.) or goat anti-mouse IgG Fab-specific FITC-conjugated antibody (Fab$_2$ flow cytometry grade antibody, Sigma Chemical Co.) and incubated on ice for 30 min in the dark. After incubation, the cells were pelleted and washed twice as stated above. The cells were fixed with 500 µl of 1% paraformaldehyde and incubated on ice for 10 min. After fixation, the cells were pelleted and re-suspended in 1 ml of sterile PBS and stored at 4°C until analysis by flow cytometry (Becton-Dickinson FACS Scan). Histograms were generated for antibody and isotype control stained cells, and were overlaid for comparison to establish positive fluorescence of the antibody stained cells.

**Adhesion Assays**

To test the specificity of purified rG3, adhesion assays were performed with all 3 cell lines according to instructions provided by the CyQuant® Cell Proliferation Assay Kit (Invitrogen Life Technologies, Carlsbad CA). The CyQuant kit is based on the use of a green fluorescent dye, CyQuant® GR dye, that emits strong fluorescence when bound to cellular DNA. Black, clear-bottomed 96-well Corning® plates (Corning Incorporated,
Corning, NY) were coated overnight at 4°C with the purified rG3 protein. The following concentrations were made by dilution in sterile PBS and added in triplicate to wells: 5, 10, 15, and 20 µg/ml, with control wells of 0 µg/ml represented only by sterile PBS. Following overnight coating, all wells were washed twice with sterile PBS and incubated for 1 hr at RT with 1% BSA/PBS. Meanwhile, all cell lines were collected by trypsinization as previously stated for flow cytometry, and a viable cell count was taken after trypan blue staining. Once wells were adequately blocked, cells were plated at 5 x 10^4 cells per well, and the plate was incubated in a 37°C humidified incubator (5% CO_2) for 1 hr. After incubation, all media was removed from the wells, and the plate was frozen at -80°C for at least 2 hr, a significant step for the cell lysing process. Following 2 hr, the plate was allowed to reach room temperature (RT) before the cells were lysed with 200 µl of the CyQuant® GR dye/cell-lysis buffer. The plate incubated at RT for 5 min, protected from light. A BioTek fluorescence microplate reader with appropriate filters for 485 nm excitation and 528 nm emission was used to determine fluorescence values. The values were expressed as fluorescent units and graphed against their appropriate control.

**Viability Assay**

To assay for cellular activity (viability), replicate MTT (3-[4,5-Dimethylthiazol – 2-yl] –2, 5-diphenyltetrazolium bromide) assays were performed with purified rG3 and appropriate controls, including non-transformed cell lysate, transformed cell lysate, or culture medium. Dilutions were made in fresh culture medium to deliver the following concentrations: 2, 4, 6, 8 and 10 µg/ml. One hundred µl of each concentration were added in triplicate to wells of a treated Falcon 96-well tissue culture plate (Falcon,
Franklin Lakes, NJ). All cell lines were collected following the same method described previously. Cells were plated at 1 x 10⁴ per well (100 µl), and the plate was incubated for 24 hr at 37°C in a humidified incubator (5% CO₂). Cells were either treated immediately with rG3, or allowed to adhere for 6 hr before treatment. Four hr prior to the end of incubation, 20 µl of MTT (Sigma-Aldrich Co., St. Louis) was added to all wells. At the end of the 24 hr incubation, the culture supernatant was removed from the wells and 150 µl of dimethylsulfoxide (DMSO) were added to solubilize cells. The plate was shaken for 15 min and absorbance values for MTT were recorded using a BioTek plate reader at dual wavelengths of 570/640 nm. Optical Density (OD) values of dissolved MTT were used to create a stimulation index (SI), which was calculated using the ratio of treatment OD ÷ control OD.

**Photomicrographs**

Photographs of all cultured cell lines treated with rG3 were taken at 3, 6, and 12 hr post rG3 addition to observe any morphological changes. The cells were collected with trypsinization and washing as previously described, and counted on a hemacytometer. The cell concentration was adjusted to 1 x 10⁴ cells (100 µl) and plated in a treated Falcon 96-well tissue culture plate (Falcon, Franklin Lakes, NJ). The rG3 was diluted in culture medium and delivered to each well to give a final concentration of 10 µg/well. The plate was placed on an inverted Olympus microscope, and photographs at each respective time were taken with a Nikon digital camera.
Caspase Assays

All cell lines were assayed for activation of key caspases (Caspase-3/7, -8, and -9), using reagents provided by the Caspase-Glo® Assay kit (Promega). Caspase-Glo® is a luminescent assay designed to generate and detect a chemiluminescent signal upon cell lysis. First, cells were collected from culture by trypsinization and washed as previously stated, and counted on a hemacytometer following trypan blue staining. The cell concentration was adjusted to $1 \times 10^4$ cells (50 µl) and plated in triplicate in wells of a non-sterile, opaque 96-well Costar plate (Corning Inc., Corning, NY). Cells were either added in conjunction with rG3 or adhered for 6 hours before rG3 treatment. Purified rG3 was diluted in culture media and 50 µl was delivered to each well, to yield a final concentration of 10 µg/well. The plate was then incubated for 12 hrs at 37°C in a humidified incubator (5% CO$_2$). After incubation, 100 µl of Caspase-Glo® reagent was added to each well, including blank, negative, and treated wells. The plate was shaken at 500 rpm for approximately 2 min and allowed to incubate at RT for at least 30 min. Luminescence values were measured using a BioTek plate-reading luminometer. The caspase activity was expressed as relative luminescence units (RLU).

Akt Western Blot

To determine specific signaling events, the phosphorylation of the serine/threonine kinase Akt (protein kinase B) was observed in MDA-MB-231 cells. Cells were trypsinized and counted as previously stated and plated at $3 \times 10^5$ cells/ml in a Falcon 6-well tissue culture plate (Falcon, Franklin Lanes, NJ). The cells were allowed to adhere overnight at 37°C in a humidified incubator (5% CO$_2$). The following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding
volume of medium. Following a 3 hr treatment, cells were lysed with 100 µl of 1X SDS sample buffer. The cells were transferred to a microcentrifuge tube and pelleted with a 30 sec spin in a microcentrifuge. To reduce sample viscosity, the cells were sonicated for 15 sec to shear DNA. In preparation for protein separation by SDS-PAGE, a 40 µl sample was heated to 95°C for 5 min. Treated and untreated lysate samples were loaded onto a 10-20% Ready Gel Precast Gel (Bio-Rad Laboratories, Inc., Hercules, CA) together with a biotinylated protein ladder (Cell Signaling Technology, Inc.). The gel was run in the Ready Gel System (Bio-Rad Laboratories, Inc., Hercules, CA) at 120 V for approximately 1 hr. Meanwhile, a sheet of Immuno-Blot™ PVDF Membrane and two pieces of 14 x 11 cm filter pads (Bio-Rad Laboratories, Inc., Hercules, CA) were soaked in chilled Western transfer buffer for at least 30 min. Following electrophoresis, the gel was also allowed to equilibrate in cold Western transfer buffer for 30 min. The filter pads, gel, and PVDF membrane were arranged in a sandwich-like fashion, and protein transfer was completed using a Trans-Blot Semi-Dry Transfer Cell. Non-specific proteins were blocked with a 1 hr Blotto incubation at RT, followed by alternating washes with PBS and TBS. An overnight primary antibody incubation at 4°C was performed with the rabbit antibodies phospho-Akt (Ser 473) and Akt (pan; C67E7) at dilutions of 1:2000 and 1:1000, respectively (Cell Signaling Technology, Inc.). Following primary antibody incubation, a 15 min wash with PBS/Tween 20 was performed to remove unbound antibody. Next, a secondary anti-rabbit IgG HRP-linked antibody was added for 1 hr at RT. Protein detection was obtained through the use of LumiGlo® reagent (Cell Signaling Technology, Inc.), a chemiluminescent substrate. The membrane was incubated for 1 min at RT with 10 ml of LumiGlo® and then wrapped in
plastic wrap. Visualization of protein bands was obtained using a FluorChem® FC2 Imaging System (Alpha Innotech Corporation, San Leandro, CA).

**Statistical Analysis**

Analysis of Variance (ANOVA) was performed on all data collected from adhesion, proliferation, and caspase assays using the Statistical Analysis System (SAS, Research Triangle Park, NC). The general linear model (GLM) procedure was used for ANOVA.
3. RESULTS

Following induction of rG3 expression and subsequent chromatography separation, SDS-PAGE analysis was performed and the gel was stained with GelCode® Blue reagent to visualize protein bands. As seen in Lane 2 of Figure 4, rG3 post separation is densely stained at roughly 27 kDa, the calculated molecular weight of rG3 with the histidine tag containing sequence. The transformed cell lysate, (Lane 3), shows strong staining of rG3 among several other higher and lower molecular weight proteins. Lane 4 demonstrates proteins found in non-transformed cell lysate, and no rG3 protein band is visible.

To confirm rG3 in transformed cell lysates, Western Blotting was performed using an anti-polyHistidine monoclonal antibody. Lanes 2 and 3 of Figure 5 show positive staining of protein bands at the predicted molecular weight of rG3. No positive staining was observed in Lane 4, of non-transformed cell lysate.

Prior to conducting cell assays with rG3, the phenotypic expression of cell surface integrin molecules was determined for all three breast cancer cell lines. Flow Cytometry histograms for MDA-MB-435, MDA-MB-231, and MCF-7 cells are seen in Figure 6. All three lines were labeled with antibodies against α3, α6, and β1 integrin cell surface receptors. Positive expression of α3, α6, and β1 integrins was observed on both MDA-MB-435 and MDA-MB-231 cells, while MCF-7 cells were only positive for expression of α6.

Adhesion assays with all three breast cancer cell lines were performed at concentrations of 0, 5, 10, 15, and 20 µg/ml. As shown in Figure 7, MDA-MB-435 and MDA-MB-231 cells displayed comparable adhesion patterns. Both cell lines exhibited a
Figure 4. GelCode® Blue stained SDS-PAGE gel with protein samples following cell lysis and purification. Lane 1 represents the Dual Precision Plus Protein Standard. Lane 2 is rG3 following purification using G50 Sephadex. Lane 3 shows Arctic cell lysate, and Lane 4 shows proteins in non-transformed lysate.
**Figure 5.** Western Immunoblot performed with a monoclonal anti-polyHistidine antibody at a 1:4000 dilution. Lanes 1 and 5 represent the Dual Precision Plus Protein Standard. Lane 2 is rG3 following G50 Sephadex purification, and Lane 3 is Arctic cell lysate. Lane 4 represents non-transformed cell lysate.
**Figure 6.** Flow cytometry histograms for the human breast cancer cell lines MDA-MB-435, MDA-MB-231, and MCF-7. The cells were labeled with anti-α3, -α6, and -β1 integrin monoclonal antibodies. Positively labeled cells are represented by solid peaks, compared with the matching isotype control immunoglobulin, demonstrated by clear peaks.
Figure 7. Adhesion of human breast cancer cell lines MDA-MB-435 (diamond), MDA-MB-231 (box) and MCF-7 (triangle) to varying concentrations of rG3. Cells were allowed to adhere for 1 hour at 37°C to rG3 coated 96-well CyQuant® plates. Cells were lysed with CyQuant® lysis buffer, and relative fluorescence units (RFU) were recorded at 485 nm excitation and 528 nm emission. Fluorescence values were graphed relative to their appropriate negative controls. *, P≤0.05 for differences from respective 0 µg/ml controls for each cancer cell line.
dose-dependent response to rG3. MDA-MB-231 cells initially increased, but soon leveled out, while MDA-MB-435 cells gradually increased, peaking at 20 µg/ml. MCF-7 cells displayed the greatest adhesion, especially at 20 µg/ml.

After observing various levels of adhesion between the three breast cancer cell lines, replicate 24-hour MTT viability assays were performed with 0, 2, 4, 6, 8, and 10 µg/ml concentrations of rG3. (Preliminary viability assays for all 3 cell lines with cell lysate controls can be seen in the Appendix, Figures A4, A5, and A6). As shown in Figure 8, rG3 exhibited a dose-dependent inhibitory effect on the viability of all three cell lines. At 10 µg/ml, the stimulation indices (SI) of all three cell lines were significantly reduced from the control. Incrementally, the cell lines exhibited sensitivity to rG3. These results indicate that although rG3 binds to the α subunit of the integrin heterodimer, this adhesion leads to reduced cell viability at 24 hr of continuous exposure.

Once inhibition of cell viability was observed with MTT assays, all three breast cancer cell lines were treated with 10 µg/ml of rG3 to examine morphological changes associated with presumed apoptosis. Photomicrographs of treated cells were taken at 3, 6, and 12 hr in culture. Seen in Figure 9, at 3 hours, most rG3 treated cells remained rounded, while control wells showed cells adhering to and spreading along the bottom of the wells. At 6 hours, visible signs of apoptosis, such as blebbing and shrinkage, became visible in both MDA-MB-435 and MDA-MB-231 cells, and continued to 12 hours. MCF-7 cells maintained a rounded appearance and increased in granularity over time, showing signs of condensed chromatin in the nuclei. Based on reduced cell viability and morphological signs of apoptosis, rG3 treated cells were assayed for the activation of key caspases including Caspase-3/7, -8, and -9. A chemiluminescent substrate specific for
Figure 8. Viability assays were performed with MDA-MB-435 (diamond), MDA-MB-231 (box) and MCF-7 (triangle) human breast cancer cell lines with varying concentrations of rG3. Optical density (OD) of dissolved MTT was recorded at dual wavelengths of 570/640 nm. Stimulation index (SI) is the calculated ratio of treatment OD ÷ control OD. **, P ≤ 0.01 for differences between denoted means and respective 0 µg/ml controls within each cell line. The denoted means significantly different from the respective controls for each cell line are bracketed and identified in the body of the graph.
Figure 9. Photomicrographs of cultured human breast cancer cells treated with 10 µg/ml of rG3 for 3, 6, and 12 hours at 37°C. As indicated by the arrows, both MDA-MB-435 and MDA-MB-231 cells exhibited blebbing and shrinkage over time. MCF-7 cells remained rounded and increased in granularity with signs of condensed chromatin in the nuclei.
each caspase was added to treated cells following a 12 hour incubation at 37°C. As seen in Figure 10, MDA-MB-435 cells displayed a drastic increase in activity of Caspase-3/7 following treatment with rG3. Caspase-8 and particularly -9 are also elevated. Figure 11 shows comparable results with MDA-MB-231 cells, with elevated caspase activity over those observed in untreated cells. As seen in Figure 12, MCF-7 cells also show all three caspases significantly activated over the levels of control cells. Specifically, the highest level of caspase activation was observed with Caspase-9.

The above biological assays were performed with cells treated simultaneously with rG3 at the start of culture. These experimental conditions mimicked cancerous cells that have detached from a primary tumor, and are free in suspension. To determine whether rG3 was capable of inducing cell death in attached cells, viability assays with adhered cells were performed. Cells were allowed to adhere for 6 hr and then treated with 0, 5, and 10 µg/ml of rG3 for 24 hr. As shown in Figure 13, viability of all three cell lines was inhibited, exhibiting a similar dose-dependent response seen with unattached cells. By 10 µg/ml, the stimulation indices (SI) of all three cell lines were significantly reduced from the control. Again, these results indicate that although rG3 binds to the α subunit of the integrin heterodimer, this adhesion leads to reduced cell viability.

Additional experiments assaying for the activation of Caspases-3/7, -8, and -9 were performed with adhered cells. Cells were allowed to adhere for 6 hours to wells of a 96-well plate before a 12 hour treatment with 10 µg/ml of rG3. Shown in Figure 14, MDA-MB-435 cells displayed a significant increase in activity of Caspase-3/7. Activation of Caspase-8 was not unlike caspase levels observed in untreated cells;
Figure 10. The activity of caspases-3/7, -8, and -9 in MDA-MB-435 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C for 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. **, P ≤ 0.01 for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
Figure 11. The activity of caspases-3/7, -8, and -9 in MDA-MB-231 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C for 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. **, P ≤ 0.01 for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
Figure 12. The activity of caspases-3/7, -8, and -9 in MCF-7 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C for 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. **, P ≤ 0.01 for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
Figure 13. Viability assays were performed with MDA-MB-435 (diamond), MDA-MB-231 (box) and MCF-7 (triangle) human breast cancer cell lines with varying concentrations of rG3. Cells were allowed to adhere for 6 hours before rG3 treatment. Optical density (OD) of dissolved MTT was recorded at dual wavelengths of 570/640 nm. Stimulation index (SI) is the calculated ratio of treatment OD ÷ control OD. **,P ≤ 0.01 for differences between denoted means and respective 0 µg/ml controls within each cell line. The denoted means significantly different from the respective controls for all cell lines are bracketed and identified in the body of the graph.
**Figure 14.** The activity of caspases-3/7, -8, and -9 in adhered MDA-MB-435 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C. Cells were adhered 6 hours and then treated 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. ***, P ≤ 0.01 for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
however, Caspase-9 levels were activated significantly over levels of control cells. Figure 15 shows comparable results with MDA-MB-231 cells, with elevated levels of Caspases-3/7 and -9 over those of control cells, while Caspase-8 failed to show enhanced activation over untreated cells. MCF-7 cells, shown in Figure 16, only showed significant activity of Caspase-3/7. Surprisingly, untreated cells displayed increased activity of Caspases-8 and -9 over treated cells.

To define a specific signaling component, the phosphorylation of Akt was investigated in MDA-MB-231 cells (3 x 10$^5$) following a 3 hr treatment with 40 µg of rG3. As seen in Figure 17, cells treated with rG3 displayed very faint protein bands for both phospho-Akt (Ser 473) and total Akt (pan; C67E7). Concerning the housekeeping protein, β-actin, treated cells showed a strong band, no different from that seen in untreated cells. Cells that did not receive rG3 presented intensely staining protein bands for both Akt antibodies as well as the control β-actin antibody. Based on these results, it is therefore obvious that rG3 is affecting not only the cell’s kinase activity to phosphorylate Akt, but is also degrading the presence of the enzyme entirely.
**Figure 15.** The activity of caspases-3/7, -8, and -9 in adhered MDA-MB-231 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C. Cells were adhered 6 hours and then treated 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. **, P ≤ 0.01 for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
Figure 16. The activity of caspases-3/7, -8, and -9 in adhered MCF-7 human breast cancer cells treated with 0 (white bar) or 10 (black bar) µg/ml of rG3 at 37°C. Cells were adhered 6 hours and then treated 12 hours. Caspase-Glo® substrate reagent, specific for each caspase, was added to both treated and control wells. Caspase activity was recorded as relative luminescence units (RLU) using a BioTek plate-reading luminometer. **, $P \leq 0.01$ for differences in respective caspase activities between untreated and rG3 treated breast cancer cells.
**Figure 17.** Akt Western Blot performed with rabbit antibodies phospho-Akt (Ser 473), Akt (pan) (C67E7), and β-actin. MDA-MB-231 cells were adhered overnight and treated with either 40 µg of rG3 or a corresponding volume of culture media for 3 hr. The left column of the figure shows protein bands from cells treated with rG3. The right side of the figure displays protein bands associated with untreated cells.
4. DISCUSSION

A soluble rG3 protein was produced and recovered for bioassays with MDA-MB-435, MDA-MB-231, and MCF-7 breast cancer cell lines. Positive identification through anti-Histidine Western blotting confirmed the recombinant protein as the G3 domain from the rat Laminin-5 α3 chain. The above mentioned cell lines were selected based on their expression levels of the integrin subunits α3, α6, and β1. Through antibody labeling, flow cytometry revealed all three cell lines as α6 expressing cells. Of interest are MCF-7 cells, for they do not express α3 or β1, as the other two cell lines do.

Although Borick (2004) successfully expressed the recombinant G3 protein and tested its activity, satisfactory recovery and purification from yeast broth was inconsistent. Likewise, additional attempts to improve production of rG3 in an E. coli system only yielded an insoluble protein that required denaturing conditions, and subsequent difficulty in determining protein activity (Turner, 2005). Current production of rG3 in ArcticExpress™ E. coli cells and recovery through G50 Sephadex separation has delivered a much more active protein than that observed by Borick (2004) or Turner (2005), greatly reducing the viability of all three breast cancer cell lines treated with rG3. Furthermore, cell death is the result of specific signaling events initiated by the binding of rG3 to its specific natural ligands, integrins α3 and/or α6.

Integrin-mediated adhesion to Laminin-5 has revealed novel insights into mechanisms regulating tumor growth and cell survival. Cell adhesion to the ECM initiates signal transduction cascades that have been shown to influence cell growth, differentiation, and death (Reddig and Juliano, 2005). Expression of certain integrins on
tumor cells is correlated with increased metastasis (Aiyer and Varner, 2005; Brakebusch et al., 2002; Mizejewski, 1999). Specifically, integrins α3β1 and α6β1 bind to the ECM protein Laminin-5. A heterotrimeric glycoprotein, Laminin-5 is naturally found in the BM of the skin, trachea, and other specialized squamos epithelia, including breast epithelium (Gagnoux-Palarios et al., 1996). Studies have demonstrated that integrin subunits α3 and α6 preferentially bind to Laminin-5 G subdomains found at the C-terminal end of the α chain to advocate tumor growth (Hirosaki et al., 2000; Kuphal et al., 2005). Although the α chain is expressed in Laminin-5, -6, and -7, proteolytic processing is only observed in Laminin-5. Moreover, the five globular domains present on the α chain are unique for each individual chain (Giannelli et al., 1991; Goldfinger et al., 1998). It is this proteolytic processing that leaves the G3 domain exposed, offering a site for tumor cells to exploit adhesion. Evidence for the supporting role of G3 in tumor development has been demonstrated through deletions and mutations within the human G3 domain (Kariya et al., 2003).

For the purpose of this study, a recombinant Laminin-5 α3 G3 protein was expressed in a genetically altered E. coli system. High-level expression of eukaryotic proteins in E. coli can lead to the production of large amounts of incorrectly folded proteins in aggregates known as inclusion bodies. Although easy to purify, recovery of an active protein requires extensive re-folding steps. To overcome this problem, ArcticExpress™ competent cells, genetically altered for enhanced protein processing at lower temperatures, were used for expression of the recombinant G3 protein. The cells co-express the chaperone proteins Cpn 10 and Cpn 60 from the psychrophilic bacterium
Oleispira antarctica. When grown at temperatures of 4-12°C, ArcticExpress™ cells are capable of increasing the yield of active, soluble rG3.

Size exclusion chromatography using G50 Sephadex provided a concentrated rG3 sample for experimental purposes, although some contaminating proteins were present. Western Blotting identified expression of the polyhistidine tagged protein at the predicted molecular weight of 27 kDa. The breast cancer cell lines MDA-MB-435, MDA-MB-231, and MCF-7 were selected based on their varying expression of α3, α6, and β1 integrins. The enriched protein was used for biological assays to assess adhesion and cellular activity following treatment with rG3.

Specificity of rG3 was first addressed by significant cell attachment with all three cell lines. Surprisingly, MCF-7 cells displayed the greatest adhesion, regardless of the lack of α3 expression. Although MCF-7 cells are deficient in α3 expression, it is obvious the dramatic adhesion to rG3 is occurring through α6. Despite this significant adhesion, it should also be noted that MCF-7 cells are considered weakly malignant, while MDA-MB-435 and MDA-MB-231 cells are highly invasive. As a non-invasive cell line, MCF-7 cells function to attach. MDA-MB-435 and MDA-MB-231 cells adhered well to rG3, but because these cell lines are invasive, certain enzymatic systems impede strong adherence, when compared to MCF-7 cells.

Tumor growth and development is directly related to the unique ability of tumor cells to escape cell death. In this study, cell viability was greatly decreased as the concentration of rG3 increased with all three cultured cell lines. Although the G3 domain of Laminin-5 is a known ECM component to which normal and breast cancer cells attach,
the majority of publications concerning this protein, and other members of the laminin family, are primarily centered on its role in cell adhesion and migration. Potential biological effects of Laminin-5, or smaller portions of the protein, have largely been overlooked. For example, a study published in 1998 observing the migratory properties of Laminin-5 on several breast cancer cell lines neglected to assay for prospective effects on cell viability (Plopper et al., 1998). Another study observing the migratory properties of Laminin-8 in human breast tumors narrowly centered research questions on this role alone. Possible biological activity of laminin and other ECM proteins has, however, been explored by those researching ovarian cancer. In 2001, Laminin-1 and smaller peptides from the α1 and β1 chains were assayed for activity. While specific peptides exerted proliferative effects on the development of ovarian tumors through upregulation of the survival genes Bcl-2 and Mdm2, other peptide sequences from the two chains decreased tumor growth and spread (Yoshida et al., 2001). While there has been a concentrated effort to exhaust the adhesive and migratory function of Laminin-5, there are currently no published data reporting reduced cell viability in breast cancer cells treated with Laminin-5 or any of the G subdomains.

Following cell viability assays, subsequent photographs of cultured cells revealed visible morphological changes associated with apoptosis. Cells undergoing necrosis do not display signs of an orderly death like apoptosis, but instead lyse, releasing cell debris. Membrane blebbing and cell shrinkage in conjunction with increased granularity over time with rG3 treatment prompted the quest for specific caspase activation, a hallmark of apoptosis.
Apoptosis, or programmed cell death, controls the lifespan of normal cells, removing excess cells from tissue during normal growth and development. Specifically, apoptosis involves the breakdown of a cell’s structural and signaling components through the activation of several proteases. Chromosomes themselves are also destroyed by nuclear digestion (Reddig and Juliano, 2005). Eventually, the cell is broken down into smaller pieces and removed by neighboring cells or professional phagocytes. Tumor development and metastasis require tumor cells to detach from the matrix or cell-cell anchors that regulate tissue architecture. Detached cells customarily undergo apoptosis when adhesion to the correct substrate is lost (Eccles and Welch, 2007; Mizejewski, 1999). The term “anoikis” was coined to signify the specific apoptosis that occurs in cells that undergo matrix detachment, and was originally described in the epithelial cell line MDCK by Frisch and Francis in 1994. Failure of cells to undergo apoptosis greatly contributes to tumorigenesis, as it helps tumor cells avoid natural elimination (Brakebusch et al., 2002).

Apoptosis is initiated by either the signaling of death receptors or through the mitochondrial release of Cytochrome c, which activates a cascade of caspases (Brakebusch et al., 2002). Caspases are crucial mediators of apoptosis, cleaving conserved aspartic acids. These proteases exist as a hierarchy of initiators and eventual executioners. Among the caspases involved in initiation of the cascade are Caspases-2, -8, -9, and -10. Those responsible for carrying out the final stages of cell death include Caspases-3, -6, and -7. Cell death initiated by cellular stress, known as the intrinsic pathway, involves the activation of Caspase-9. Central to the intrinsic pathway is
permeabilization of the mitochondrial outer membrane and subsequent release of mitochondrial proteins like Cytochrome c. Cytosolic Cytochrome c associates with Apoptotic protease factor 1 (Apaf-1) to activate Caspase-9, a structure now denoted the apoptosome. Caspase-3 is then stimulated to execute the cleavage cascade (Reddig and Juliano, 2005).

Cells treated immediately with rG3 demonstrated increased activation of Caspases -3/7, -8, and -9 over levels observed in untreated cells. Significant activation of Caspase -3/7 was crucial in supporting an apoptotic event, as Caspase-3/7 is associated with the final stages of programmed cell death. More important is the upregulation of Caspase-9. This observation confirms cell death through an intrinsic apoptotic pathway, where Caspase-3/7 is stimulated by the association of Caspase-9 and Cytochrome c. Also noteworthy is the elevated Caspase-8 levels. Although associated with receptor mediated activation of apoptosis through a known death receptor like Fas, Caspase-8 can facilitate the release of Cytochrome c from the mitochondria to amplify the intrinsic caspase cascade (Reddig and Juliano, 2005). Activation of Caspases-3/7 and -9 was also observed with adhered cells. While all three cell lines produced significant activation levels of Caspase-3/7, MCF-7 cells failed to display enhanced activation of Caspases-8 and -9. This observation is in light of different experimental conditions, as opposed to previous caspase assays where rG3 was added simultaneously to cells at the start of culture. The discovery of specific caspase activation was paramount in confirming an apoptotic cell death. Because necrotic cells lack cell signaling events, it can only be concluded that rG3 treated cells are initiating specific events causing cell death.
To establish down-regulated phosphorylation of the kinase Akt following rG3 treatment, only MDA-MB-231 cells were chosen to investigate this occurrence. MDA-MB-435 cells are currently the topic of heated debate concerning their origin. A study published in 2000 by Ross et al. surveying global mRNA expression patterns of the National Cancer Institute’s Developmental Therapeutics Program 60 (NCI 60) cell line panel, found the MDA-MB-435 cell line to express a large number of melanoma associated genes. The study further suggested that the patient from whom this cell line was established most likely suffered from an undiagnosed melanoma. Another report, however, claims the more likely possibility that MDA-MB-435 cells were unintentionally lost due to contamination by another cell line, an unfortunate phenomenon not uncommon in cell culture. The study specifically stated that while the cells may have initially been of breast cancer origin, at some point after their establishment, they were contaminated with M14, a melanoma cell line. Alternatively, the study also suggested a potential labeling error resulting in the misidentification of these cells (Rae et al., 2007). The storm surrounding the origin of these cells is in part persisted by conflicting reports. For example, Sellappan et al. (2004) strongly support the likelihood that MDA-MB-435 cells have undergone lineage infidelity, an event where cells co-express protein markers of different cell lineages. Here, the authors demonstrate these cells as breast epithelial cells, since they maintain the fundamental and functional markers of breast epithelial cells, such as β-casein and α-lactalbumin. Unlike most melanoma cell lines, it was found that MDA-MB-435 cells lack expression of melanocyte-specific markers including MITF and HMB45. Ultimately, this study concluded that this cell line originated from an
advanced breast cancer, and provides a tremendous model for studying highly malignant and dedifferentiated breast cancers. MCF-7 cells are not without controversy, although it is not the origin of these cells that is being disputed, but rather the metastatic capabilities. Recent publications describe MCF-7 cells as a weakly malignant or completely non-invasive cell line (Plopper et al., 1998; Pucci-Minafra et al., 2002; Vercoutter-Edouart et al., 2001).

Because certain characteristics of both MDA-MB-435 and MCF-7 cells have come into question, further signaling investigations were only conducted with MDA-MB-231 cells, as they are currently not under speculation. Following the discovery of caspase-mediated cell death, the phosphorylation of Akt upon rG3 treatment was explored. The phosphorylation of Akt is widely accepted as a promoting event in cancer cell progression. MDA-MB-231 cells \((3 \times 10^5)\) were adhered overnight and then treated with 40 \(\mu\)g of rG3 for 3 hr. Western blotting performed with a specific antibody against the phosphorylated form of Akt shows that cells treated with rG3 succumb to a reduction in phosphorylation of Akt compared to untreated cells. Furthermore, complete degredation of the entire enzyme complex is supported by the lack of total Akt, phosphorylated and unphosphorylated, displayed in rG3 treated cells. Because the housekeeping protein, \(\beta\)-actin, was not affected by rG3 treatment compared to untreated cells, it is obvious that a very essential mediator of cancer cell progression is effectively being shut down following treatment with rG3.

Cell adhesion to the ECM stimulates signal transduction cascades that are known to influence cell growth, differentiation, and death. Particularly, investigations have
revealed the important role of cell adhesion in cell survival and apoptosis. Integrin signaling can regulate apoptotic induction leading to increased cell survival (Brakebusch et al., 2002). Integrins can promote survival and suppress apoptosis through widely accepted kinases such as phosphoinositide 3-kinase (PI3-K), integrin-linked kinase (ILK), and Akt (Mizejewski, 1999). Shown in Figure 18A, upon integrin binding to the intact ECM protein Laminin-5, activated PI3-K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), promoting the phosphorylation, and therefore activation, of the serine/threonine kinase Akt. Activated Akt mediates cell survival through several different pathways, including phosphorylation, and therefore inactivation, of several substrates including a number of proapoptotic molecules such as Bad and Caspase-9. Akt is also responsible for activating the transcription factor NF-κB, via phosphorylation of its inhibitor IκB to induce the expression of survival genes (Redigg and Juliano, 2005).

**Figure 18A.** Activation of the Akt pathway in cancer cells through integrin heterodimer binding to the extracellular matrix protein Laminin-5 for cell survival.
The tumor suppressor function of p53, predominately mediated through its interaction with the ubiquitin ligase Mdm2, is also affected by Akt signaling. Mdm2-regulated shutdown of p53 activity occurs through activation of Mdm2 and consequent down-regulation of pro-apoptotic p53 (Gudkov and Komarova, 2007).

Based on results reported herein, the scheme seen in Figure 18B is envisaged. Because the α subunit has been shown to be largely responsible for specific ligand binding of the integrin heterodimer (Alghisi and Ruegg, 2006), the recombinant G3 domain protein exclusively binds the α3 and/or α6 subunit expressed on breast cancer cells. While the α subunit may be engaged, the heterodimer fails to become fully activated, for the β1 subunit remains in a low affinity state, unengaged, and bent towards the cell membrane. Unable to properly associate with cytoplasmic signaling molecules, the cell survival cascade presented in Figure 18A is compromised. Without phosphorylation of Akt, the cell defaults to the activation of pro-apoptotic molecules, such as Bad and Caspase-9, to set in motion events leading to DNA fragmentation and eventual apoptosis. Likewise, failure to phosphorylate IKK and IκB prevent NFκB from promoting cell survival. Without activation of Akt, phosphorylation events leading to the inhibition of the tumor suppressor p53 are no longer present, allowing this guardian of cell cycle progression to initiate a signaling cascade resulting in apoptosis. Supporting evidence reported herein corroborate the events seen in Figure 18B. Because rG3 treatment essentially inhibits the activation of cells to phosphorylate Akt, pro-apoptotic Caspases-3/7, -8, and -9 are free to initiate cell death. It is therefore specific binding to α3 and/or α6 by rG3 that disrupts cancer cell signaling for survival and growth.
Figure 18B. Inactivation of the Akt pathway following binding of rG3 to the alpha component of the integrin heterodimer leading to induction of apoptosis.

The β1 integrin cytoplasmic domain is known to regulate survival signaling via Akt (Reddig and Juliano, 2005). ILK, a serine/threonine kinase, is known to associate with the cytoplasmic tail of β1, and has been reported to increase phosphorylation of Akt. 

*In vitro* studies on apoptosis in mammary epithelial cells revealed activation of caspases in the absence of a β1-mediated integrin signal (Pinske et al., 2005). Likewise, *in vivo* data using a dominant negative receptor inhibiting β1 function in the mammary gland lead to increased cell death and reduced phosphorylation of Akt and its substrates Bad and Caspase-9. Evidence here also supported the phosphorylation of Akt through ILK (Brakebusch et al., 2002).
Integrins are currently under intense investigation, as they are involved in the resistance of tumor cells to chemotherapy-induced apoptosis, specifically those expressing β1. For example, small-cell lung cancer cells expressing β1 for adhesion to ECM molecules are protected from apoptosis-inducing agents. The β1 subunit reacts to chemotherapy-induced DNA damage by activating phosphotyrosine kinases. Also, it has been observed in breast cancer cells expressing β1 that activation of PI3-K increases the resistance of tumor cells to apoptosis inducing agents (Brakebusch, et al., 2002). In light of these and many more discouraging cancer therapeutics, rG3 offers a novel approach to breast cancer treatment. Beginning in 2004 (Borick), a recombinant G3 domain protein from the rat Laminin-5 α3 chain was expressed. Although crude, the production of this protein and its possible use in cancer therapy was original. In 2005 (Turner), the production was further enhanced and activity increased. Now, further efforts to explore the potential of this recombinant protein have lead to immense discoveries in stimulating the death of breast cancer cells through a specific signaling cascade. It is apparent that this recombinant protein warrants further research, as does the model presented here. Once seen as an accomplice in tumor growth and development, the G3 domain of rat Laminin-5 in an isolated form, free of the assistance provided by G1 and G2, breathes new hope into potential cancer therapies and provides a greater understanding of cancer biology.
Figure A1. Diagnostic gel of PCR amplification of G3 from the plasmid pHB9. Lane 1 represents the 650 bp product, and Lane 2 is the 1 kb standard used for size verification.
Figure A2. Diagnostic gel of PCR amplification with a plasmid specific forward and gene specific reverse primer to verify positive transformants. Lane 1 represents the 1 kb standard. Lanes 2, 4, and 5 illustrate positive transformants, while Lane 3 is negative for successful ligation and cloning into the pET100 TOPO vector.
Figure A3. Nucleic acid and predicted 231 amino acid (M.W. 25 kDa) sequences of the G3 cDNA insert obtained from NCBI Open Reading Frame (ORF) Finder. The 6 histidines appear in bold letters, and the arrow designates the start of the G3 sequence. The * illustrates the stop codon.

156 atgcggggtttcctatcatcatcatcatcatcatatggatgctagcatg
   M   R   G   S   H   H   H   H   H   H   G   M   A   S   M
201 actgggtggacagcaaatgggtctgggtcatctgacgatgacgat
   T   G   G   Q   Q   M   G   R   D   L   Y   D   D   D   D
246 aaggatcatccccttcaccaagaatctgaagaaaccagtggggtt
   K   D   H   P   F   T   K   N   L   K   K   T   S   G   V
291 gtcaggtttgaatgtactgtgggtgtgtaaccaagaatgctcagaa
   V   R   L   N   D   T   V   G   V   T   K   K   C   S   E
336 gactggaagctttgtgcgaaaccgcctcgtttctccaggagggcag
   D   W   K   L   V   R   T   A   S   F   S   R   G   G   Q
381 atgagcttttacaaactttgacgctgcctgacgctctgctcagag
   M   S   F   T   N   L   D   V   P   S   T   D   R   F   Q
426 ctctccctttggttttcagacttttcaaccccagtggtcactgtgac
   L   S   F   G   F   Q   T   F   Q   P   S   G   T   L   L
471 aatcatcagacncggacaagcagcctgcgtgtgtcaccctgagaagat
   N   H   Q   T   R   T   S   S   L   L   V   T   L   E   D
516 ggcacattgagttgagcacttagggagcacagcaacatccccaattttc
   G   H   I   E   L   S   T   R   D   S   N   I   P   I   F
561 aagtctccaggacctacatggacggtttactgcacatgtaatct
   K S P G T Y M D G L L H V S
606 gtaataaagtgcaccctcaggtctcgccttctcatcgatgaccag
   V I S D T S G L R L D D Q
651 gtcctgagaaggagcaggctctctagctctcttaacgcccag
   V L R R N Q R L P S F S N A Q
696 cagtcgctccgcctggaggagttcatttcgagggtgtctatcagc
   Q S L R L G G G H F E G C I S
741 aatgttttagtccaaaaggttttcacaggtccagaagtcctgga
   N V L V Q R F S Q S P E V L D
786 tctggccagtaaatctaccaagaaggatgcattacccctaggaggctg
   L A S K S T K K D A S L G G C
831 cagtttaaacaagccacctttttctttagtttgttttaaatcccaaa
   S L N K P P F L M L F K S P K
876 gagatttaaacaagggccggttttcttttgatttaatcagtag 915
   R F N K G R I F N V N Q *
Figure A4. Preliminary cell viability assay with MDA-MB-435 cells and non-transformed cell lysate (Lys), Arctic cell lysate containing rG3 (LysG3), and rG3 following G50 Separation.
Figure A5. Preliminary cell viability assay with MDA-MB-231 cells and non-transformed cell lysate (Lys), Arctic cell lysate containing rG3 (LysG3), and rG3 following G50 Separation.
Figure A6. Preliminary cell viability assay with MCF-7 cells and non-transformed cell lysate (Lys), Arctic cell lysate containing rG3 (LysG3), and rG3 following G50 Separation.
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