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Kari Tyler

Determining the role of the galectin-3 N-terminus
in tumor-specific apoptosis of human glioblastoma cells

By

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Graduate Division of Biological and Biomedical Sciences

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An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in the Graduate Division of Biological and Biomedical Sciences, Cancer Biology, 2016.

Abstract

Determining the role of the galectin-3 N-terminus in tumor-specific apoptosis of human glioblastoma cells

By Kari C. Tyler

Among malignant cancers, glioblastoma multiforme (GBM) is one of the most aggressive and lethal; patients typically die within a year of diagnosis despite current treatments, which include surgery, chemotherapy and radiation. Because of its treatment-resistant nature, it is important to develop novel, selective therapeutics that can undermine the mechanisms used by this cancer to progress despite an aggressive therapeutic regime. Members of the Van Meir Laboratory at the Winship Cancer Institute of Emory University identified glycoprotein galectin-3 as a candidate for a novel therapeutic option for GBM. A modified form of galectin-3 containing a 37 amino-acid signal peptide sequence at its N-terminus demonstrates powerful, tumor-specific apoptotic ability; notably, against several GBM cell lines. Here we develop additional galectin-3 variants containing distinct N-terminus alterations in an effort to better understand how addition of a signal peptide to galectin-3 enhances its pro-apoptotic ability. More specifically, we aim to understand whether N-terminus elongation or an innate quality of the signal peptide itself enhances the ability of galectin-3 to induce apoptosis of tumor cells. We developed four recombinant galectin-3 variants through PCR-based cloning methods, expression in bacteria and affinity chromatography protein purification. We assessed the effects of each on the cell viability of human GBM cells and found that both full-length-recombinant galectin-3 and a truncated form of the protein lacking several collagen-like domains within the protein's N-terminus led to a slight increase in the GBM cell viability. Elongated galectin-3 had no effect on the viability of the cells and sGal3, engineered with a signal peptide at the N-terminus, led to a drastic decrease in cell viability within 24 hours. These findings suggest that the specific signal peptide addition to sGal3 grants the protein with enhanced apoptotic ability and cannot be mimicked by generic additions to the protein's N-terminus. The variants produced in this work will serve as useful tools for discovering more about the function of the N-terminus of the galectin-3 protein, which may prove useful in further unearthing its roles in cancer.

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Table of Contents

Table of Figures

Introduction

The Clinical Characteristics of Glioblastoma (GBM): Incidence, Survival, Current Treatments, and Future Outlook

Glioblastoma, like all cancers, is a horrible disease. Based on both the incidence of the disease and its poor prognosis, the burden of glioblastoma on our society is significant. In 2015, the National Cancer Institute (NCI) estimated new brain and central nervous system cancer diagnoses to be 22,850, about 1.4% of all cancer diagnoses. NCI also estimated the percentage of deaths resulting from these diagnoses to be 2.6% of all cancer deaths in 2015 (NCI, 2015). Itself, GBM accounts for 82% of malignant gliomas, a category of cancers that makes up the vast majority (80%) of all primary brain tumors with a current diagnosis rate of approximately 17,000 new cases per year (Omuro and Deangelis, 2013).

Not only is the volume of new cases of this disease alarming, but the prognoses are also very poor and cause for concern. The most cited median survival statistic for patients treated by standard-of-care therapy is only 14.6 months post-diagnosis and little improvement has been made in the last 10 years (Stupp et al., 2005). Recurrence is a major problem in combatting GBM and contributes greatly to the low survival rates; despite extensive surgical resection and other therapies, nearly all GBM tumors recur and median survival after recurrence drops to only 6.9 months despite surgical and other medical interventions (Wen and Kesari, 2008).

In addition to the short survival time post-diagnosis for GBM patients, quality of life also presents a major issue for those diagnosed with the disease. Because of the

location of the cancer, neurological and cognitive function can be greatly impaired by both GBM and its treatment resulting in a decrease in health-related quality of life. Health-related quality of life is measured by criteria such as social and mental functioning, motor and visual functioning, fatigue, seizure, headaches and many others that often appear as symptoms of GBM (Taphoorn et al, 2015). While tumor progression contributes most to decreased quality of life for GBM patients, treatment is also a huge factor, which makes decisions to continue treatment that may decrease quality of life a great ethical problem for physicians. Problems such as these demonstrate the continued need for safer therapeutic options for GBM.

Current treatments for GBM most commonly include surgery followed by a course of radiotherapy and adjuvant temozolomide chemotherapy. Temozolomide works as a DNA alkylating agent capable of interfering with DNA replication. Carmustine (BCNU), another alkylating chemotherapeutic agent is also included in standard-of-care treatment and is implanted at the tumor site after surgical resection in the form of a wafer (Adamson et al., 2009). Bevacizumab, an anti-angiogenic, is also used clinically in GBM treatment. Unfortunately, GBM cells prove to be quite resistant to these therapies at recurrence making progression-free survival at this stage very rare. Because of this, the research being done on this disease is, and will continue to be, critical in developing better therapeutics.

Pathological Hallmarks of GBM

GBM has been characterized pathologically by hypercellularity, the presence of small anaplastic cells with nuclei of many different sizes, vascular infiltration in the tumor domain, and necrosis surrounded by an area of dense pseudopalisading cells (Burger et al., 1985). Necrosis is a key factor in distinguishing GBM from lower grade astrocytomas, and often the necrotic region makes up most of the tumor mass (Kleihues, 2000). Most of the cells composing a GBM tumor are poorly differentiated and share many characteristics with stem cells which may be aiding in their tumorigenic mechanisms (Kleihues, 2000). Hypercellularity, suggestive of increased proliferation and decreased apoptosis, is coupled to an increase in the Ki-67 proliferation marker in GBMs compared to lower grade gliomas (Schiff and O'Neill, 2005). In addition, GBM is characterized as a diffuse tumor, which infiltrates itself deep into brain tissue distant from the site of the malignancy, in turn making complete surgical removal of the tumor nearly impossible. Overall, the characteristics of GBM are quite diverse across patients and this reason led to the naming of the disease as "*multiforme*" (Schiff and O'Neill, 2005). The heterogeneity displayed by this cancer, as well as its diffuse nature, represents a huge reason for the failure of many GBM therapeutics. Because of this, generating a therapy that is capable of specifically targeting cancer cells could work as a complementary therapy to surgical resection and would be greatly beneficial in targeting cells deep in the brain tissue, without harm to normal cells.

Genetics of GBM

GBM has been classified into primary and secondary subtypes. Secondary GBMs are defined as those that arise from lower grade gliomas, while primary GBMs arise

spontaneously. Primary GBM is further classified into four subtypes: classical, mesenchymal, neural and proneural, each of which is characterized by different genetic events. Most commonly, GBMs are characterized by genetic mutations involving epidermal growth factor receptor (*EGFR),* p53 (*TP53),* isocitrate dehydrogenase 1 *(IDH1)* and the phosphatase and tensin homolog (*PTEN)*. *EGFR* mutations, overexpression, and amplification most commonly occurs in the classical and neural subgroups of GBM while p53 loss generally occurs in the mesenchymal and proneural subgroups. Overall, the proneural subtype of primary GBMs shares many genetic events with secondary GBM, and this entity is now defined by the presence of IDH mutations. Loss of *PTEN* is common among all GBM subtypes, except for neural (Van Meir et al., 2010).

The classification of GBM allow researchers to develop better-targeted therapies to different subtypes of the disease, and allow clinicians to give better diagnoses and choose better treatment options. These genetic events also serve a prognostic role. For example, patients with IDH1/2 mutations often have better prognoses than those with wild-type IDH (Cohen et al., 2013)

Cancer Glycobiology

Changes in cell glycobiology are extremely common in cancer cells; certain glycan structures are even prognostic for tumor progression. For example, increased expression of galectins, including galectin-3, correlate to tumor progression. Cancer cells nearly always have altered glycosylation patterns compared to normal cells and study of

these cancer-associated carbohydrates has elucidated their roles in oncogenic transformation, tumor progression and metastasis (Moskal, Kroes, Dawson, 2009). For these reasons, it may be beneficial to investigate glycoproteins and oligosaccharides as possible targets for cancer treatment.

Over half of all human proteins are glycosylated, making glycosylation the most common post-translational modification. Not only is it frequent, but glycosylation plays an extremely important role in the communication between a cell and its extracellular environment and may have roles in signal transduction, protein assembly, growth, apoptosis, and others (Moskal, Kroes, Dawson, 2009).

In gliomas specifically, glycosylation seems to play a role in the invasiveness of tumor cells, which may contribute to the diffuse nature of these cancers. Changes in glycobiology of GBM include not only differences in levels of expression of glycoconjugates in tumor versus normal tissue, but also differences in the structure of the sugars expressed. In GBM, simpler glycan structures are observed compared to those in normal brain tissue. Additionally, GM1 and GM3 gangliosides that are highly expressed in GBM are major ligands for galectins, including galectin-3. The changes related to GBM glycobiology impact the interactions of galectin-3 with cell-surface proteins such as integrins leading to changes in cell adhesion and signaling that may promote invasiveness (Moskal, Kroes, and Dawson, 2009).

Galectins and Gliomas

It is known that gliomas display decreased levels of apoptosis, which makes them more resistant to drugs and other treatments that rely on this cell death mechanism (Mercier, 2010). Expression levels of galectin-3 have been found to positively correlate to grade of glioma. For example, galectin-3 has been found to be a marker of early neoplastic proliferation in gliomas (Binh et al, 2013), and galectin-3 staining was highly present in high grade astrocytomas and absent in lower grade tumors (Bresalier et al., 1997). In addition, evidence for galectin-3 in tumor infiltrating macrophages, lymphocytes, and endothelial cells suggest that galectin-3 expression in tumors may not be directly correlated to galectin-3 expression in cancer cells alone, but to cells making up the tumor micro-environment.

The role of galectins in the extracellular-matrix (ECM) is thought to be an important regulatory mechanism in galectin-mediated adhesion and invasion properties. Galectins have been implicated as binding partners of various integrin subunits (Fukushi et al., 2004). Galectin-3 has been shown to bind to $\alpha 1\beta 1$ integrins and, in this way, is thought to disrupt interaction of these integrins with the ECM leading to enhanced invasion ability of cells (Fukushi et al., 2004). In fact, increased galectin-3 expression is linked to more invasive areas of GBM tumors. Galectin-3 interaction with $\alpha 3\beta 1$integrins in endothelial cells may play a role in the angiogenic ability in certain cancers including GBM. In addition, galectin-3 has been shown to interact with NG2, a proteoglycan on pericytes surrounding endothelial cells, leading to an enhancement of angiogenesis in GBMs (Fukushi et al., 2004).

Galectin-3

Galectin-3 is a 26-30kDa protein with lectin activity and has glycan-binding abilities. It is a member of the galectin family, which represents a subgroup of lectins that bind specifically to glycans containing beta-galactoside sugar residues. All galectins are characterized by carbohydrate recognition domains (CRD) that lie at the C-terminus. Galectin-3 consists of three specific domains: an N-terminus leader sequence, a series of nine-collagen like domains, and the carbohydrate recognition domain, which shares homology to other galectins (Figure 1). The N-terminus "tail" of galectin-3, rich in proline and glycine, links the leader sequence to the CRD and makes the protein structurally distinct enough to be singularly categorized into a "chimeric" class of galectins (Figure 2). The long N-terminus tail of galectin-3 has been difficult to characterize by traditional structural determination methods, and thus many of its functions have yet to be elucidated.

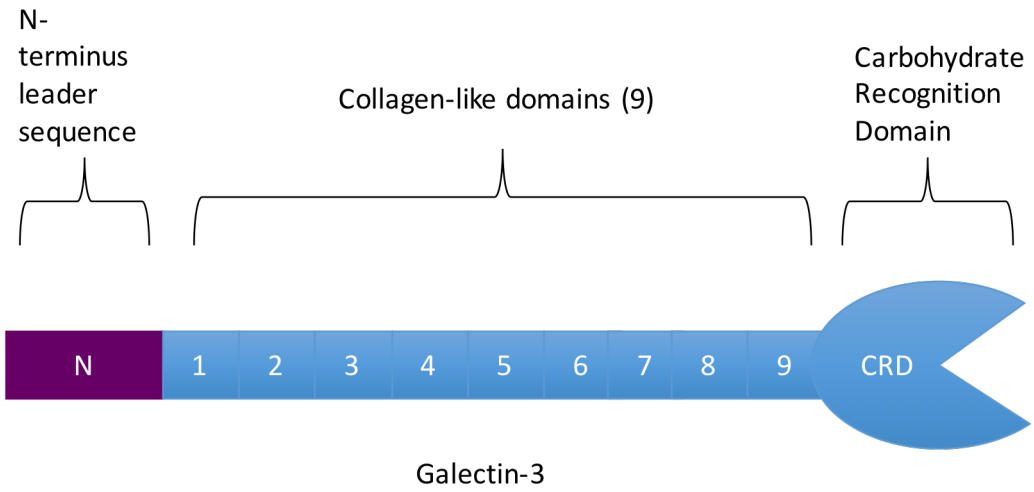

Figure 1. Structural domains of Galectin-3. Galectin-3 consists of three distinct domains, an N-terminus leader sequence, a collagen-like linker region, and a carbohydrate-recognition domain (CRD) at the C-terminus.

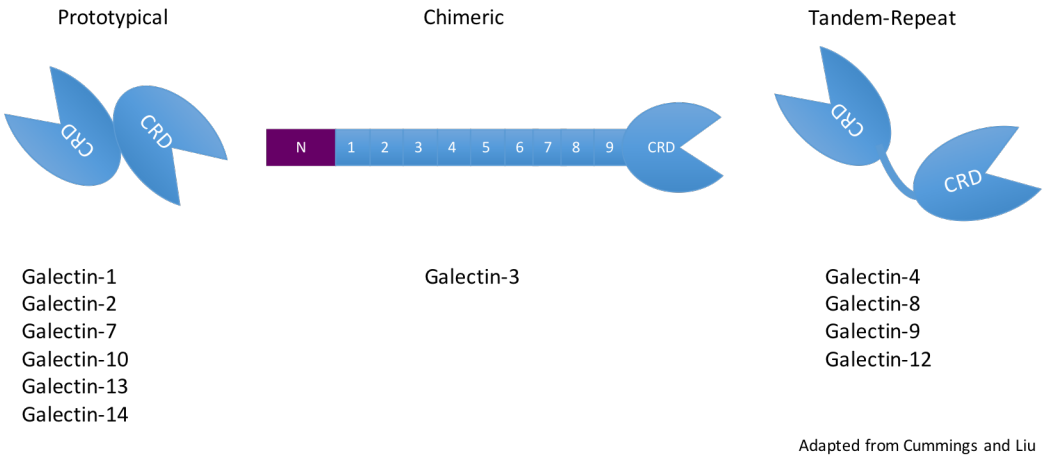

Figure 2. Classes of human galectins. Prototypical galectins contain a single CRD and tend to homodimerize. Galectin-3 is the only member of the chimeric class of galectins, and contains an N-terminal leader sequence, a collagen-like sequence, and a carbohydrate recognition domain at the C-terminus. Tandem-repeat class galectins contain more than one carbohydrate-recognition domain within the peptide sequence.

Conversely, the carbohydrate recognition domain (CRD) has been well characterized and its crystal structure has been determined (Seetharaman, 1998). Its functions cover a broad spectrum of activities including cell-cell adhesion leading to metastasis (Zou, 2005), and oligomerization or "self-association" in what is termed Type-C association (Lepur, 2012). While much clarity exists about the importance of galectin-3's CRD and its function, evidence has been found to suggest that the CRD does not work alone. For example, while the carbohydrate recognition domain is capable of binding the many important ligands of the protein, it lacks the ability to bind multivalent ligands on its own as suggested by the ability of full-length, but not CRD-only truncations of the galectin-3 protein to bind to these multivalent ligands. In addition, several studies have reported that the N-terminal tail may also have an important role in the glycan-binding ability of the galectin-3 protein. In fact, elimination of portions of the N-terminus from the galectin-3 protein can reduce its binding ability to glyco-components of the extracellular matrix (Barboni, Bawumia, Henrick, & Hughes, 2000). In addition, unlike galectins-1 and -2, galectin-3 forms monomers in equilibrium with higher order oligomers at concentrations up to 0.1mM suggesting that the common oligomerization-promoting CRD motifs that exist in galectin-1 and galectin-2 may serve a different purpose in galectin-3 (Barboni, Bawumia, Henrick, & Hughes, 2000).

In addition to the role of different structural motifs of the protein, galectin-3 localization also correlates to its function. Galectin-3 is expressed ubiquitously in normal and tumor cells, localized to both the cytoplasm and nucleus, and has roles in cell proliferation, cell growth, and tumor progression when expressed intracellularly (Table

1). Intracellular Gal-3 is highly expressed in tumor cells from several different cancers and has anti-apoptotic properties that have a significant role in tumorigenesis (Nakahara, 2005). While intracellular Gal-3 may aid tumorigenesis, extracellular Gal-3 may have both pro- and anti-tumor properties (Table 1). Extracellular Gal-3 has been found to induce apoptosis in several cell types including human T-cells, which may be a method by which the protein facilitates tumorigenesis by enhancing immune evasion (Nakahara, 2005). While galectin-3's apoptotic role in T-cells has already been recognized, recent evidence in our lab suggests that the apoptotic ability of galectin-3 may extend to tumor cells as well.

Table 1: Galectin-3 has many different roles in cancer and its function is often dependent on its location. Galectin-3 function is dictated by whether it is inside or outside of the cell. Intracellular galectin-3 has been shown to be anti-apoptotic as well as have roles in pre-mRNA processing, cell proliferation, cell cycle regulation and metastasis. Extracellular galectin-3 has been shown to actually induce apoptosis in several cell types and also has roles in cell proliferation, adhesion and metastasis. This pro-apoptotic quality of galectin-3 is thought to be mediated by its carbohydrate recognition domain interacting with glycans on cell surface proteins. The asterisk beside "pro-apoptotic" denotes the importance of this property of galectin-3 in this work.

Intracellular	Extracellular
Anti-apoptotic (Nakahara et al., 2005; Akahani et al, 1997)	***Pro-apoptotic** (Nakahara et al., 2005; Fukumori et al., 2003)
pre-mRNA processing (Liu et al., 2002)	**Cell proliferation** (Inohara et al., 1998)
Cell proliferation (Moutsatsos et al., 1987; Yang et al., 1996)	**Cell adhesion** (Mattarrese et al., 2000)
Cell cycle regulation (Kim et al., 1999)	**Metastasis** (Iurisci et al., 2000)
Metastasis (Raz et al., 1987)	

Extracellular localization of galectin-3 likely occurs through a non-classical secretion pathway involving vesicular transport, as it lacks a classical signal peptide that would guide the protein through Golgi apparatus-dependent secretion. Unpublished work in the Van Meir laboratory has shown that secreted Gal-3 binds to β1-integrins on nearby cells, and this paracrine signaling leads to cell death by apoptosis. The exact mechanism for this process remains largely unknown: however, they identified caspase-9 and caspase-3 as components of the apoptotic pathway in the affected cells. They also found that this Gal-3 mediated apoptosis specifically targeted cancer cells and left normal cells largely unaffected and hypothesized that tumor cells have increased post-translational glycosylation on β1 integrins that facilitates Gal-3 binding and the induction of cell death. Glycosylation-dependent changes in tumor cells may prove to be useful targets for novel cancer therapeutic options.

Galectin-3 as a potential therapeutic for GBM

Van Meir lab members identified galectin-3 as a possible mediator of a tumor-specific apoptotic effect and developed a variant of galectin-3 that would target the protein through a classical secretion pathway through the Golgi apparatus and the endoplasmic reticulum (ER) of the cell. While galectin-3 is normally secreted through a non-classical, exosome-dependent pathway, the galectin-3 variant that they produced contains a signal sequence at its N-terminus directing it through the classical pathway for its secretion. They produced this protein by transfecting a vector containing a signal peptide sequence derived from the protein tissue plasminogen activator (tPA) into HEK293 cells. While they expected the signal peptide to be cleaved upon maturation through the ER, it instead remained intact leading to a distinct galectin-3 variant with an

additional 37 amino acid peptide sequence at its N-terminus, termed secreted galectin-3 (sGal3).

Treatment of a panel of cancerous and normal cell lines with conditioned media from sGal3-transfected HEK293 cells induced a tumor-specific killing, leaving the viability of normal cell lines largely unaffected. It is known that the C-terminus of galectin-3 is necessary for inducing this cell-death, as the effect is blocked by high concentrations of lactose. Lactose binds to the CRD of the protein and prevents binding to other ligands through competitive inhibition. It is unknown whether the galectin-3 N-terminus may play roles in facilitating this effect, and its manipulation may prove valuable in better understanding the function of the protein as a whole, especially because the signal peptide was added at the protein's N-terminus.

In addition, because of the N-terminal's proposed role in glycan-binding, the addition of the signal peptide to the galectin-3 protein's N-terminal could result in a variant with altered binding affinity that could lead to a change in the potency of sGal3 in the treatment of GBM cells. Likely due to the altered glycosylation pattern known to be associated with cancer cells, the specificity of sGal3-mediated tumor cell death suggests its possible role as a non-toxic therapy to GBM and other cancers.

Scope of the Thesis

This thesis will focus on the investigation of the role of the galectin-3 N-terminus in tumor-specific apoptosis, in regards to both length of the domain and the role of the additional signal peptide through evaluation of recombinant forms of galectin-3 variants with distinct N-terminus alterations in order to better understand the functions of sGal3 as a cancer therapeutic. The signal peptide addition to the galectin-3 protein changes the protein in two ways: (i) through the innate, specific qualities of the tPA peptide addition and (ii) non-specific qualities that are a consequence of added protein length. Studies have demonstrated the role of galectin-3 N-terminus length on binding properties of the protein, suggesting the possibility for alterations of galectin-3 N-terminus length to have other functional consequences. I hypothesize that (i) the apoptotic abilities granted by the tPA signal peptide addition to the N-terminus of galectin-3 are a consequence of N-terminus elongation and not innate qualities of the signal peptide itself; (ii) generic elongation of the galectin-3 N-terminus will lead to enhanced apoptotic ability of the galectin-3 protein; and (iii) truncation of the galectin-3 N-terminus tail will result in lack of apoptotic ability of the protein. In this study, I also aim to produce recombinant versions of full-length galectin-3 and sGal3 in order to develop a reliable, efficient production system to use in further translational studies. Production of a recombinant form of the protein will allow us to study the influence of a pure solution of galectin-3, without the influence of other proteins co-secreted into the conditioned medium. Also, mass production of the recombinant protein is much more efficient and cost-effective than protein isolation from mammalian cells, both of which are characteristics that are extremely beneficial when realistically considering a protein's therapeutic value.

To investigate my hypotheses, I aimed to develop several mutant galectin-3 variants to address whether added protein tail-length or an innate quality of the tPA signal peptide addition to the galectin-3 N-terminus is responsible for sGal3's apoptotic abilities (Figure 3). Due to time constraints only four of the seven planned recombinant galectin-3 variant proteins were produced. By investigating the N-terminus of galectin-3 and developing recombinant versions of the protein, I hope to better evaluate its functional role in the activation of apoptosis, and use this information to design an optimized form of Gal-3 that could become a plausible therapeutic agent for GBM.

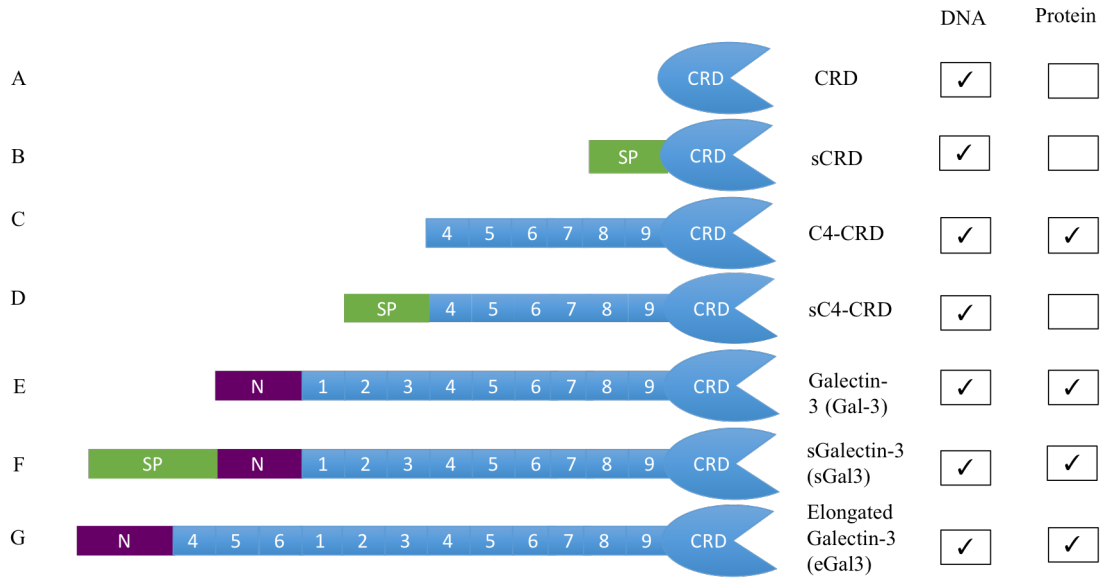

Figure 3. Planned Galectin-3 variants. (A) I aimed to produce CRD-only variant of galectin-3 to investigate the effects of the absence of the N-terminus tail of galectin-3 on the protein's tumor-specific killing. **(B)** I aimed to produce a CRD-only variant with a tPA signal peptide addition at the N-terminus to investigate whether the signal peptide addition was sufficient in granting the galectin-3 CRD with apoptotic abilities. **(C)** I successfully produced a truncated galectin-3 protein (C4-CRD) lacking the N-terminus leader sequence and the first three collagen-like domains to in investigate the effects of N-terminus tail shortening on the protein's apoptotic ability.
(D) I aimed to produce a truncated galectin-3 variant with a signal peptide addition at the N-terminus to assess the role of the signal-peptide addition, when it did not result in an elongated N-terminus of the galectin-3 protein. **(E)** I successfully produced recombinant galectin-3. **(F)** I successfully produced recombinant sGal3. **(G)** I successfully produced elongated galectin-3 (eGal3) to test the effect of a generic peptide addition to elongate the N-terminus tail and compare to the effects of sGal3.

Materials and Methods

Plasmids and Protein Purification

The DNA for Galectin-3 mutants was produced by amplification of sGal3 from a mammalian puCMV-7 vector. Desired segments of the sGal3 DNA sequence were amplified by PCR using primers with added restriction enzyme cleavage sites and ligated into the pGEX2T, in the case of GST-tagged proteins and pET15b in the case of his-tagged proteins expression vectors (see appendix for details). Truncated Galectin-3 lacking the first three collagen-like domains, termed C4-CRD, was amplified by PCR and the resulting 603bp fragment was cloned into a pGEX-2T vector (see appendix for map and sequence). The resulting GST-fusion protein was expressed in BL21 (DE3) *E. Coli* (New England Biolabs) and purified using glutathione agarose beads (Thermo-Fisher Scientific Pierce) (see appendix for details).

The pGEX-2T-Galectin-3 vector was provided by Drs. Liquan Yang and Sok-Hyong Lee (see appendix for map and sequence). The protein was expressed in BL21 (DE3) *E. Coli* (New England Biolabs), induced with 400μM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and purified with glutathione-agarose beads (Thermo-Fisher Scientific Pierce) (see appendix for details).

The pET15b-sGal3 vector (see appendix for map and sequence) provided by Dr. Liquan Yang and Sok Hyong-Lee was transfected in BL21 (DE3) *E.Coli* to produce recombinant His-sGal3. The protein was purified with Nickel-nitrilotriacetic acid (Ni-NTA) agarose beads and eluted from the beads with 400mM imidazole (see appendix for details)

Elongated-Galectin-3 with three additional collagen-like domains was amplified in three regions by overlap extension PCR (i) the N terminus leader sequence (ii)

collagen domains 4-6, and (iii) the full-length Galectin-3 protein so that the final protein contained addiltional copies of collagen-like domains 4-6 between the N-terminus leader sequence and the rest of the protein. Using primers with extensive overlapping sequences, the three fragments were joined together through an overlap-extension PCR procedure (Lee et al., 2010). The 828bp elongated Galectin-3 DNA sequence was cloned into the pET15b vector and expressed in BL21 (DE3) *E. Coli,* followed by purification with Ni-NTA agarose beads and elution by imidazole (see appendix for details).

ELISA

Following protein purification, Galectin-3 proteins were quantified by ELISA (Ray Biotech) according to the manufacturer's instructions.

Cell Culture and Crystal Violet Assay

LN229 GBM cells were plated at 50,000 cells per well in a 24-well cell culture plate in DMEM containing 10% FBS. After cells reached 70-80% confluency, cells were washed and serum-containing DMEM was replaced with serum-free media containing the Galectin-3 variant proteins in concentrations between 0 and 1.25ug/ml or a buffer control. LN229 cells were then assessed for viability after 24 or 48 hours with the crystal violet assay and quantified by absorbance of crystal violet dye at 595nm. Significance was assessed by a paired t-test (see appendix for details).

Results

Galectin-3 can be purified using a GST purification protocol

Because commercially produced proteins may lack functionality, we wanted to produce a recombinant form of Galectin-3 in a native conformation that maintained functionality and could be used to induce tumor cell death. A recombinant production method would allow for efficient production of large amounts of the protein that could be used for our studies, and translationally, would be useful in producing quantities of the protein that would be necessary in a therapeutic setting. In order to produce recombinant Galectin-3 (rGal3), I utilized a GST fusion protein system. Briefly, pGEX-2T-Galectin-3 transformed BL21 *E. Coli* were grown in a 1L batch of Luria Broth and protein production of rGST-Gal3 by the bacteria was induced with IPTG. The protein was then isolated using glutathione-conjugate agarose beads, resulting in a full-length recombinant Galectin-3 protein attached to a GST motif at its N-terminus. The isolated protein was run on a gel and rGST-Galectin-3 could be observed by coomassie staining after gel electrophoresis at 50kDa corresponding to the combined molecular weights of the GST (~25kDa) and the Galectin-3 (~26kDa) proteins (Figure 4). Galectin-3 was later cleaved from both the agarose beads and the GST tag through incubation with thrombin protease, for which there is a cleavage motif between the GST and Galectin-3 proteins. Further, thrombin was removed from the solution using p-aminobenzamidine agarose beads. The quality of the purified rGal3 was assessed with both coomassie blue and western blot. Our recombinant Galectin-3 displayed remarkable purity compared with original whole-cell lysate (not shown) and appeared at 26kDa, in agreement with the molecular weight observed for commercial Galectin-3 (Peprotech) used as a control (Figure 4).

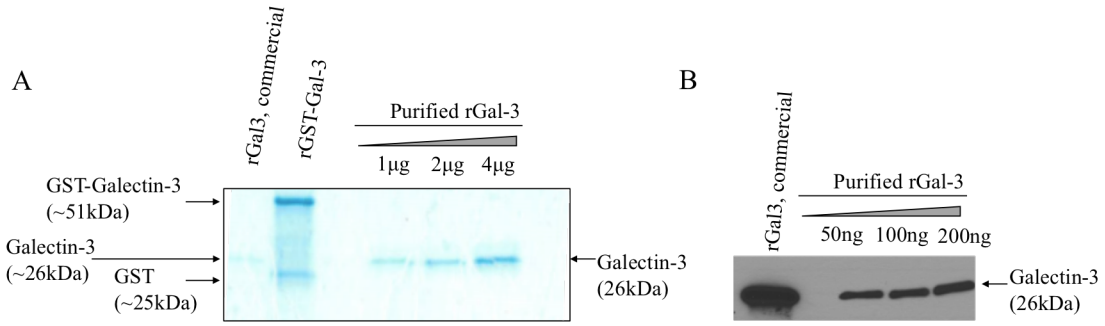

Figure 4. Galectin-3 can be purified through GST-purification. (A) 200ng of recombinant galectin-3 (Peprotech) was loaded into the 12% acrylamide gel and used to approximate the amount of galectin-3 purified from GST. GST-galectin-3 was observed at ~50kDa prior to cleavage of galectin-3 from the GST motif using by the protease thrombin. Galectin-3 cleaved from GST by thrombin was loaded in increasing concentrations approximated to be 1ug, 2ug, and 4ug from left to right. **(B)** Western blot of GST-purified galectin-3 with rabbit-anti-Galectin-3 antibody (Santa Cruz). 1ug of commercial galectin-3 (Peprotech) was loaded onto the 12% polyacrylamide gel. Concentration of Galectin-3 purified from GST was approximated by comparison to the commercial protein and estimated to be 50, 100, and 200ng from left to right.

Secreted Galectin-3 was not successfully purified using GST purification methods

We wanted to produce a recombinant version of sGal3 as well in order to have an easier, more efficient and cost-effective production method for the protein, as with rGalectin-3. pGEX2T-sGal3-transformed BL21 *E. Coli* coding for recombinant GST-tagged sGal3 were used; however, the protein could not be successfully purified in the same manner as galectin-3 using either the same, or a slightly modified protocol. Coomassie Blue staining of GST-sGal3 fusion protein revealed a band, corresponding to an unexpected molecular weight at ~33kDa while the expected size of this fusion protein was ~57kDa (Figure 5) and this problem was not remedied. Purification of sGal3 using the GST-method was abandoned, and another purification system was utilized.

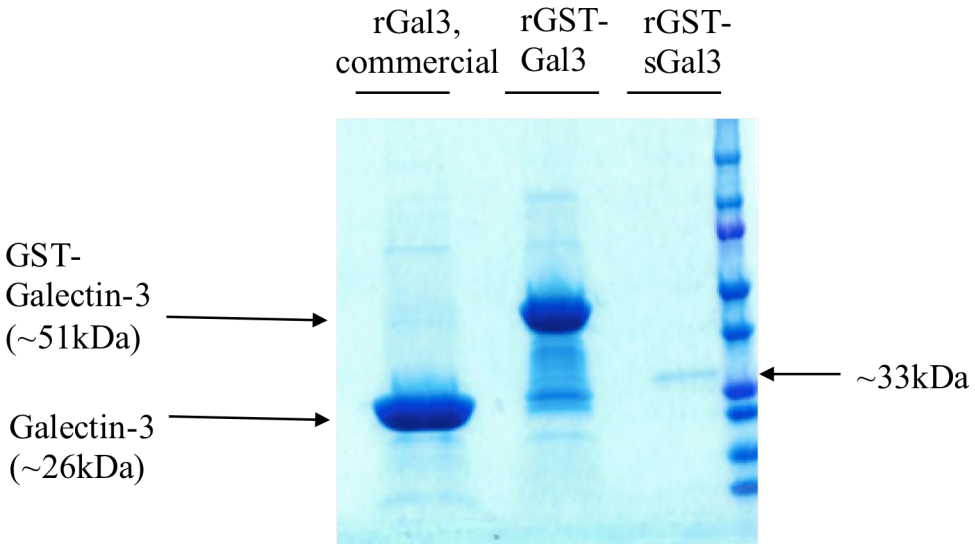

Figure 5. **rGST-sGal3 was not successfully produced in BL21 *E. Coli*.** Commercial galectin-3 and rGST-galectin-3 show prominent bands at equalized protein levels corresponding to expected molecular weights of about ~26kDa (galectin-3) and ~51kDa (rGST-galectin-3) while rGST-sGal3 shows a weak band at approximately ~33kDa, while the expected size was ~57kDa. Size of protein standards are as follows (from bottom to top): 10kDa, 15kDa, 20kDa, 25kDa, 37kDa, 50kDa, 75kDa, 100kDa, and 150kDa.

sGal3 can be purified using a Histidine-tag purification protocol

After failed expression of r-sGal3 using a GST vector system, we moved to a Histidine-tag expression system. The sGal3 cDNA was ligated into a pET15b vector containing an N-terminus Histidine-tag and used to transform BL21 *E. Coli.* sGal3 was purified from the bacterial whole cell lysate using Ni-NTA beads and eluted with imidazole, after which elution fractions were assessed by coomassie gel (not shown) and western blot with both an anti-Galectin-3 antibody and an anti-histidine tag antibody (Figure 6). Recombinant His-sGal3 showed the same molecular weight as that produced in HEK293 cells (~33kDa), suggesting post-translational modification in the mammalian-produced protein is absent, or minimal, which is important when considering recombinant sGal3's potential substitution for the mammalian protein.

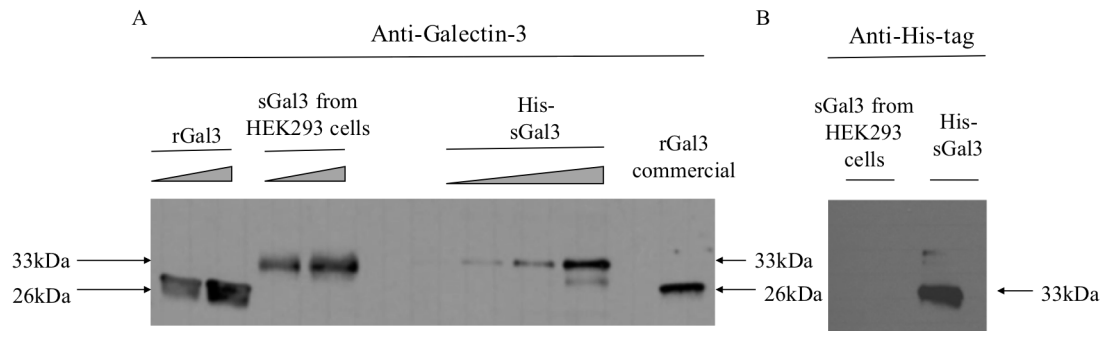

Figure 6. sGal3 can be efficiently purified with the His-tag purification method and detected via western blot. (**A**) rGal3 was purified from *E. Coli* using GST purification techniques, and is shown in increasing concentrations (from left to right). sGal3 was expressed in HEK293 cells to be secreted into the conditioned medium. The sGal3-containing conditioned medium from these cells (sGal3 from HEK293 cells) is seen here at approximately 33kDa and is shown in increasing concentration as well (from left to right). His-sGal3 was loaded onto the gel in increasing concentrations (from left to right) and its size correlates to that of sGal3 from 293 cells. Commercial galectin-3 (rGal3, 200ng, Peprotech) was used to approximate concentration of rGal3, sGal3 and His-sGal3. (**B**) His-sGal3 can also be detected with His-tag antibody at ~33kDa.

Recombinant Galectin-3 does not reduce the cell viability of LN229 GBM cells, while recombinant sGal3 does

In order to assess the effects of rGal3 produced from the GST-expression system, we tested the cell viability of LN229 cells in response to treatment with this protein. While commercially produced recombinant galectin-3 failed to elicit cell death, as described earlier, we wanted to assess whether recombinant galectin-3 purified through GST, could elicit any changes in cell viability in GBM cells. Briefly, LN229 cells were incubated for 48 hours in serum-free DMEM supplemented with 125, 250, 500, 1,000 or 2,000ng/ml of rGal3. After treatment, the cells were assessed via crystal violet or SRB staining to measure cell viability. Treatment with rGal3 led to an increase in cell viability at concentrations up to 1,000ng/ml, as demonstrated by increased SRB uptake compared to control cells. The increase in cell viability was statistically significant, as determined by unpaired t-tests, at concentrations of 125ng/ml ($p<.0001$, $df=22$), 250ng/ml ($p=.0148$,

df=22), 500 (p=.0049, df=22) and 1,000ng/ml (p=.0060, df=22). Cell death was observed at 2,000ng/ml and the difference from control was statistically significant (p=.0032, df=22) (Figure 7B).

We also wanted to assess the effects on cell viability of recombinant His-sGal3, produced from the His-expression system to see if it could mimic the cell death effects of its mammalian-produced counterpart. Unlike rGal3, recombinant sGal3 leads to an obvious decrease in cell viability of LN229 in a dose-dependent manner compared to the imidazole control at concentrations as low as 125ng/ml. This demonstrates an unexpected property of the signal-peptide with roles related to tumor cell viability (Figure 7).

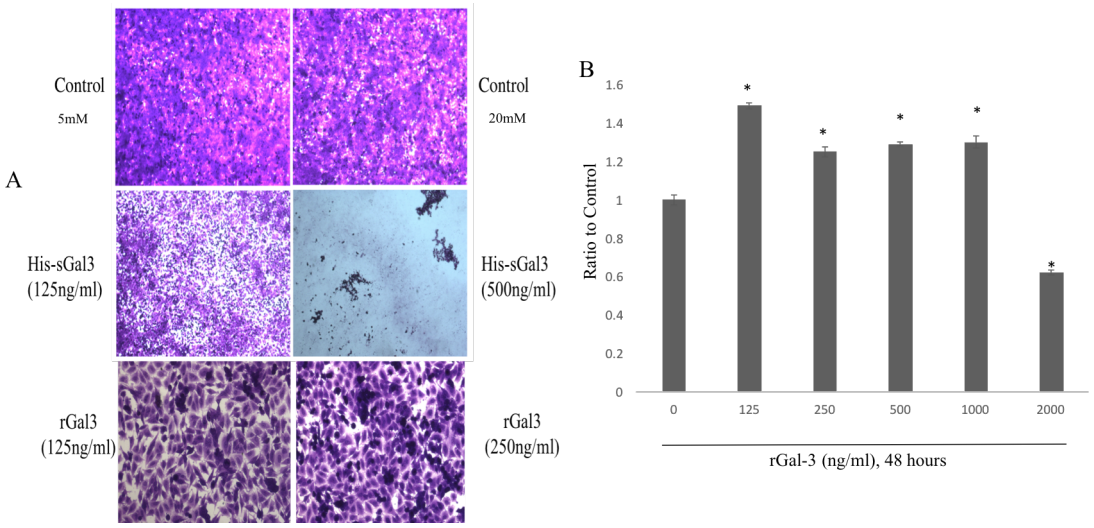

Figure 7. rGal3 increases cell viability of GBM cells (LN229) cells at low concentrations and kills at high concentrations. His-tag sGal3 kills GBM cells (LN229) in a concentration-dependent manner. (A) (***Top***) Control cells were treated with either 5mM imidazole or 20mM imidazole to control for any remaining imidazole in His-sGal3 preparations. High concentration Imidazole was used to elute His-sGal3 from Ni-NTA beads based on the similar affinities of imidazole and histidine for the nickel beads. (***Middle***) His-sGal3 drastically decreased cell viability of LN229 cells following 24-hour treatment. (***Bottom***) rGal3 slightly increased cell viability of LN229 cells following 24-hour treatment. **(B)**LN229 cells were treated with rGal-3 for 48 hours in serum free DMEM at 37°C and assessed by crystal violet staining. Values are expressed as ratio to control absorbance of solubilized SRB stain at 510nm corresponding to number of viable cells.

Truncated Galectin-3 was purified using a GST purification method

To better understand how sGal3 treatment results in decreased cell viability of LN229 cells, I aimed to construct distinct N-terminus alterations to better assess the function of the signal-peptide addition to sGal3 in the protein's ability to affect cell viability. The signal peptide addition grants the protein not only with an additional domain, but also with added length. Because several studies have speculated the role of N-terminus length in galectin-3's function, it was useful to determine the role of the N-terminus length here on the protein's tumor-specific killing abilities. (Kopitz et al., 2013; Barboni et al., 2000; Nangia-Makker et al., 2013).

To better understand the role of the N-terminus length on galectin-3's functions, I constructed both an elongated and truncated form of the protein, with either alteration occurring at the N-terminus (Figure 3). Truncated galectin-3 lacking the N-terminus leader sequence, as well as the first three collagen domains, termed C4-CRD, was produced using a glutathione s-transferase (GST) purification system. Truncated-galectin-3 has a size of approximately 17kDa as assessed by coomassie gel and western blot of the protein using an anti-galectin3 antibody (Figure 8A-B). Following cleavage from GST, the protein could be further purified from contaminating proteins using size exclusion centrifugal filtering with a 50kDa molecular weight cut-off. (Figure 8B).

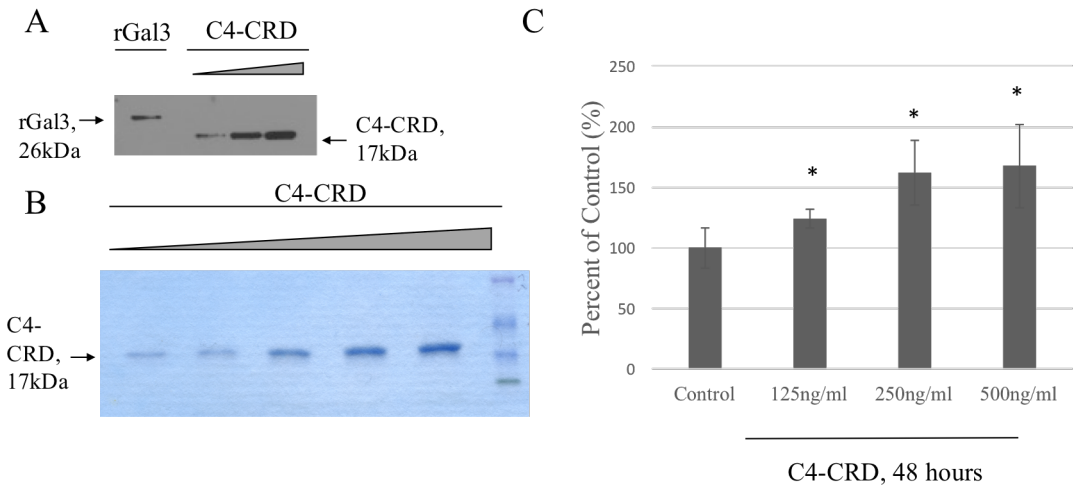

Figure 8. Truncated galectin-3 produced by GST-purification increases LN229 cell proliferation
(**A**) A truncated variant of galectin-3 (C4-CRD) lacking the first three collagen-like domains was produced using a GST-purification system in BL21 *E. Coli* and then assessed by western blot with an anti-Gal-3 antibody targeting the N-terminus of the protein (Santa Cruz, sc-20157).
(**B**) C4-CRD purity was assessed by coomassie stained 12% polyacrylamide gel after centrifugal filtration purification. The protein (17kDa) was loaded onto the gel in increasing concentrations 1.9, 3.8,7.5, 11.3, and 15µg from left to right. Protein standards are as follows (from bottom to top): 15kDa, 20kDa, 25kDa, 37kDa
(**C**) C4-CRD led to a dose-dependent increase in cell viability of LN229 cells during 48hr incubation of the protein in serum-free DMEM. Values are expressed as percentage of control absorbance of crystal-violet stain at 595nm corresponding to number of viable cells.

Truncated Galectin-3 (C4-CRD) increases the cell viability of LN229 cells

To test the ability of truncated galectin-3 to affect the cell viability of LN229 cells, they were treated with 125, 250, or 500 ng/ml of the C4-CRD protein diluted in serum free medium and assessed for cell viability after 48hrs at 37°C by crystal violet staining. Truncated galectin-3 led to a clear dose dependent increase in cell viability as assessed by the absorbance of solubilized crystal violet at 595nm (Figure 8C). Difference from control was statistically significant at 125ng/ml ($p<.0001$), 250ng/ml ($p<.0001$), and 500ng/ml ($p<.0001$)

Elongated Galectin-3 (eGal3) can be purified using histidine-tag purification methods

To understand whether sGal3 confers galectin-3 with apoptotic ability due to the added length to the N-terminus, I built eGal3 which also has added N-terminal length, but from a different peptide sequence. Elongated galectin-3 was constructed to have three additional collagen-like domains within the collagen-like region of the galectin-3 protein to mimic the length of the N-terminus of sGal3. The size of the elongated galectin-3 protein is 275aa, while sGal3 is 287aa. Using Ni-NTA beads to purify the histidine-tagged protein from the bacterial whole cell extract (BWCE), we purified eGal3 in the elution fractions at a molecular weight of approximately 34kDa as assessed by coomassie-stained gel and western blot (Figure 9A-B) similar in size to sGal3 (~33kDa).

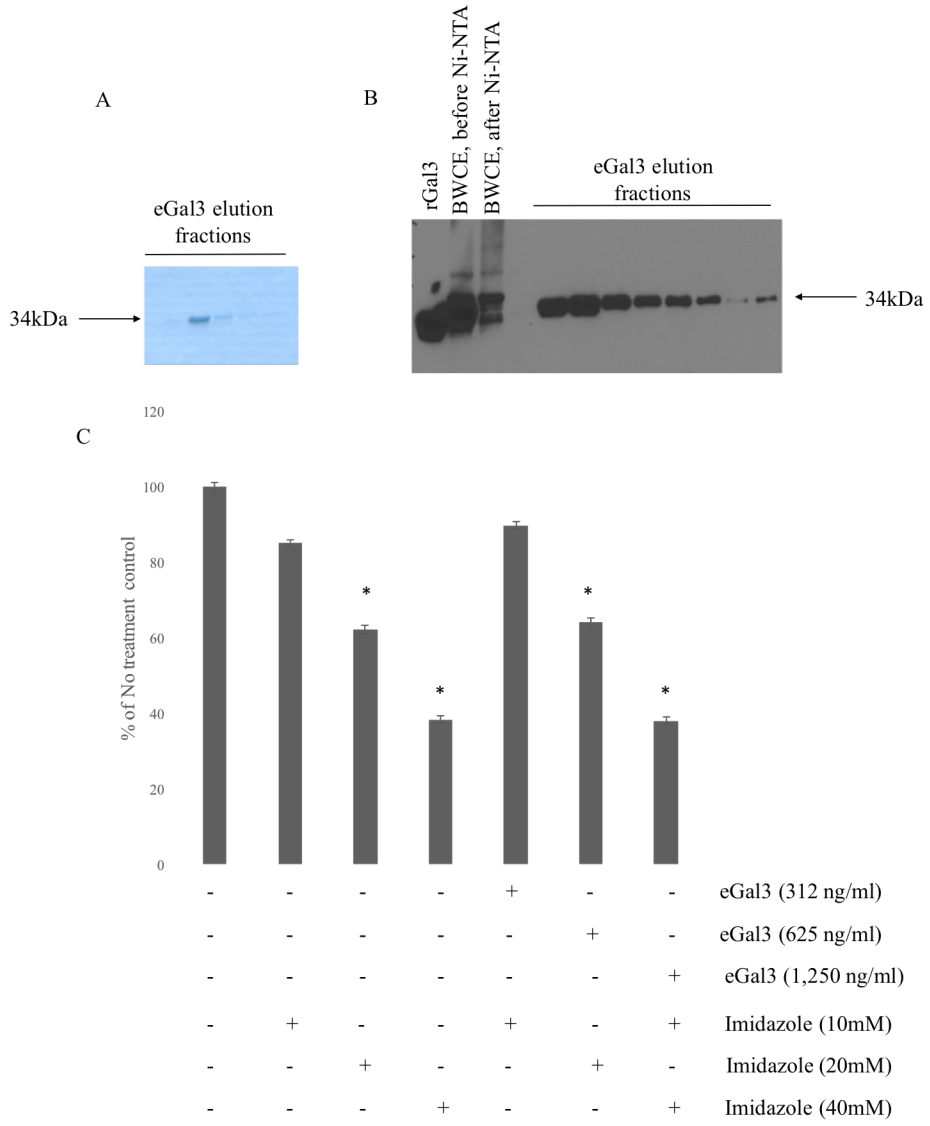

Figure 9. His-tagged elongated galectin-3 has no effect on LN229 cells compared to control (**A**) An elongated variant of galectin-3 (eGal3) containing three additional collagen-like domains was produced using a histidine-tag purification system in BL21 *E. Coli* and then assessed by coomassie staining on a 12% polyacrylamide gel following elution from Ni-NTA beads. The elution fractions were loaded onto the gel in the order eluted. Each elution fraction contains a different concentration of the protein as observed by staining (**B**). eGal3-containing elution fractions were then assessed by western blot with an anti-Gal3 antibody targeting the N-terminus of the protein (Santa Cruz, sc-20157) along with the BWCE and GST-purified rGal3 as controls. (**C**) eGal3 in elution fractions still containing the elution buffer were used to treat LN229 cells in triplicate. eGal3 fractions were diluted to contain 10, 20, or 40mM imidazole, while elution buffer without eGal3 was used as control (Imidazole). Cell viability of LN229 cells during 48hr incubation of the protein in serum-free DMEM was affected by both the elution buffer and the eGal3-containing elution buffer to the same degree ($p=0.8207$). Values are expressed as percent of the no-treatment control with respect to absorbance of crystal-violet stain at 595nm. * denotes statistical significance from no-treatment control.

Elongated Galectin-3 (eGal3) does not decrease the cell viability of LN229 cells compared to imidazole control

Similarly to testing of the other galectin-3 variants, eGal3 was used to treat LN229 cells at different concentrations (312ng/ml, 625ng/ml, or 1.25ug/ml), and the viability of these cells was assessed by crystal violet assay after three days. The cells were also treated with three concentrations of imidazole, which is used to elute His-eGal3 from the nickel beads during the purification procedure. These correspond to the amount of imidazole present in the diluted eGal3 samples used for cell treatment. Both eGal3 and the imidazole controls were statistically different from the no-treatment control for imidazole concentrations 20mM and 40mM ($p=0.0228$; $p=0.0178$ respectively). However, eGal3 did not show any additional effects on cell viability compared to imidazole control ($p=0.8207$). The elution buffer used to purify eGal3 was diluted 10, 20, or 40 fold for control imidazole treatment, as was the eGal3 in elution fractions, in order to equalize imidazole concentrations between the two groups (Figure 9C).

Discussion

In this thesis, I had multiple goals. First, I aimed to produce recombinant forms of both the galectin-3 and the sGalectin-3 protein—a galectin-3 variant which contains an additional signal peptide sequence at its N-terminus—and test the effects of these proteins on the viability of human GBM cells. Secondly, in order to determine whether the signal peptide uniquely granted galectin-3 with apoptotic qualities, or whether similar effects can be mimicked with another peptide, I wanted to test the effects of a generic peptide addition to the galectin-3 N-terminus in granting the protein the ability to elicit cell death of human GBM cells. Lastly, I wanted to further dissect the role of the galectin-3 N-terminus length by testing the effects of an N-terminus truncated variant of galectin-3 on GBM cell viability.

I have shown that recombinant galectin-3 can be produced using a GST purification system in order to better assess the role of extracellular galectin-3 in inducing death of cancer cells. Production of a recombinant form of the protein allowed us to study the influence of a pure solution of galectin-3, without the influence of other proteins co-secreted into the conditioned medium and also to produce the protein in mass. The GST-purification system, allowed us to generate a relatively pure protein from the bacterial whole cell extract, after thrombin cleavage, that enables us to more confidently attribute the effects of endogenous galectin-3 to the protein itself and not to other factors. Effects elicited by the recombinant protein suggest that the protein itself is, in fact, responsible.

I have also shown that recombinant sGal3 can be produced using a histidine-tag purification method. Histidine-tag purification also allows us to generate a pure protein from the bacterial whole cell extract, however this method has drawbacks. Ni-NTA agarose beads are not as specific at targeting only 6xHis-taggeed proteins, but may also pull-down several bacterial proteins containing sequential Histidine-residues. Protein purity was assessed by coomassie stain, but because of the sensitivity of the assay, we may not be able to properly detect proteins that were pulled-down at lower concentrations. While unlikely, due to the inhibition of cell-death effects by lactose presence of other proteins in the recombinant mixture could be responsible for the decrease in cell viability observed with galectin-3 treatment.

The effects of rGal-3 and rsGal3 on the cell viability of LN229 cells were assessed, and rGal-3 was found to stimulate a slight increase in cell proliferation while sGal3 lead to a drastic decrease in viability. These findings are concordant with the existing literature on Gal-3. Documentation of galectin-3's mitogenic potential dates to the late 1990s and has been verified in several cell types (Inohara et al., 1998; Maeda et al., 2003). The effects of recombinant sGal3 mimic the effects of the mammalian, secreted form of the protein, suggesting that the protein can elicit cell death independently of co-secreted protein of HEK293 cells.

Similarly, a truncated form of galectin-3 was also successfully produced using a GST purification system and it also stimulated an increase in cell viability of LN229 cells. This is consistent with results from a 2014 study by Kopitz et al., that shows that galectin-3 lacking the N-terminus leader sequence and the first three collagen domains is similar in functionality to the full-length protein with respect to dissociation to ECM

glycoproteins and competitive inhibition of galectin-1. The behavioral similarities of cells treated by rGal3 or C4-CRD also suggest similar function between the two proteins with regards to effects on tumor cell viability.

A recombinant elongated form of galectin-3 was successfully produced using a His-tag purification system. Elongated galectin-3 did not elicit cell death as sGalectin-3 was able to, supporting the idea that the N-terminus signal peptide addition in sGal3 has unique characteristics that enable the protein to induce tumor-specific apoptosis. However, cell death observed by imidazole compared to no-treatment control of GBM cells did not occur with sGal3 treatment, although an imidazole concentration of 20mM was used in both cases. This could be explained by inconsistencies in elution buffer preparation, but makes it difficult to readily assess the true viability effects of eGal3 on LN229 cells. Preparation of eGal3 with lower imidazole concentrations may be more useful in determining the true functionality of this protein. This tPA signal peptide addition to galectin-3 may not be the only addition that changes the protein's ability to induce tumor-specific cell death and the properties that grant sGal3 with apoptotic ability should be further investigated. Whether it is a specific property of the peptide, extends to all signal-motifs, or can be elicited with a broad spectrum of N-terminus additions is yet to be elucidated. Regardless of this, sGalectin-3's apoptotic role in GBM cell viability makes it clear that the protein's structure and function should continue to be investigated as a potential therapeutic for this devastating disease.

Outside of viability studies, both elongated and truncated galectin-3 will be useful in investigating N-terminus function of galectin-3, especially as it pertains to the collagen-like domains. The N-terminus is thought to be involved in protein

oligomerization. If the collagen-like domains are important in facilitation of protein multimerization, it is plausible that multimers will exist in lower concentrations with the N-terminus truncated protein and multimers may be stabilized in the elongation mutant. Studies investigating the multimerization of galectin-3 have found that truncations of galectin-3 by endogenous proteases such as MMP-2 and MMP-9 inhibit full-length galectin-3 multimerization and biological function (Cardoso et al., 2016).

Comparative studies between rGal-3 and rsGal3 could also be hugely beneficial to understanding Gal-3's multimerization patterns. A 2004 study developed models for two different conformations of galectin-3, one of which has an outstretched N-terminus, and the other has an N-terminus more proximal to the CRD (Ahmad et al., 2004). The authors suggested that the conformer with the outstretched N-terminus facilitated multimerization, especially in the presence of multivalent ligands. It is possible then, that the signal-peptide addition to galectin-3 seen in sGal3 facilitates one or the other conformations, which in turn affects the protein's multimerization and other biological functions.

I was unable to produce the three planned proteins CRD, sCRD, and sC4-CRD. For the latter two proteins, it is possible that the signal peptide causes difficulty in bacterial expression, which also explains the difficulty in isolating sGal3 with the originally planned GST expression system. For the CRD alone protein, it is unclear why expression failed, but is possible that the N-terminus could play a role in galectin-3 stability.

Future Directions

Further research should aim to address some of the drawbacks of this research, including remedying imidazole toxicity interfering with assessment of His-tagged proteins, and using additional concentrations of C4-CRD in order to assess its effects at higher concentrations.

Another important future direction for research is to determine the mode of cell death caused by these proteins. While apoptosis was induced by mammalian sGal3, we need to investigate the cell death mechanism of the recombinant form of this protein and the other galectin-3 variants.

Studies assessing the role of N-terminus alterations on the multimerization of galectin-3 would also be useful future studies, in order to determine the necessary domain for the proteins multimer conformations.

Additionally, further research should be done to evaluate the necessity of the CRD of the various galectin-3 proteins by lactose inhibition or mutagenesis studies.

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Appendix I: Protocols

A portion of this work has been published in *Bio-protocol* (2016).

Tyler, K., Lee, S. and Van Meir, E. G. (2016). Preparation of Recombinant Galectin-3 for Cancer Studies. *Bio-protocol* 6(1): e1696. http://www.bio-protocol.org/e1696

Protocols used for experiments

Protocol for GST-Gal3 and GST-C4-CRD purification (partially adapted from Harper and Speicher, 2011)

Part I.

Materials and Reagents

1. Microcentrifuge tubes (Denville Posi-Click Tubes, catalog number: C-2170)
2. 15 ml conical tubes (Thermo Fisher Scientific, Corning Centristar, catalog number: 05-538-59A)
3. 10 ml serological pipettes (Thermo Fisher Scientific, Corning Costar, catalog number: 4488)
4. BL21 (DE3) competent *E. Coli* (New England Biolabs, catalog number: C2527H)
5. pGEX 2T-Gal-3 vector (kindly provided by Dr. W. Stallcup, UCSD, CA)
6. L-broth (Thermo Fisher Scientific, catalog number: BP1426-2)
7. Glutathione-Agarose Beads (Thermo Fisher Scientific, Pierce™, catalog number: 16100)
8. Protease Inhibitor Tablets, EDTA-free (Thermo Fisher Scientific, Pierce™, catalog number: 88665)
9. Ice
10. Purified Water
11. 10x PBS, diluted to 1x (Thermo Fisher Scientific, Hyclone, catalog number: BP3994)
12. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Gold Biotechnology, catalog number: 12481C25)
13. Tris-Cl (pH 7.5)
14. 150 mM NaCl
15. 0.75% CHAPS powder (AG. Scientific, catalog number: C-1019)
16. 0.75% CHAPS buffer (see Recipes)

Equipment

1. Microfuge (Beckman Coulter, model: Microfuge 16)
2. 37 °C shaker (FormaOrbital Shaker)
3. Shaker (GMI, Lab Companion, model: SK-300 Shaker)
4. Rotating Platform (24 rpm) (Benchmark model: MiniMixer)
5. Vortex (Thermo Fisher Scientific, catalog number: 12-812)
   Note: Currently, it is "LabX, catalog number: 12-812".
6. Centrifuge (Beckman Coulter, model: Avanti J-20 XP)
7. Centrifuge rotor (Beckman Coulter, model: JLA-16.250)
8. Centrifuge (Beckman Coulter, model: Allegra 25R)
9. Centrifuge rotor (Beckman Coulter, model: TS-5.1-500)
10. 250 ml plastic centrifuge bottles (Sigma-Aldrich, Nalgene®, model: B1033)
11. 2 L flask (Corning, Pyrex®, model: No. 4980)
12. 10 ml serological pipettes (Corning, Costar®, catalog number: 4488)

Procedure

1. Transform BL21 bacteria with pGEX-2T-Gal-3 expression vector, pick colonies and verify plasmid DNA with appropriate restriction digests (BamHI and EcoRI). The size of the plasmid is 5,697 bp; the size of the galectin-3 insert is 753 bp.
2. Use one colony to inoculate 3 ml clean, autoclaved Luria-broth and grow overnight at 37 °C at 250 rpm until OD_{600}=1.0.
3. In a 2 L flask, add 1,000 ml water. Measure 25 g Luria Broth and add to water in flask. Autoclave before use (see step 2).
4. Add 0.3 ml of the transformed bacteria to Luria Broth.
5. Grow at 37 °C at 250 rpm for 4-5 h until OD_{600}=0.6.
6. Next, induce bacteria with 0.5 mM IPTG (119.2 mg) and allow growth on shaker at room temperature at 200 rpm overnight for 16-18 h.
7. Spin down bacteria into four, 250 ml plastic centrifuge bottles at 5,000 rpm for 15 min at 4 °C until pelleted. Discard supernatant. Place pellets on ice. (If you wish to stop here, the pellets may be stored at -80 °C until lysis. Prior to lysis, warm frozen pellets in 37 °C water bath for 10 min.)
8. On ice, prepare CHAPS lysis buffer solution.
9. Add two protease inhibitor tablets to 50 ml CHAPS lysis buffer solution and dissolve the tablets into the buffer by vortexing.
10. On ice, add 12 ml CHAPS lysis buffer solution to each pellet.
11. Break up the pellet by pipetting and vortexing until mixture is smooth. Vortex first to detach pellet from the wall of the centrifuge bottle, then use a 10 ml serological pipette to further disrupt the pellet by pipetting up and down to form a homogenous solution.
12. Allow lysis to continue on ice for 30 min. Vortex every five minutes for the duration of the lysis (six times total).
13. While bacterial lysis is taking place, add 2 ml glutathione-agarose bead slurry to 13 ml of purified water in 15 ml conical tube. Mix by inverting tube 2 to 3 times. Allow beads to swell for thirty minutes on ice.
14. Distribute the bacterial lysate to microfuge tubes (about 1.5 ml lysate per tube, approximately 30 tubes). Alternatively, the lysate can be placed into a 50 ml centrifuge tube.
15. Spin microfuge tubes at 14,000 rpm at 4 °C for 15 min. Supernatant should be clear. If using 50 ml centrifuge tube, spin at 3,500 rpm for 15 min at 4 °C. It is recommended, then, that the resulting supernatant be transferred to microfuge tubes and spun at 14,000 rpm for 10 additional minutes.
16. Using a micropipette, separate supernatant from the pellets. Discard pellets.
17. Pool supernatant from all microfuge tubes into two15 ml conical tubes.
18. Gently aspirate excess water from swelled agarose-beads (beads should be settled at bottom of vial; if not centrifuge briefly for 10-20 sec without exceeding 1,000 rpm as high speeds can damage the beads).
19. Add 10 ml CHAPS lysis buffer to beads to equilibrate. Mix by inverting the tube several times. Centrifuge briefly without exceeding 1,000 rpm.
20. Gently aspirate excess buffer from the agarose beads.
21. Add ~1 ml CHAPS lysis buffer to the agarose beads so that bead volume and buffer volume are equal (50% v/v). Mix beads and buffer by inverting the tube several times.

22. Add 1 ml of the bead/buffer solution to each 15 ml conical tube containing bacterial lysis supernatant. Tip of pipette should be cut for beads to fit through.
23. Mix beads and lysis supernatant by inverting the tube several times.
24. Incubate overnight at 4 °C on slowly rotating platform (24 rpm).
25. The next day, spin down supernatant and beads briefly without exceeding 1,000 rpm for ~10 sec at 4 °C to settle beads. Remove supernatant and save as it may still contain some of your protein and may be useful if further analysis is necessary.
26. Wash GST-protein-bound beads by adding 14 ml 1x PBS to each tube. Mix by inverting. If beads are stuck at the bottom of the tube, gently flick the tube with your finger to mobilize the beads.
27. Centrifuge briefly without exceeding 1,000 rpm to allow beads to settle. Remove 1x PBS, and wash again with another 14 ml 1x PBS.
28. Remove 1x PBS. Beads should contain your GST-fusion protein.
29. Assess the integrity and yield of your rGST-Gal-3 or rGST-C4-CRD protein with a Coomassie stained 12% polyacrylamide SDS-PAGE gel and/or Western blot.
30. Store beads in 50% v/v CHAPS buffer solution (~1 ml beads, ~1 ml CHAPS buffer) at 4 °C until further use or proceed with thrombin cleavage to separate galectin-3.

Recipes

1. 0.75% CHAPS buffer
   1.2 g (30 mM) Tris-Cl (pH 7.5)
   2.2 g (150 mM) NaCl
   1.9 g (0.75%) CHAPS powder
   Fill to 250 ml with water

Part II: Protocol for thrombin-mediated cleavage of GST from GST-Gal-3

Materials and Reagents

1. Microfuge tubes (DENVILLE SCIENTIFIC, catalog number: C-2170)
2. 375 U Thrombin (Merck Millipore Corporation, Calbiochem®, catalog number: 605195)
3. Glutathione-agarose beads bound with rGST-Gal-3
4. p-aminobenzamidine agarose beads [50% (V/V)] (Sigma-Aldrich, catalog number: A7155)
5. Tween-20 (Thermo Fisher Scientific, Fisher Bioreagent, catalog number: BP337-500)
6. Ice
7. 10x PBS, diluted to 1x (Thermo Fisher Scientific, Hyclone, catalog number: BP3994)
8. Commercial galectin-3 (Peprotech catalog number: 450-38, 50 ng)
9. Anti-galectin-3 primary antibody (1:800 in PBST with 2.5% BSA, Santa Cruz, 20157)
10. Chicken anti-rabbit secondary antibody (1:10,000 in PBST with 2.5% BSA, Santa Cruz, sc-2955)
11. PBST (0.1% Tween 20) (see Recipes)

Equipment

1. Rotating Platform (24 rpm) (Benchmark Scientific, model: MiniMixer)
2. Microfuge (Beckman Coulter, model: Microfuge 16)

Procedure

1. On ice, add 500 µl PBST to 100 µl of the 50% v/v rGST-Gal-3 bound agarose beads in CHAPS buffer in microfuge tubes (600 µl total volume/tube).
2. On ice, prepare a thrombin solution by diluting 375 U of thrombin into 800 µl PBST.
3. Add 400 µl of thrombin solution to each microfuge tube for a final volume of 1 ml per microfuge tube.
4. Place tubes on rotating platform (24 rpm) and allow incubation overnight at room temperature.
5. The next day, wash p-aminobenzamidine beads in 1x PBS by adding necessary amount of p-aminobenzamidine slurry (250 µl bead slurry per 100 µl rGST-Gal-3 or rGST-C4-CRD bound agarose beads) into a 15 ml centrifuge tube then filling the tube to 15 ml with 1x PBS. Mix by inverting then centrifuge briefly without exceeding 1,000 rpm.
6. Remove 1x PBS then repeat wash with fresh 1x PBS.
7. Remove PBS, then add enough fresh 1x PBS so that beads and 1x PBS form a 50% v/v solution.
8. Centrifuge the bead/thrombin solution at 3,500 rpm for 1 min in tabletop microfuge. Remove supernatant and place into fresh 1.5 ml microfuge tubes.
9. Add 250 µl p-aminobenzamidine agarose bead slurry to each supernatant-containing microfuge tube in order to remove thrombin from the solution.
10. Incubate the p-aminobenzamidine beads with supernatant for 1.5 h at room temperature on rotating platform (24 rpm).
11. Microfuge tubes at 3,500 rpm for ~30 sec.
12. Collect supernatant. Proteins will be in supernatant while thrombin, attached to the beads, will be eliminated with the pellet.
13. Assess supernatant protein composition with a Coomassie-stained SDS-PAGE and Western blot.

Protocol for production and purification of Histidine-tagged sGal3 and eGal3 (adapted from MCLab)

Materials and Reagents

1. Microcentrifuge tubes (Denville Posi-Click Tubes, catalog number: C-2170)
2. 15 ml conical tubes (Thermo Fisher Scientific, Corning Centristar, catalog number: 05-538-59A)
3. 10 ml serological pipettes (Thermo Fisher Scientific, Corning Costar, catalog number: 4488)
4. BL21 (DE3) competent *E. Coli* (New England Biolabs, catalog number: C2527H)
5. pET15b-sGal3 vector (kindly provided by Dr. Sok-Hyong Lee) or pET15b-eGal3 (see plasmid maps)
6. L-broth (Thermo Fisher Scientific, catalog number: BP1426-2)
7. Ni-NTA Agarose Beads (MCLab, catalog number: Ni-NTA300)
8. Ice
9. Purified Water
10. 10x PBS, diluted to 1x (Thermo Fisher Scientific, Hyclone, catalog number: BP3994)
11. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Gold Biotechnology, catalog number: 12481C25)

Equipment

1. Microfuge (Beckman Coulter, model: Microfuge 16)
2. 37 °C shaker (FormaOrbital Shaker)
3. Shaker (GMI, Lab Companion, model: SK-300 Shaker)
4. Rotating Platform (24 rpm) (Benchmark model: MiniMixer)
5. Vortex (Thermo Fisher Scientific, catalog number: 12-812)
   Note: Currently, it is "LabX, catalog number: 12-812".
6. Centrifuge (Beckman Coulter, model: Avanti J-20 XP)
7. Centrifuge rotor (Beckman Coulter, model: JLA-16.250)
8. Centrifuge (Beckman Coulter, model: Allegra 25R)
9. Centrifuge rotor (Beckman Coulter, model: TS-5.1-500)
10. 250 ml plastic centrifuge bottles (Sigma-Aldrich, Nalgene®, model: B1033)
11. 2 L flask (Corning, Pyrex®, model: No. 4980)
12. 10 ml serological pipettes (Corning, Costar®, catalog number: 4488)
13. 10 ml plastic chromatography column (Bio-Rad, catalog number: 7311550)

Procedure

1. Transform BL21 bacteria with pET15b-sGal3 expression vector, plate on ampicillin-containing LB plate, incubate at 37°C overnight, pick colonies, inoculate 5ml culture of LB, incubate at 37°C overnight at 250rpm, miniprep and verify plasmid DNA with appropriate restriction digests (BamHI and NdeI). The size of the plasmid for His-sGal3 is 6,564bp; the size of the sGal3 insert is 864 bp. The size of the plasmid for eGal3 is 6,528bp, the size of the eGal3 insert is 828bp (see "Transformation," "Miniprep," and "Digestion" protocols for details).

2. Use one colony to inoculate 3 ml clean, autoclaved Luria-broth and grow overnight at 37 °C at 250 rpm until OD_{600}=1.0.
3. In a 2 L flask, add 1,000 ml water. Measure 25 g Luria Broth and add to water in flask. Autoclave before use (see step 2).
4. Add 0.3 ml of the transformed bacteria to Luria Broth.
5. Grow at 37 °C at 250 rpm for 4-5 h until OD_{600} is between 0.4 and 0.8.
6. Next, induce bacteria with 0.4 mM IPTG and allow growth on shaker at room temperature at 200 rpm overnight for 16-18 h.
7. Spin down bacteria into four, 250 ml plastic centrifuge bottles at 5,000 rpm for 15 min at 4 °C until pelleted. Discard supernatant. Place pellets on ice. (If you wish to stop here, the pellets may be stored at -80 °C until lysis. Prior to lysis, warm frozen pellets in 37 °C water bath for 10 min.)
8. On ice, prepare phosphate/triton-x lysis buffer solution.
9. On ice, add 12 ml lysis buffer solution to each pellet.
10. Break up the pellet by pipetting and vortexing until mixture is smooth. Vortex first to detach pellet from the wall of the centrifuge bottle, then use a 10 ml serological pipette to further disrupt the pellet by pipetting up and down to form a homogenous solution.
11. Allow lysis to continue on ice for 30 min. Vortex every five minutes for the duration of the lysis (six times total).
12. While bacterial lysis is taking place, add 4 ml Ni-NTA agarose bead slurry to 11 ml of purified water in 15 ml conical tube. Mix by inverting tube 2 to 3 times. On ice, allow beads to settle back to the bottom of the tube.
13. Distribute the bacterial lysate to microfuge tubes (about 1.5 ml lysate per tube, approximately 30 tubes). Alternatively, the lysate can be placed into a 50 ml centrifuge tube.
14. Spin microfuge tubes at 14,000 rpm at 4 °C for 15 min. Supernatant should be clear. If using 50 ml centrifuge tube, spin at 3,500 rpm for 15 min at 4 °C. It is recommended, then, that the resulting supernatant be transferred to microfuge tubes and spun at 14,000 rpm for 10 additional minutes.
15. Using a micropipette, separate supernatant from the pellets. Discard pellets.
16. Pool supernatant from all microfuge tubes into two15 ml conical tubes.
17. Gently aspirate excess water from swelled agarose-beads (beads should be settled at bottom of vial; if not centrifuge briefly for 10-20 sec without exceeding 1,000 rpm as high speeds can damage the beads).
18. Add 6 ml lysis buffer to beads to equilibrate. Mix by inverting the tube several times. Allow beads to settle on ice.
19. Gently aspirate excess buffer from the agarose beads.
20. Repeat steps 18-19
21. Add ~2 ml of lysis buffer to the agarose beads so that bead volume and buffer volume are equal (50% v/v). Mix beads and buffer by inverting the tube several times.
22. Add 1 ml of the bead/buffer solution to each 15 ml conical tube containing bacterial lysis supernatant. Tip of pipette should be cut for beads to fit through.
23. Mix beads and lysis supernatant by inverting the tube several times.
24. Incubate for 2 hours at RT on slowly rotating platform (24 rpm).

25. Allow beads to settle to the bottom of the vial on ice. Remove supernatant and save as it may still contain some of your protein and may be useful if further analysis is necessary.
26. Wash protein-bound beads by adding 14 ml 1x PBS to each tube. Mix by inverting. If beads are stuck at the bottom of the tube, gently flick the tube with your finger to mobilize the beads.
27. On ice, allow beads to settle. Remove 1x PBS, and wash again with another 14 ml 1x PBS.
28. Remove 1x PBS. Beads should contain your Histidine-tag protein.
29. Add 1x PBS so that the beads are suspended in 50%v/v PBS solution.
30. Transfer bead slurry to a 10ml plastic chromatography column.
31. Allow excess PBS to drip from the beads from chromatography into a waste container.
32. Add ~10ml His-tag wash buffer to chromatography column and collect the buffer in 1ml fractions in microcentrifuge tubes until the column stops dripping.
33. Add ~10ml His-tag elution buffer to chromatography column and collect the buffer in 1ml fractions in microcentrifuge tubes until the column stops dripping.
34. Assess the wash and elution fractions for His-sGal3 and assess purity by Coomassie-stained SDS-PAGE and Western blot.

Recipes

His-tag wash buffer
40mM Na_2HPO_4
400mM NaCl
20mM Imidazole

His-tag elution buffer
40mM Na_2HPO_4
400mM NaCl
400mM Imidazole

Phosphate-lysis buffer
40mM Na_2HPO_4
400mM NaCl
0.2% Triton-X
pH8.0

Protocol for Transformation of Bacteria with plasmid DNA (adapted from Addgene and NEB)

Materials and Reagents

1. Competent DH5α or BL21 (DE3) *E. Coli* (New England Biolabs, catalog numbers: C2897H, C2527H)
2. Ice
3. L-broth (Thermo Fisher Scientific, catalog number: BP1426-2)
4. Purified water
5. Agar (Fisher Bioreagents, BP1423-500)
6. Ampicillin (Sigma, catalog number: A0166)
7. Petri-Dishes (Weber Scientific)
8. Disposable Pasteur pipettes (VWR, catalog number: 14673-043)
9. Plasmid DNA
10. SOC media (New England Biolabs, catalog number: B9020S)

Equipment

1. 42°C water bath (Fisher Scientific, Isotemp)
2. Micropipettes
3. 37 °C shaker (FormaOrbital Shaker)
4. 37 °C incubator (Thermolyne, I42300)
5. Bunsen Burner (Humboldt)
6. 1 L flask (Pyrex, catalog number: 5100)

Procedure

1. One day prior to transformation, prepare LB-agar plates. Add 12.5g L-broth (LB), and 7.5g agar powder to a 1L flask. Fill flask to 500mL with purified water.
2. Autoclave according to machine settings.
3. Allow autoclaved LB/agar solution to cool to ~50°C, then add 50mg ampicillin to flask and swirl to evenly distribute.
4. Immediately pour solution into petri dishes (usually ~25 dishes for 500mL LB/agar batch, approximately 20mL per dish).
5. Allow LB/agar solution to set in the petri dishes for about one hour at room temperature.
6. Store plates at 4°C for up to one month.
7. The next day, thaw one 50ul aliquots of either DH5α or BL21 (DE3) competent cells from -80°C on ice for 10 minutes.
8. Add between 1-5ul of purified plasmid DNA or heat-inactivated ligation reaction mix containing up to 100ng of DNA to one 50ul bacterial aliquot and flick the tube gently to mix.
9. Incubate on ice for 30 minutes.
10. Heat-shock transformation tube at 42°C in water bath for 30 seconds for DH5α or 10 seconds for BL21 (DE3) *E. Coli.*
11. Return transformation tubes to ice for five minutes.

12. Add 950ul SOC media to the transformation tube and incubate at 37°C, 250rpm in shaker for one hour for outgrowth. While bacteria are shaking, warm LB-amp plates to 37°C in incubator.
13. Dilute 100ul of the bacteria from outgrowth step 1:10.
14. Plate 100ul of bacteria of non-diluted bacteria onto an LB amp plate. Working near a flame for sterility, pipette the bacteria onto the center of the warmed plate. Create a bacterial spreader with a Pasteur pipette by placing the pipette over Bunsen burner flame at two points, about an inch apart, so that it bends into an open triangular shape. Spread bacteria using this spreader, avoiding the outer 1cm of the plate.
15. Plate 100ul of diluted bacteria onto an LB amp plate.
16. Place the plates upside down in 37°C incubator and incubate overnight.
17. The next day, check for transformants.

Protocol for Generation of cDNA for all Galectin-3 variants by Polymerase Chain Reaction

Materials and Reagents

1. 0.2ml PCR tubes (Bio-Rad, catalog number: TWI0201)
2. 2X PCR MasterMix (New England Biolabs, catalog number: M0271L)
3. Template DNA containing sGal3 (provided by Dr. Bing Yu)
4. Primers, each diluted to 10uM.
5. Agarose (Bio-Rad, catalog number: 1613100)
6. 50X TAE buffer diluted to 1X (Fisher Scientific, catalog number: BP13324)
7. Water
8. Ice
9. Ethidium Bromide (10mg/ml) (Bio-Rad, catalog number: 1610433)
10. DNA ladder (New England Biolabs, catalog number: N0467S)
12. PCR purification kit (Thermo-Fisher, catalog number: K0701)

Equipment

1. Thermocycler (Biometra)
2. Transparent Gel Tray (Bio-Rad, catalog number: 1704436)
3. Gel Caster (Bio-Rad, catalog number: 1704422)
4. 8-well Gel Comb (Bio-Rad, catalog number: 1704463)
5. Horizontal Gel Electrophoresis system (Bio-Rad, catalog number: 1704406)
6. 250ml Flask (Pyrex, catalog number: 4980)
7. UV Gel Imaging system (Syngene, Ingenius 3)

Procedure

1. On ice, combine 1ul of each primer, up to 1ug of template DNA, 25ul PCR MasterMix then add water so that the final volume is 50ul in a 0.2ml PCR tube.
2. Tap the tube on the benchtop to settle contents to the bottom of the tube.
3. Place the tube into a thermocycler and set the cycler to perform one five-minute cycle at 95°C, followed by 29 cycles of 95°C for 30 seconds, 52°C for 45 seconds, and 72°C for 45 seconds, followed by one final five-minute cycle at 72°C.
4. During PCR cycling, prepare agarose gel for electrophoresis by combining 0.5g agarose with 50ml 1x TAE buffer in a 200ml flask.
5. Microwave the agarose solution on high for one-minute.
6. Cool slightly under cold water until flask is comfortable to touch.
7. Add 3ul of Ethidium Bromide to agarose solution and swirl to mix.
8. Tightly secure gel tray into gel caster and pour agarose solution into tray. Place 8-well comb into the solution and into notches of gel tray for stability.
9. Allow the gel to set at room temperature for at least 20 minutes.
10. Following PCR cycling, remove the comb from the gel and transfer the gel tray to the gel electrophoresis chamber and fill chamber to fill line with 1x TAE buffer.
11. Load 5ul DNA ladder into one lane of gel. In another lane, load 25ul of the PCR mixture into the agarose gel.

12. Connect the chamber to the electrophoresis power supply at 95 V for 30-45 minutes.
13. Disconnect the chamber from the power supply.
14. Remove the gel tray from the electrophoresis chamber and place the tray inside a UV gel imaging system.
15. Image the gel and assess PCR product for size and yield
16. Purify the remaining 25ul of the PCR mixture, using a PCR purification kit, following manufacturer instructions.
17. Store purified PCR product at -20°C.

Protocol for Restriction Digestion

Materials and Reagents

1. Plasmid DNA
2. Fast Digest Restriction enzymes and green buffer (NdeI, BamHI, EcoRI, Thermo Scientific, catalog numbers FD0584, FD0054, FD0274)
3. Microcentrifuge Tubes (Denville Posi-Click Tubes, catalog number: C-2170)
4. Agarose (Bio-Rad, catalog number: 1613100)
5. 50X TAE buffer diluted to 1X (Fisher Scientific, catalog number: BP13324)
6. Water
7. Ice
8. Ethidium Bromide (10mg/ml) (Bio-Rad, catalog number: 1610433)
9. DNA ladder (New England Biolabs, catalog number: N0467S)
10. Gel Extraction Kit (Qiagen, catalog number: 28704)

Equipment

1. 37°C water bath (Fisher Scientific, Iso-Temp 210)
2. Heat block (Fisher Scientific, Iso-Temp)
3. UV Gel Imaging system (Syngene, Ingenius 3)
4. UV light box (Fotodyne)

Procedure

1. At room temperature in a microcentrifuge tube, combine 2ul of digest buffer, up to 1ug of plasmid DNA, and 1ul of fast digest enzyme. For double-digestions, use only 0.5ul of each enzyme and mix gently.
2. Place mixture in 37°C water bath for 10 minutes.
3. Set heat block to 65°C .
4. Place digest reactions in 65°C heat block to inactivate enzymes then place on ice.
5. Prepare agarose gel for electrophoresis by combining 0.5g agarose with 50ml 1x TAE buffer in a 200ml flask.
6. Microwave the agarose solution on high for one-minute.
7. Cool slightly under cold water until flask is comfortable to touch.
8. Add 3ul of Ethidium Bromide to agarose solution and swirl to mix.
9. Tightly secure gel tray into gel caster and pour agarose solution into tray. Place 8-well comb into the solution and into notches of gel tray for stability.
10. Allow the gel to set at room temperature for at least 20 minutes.
11. Following PCR cycling, remove the comb from the gel and transfer the gel tray to the gel electrophoresis chamber and fill chamber to fill line with 1x TAE buffer.
12. Load 5ul DNA ladder into one lane of gel. In another lane, load 25ul of the PCR mixture into the agarose gel.
13. Connect the chamber to the electrophoresis power supply at 95 V for 30-45 minutes.
14. Disconnect the chamber from the power supply.
15. Remove the gel tray from the electrophoresis chamber and place the tray inside a UV gel imaging system.
16. Image the gel and assess for proper digestion.

17. Assisted by UV light for DNA visualization, excise the desired digestion fragment from the agarose gel using a sharp razor blade the extract the DNA form the agarose slice using a Gel Extraction Kit according to manufacturer instructions.
18. Store digested DNA at -20°C.

Protocol for DNA Ligation

Materials and Reagents

1. T4 DNA Ligase and 10X T4 DNA ligase buffer (New England Biolabs, catalog number: M0202L) or 2X rapid ligation buffer and T4 DNA ligase (Promega, catalog number: A3600)
2. Digested insert DNA or purified PCR product
3. Digested plasmid DNA or pGEM-T vector (Promega, catalog number: A3600)
4. Water
5. Ice
6. Microcentrifuge tubes (Denville Posi-Click Tubes, catalog number: C-2170) or 0.2ml PCR tubes (Bio-Rad, catalog number: TWI0201)

Equipment

1. Heat block

Procedure

1. For non-T vector ligations, thaw 10X T4 DNA ligase buffer at room temperature, until fully thawed, then place on ice.
2. On ice, combine 2ul 10X DNA ligase buffer, 50ng of plasmid DNA, insert DNA in a 3:1 ratio with plasmid DNA, 1ul T4 DNA ligase, and water to 20ul in a microcentrifuge tube and mix. For pGEM-T vector ligations, combine 5ul 2X rapid ligation buffer, 1ul (50ng) pGEM-T vector DNA, purified PCR product DNA in 3:1 ratio with pGEM-T vector, 1ul T4 DNA ligase, and water to 10ul in a 0.2ml PCR tube.
3. Incubate at room temperature for one hour.
4. Heat inactivate reaction mixture at 65°C for 10 minutes.
5. Use between 1-5ul of the ligation mixture for transformation.

Protocol for SDS-PAGE

Materials and Reagents
1. 10X Running Buffer diluted to 1X (see recipe)
2. Pre-Cast poly-acrylamide gels (Bio-Rad Criterion-TGX)
3. Precision Plus Protein Kaleidoscope Pre-stained Protein Standards (Bio-Rad, catalog number: 1610375)
4. B-mercaptoethonol Laemmli buffer (see recipes)
5. Microcentrifuge Tubes (Denville Posi-Click Tubes, catalog number: C-2170)

Equipment
1. Heat Block (Techne, Dri-Block DB3)
2. Vertical Electrophoresis Chamber (Bio-Rad Criterion, 1656001)
3. Electrophoresis power supply (Bio-Rad model 3000Xi)
4. Microcentrifuge (Beckman Coulter, Microfuge 16; Rotor: A46544)

Procedure
1. Place pre-cast poly-acrylamide gel into vertical electrophoresis chamber and add 400ml 1X running buffer. Then, remove the comb from the pre-cast gel.
2. Prepare your proteins for SDS-PAGE analysis by combining your protein solution and 3Xbeta-mercaptoethanol laemmeli buffer in a microcentrifuge tube, so that buffer volume is diluted by three. Mix and microcentrifuge briefly.
3. Place microcentrifuge tubes in a heat block set to 95°C for 5 minutes.
4. Add each protein solution you are analyzing into separate lanes of the precast gel leaving at least one lane open for loading the protein standard.
5. Add the protein standard to at least one lane of the gel.
6. Connect the chamber to the power supply and adjust voltage. For 4-20% gel, set voltage to 130V for 1.5 hours.
7. Following electrophoresis, remove the pre-cast gel from the cassette and evaluate by coomassie or western blot.

Recipes

Running Buffer
30g Tris
144g Glycine
10g SDS
adjust volume to 1L with water

3X B-mercaptoethanol Laemmli Buffer
188mM Tris-HCl (pH 6.8)
3% SDS
30% glycerol
0.01% bromophenol-blue
15% B-mercaptoethanol

Protocol for Coomassie staining of poly-acrylamide Gel

Materials and Reagents

1. Coomassie Staining Solution (see recipe)
2. Coomassie Destaining Solution (see recipe)
3. Electrophoresed poly-acrylamide gel
4. Plastic Wrap
5. Paper Towel
6. Blotting Paper

Equipment

1. Rocker (Arma Lab, Arma Rock)
2. Gel Dryer and Vacuum (Fisher Scientific, FB GD 45; Fisher Scientific MaximaDry)

Procedure

1. Following electrophoresis, remove poly-acrylamide gel from gel cassette and place in a shallow container, add 75-100ml coomassie staining solution and cover with plastic wrap.
2. Place container on rocking platform for 1-2 hours.
3. Decant staining solution and blot excess with a paper towel.
4. Add 75-100ml of coomasie staining solution and destain overnight.
5. Decant destaining solution and place blotting paper onto gel. Cover the gel and blotting paper with plastic wrap and place gel on gel dryer.
6. Set temperature control to 80°C and time control to 2hrs and connect gel dryer to vacuum. Allow gel to dry.

Recipes

Coomassie Stain
10% Acetic Acid
40% Methanol
Water 50%
1g Coomassie Brilliant Blue

Coomassie Destain
10% Acetic Acid
40% Methanol
Water 50%

Protocol for Western Blot (Adapted from Provost and Wallert Lab and Dr. Sok-Hyong Lee)

Materials and Reagents

1. 10X Transfer Buffer diluted to 1X (see recipe)
2. Blotting paper
3. Electrophoresed Poly-acrylamide Gel
4. Nitrocellulose membrane (Maine Manufacturing, catalog number:1215483)
5. Ice
6. Water
7. 5% Milk (see recipe)
8. PBS (Corning, catalog number: 21-031-CV)
9. Primary antibody (anti-Gal-3: Santa Cruz, sc-20157; His: sc-804)
10. Secondary antibody (Santa Cruz, sc-2963)
11. West Pico Chemiluminescent substrate (luminol enhancer solution and (Thermo-Fisher, catalog number: 34080)

Equipment

1. Western Blot Transfer Apparatus with cassette and fiber pads (Bio-Rad, catalog number: 1704071)
2. Electrophoresis power supply (Bio-Rad, Model 200)
3. Western Blot Transfer Tray (Bio-Rad)
4. Rocker (Arma Lab, Arma Rock)
5. Film Developer (Konica-Minolta, SRX-101A)

Procedure

1. Fill transfer tray with 1X transfer buffer so that the transfer cassette is fully submerged.
2. Place transfer cassette, black side down inside of the transfer tray and place one filter pad on the cassette. Smooth to avoid any bubbles.
3. Place two pieces of blotting paper, the size of the gel, on top of the filter pad. Smooth to avoid any bubbles.
4. On top of the blotting paper, place the electrophoresed poly-acrylamide gel. Orient so that proteins transfer to membrane in the order desired. Smooth to avoid any bubbles.
5. Place nitrocellulose-membrane, the size of the gel, on top of the gel. Smooth to avoid bubbles.
6. Place an additional two pieces of blotting paper on top of the membrane. Smooth to avoid bubbles then place the other filter pad on top of the blotting paper and smooth again. Close the transfer cassette carefully and place the cassette inside of the transfer chamber with the black side of the cassette oriented to the black side of the transfer chamber.

7. Fill the transfer chamber to the fill line with 1X transfer buffer.
8. Place lid onto the transfer chamber and connect to the power supply. Carry out transfer at 50V for 1.1 hours at 4°C.
9. Disconnect from the power supply and remove the transfer cassette. Open the cassette and remove the nitrocellulose membrane. Check for successful protein transfer by looking for the dyed protein standard ladder on the membrane.
10. Following transfer, incubate the membrane in 5% milk for one hour at room temperature to block non-specific proteins.
11. Add primary antibody in a 1:800 ratio to 5% milk in PBS. Incubate primary antibody solution with the nitrocellulose membrane overnight at 4°C on rocking platform.
12. The next day, wash the membrane in PBS three times, five minutes each time.
13. Prepare secondary antibody in a 1:10,000 ratio in 5% milk in PBS.
14. Incubate the membrane with the secondary antibody solution for one hour at room temperature on rocking platform.
15. Wash the membrane three times, five minutes each time in PBS.
16. Combine luminol/enhancer solution and peroxide buffer in a 1:1 ratio. Incubate nitrocellulose membrane in the peroxide solution.
17. Place membrane in a clear, plastic sheet protector inside of a film cassette. Then, expose the membrane to X-ray film inside of film cassette in a dark room, under only red light.
18. Expose blot to X-ray film. Length of exposure to X-ray film can range from 5 seconds to several minutes.
19. Pass X-ray film through a film developer to visualize the protein and choose the clearest blot as a representative image.

Recipes

Transfer Buffer
30g Tris
144g Glycine
1g SDS
adjust volume to 1L with water

5% milk
2.5 g Non-fat milk powder (Nestle Carnation)
Water to 50mL

Protocol for Cell Culture

Materials and Reagents

1. Frozen LN229 GBM cells
2. DMEM (Corning, catalog number: 10-013-CV)
3. FBS (Hyclone, catalog number: SH30396.03)
4. Pen/Strep (Lonza, catalog number: 17-745E)
5. Non-essential amino acids (Hyclone, catalog number SH40003-12)
6. T-25 flask (Corning, catalog number: 430639)
7. 10cm culture Dish (Corning, catalog number: 430167)
8. 24-well culture dish (Corning, catalog number: CLS3524)
9. 15ml centrifuge tubes (Corning, catalog number: 430791)
10. Serological Pipettes
11. 0.25% Trypsin (Corning, catalog number: 25-053-CI)
12. Trypan Blue (Sigma, catalog number: T8154)
13. 0.45-micron filter (Corning, catalog number: 431225)

Equipment

1. Hematocytometer (Reichert)
2. 37°C Incubator (Thermo-Forma, Steri-Cycle)
3. Cell Culture Hood (Thermo-Scientific, 1300 series A2)
4. Water Bath (Precision Scientific, Dual Chamber Water Bath 188)
5. Centrifuge (Beckman, GS-154 Centrifuge; Rotor: S4180)

Procedure

1. Prepare DMEM solution with serum by combining 500ml DMEM, 50mL FBS, 5ml pen/step, and 5ml non-essential amino acids. Warm to 37°C in water bath.
2. Thaw LN229 eclls in 37°C water bath for ~30 seconds.
3. Add 1ml of thawed cells and 3ml of the DMEM solution to a 15ml centrifuge tube.
4. Spin for 1 minute at 1,000 rpm.
5. Aspirate media from cells and add 2ml of fresh media to the cells and re-suspend the cells slowly by pipetting up and down. Add the cells the a T-25 culture flask and incubate at 37°C until confluency reaches 70-80%.
6. Incubate plate with 1ml trypsin and incubate at 37°C for three minutes. Add 3ml of the DMEM solution to the cells.
7. Add 100ul of the cell suspension to 100ul Trypan Blue in a microcentrifuge tube and mix well.
8. Add 10ul of the cell/trypan blue solution to each side of the hematocytometer and calculate the number of cells per milliliter in the original solution.
9. Dilute solution with DMEM and plate the cell solution into the inner 8 wells of a 24-well plate so that there are 50,000 cells per well at seeding in a total volume of 500ul per well.
10. Next, incubate the plate until it reaches 70-80% confluency.
11. Next, using serum-free DMEM, dilute treatment proteins (eGal3, sGal3, Gal-3, or C4-CRD) to the desired concentrations, usually 125, 250, and 500ng/ml after

measuring concentration with ELISA. Pass the solution through at 0.45-micron filter to remove any debris and large particles.

12. Add the treatments to the wells of the 24-well plate in differing concentration along with a no-treatment control and a buffer control.
13. Incubate the plates for 24-48 hours and assess cell viability by crystal violet assay.

Protocol for Crystal Violet Assay

Materials and Reagents

1. Crystal Violet Solution (see recipes)
2. Sorenson's Buffer (see recipes)
3. 96-well plate
4. LN229 cells in 24-well plate
5. Water
6. Paper Towels

Equipment

1. Rocker (Arma Lab, Arma Rock)
2. Spectrophotometer with microplate reader (SpectraMax Plus 384)

Procedure

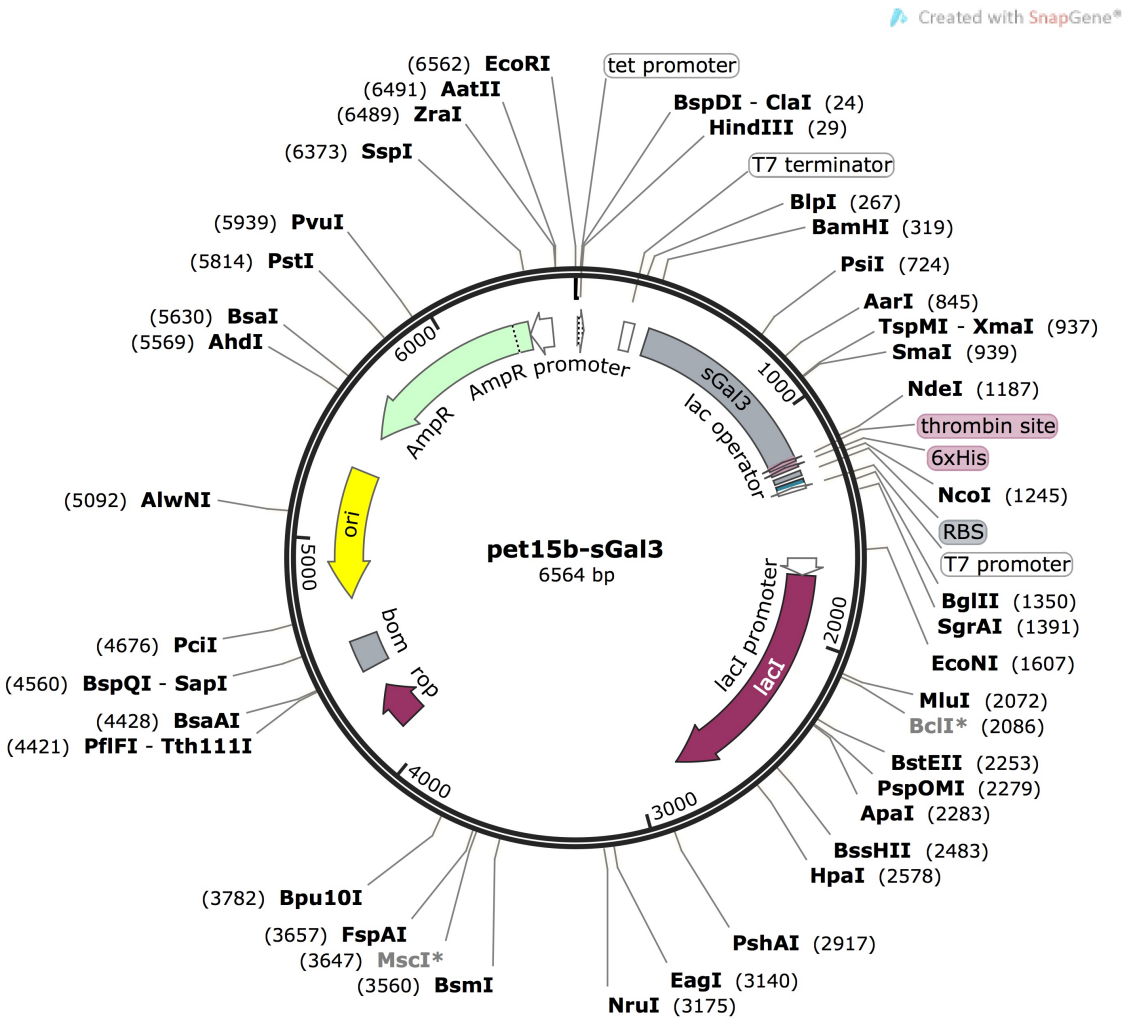
1. Remove media from cells in 24-well plate by decanting.
2. Allow the plate to dry upside on a paper towel for one hour.
3. Add 200ml of crystal violet solution to each well with cells of the 24 well plate and incubate on a rocking platform at room temperature for 15 minutes.
4. Prepare warm water bath.
5. Remove excess crystal violet by decanting, then submerge the plate well-side down in the warm water bath. Pour out warm water bath and replace with clean, warm water. Submerge plate and replace water repeatedly until water runs clear.
6. Allow plate to dry for at least one hour or overnight upside down at room temperature.
7. Re-suspend dried crystal violet solution in 800ml Sorenson's buffer and incubate at room temperature on rocking platform until the dye is distributed evenly throughout the well.
8. Transfer the solution to wells of a 96-well plate, adding ~200ml per well. Each well of the 24-well plate will be read from 4 wells of a 96 well plate.
9. Read the intensity of the crystal violet stain using a spectrophotometer with a microplate reader at 595nm.

Recipes

Crystal Violet Stain
25ml Methanol
500mg crystal violet
75ml water

Sorenson's Buffer
0.133 M Na_2HPO_4
0.133 M KH_2PO_4

Appendix II: Plasmids



The sGal3 cDNA sequence was cloned into the pET15b expression vector using the NdeI and BamHI restrictions sites. This plasmid was created and generously provided to me by Drs. Liquan Yang and Sok-Hyong Lee.

Sequence:
Red: Restriction Sites
Blue: sGal3 sequence

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aaacatgagaa3’

55 nt per line

The elongated galectin-3 variant containing additional collagen-like domains, termed eGal3, was amplified from a mammalian vector containing sGal3 cDNA using an overlap-extension PCR method (Lee et al., 2010). This PCR method utilizes six primers: a forward and reverse primer, as well as 4 chimeric primers. eGal3 was amplified using the following primers:

(1) 5’ AGAAGAAGACATATGGCAGACAATTTTTCG 3’ (forward)

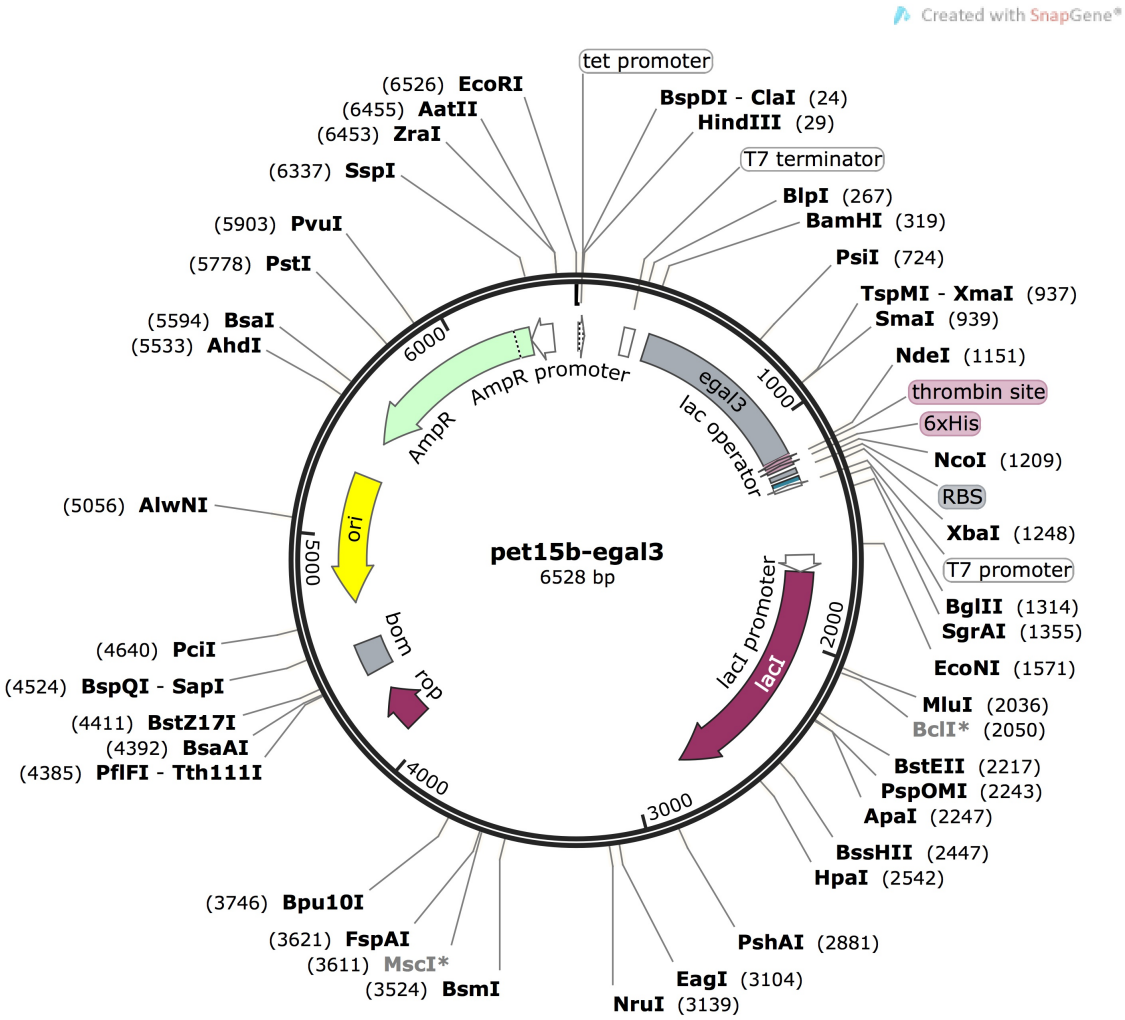
(2) 5’AGCCCCTGGCCATCCTTGAGGGTTTGG 3’

(3) 5’ CAAGGATGGCCAGGGGCTTATCCTGGA 3’

(4) 5’ TGCGCCAGGTGCAGGTGCTCCGGGATA 3’

(5) 5’ GCACCTGCACCTGGCGCATGGGGGAAC 3’

(6) 5’ AGAAGAAGAGGATCCTTATATCATGGTATATGAAGC 3’ (reverse) with the forward and reverse primers containing recognition sites for NdeI and BamHI, respectively.



Sequence:
Red: Restriction Sites
Blue: eGal3 sequence

5’

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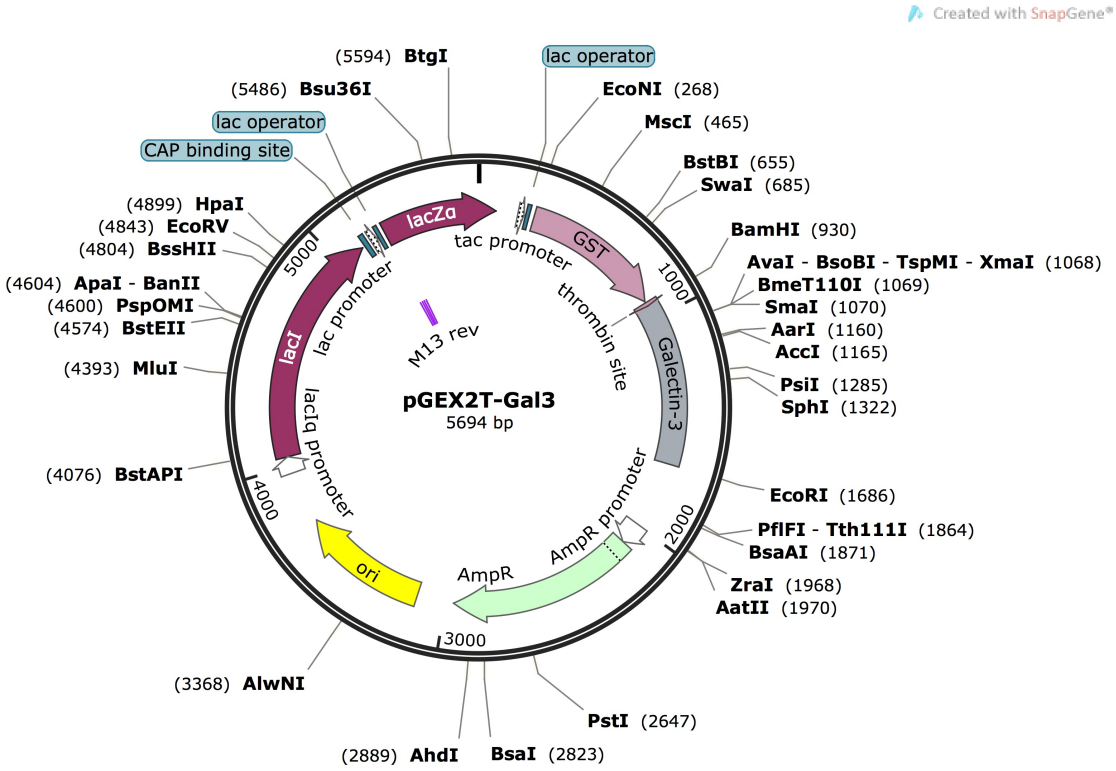

The galectin-3 sequence was ligated into pGEX-2T plasmid using the NdeI and BamHI restrictions sites. This plasmid was created and generously provided to me by Drs. Liquan Yang and Sok-Hyong Lee.

Sequence:
Red: Restriction Sites
Blue: Gal-3 sequence

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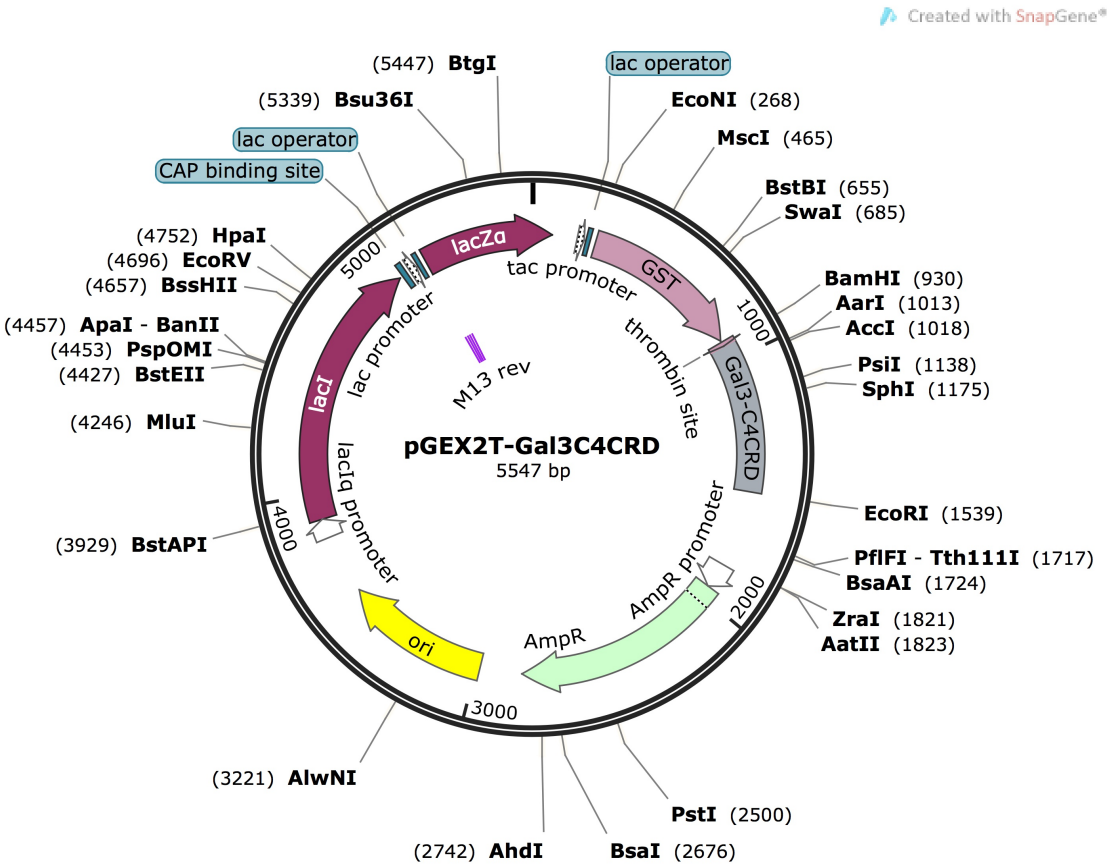

The truncated galectin-3 variant lacking the first three collagen-like domains, termed C4-CRD, was built by amplifying the sGal3 cDNA present in mammalian vector pUCMV7 with forward primer 5'-AGAGGATCCCCCCAGGGGCTTATCCT-3' containing a recognition site for restriction enzyme BamHI and reverse primer 5' AGAGAATTCTTATATCATGGTATATGAAGC-3' containing a recognition site for EcoRI. C4-CRD cDNA was then digested by the aforementioned enzymes and ligated into a compatibly digested pGEX-2T vector. Digests were performed using fast-digest enzymes (Thermo Fisher Scientific) according to manufacturer instructions (see appendix I for full protocols).

Sequence:
Red: Restriction Sites
Blue: C4-CRD sequence
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55nt per line