Non-Neoplastic Extracellular Matrix Components As A Therapeutic For Glioblastoma Multiforme

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Bachelor of Science, Brigham Young University, 2014

Submitted to the Graduate Faculty of the

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

UNIVERSITY OF PITTSBURGH

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Mark Hikaru Murdock PhD University of Pittsburgh, 2022

Glioblastoma multiforme (GBM), a type of glioma arising in the central nervous system, is one of the most aggressive and lethal types of cancer with a two-year survival rate of ~15%, and a five-year survival rate of just ~5%. The current standard of care for GBM is aggressive surgical resection followed by radiotherapy with concomitant daily temozolomide, both of which are associated with morbidity and decreased quality of life. In 2005 clinical testing for this approach showed an increase in median survival of only 2.5 months. New methods of treating GBM are desperately needed.

Suppressive and/or lethal effects of non-neoplastic mammalian extracellular matrix (ECM) biomaterials upon various cancer cell types including breast, urinary bladder, prostate, esophageal, melanoma, and colon cancer have been reported. Though the mechanisms of action are unclear, ECM biomaterials purportedly exert their anti-cancer effects both directly through multiple effector molecules existent in the ECM microenvironment and indirectly by modulating the immune system. The present dissertation shows that there is potential therapeutic value for GBM in the salinesoluble fraction of non-neoplastic mammalian ECM biomaterials.

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The type of source tissue from which ECM biomaterials were isolated was a determinant factor in the anti-cancer potency of the resultant microenvironment as evidenced by in vitro viability assays of primary and cancer cell lines. Saline-soluble fractions of these ECM biomaterials were sufficient to reproduce the cancer-killing effect enacted by whole pepsin-digested ECM with porcine urinary bladder matrix being the most potent. The saline-soluble fraction of urinary bladder matrix (UBM-ECM-SF) induced apoptosis in primary GBM cells in vitro, showed neurosupportive function with astrocytes and neurons in vitro, showed low toxicity with non-neoplastic cells in vitro, and good biocompatibility intravenously and intracranially in rodents. Furthermore, treatment with UBM-ECM-SF decreased intracranial tumor growth rate leading to smaller final tumor volumes while doubling the median survival in the commonly used C6-Wistar rat glioma model. The proteomic composition of UBM-ECM-SF was explored though specific effector molecules remain unknown.

The findings from this work show that non-neoplastic ECM components have potent anti-glioma effects and may represent an alternative or complementary approach to conventional therapy for GBM.

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1.0 Introduction

1.1 Glioblastoma Multiforme and the Current Standard of Care

Gliomas are a common type of primary tumors originating from the glial tissue in the brain and spinal cord. The World Health Organization (WHO) has designated a grading system I-IV based on their histological characteristics which indicate increasing levels of malignancy. Grade IV glioma, also known as glioblastoma multiforme (GBM), is the most aggressive and lethal type of brain cancer. GBM has a relative 2-year survival rate of ~15%, and a 5-year survival rate of ~5%, one of the lowest of any type of cancer, see (1, 2). Up to 81% of malignant brain tumors are glioma and up to 45% of all glioma are GBM (3). The annual incidence of GBM is 3.2 per 100,000 people in the United States resulting in approximately 12,000 new cases diagnosed every year (4). The incidence of GBM increases with age and approaches 13.1-15.0 cases per 100,000 persons for ages 65-84 (3).



Figure 1 Relative survival rates of various cancers at 5-years in the USA measured from 2008-2014. GBM is one of the most lethal types of cancer with a 5-year survival rate of less than 5%.

The current standard of care for GBM is aggressive surgical resection followed by chemoradiation therapy, specifically the treatment regimen known as the Stupp protocol which consists of radiotherapy with concomitant daily temozolomide (TMZ) doses (5, 6). This method was adopted in 2005 following a study from Stupp et al showing that this combination therapy increased median survival from 12.1 months with radiotherapy alone to 14.6 months (7). TMZ is an indiscriminate alkylating agent which operates by methylation of the purine residues of DNA (adenine and guanine) causing mispairing of bases during DNA replication and ultimately persistent strand breakage and apoptosis. In this manner it targets any cells which are actively undergoing cell division and as a result can cause a host of side effects- some of which may not occur

until months or years after use. There are dozens of side effects ranging from mild to life threatening in seriousness (see Temodar label,

https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/021029s031lbl.pdf).

The results from the 2005 Stupp study showed that 49% of the 287 patients who received TMZ reported one or more "severe" or "life-threatening" reactions during the study according to according to the National Cancer Institute Common Toxicity Criteria, version 2.0. The rapid lethality of GMB combined with the limited survival benefits of TMZ and associated side effects sometimes prompt newly diagnosed patients to electively refuse chemoradiation therapy altogether. In the words of the Roman poet Virgil, aegrescit medendo, which can be translated to "the remedy is worse than the disease". While researchers have developed promising new drugs and administration techniques in recent years, most efforts have not achieved long-term remission in clinical trials and improved therapeutic options are desired.

While the pathogenesis of GBM is complex and largely unknown, it is well established that the glioma tumor microenvironment, and the microenvironments of all solid tumors, have a major supportive role in tumor growth, invasion, and metastasis (8-10). This glioma microenvironment, largely comprised of dysregulated extracellular matrix (ECM), promotes local immunosuppression and neo-vascularization, and impairs blood-brain-barrier integrity (11). The biochemical composition of the glioma microenvironment can differ between patients and this variability can lead to differential prognoses (12). Since the tumor microenvironment is closely associated with multiple aspects of tumor growth, it has been proposed that targeting the microenvironment itself or tumor microenvironment-associated cells may be a viable strategy (13, 14).

1.2 Non-neoplastic Extracellular Matrix and Dynamic Reciprocity

The extracellular matrix (ECM) consists of the structural and functional molecules secreted by cells to impart structure and biochemical support to a tissue. Collagens are the major structural component of the ECM and may produce bioactive matricryptic peptides following proteolysis (15). The biochemical support includes molecules for matrix-cell signaling which activate classic signal-transduction pathways and modulate cell growth, proliferation, and gene expression (16). These components classically consist of the ligand landscape including cytokines and growth factors, and matricryptic peptides formed by proteolysis of structural molecules (Figure 2) which function individually and in concert to support and modulate cells (17). A novel bioactive ECM component was recently described by the Badylak laboratory, the matrix-bound nanovesicle (MBV), which will be further discussed in later sections (18). All multicellular organisms produce an ECM but its biochemical composition varies depending on species, tissues, and even the age of the organism (19, 20).



Figure 2 Bioactive components of ECM. A diverse population of ligands, matricryptic peptides derived from structural molecules, and vesicles work individually and in concert to support and modulate resident cells of the ECM. Figure adapted from "The extracellular matrix of the gastrointestinal tract: a regenerative medicine platform" (21)

The concept of "dynamic reciprocity" can be defined as the hypothesis that the ECM influenced gene expression which in turn resulted in modification of cell secreted products (Figure 3). Multiple groups have since demonstrated that the ECM can directly affect cell signaling not only by acting as a reservoir for growth factors and other cell-secreted molecules, but also directly through mechanotransduction (22). Tissue-resident cells create the ECM upon which they reside and thus dictate the composition, mechanics, and geometry of the ECM. The ECM, in turn, can affect the morphology, phenotype, and even survival of resident cells.



Figure 3 ECM-cell interactions are a feedback loop. Resident cells create ECM by secretion, degradation, and remodeling, which then affects cell phenotype and behavior in various contexts such as development, homeostasis, injury, or disease.

1.3 Neoplastic Extracellular Matrix and Theories of Carcinogenesis

Just as non-neoplastic ECM confers specific, modulating cues to resident cells, neoplastic ECM, the acellular portion of the tumor microenvironment, contains aberrant signals that support cancer cells (8, 9). Cancer cells and tumor-associated cells both secrete products which contribute to the cancer microenvironment, enhancing cancer cell survival and even promoting cancer cell colonization at distant sites (23). Neoplastic ECM has been implicated in driving heterogeneity in cancer cells, supporting growth, proliferation, and migration, and recruiting tumor-associated cells to derail a proper immune response (24). Neoplastic ECM has also been shown to decrease the effectiveness of radio-, chemo-, and immunotherapy (25).

Biochemically, many of the matrix components in the neoplastic ECM vary significantly from their non-neoplastic ECM counterparts in terms of relative abundance or post-translational modification (25). Classically, neoplastic ECM contains an elevated

presence of matrix proteases which results in a rapid turnover/remodeling rate and an increased release of matrix-bound growth factors within the tumor, as well as macrophage colony-stimulating factor which attracts and activates blood circulating monocytes into the infamous tumor-associated macrophage (25). Neoplastic ECM is strongly implicated in cancer progression leading some research groups to believe that the aberrant ECM presents itself as a therapeutic target for treatment of cancers, including glioma (25-28).

The current dogma of carcinogenesis, somatic mutation theory (SMT), states that cancer arises as a cell-centric disease and attributes the sole cause to the accumulation of DNA damage and subsequent mutation, leading to a cell population with all the genetic changes to sustain proliferative signaling, ignore growth suppressors, resist cell death, induce angiogenesis, and invade/metastasize to other tissues (29). The basis for SMT originated in 1914 when Theodor Boveri proposed that chromosomal defects led to cancer (30). Subsequent discoveries such as that of the DNA double helix and its ubiquitous role in biology, that a number of carcinogenic chemicals were also mutagenic, and that certain viruses with "transforming genes" led to tumors in some animal models led to the widespread adoption of SMT which has dominated the cancer-research community. SMT becoming the leading theory of carcinogenesis resulted in modern cell-centric cancer therapeutics which combat the symptoms of the disease, not necessarily the cause of it.

While SMT led to a deeper knowledge of cancer biology and some cancers have strong genetic causal components, it has become clear that the premises of SMT do not apply to all instances of cancer (31, 32). Somatic mutation is sometimes present only

after the early cues of carcinogenesis are manifest (33). Several cancers are not associated with mutations but are driven solely by epigenomic alterations or fusion proteins (34, 35). Biochemical and physiological processes (notably inflammation) and cell-cell signaling have been implicated as causal cancer drivers (36, 37). The normal rate of mutations in somatic cells does not account for the number of mutations found in many neoplasms, especially in young people (38). Comparison of 31,717 cancer cases and 26,136 cancer-free controls from 13 genome-wide association studies showed "the vast majority, if not all, of aberrations that were observed in the cancer-affected cohort were also seen in cancer-free subjects" (39). Altogether there is evidence that somatic mutations are epiphenomena or post-carcinogenesis events rather than immutable causes.

Those studying the theory of carcinogenesis have not overlooked the strong association between components of the non-neoplastic ECM and the rise and progression of cancerous tissue. Tissue organization field theory (TOFT) proposes a tissue-centric (rather than a cell-centric) model in which the microenvironment, of which the ECM is a major part, has gone awry and carcinogenesis is a problem of tissue organization, see Figure 4 (40, 41). TOFT harmonizes many observations that are problematic for SMT, often displaying the principles of dynamic reciprocity. Early embryos transplanted ectopically acquire properties of malignant neoplasms (teratomas) with no exogenous carcinogens or mutagens. Neoplasia occurring without carcinogens or mutagens can occur outside of experimental conditions as well; ectopic pregnancies are seen to have teratogenic characteristics (42, 43). Conversely, teratoma cells injected into the blastocyst stage of early embryos generated normal organs,

tissues, and cells, including oocytes and sperm which generated normal progeny (44). Nuclei from frog renal carcinoma cells were transplanted into enucleated ova and developed into normal tadpoles (45).

Other experiments specifically showcase the ability of ECM biomaterials to suppress or modulate the cancer phenotype. Breast cancer cells placed in nonneoplastic ECM reverted from their malignant phenotype (46). Porcine small intestinal submucosa ECM (SIS-ECM) did not hasten the formation of primary tumors and decreased the volume of recurring tumors after surgical resection (47). High grade metastatic bladder cancer cells grown on SIS-ECM hydrogel decreased invasion and showed more organized growth than when grown on Matrigel or collagen (48). Porcine urinary bladder ECM (UBM-ECM) and esophagus-derived ECM both decreased proliferation of esophageal cancer cells and UBM-ECM differentially increased apoptosis (49). A solid UBM-ECM scaffold inhibited melanoma tumor formation in a macrophage-dependent manner in mice (50). Taken altogether these observations show genotype is secondary to microenvironmental cues in the context of carcinogenesis and certain non-neoplastic extracellular matrices contain sufficient cues to impede various types of cancer at the organ-, tissue- and cellular-levels.



Figure 4 Somatic mutation theory vs tissue organization field theory. SMT posits a cell-centric view of carcinogenesis starting with somatic mutations of a single cell. TOFT posits a tissue-centric view of carcinogenesis reliant on cell-ECM interactions and dynamic reciprocity. Figure adapted from "Competing views on cancer" (29)

1.4 Therapeutic Potential of Extracellular Matrix Biomaterials

Adopting TOFT as a premise suggests that if aberrations in the native microenvironment are sufficient to gradually nudge a tissue toward the cancer phenotype, then restoring elements of the original, non-neoplastic microenvironment may dissuade oncogenic behavior. Rather than inserting select components from the microenvironment which may be impotent without cofactors, one can introduce nearly the whole native microenvironment by isolating ECM from non-neoplastic tissue through decellularization. This approach to cancer treatment is heretofore unseen in clinical practice yet represents a new generation of cancer therapeutics which may sidestep the side effects of chemoradiation therapy. Taking this one step further, by generalizing TOFT and applying the principles of dynamic reciprocity we might presume that a host of other progressive diseases, conditions, and injury models may benefit from having a "normal" ECM microenvironment restored at crucial time points.

The therapeutic potential of ECM was studied through the advent of the field of tissue engineering when researchers used decellularized ECM bioscaffolds as primarily mechanical supports to seed stem and supporting cells in efforts to regrow tissue (51). Over time some studies showed that the cellular component had little effect on the tissue in question and that the implanted acellular bioscaffolds were sufficient to induce some level of functional remodeling directly through bioactivity of components intrinsic to the ECM and indirectly through recruitment of progenitor cells and modulation of the host immune response (18, 52-56). For some, ECM came to be viewed as Mother Nature's intended microenvironment for a given tissue type replete with a complex and synergistic milieu of homeostatic components. Isolating individual components from the whole may result in reduced efficacy by inadvertently disturbing some evolutionary preserved, natural balance of co-dependent constituents. This perspective led to numerous basic and clinical research studies showing therapeutic effects of whole ECM in diverse injury and disease models, including several models of cancer.

Basic research has shown therapeutic effects of ECM in injury models for nearly every tissue type and organ system through common features such as induction of angiogenesis, recruitment of progenitor cells, and modulation of the immune system away from the typical inflammation response (15, 53, 55, 57, 58). ECM interventions

with no added cellular component have been used to improve functional reconstruction in injury models for skin (59-62), muscle (63-65), ligament (61), cartilage and bone (66-69), urinary bladder (70, 71), corneal stroma (72), tunica albuginea (73), dura mater (74-77), spinal cord (78-80), brain (81-84) and others. The host response to ECM in injury models is associated with functional remodeling and is distinct from typical scar tissue formation.

Disease model outcomes can also be improved with ECM-derived materials and components as has been shown for models of Type I diabetes (85, 86), complex chronic wounds (87-90), stroke (81-84, 91, 92), ulcerative colitis (93), liver disease (94-96), cardiovascular disease (97-99), lung disease (100-102), and neurodegenerative diseases (103-105).

Multiple forms of cancer have been shown in various models to be modulated in their phenotype and progression by ECM-based biomaterials, often in the form of an injectable hydrogel, including breast cancer (46, 50, 106), urinary bladder cancer (48, 107-109), prostate cancer (47, 110), esophageal cancer (49, 111-113), sinonasal cancer (114), melanoma (50), and colon carcinoma (50). While this phenomenon is relatively new and the mechanisms of action have yet to be elucidated, it is known that dysregulated matrix-cell signaling in cancer can be corrected to produce therapeutic results. For example, in the microenvironment of breast cancer the aberrant signaling of integrin β 1 and β 4 can be remediated by inhibitory antibodies which then induce the cancer cells to revert morphologically and functionally toward a normal phenotype (115, 116). Re-establishing the proper, non-neoplastic microenvironmental signaling was sufficient to override the neoplastic cancer cell genotype. ECM biomaterials have even

been shown to improve the efficacy of tissue vaccines for cancer and are thought to have potential utility as vaccine adjuvants (110). Improved outcomes for various injury, disease, and cancer models show the significant therapeutic potential that ECM and ECM components have across a diversity of physiologic ailments.

These types of studies fuel the clinical studies pipeline that is establishing the next generation of ECM-biomaterial based therapies. In fact, ECM is so strongly supported by clinical research to have beneficial effects in injury models that there are already dozens of ECM-based products on the market with indications ranging from generic "soft tissue" and "wound" repair to specific applications such as tendon, nerve, cardiac tissue, tendon, and dura mater repair (Table 1).

Year	Title	Ref
2020	Esophageal extracellular matrix hydrogel mitigates metaplastic change in a dog model of Barrett's esophagus	(112)
2019	A biologic scaffold-associated type 2 immune microenvironment inhibits tumor formation and synergizes with checkpoint immunotherapy	(50)
2019	Extracellular Matrix Degradation Products Downregulate Neoplastic Esophageal Cell Phenotype	(49)
2014	Porcine small intestine submucosal grafts for post-tumor resection orbital reconstruction	(114)
2013	Suppression and activation of the malignant phenotype by extracellular matrix in xenograft models of bladder cancer: a model for tumor cell "dormancy"	(48)
2011	Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold	(111)
2008	Use of an extracellular matrix material as a vaccine carrier and adjuvant	(110)
2006	Analysis of the interaction of extracellular matrix and phenotype of bladder cancer cells	(108)
2006	Proteome-level display by 2-dimensional chromatography of extracellular matrix-dependent modulation of the phenotype of bladder cancer cells	(109)
2004	Small intestinal submucosa does not promote PAIII tumor growth in Lobund-Wistar rats	(47)
2003	Regulation of Breast Cancer Progression by Extracellular Matrix Mechanics: Insights from 3D Culture Models	(106)
2003	Matrix-dependent plasticity of the malignant phenotype of bladder cancer cells	(107)
2002	The organizing principle: microenvironmental influences in the normal and malignant breast	(46)

Table 1 Select Studies Utilizing ECM to Modulate Cancer

2.0 Considerations for an Extracellular Matrix-Based Glioblastoma Therapy

2.1 Rationale for and Production of Extracellular Matrix Test Articles

2.1.1 ECM as a Biomaterial

At this point it is necessary to define the scope of the ECM biomaterials of interest for this work and to distinguish them from other types of ECM or other biomaterials. In the broadest sense, biomaterials are any materials or substances that have been isolated, created, or engineered for the purpose of operating in close contact with biological systems. They are often intended to confer a therapeutic benefit though some biomaterials serve a diagnostic purpose. Biomaterials may either be synthetic or natural. Synthetic biomaterials include groups such as metals, ceramics, and polymers, and are usually physiologically inert by design or are associated with problems like infection and inflammation due to poor biocompatibility. Natural biomaterials come in several types including protein-based like collagen and silk, polysaccharide-based like cellulose and chitin, and decellularized tissue-based which can come from plant or animal tissue (117). For the purposes of this work only animal-derived ECM will be discussed.

Decellularizing a tissue properly can leave the ECM intact, producing an ECM with a bioactive signature and milieu that conceptually represents the culmination of millions of years of evolutionary trial and error, biochemically balanced to support normal cell growth and homeostasis for the cells within that given tissue type.

Heterologous ECM has serendipitously been shown to exert positive remodeling or other therapeutic effects on tissues from which they did not originate (111, 118-120). For example, ECM from porcine urinary bladder implanted into volumetric muscle loss defects led to constructive tissue remodeling (65, 118). Therefore, a therapy for a human physiological ailment would benefit from a microenvironment that has many of the common features evolutionarily conserved, i.e. mammalian tissue ECM. Lastly, the ECM should be derived from non-neoplastic tissue. Neoplastic ECM, like Matrigel® derived from Engelbreth-Holm-Swarm sarcoma cells, has utility in the culturing of stem cells perhaps because stem and cancer cells have so much in common (121) but it has also been shown to drive cancer progression and inspire a cancer-phenotype in otherwise non-neoplastic cells (23, 24). For these reasons a non-neoplastic mammalian ECM biomaterial suits the experimentation found in this work.

2.1.2 Tissue Procurement

Intact ECM can be isolated from virtually any animal and any tissue type. ECM is routinely harvested for research purposes and for clinical application from different species including human, pig, cow, and horse (122). Porcine tissues are especially useful given their physiological similarity to humans and their ready availability from abattoirs and industry sources. Though source species has not been shown to be a factor in ECM bioactivity or host response, other factors such as age (19), diet (123), and tissue type (124) can have an effect. We therefore expect a certain level of biological variability in the source material.

Another source of variability is the manual nature of the decellularization process (see next section), necessitating the use of standard operating procedures within the laboratory to decrease variability as much as possible. Decellularization protocols have been established for heart valves, blood vessels, skin, nerves, central nervous system, skeletal muscle, tendons, ligaments, ovaries, testes, small intestine, urinary bladder, liver, and other tissues. The Badylak laboratory has extensive experience and established protocols for decellularizing various tissues and has set the standard for assessing decellularization efficiency (125).

ECM scaffold materials harvested from different tissues have unique biochemical compositions which can affect ECM-bioscaffold functionality. For example, ECM scaffolds derived from porcine small intestinal submucosa (SIS–ECM) consist of ~90% of collagen, the majority being collagen type I, and minor amounts of other collagen types III, IV, V, and VI (126). On the other hand, ECM scaffolds composed of porcine urinary bladder matrix (UBM–ECM), while containing the same collagen types as SIS– ECM, contain greater amounts of Col III, as well as Col VII, an important component of the epithelial basement membrane (127). Taken together these tissue procurement considerations support a research strategy comparing the effects of ECM derived from several porcine tissue types with well-established decellularization protocols. ECM derived from porcine dermis, small intestine, and urinary bladder will be evaluated.

2.1.3 Decellularization

Conceptually, intact ECM consists of all the structural and functional components secreted by the resident cells in their native 3D microarchitecture. The content and microarchitecture of intact ECM is invariably altered during the decellularization process. ECM will necessarily lose some of its functional components as they are washed out by detergents or other liquid washes. Decellularization processes are designed to balance the removal of cellular material with the preservation of the composition, mechanical integrity, and biological activity of the remaining ECM.

Decellularization processes attempt to achieve the metrics or criteria of decellularization established in 2011 by Crapo et al.; <50ng of double stranded DNA per milligram of dry weight ECM, no visible nuclei in H&E stained histological sections, any residual DNA being fewer than 200 base pairs in length (125). The consequences of an insufficiently decellularized tissue implanted in vivo can include fibrosis, a severe inflammatory response, chronic inflammation, and seroma or abscess formation (128).

The main ECM components are highly conserved between species and are well tolerated by xenogeneic recipients (129, 130). Millions of patients have received ECM-based materials in clinical settings, the majority of ECM products being of xenogeneic origin, and no immune rejection complications have been reported. Residual amounts of DNA and certain immunogenic species-specific antigens, such as galactosyl- α -1,3-galactose (α -Gal epitope), have been shown to be present in ECM scaffolds, but fail to activate complement or bind IgM antibody, possibly due to the small amount and widely scattered distribution of the antigen (131-134).

The ECM isolated by the Badylak laboratory using in-house standard operating procedures have been successfully utilized in many experiments. Tissue sources include porcine dermis (135), small intestine (136-138), urinary bladder (127, 139), and others.

2.1.4 ECM Physical Specifications

Following decellularization and post-processing steps the final form of ECM biomaterials can vary based on the intended application. Such physical specifications, or form factors, include whole organs, two dimensional sheets, powders, hydrogels, and newer generations of "liquid" ECM (140). It is important to consider which form factor is best suited to the application of GBM. Solid ECM sheets were utilized in the esophageal preservation of patients receiving resection after onset of esophageal cancer, resulting in good constructive remodeling and no recurrence of cancer (111). A mucoadhesive ECM hydrogel was used in a dog model of precancerous Barrett's esophagus and mitigated metaplastic change (112). For ease of use between in vitro experiments and in vivo glioma models likely requiring ECM injection, hydrogels and liquid ECM will be the form factors of choice.

Herein "liquid ECM" or similar terms will refer to liquid fractions derived from an ECM precursor not to the resolution of single components but rather containing many components with shared characteristics such as molecular weight. One example of liquid ECM in this context would be Spang et al. starting with a myocardial hydrogel and centrifuging, dialyzing, and sterile filtering it to remove high molecular weight proteins,

resulting in a liquid ECM which could be infused intravenously without the large submicron particulates present in injectable hydrogels which are too large to pass through leaky vasculature at sites of injury or inflammation (140). Another form of liquid ECM may be the ECM fractions containing matrix-bound nanovesicles (MBV) which may be obtained through methods of KCl extraction or ultracentrifugation and which contain bioactive protein, miRNA, and lipid components (141). The release of soluble ECM factors appears to be essential processes for constructive remodeling to occur. This fact is highlighted by an altered remodeling profile in clinical applications using scaffolds that have been chemically crosslinked using glutaraldehyde, carbodiimide or hexamethylene-diisocyanate, or non-chemical methods (142-144).

A trend of increasing amounts of manipulation and processing is seen as form factors go from whole organ to liquid ECM fractions. In terms of the in situ microenvironment each additional phase of processing necessarily (though not deleteriously) loses some characteristic of the previous form factor. In milling a sheet of ECM into powder, the ultrastructure is disrupted; the directionality of the collagen fiber network is lost, as is the luminal-serosal orientation. In proteolytic digestion or sonication of a powder into a hydrogel, features of microarchitecture like porosity and stiffness are lost. And in fractionating ECM to exclude macromolecular components, resulting in a liquid ECM, additional elements of biocomposition and 3D orientation navigable by cells are lost. Despite the ECM microenvironment losing features through increased processing, the capacity to elicit several common host-responses such as promoting tissue repair and modulating inflammation, seems to be retained. Hydrogels and even liquid preparations ECM-derived MBS are capable of modifying macrophage

phenotype (93, 145). Though the natural conclusion may be to exclude an increasing number of ECM components to isolate single effector molecules, the entire ECM microenvironment evolved together, holistically, and important interactions between components or balances between ratios of components may be lost if the ECM is processed to a purely reductionist end.


Figure 5 ECM form factors. All ECM bioscaffolds come from source tissue and can take various form factors after decellularization. Each form factor has strengths and weaknesses in a given application.

2.2 Host Response to Extracellular Matrix Biomaterials

ECM biomaterials, seemingly regardless of physical form factor, elicit a favorable host response. The lack of an adverse immune response to xenogeneic ECM implants has been attributed to the common nature and evolutionary conservation of amino acid sequences and surface epitopes between species (129, 130). Xenogeneic ECM used in research are often of porcine origin, from large, easily decellularized tissues such as the dermis, small intestine, or urinary bladder. ECM-induced positive functional remodeling has been observed in many tissue types including skin (88), tendon/ligament (146), urinary bladder (147), skeletal muscle (119), heart (148), esophagus (149), brain (84, 150, 151), and others. The exact mechanisms of action for ECM biomaterials' favorable host response are unknown, although several observations each provide a partial explanation.

Bioactive cell- and tissue-supportive molecules have been found in ECM including intrinsic growth factors and matricryptic peptides (152, 153). For example, vascular endothelial growth factor has been identified in porcine small intestinal submucosa ECM which induced angiogenesis in animal models of wound repair (154). In another study this same ECM was found to contain a functional TGF- β related component and FGF-2 which stimulated fibroblast cells toward proliferation, though importantly the isolated FGF-2 was found to be responsible for 60% of the stimulating effect suggesting other active components or cofactors present, again precluding a total reductionist view of ECM (155). Externally supplied ECM has also been implicated in inducing innervation in animal models (156, 157) and enhancing mitogenesis in certain cell populations (158).

ECM biomaterials have been shown to attract and/or facilitate differentiation of host cell populations including endogenous stem/progenitor cells (53). In the above study regarding innervation induction, it was found that Schwan cells specifically had migrated into the area due to ECM cues. In a clinical trial studying volumetric muscle loss it was noted that the ECM scaffolds attracted muscle and nerve progenitor cells to the wound area, thus assembling the key players for muscle regeneration (65). In another study, an ECM hydrogel was applied to the volumetric lesion of a stroke infarct and within 24 hours the host cell infiltrate was characterized. Approximately 60% of the cells were found to be of brain-derived phenotypes, including neural progenitor cells, which upon histological staining appeared to be differentiating into neuronal networks (83). Another 30% of infiltrating cells were peripheral macrophages, and importantly, the ECM hydrogel directed their phenotype away from the M1, classical pro-inflammatory activation, and toward the M2, alternatively activated anti-inflammatory phenotype, putatively aiding in remodeling. The initial host immune response to ECM scaffolds appears to be critically important in determining subsequent processes including scaffold degradation, release of matricryptic peptides, host cell recruitment, and angiogenesis, among others (53, 159).

This phenomenon of ECM components shifting the innate immune response away from inflammation and towards remodeling is well characterized and seems to be present in almost all therapeutic applications of ECM (143). Macrophages are a subset of the mononuclear cell population which are a major part of the immune system arriving to the site of injury (or remodeling) quickly and influencing the remainder of the host response. In response to inflammatory stimuli, such an insufficiently decellularized

ECM, activated macrophages produce a variety of cytokines such as tumor necrosis factor, IL-1, IL-6, IL-8, and IL-12 which in concert with other secreted chemokines, leukotrienes, prostaglandins, and complement increase vascular permeability, recruit inflammatory cells, and can cause fever and the production of acute inflammatory response proteins (160). This inflammatory reaction of macrophages is the classical activation state, termed M1. However, in response to non-inflammatory stimuli such as properly decellularized ECM biomaterials, macrophages have an alternative activation pathway and activate toward the M2 state which is involved in wound healing tissue repair/remodeling. M2 macrophages secrete anti-inflammatory cytokines like Arginase-I, IL-10 and TGF- β (161). The M1 and M2 activation states are not a simple binary system. There is a spectrum of activation states that span the M1 and M2 responses, each with their own distinct profile of secretion products and range of inflammatory vs anti-inflammatory effects.

It is known that ECM components and degradation products have various effects on immune cells other than macrophages as well. For example, ECM components such as entactin, nidogen, and fragments of laminin, elastin, and type IV collagen have been shown to attract neutrophils, a microbicidal cell type (162, 163). It was also found that TNF-alpha linked to fibronectin affected the signal transduction and cell adhesive properties of CD4+ T cells, possibly helping to direct these immune cells toward inflammatory sites (164). Another study shows that a three-dimensional collagen matrix was essential for naïve T cells to interface meaningfully with antigen-presenting dendritic cells, leading to signal induction and T cell activation (165). These studies

illustrate how ECM bioscaffolds promote regenerative conditions and tissue development by priming the local immune response.

2.3 Commercial Considerations

2.3.1 Commercially Available ECM

As the science behind the generation and characterization of ECM biomaterials has expanded, the applications for these materials have greatly increased. Presently, the majority of commercially available ECM scaffolds are used as a surgical mesh for soft tissue reconstruction or topical wound healing (see Table 1). Preclinical research has demonstrated the promise of using ECM scaffolds (and recellularized ECM scaffolds) for cardiac patches, vascular cuffs, heart valve replacement, tracheal reconstruction, and potentially even organ transplantation. among numerous other uses. Future research may pave the way for ECM product application utilized in disease modulation, possibly including treatment of various cancers. To this end it is important to keep in mind the commercial landscape of ECM therapeutics in tandem with regulatory considerations while developing a prospective therapy.

Table 2 Examples of Extracellular Matrix Biomaterial-based Products

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*SIS = small intestinal submucosa

Table 2 continued				
Glyaderm®	Euro Skin Bank	Human dermis	Preserved in 85% glycerol	Dermal repair and plastic surgery
GraftJacket®	Wright Medical	Human	Natural, dry	Tendon and
	Tech	dermis	sheet	ligament repair
Integra®	Integra Life	Human	Natural, dry	Soft tissue
HuMend™	Sciences	dermis	sheet	reconstruction
Integra®	Integra Life	Porcine	Natural, dry	Soft tissue
Reinforcement	Sciences	dermis	sheet	reconstruction
Matrix				and tendon
			-	repair
MIRODERM®	MiroMatrix	Porcine liver	Sheet cut from a	Wound repair
	Medical Inc		decellularized	
			organ	0.6.6
MIROMESH®	MiroMatrix	Porcine liver	Sheet cut from a	Soft tissue
	Medical Inc		decellularized	repair
	Quesith and		organ	
	Smith and	Porcine SIS	Natural, dry	wound repair
	Nepnew	Dovino	Sheet Crocolinkod dry	Dericardial and
Pen-Guard®	Synovis	Bovine	crossiinkea, ary	Pericardial and
 PorioDorm™	Musculoskolotal		Notural dry	Solt lissue repair
FenoDenn	Transplantation	dermis	sheet	integumental
	Foundation	dennis	51000	and soft tissue
				repair
Permacol™	Medtronic	Porcine	Crosslinked	Soft tissue
	modulomo	dermis	sheet	repair
PriMatrix®	Integra Life	Fetal bovine	Natural. drv	Wound repair
	Sciences	dermis	sheet	•
SurgiMend®	Integra Life	Fetal bovine	Natural, dry	Soft tissue
U	Sciences	dermis	sheet	repair
Suspend®	Coloplast	Human	Natural, dry	Prolapse and
-		fascia lata	sheet	stress urinary
				incontinence
TissueMend®	Stryker	Fetal bovine	Natural, dry	Tendon repair
		dermis	sheet	
Vascu-	Synovis	Bovine	Crosslinked,	Vascular
Guard®	Surgical	pericardium	hydrated sheet	reconstruction
Veritas®	Baxter	Bovine	Crosslinked,	Soft tissue
	Healthcare	pericardium	hydrated sheet	repair
Xenform™	Boston	Fetal bovine	Natural, dry	Soft tissue
	Scientific	dermis	sheet	repair
XenMatrix™	Bard (Davol)	Porcine	Natural, dry	Soft tissue
		dermis	sheet	repair

Table 2 continued					
Zimmer® Collagen Repair Patch	Zimmer	Porcine dermis	Crosslinked, hydrated sheet	Tendon repair	

2.3.2 Current Clinical Trials Utilizing ECM

This section is an overview at the time of writing of the types and applications of various ECM biomaterials being developed into therapeutics as evidenced by their inclusion in clinical trials. Information was gathered throughout August 2021 from an official website from the U.S. National Library of Medicine within the National Institute of Health: https://clinicaltrials.gov/ct2/home.

The search terms "extracellular matrix" and "ECM" bring up 363 unique studies assessing 603 conditions from Abdominal Aortic Aneurism and to Wounds and Injuries. Not every entry represents an ECM biomaterial being tested but the majority of them do. To illustrate, all 20 of the top entries are shown and each one utilizes an ECM biomaterial, including one derived from fish skin and one assessing utility in high grade dysplasia (see Table 3).

The following statements refer only to the 361 studies brought up with the latter search term, "ECM". 92 of these studies are classified as Recruiting, Enrolling, or Active, meaning they are currently collecting data. 256 are classified as Interventional studies, which is defined on the site as follows "A type of clinical study in which participants are assigned to groups that receive one or more intervention/treatment (or no intervention) so that researchers can evaluate the effects of the interventions on biomedical or health-related outcomes. The assignments are determined by the study's protocol. Participants may receive diagnostic, therapeutic, or other types of interventions." These interventional studies are precursors to new therapies that will become commercial products or expand the current indication of current commercial products. To underscore the interest of industry in developing such therapies, 107 of the studies are marked as having Industry Funding.

Table 3 Top 20 Clinical Trials for "ECM"

<u>#</u>	Study Title	Conditions	Interventions
1	CorMatrix Cor TRICUSPID ECM Valve Replacement Study	Tricuspid Valve Disease	Device: CorMatrix Cor TRICUSPID ECM Valve
<u>2</u>	Investigation of A-ECM for the Correction of Soft Tissue Defects	Disorder of Soft Tissue of Body Wall Region	Device: Adipose- derived ECM
<u>3</u>	Restore Myocardial Function With CorMatrix® ECM® Particulate (P-ECM)	Heart Failure	Device: P-ECM Implant
4	Epicardial Infarct Repair Using CorMatrix®-ECM: Clinical Feasibility Study	Acute Coronary Syndrome Heart Failure	Device: Epicardial Infarct Repair with CorMatrix- ECM Procedure: Coronary Artery Bypass Grafting Surgery
5	TECR & ECM Placement for Esophageal High-Grade Dysplasia	Barrett Esophagus Esophagus High Grade Dysplasia	Device: Subjects undergoing TECR with ECM placement (ACell MatriStem, Boston Scientific WallFlex))
<u>6</u>	Examining the Effectiveness of a Connective Tissue Matrix Implant in Reducing Post- operative Pain and Narcotic After Outpatient Arthroscopic Shoulder Surgery	Arthroscopic Shoulder Surgery Pain, Postoperative	Device: Extracellular Matrix Graft Injectable Implant

	Table 3 continued					
<u>7</u>	CorMatrix ECM Study: To Identify Inflammatory Markers Following CABG With/Without ECM	Coronary Artery Disease	Device: CorMatrix extra cellular matrix (ECM)			
<u>8</u>	A Study on the Use of CorMatrix® ECM® for Femoral Arterial Reconstruction	Peripheral Vascular Disease	Device: CorMatrix ECM for Vascular Repair			
<u>9</u>	A Study to Obtain Additional Information on the Use of CorMatrix® CanGaroo ECM® Envelope	Coronary Disease	Device: CorMatrix CanGaroo ECM Envelope			
<u>10</u>	Cellular Therapy for In Utero Repair of Myelomeningocele - The CuRe Trial	Myelomeningocele	Biological: Placental Mesenchymal Stem Cells seeded on a commercially available dural graft extracellular matrix			
<u>11</u>	CorMatrix ECM for Carotid Repair Following Endarterectomy Registry	Carotid Stenosis	Device: CorMatrix ECM for Carotid Repair			
<u>12</u>	ECM and Blood Components for Wound Healing	Ulcer Venous Ulcer Foot Ulcer, Diabetic	Device: ECM Biological: Blood Products Device: ECM in Blood Products			
<u>13</u>	Obtain Additional Information on Use of CorMatrix ECM (Extracellular Matrix)	Coronary Disease	Device: CorMatrix ECM			
<u>14</u>	Evaluation Of New Onset Postoperative Atrial Fibrillation	Atrial Fibrillation New Onset	Device: CorMatrix ECM for Pericardial Closure			
<u>15</u>	Treatment of Wounds Using Oasis® ECM	Non-healing Wound	Device: Oasis Extracellular Matrix			
<u>16</u>	Effect of Hyaluronic Acid ECM on Venous Ulcers	Venous Ulcers	Device: Hyalomatrix extra- cellular matrix			
<u>17</u>	A Prospective Randomized, Double-Blind, Placebo- Controlled Trial Comparing VitroGro® ECM to Placebo in Patients With Venous Leg Ulcers	Venous Leg Ulcer	Drug: VitroGro® ECM Drug: Dulbecco's Phosphate Buffered Saline			
<u>18</u>	Use of Fish Skin Extracellular Matrix (ECM) to Facilitate Chronic Wound Healing	Pressure Ulcers Venous Ulcers Diabetic Ulcers	Device: Marigen Wound Dressing			

Table 3	continued
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<u>19</u>	LifeNet: Extracellular Matrix Graft in Rotator Cuff Repair	Rotator Cuff Tear	Biological: ArthroFLEX ECM scaffold graft
			Procedure: Control Biological: Crossover
<u>20</u>	A Post Market Study on the Use of Cormatrix® Cangaroo ECM® (Extracellular Matrix) Envelope	Cardiovascular Disease	Device: Cormatrix® Cangaroo ECM®

2.3.3 Regulatory Considerations

ECM biomaterials have a long history of commercial and clinical application. Most of these products have been classified as a medical device by the Food and Drug Administration (FDA). This is in contrast to drugs classified by the FDA as a product achieving its primary intended goal by means of chemical action or being metabolized by the body. In 1997, the FDA created the Tissue Reference Group (TRG) to assist in the categorization of products as a device, biologic, or combination, specifically in the realm of the regulation of human cells, tissues, and cellular- and tissue-based products (HCT/P). The TRG is composed of representatives from the FDA's Center for Biologics Evaluation and Research, the Center for Devices and Radiological Health, the Office of Combination Products, and the Office of the Chief Counsel. The TRG continues to update their recommendations on the classification of products. As of the time of writing, the TRG indicated that secreted or extracted human products, such as collagen, are not considered an HCT/P. In 2014, ground, defatted, decellularized adipose tissue was determined not to be an HCT/P because it is more than minimally manipulated. As a final example, in 2012, allogeneic, processed acellular dermis for breast tissue defects was determined not to be an HCT/P because it is used in a non-homologous site. It is apparent that FDA regulation of commercial/clinical products is changing as the field of tissue engineering and regenerative medicine advances. For the present, new ECM biomaterial-based products will likely be classified based on their predecessors.

3.0 Objectives

While carcinogenesis is a complex phenomenon that is not yet fully understood, it is certain that dysregulated extracellular matrix (ECM) forms a tumor microenvironment which drives glioblastoma multiforme (GBM) progression. Through dynamic reciprocity the microenvironment can affect many facets of cellular behavior including proliferation, migration, differentiation, and even survival. Studies have shown various types of cancer cells can be modulated away from the neoplastic phenotype when in the presence of non-neoplastic, mammalian ECM biomaterials. GBM is among the most lethal types of cancers and improved therapeutic options are desired.

ECM biomaterials are rapidly gaining popularity as a therapeutic substance and have had promising application in central nervous system injuries such as stroke and traumatic brain injury. Several reports have shown ECM biomaterials elicit anti-cancer effects in vitro and in vivo though their potential for GBM treatment is unknown. Herein, the use of ECM biomaterials as a therapy for GBM is proposed and evaluated. The objectives of the present dissertation were to experimentally confirm results found in scientific literature to verify that non-neoplastic mammalian ECM modulates the phenotype and progression of non-GBM cancer cells, to characterize the effects of these materials on GBM cells in vitro, to determine whether any therapeutic benefit is gained by ECM biomaterial intervention in vivo, and finally to characterize the proteomic profile of the ECM itself.

This work is intended to lay the groundwork for producing the first ECM biomaterials-based therapeutic for GBM which would represent a game-changing update for this devastating disease.

4.0 Central Hypothesis and Specific Aims

The central hypothesis of the present work is that non-neoplastic mammalian extracellular matrix (ECM) will have distinct anti-neoplastic effects on various models of glioblastoma multiforme (GBM). A corollary to this hypothesis is that delivery of ECM components will bestow a clear therapeutic benefit in a preclinical model of GBM.

Aim 1: Experimentally confirm results described in scientific literature to verify that pepsin-solubilized non-neoplastic mammalian ECM possesses anti-cancer properties in non-glioma cancer cells in vitro.

Aim 2: Characterize the effects of non-neoplastic mammalian ECM on primary glioma cells and glioma cell lines in vitro.

Sub-Aim 2.1: Identify a preferential source of ECM for glioma treatment based on bioactivity and logistics for downstream application.

Sum-Aim 2.2: Determine whether a liquid fraction of ECM (liquid ECM) elicits the same effects as whole, pepsin-solubilized ECM.

Sub-Aim 2.3: Characterize the effects of ECM on non-neoplastic CNS cells.

Aim 3: Characterize any therapeutic benefits intervention with liquid ECM provides in a C6-Wistar rat intracranial model of GBM.

Sub-Aim 3.1: Verify the in vivo safety of this liquid ECM in a rat model.

Aim 4: Contrast the proteome of the liquid ECM with its parent ECM and native tissue of origin.

5.0 Validating Reports of Extracellular Matrix-Based Biotherapy for Non-Glioma Cancers

5.1 Introduction

The earliest cancer therapy that still has modern application was based in the field of physics with radiotherapy gaining traction as early as 1901. Starting in the 1940s this growing sector of healthcare has been dominated by contributions from the field of chemistry, with chemotherapy being almost synonymous with cancer therapy. Only recently has the field of biology supplied viable materials or techniques for treating cancer- a growing source of therapies called biological therapy, or biotherapy (166, 167). While not yet in widespread clinical use, putative biotherapies generally fall into several categories including gene therapy, and biological response modifier therapy (including immunotherapy). Traditional radio- and chemotherapeutic methods are often pan-cytotoxic, creating the hallmark host of serious side effects associated with cancer therapy, notably immune suppression. Biotherapies often operate through cancerspecific mechanisms, and some enact an effect by directly boosting the immune response to cancer. The use of ECM to modulate cancer progression would be classified as a biological response modifier (BRM) therapy, being defined as a therapy based on substances made from living organisms. Examples of BRMs include cancer treatment vaccines, interferons, interleukins, macrophage colony stimulating factors, growth factors, and other similar molecules (168). Note that many of these molecules are naturally found in the ECM.

The extracellular matrix (ECM) represents a major portion of the tissue microenvironment and is composed of structural and functional molecules such as collagens, proteoglycans, cytokines, growth factors, and matrix-bound nanovesicles, among others (18, 169, 170). The microenvironment markedly influences cell behavior, gene expression, and phenotype, and is a major factor in the dynamic and continuous cell-matrix crosstalk referred to as "dynamic reciprocity" (171, 172).

As cancer research progresses, the prevailing theoretic framework of carcinogenesis, somatic mutation theory, has failed to adequately explain an increasing number of observations and datapoints, leading to alternative theories such as tissue organization field theory (TOFT) (33). These theories are not necessarily mutually exclusive to somatic mutation theory and hybrid models are being considered (32). TOFT posits that metaplastic and neoplastic transformation of cells is directly related to changes in the microenvironment/ECM (40, 41). Therefore, if this theory is accurate, manipulation of the tumor microenvironment could modulate neoplastic cell behavior. The specific components of the ECM milieu that modulate cell phenotype are only partially understood.

Several studies have documented the potential transformative effects of mammalian non-neoplastic ECM upon neoplastic cells in vitro and in vivo. These studies have included various types of cancer including breast, urinary bladder, prostate, colon, skin, and esophageal, among others (46-50, 107, 111, 113). A recent study showed the beneficial therapeutic effects of an ECM hydrogel derived from normal esophageal tissues upon dysplastic esophageal mucosa (i.e. Barrett's esophagus) in which metaplastic cells regressed to a normal, squamous epithelial

phenotype in a canine model (112). A study by the same group found increased late apoptosis in a metaplastic and two neoplastic cell lines exposed to urinary ladder matrix ECM (49).

The present chapter aims to experimentally confirm the results found in scientific literature that support the claim that ECM biomaterials modulate the phenotype of non-GBM cancer cells. These preliminary experiments are a critical step before interrogating the effect of ECM on glioma cells. The salient existing literature in question includes both in vitro and in vivo experiments but for the purposes of these experiments limiting methodology to cell culture is sufficient. Cancer cell and ECM types were opportunistically used according to availability to rapidly validate or invalidate further experimentation with glioma cells.

5.2 Materials and Methods

5.2.1 Overview of Experimental Design

Tissues from adult porcine sources were opportunistically obtained and decellularized in accordance with established standard operating procedures to obtain extracellular matrix (ECM). ECM was lyophilized, comminuted, and digested in pepsin to produce ECM hydrogels. Two esophageal cancer cell lines were seeded onto ECM hydrogels and agar and cell morphology and colony number/morphology were observed. ECM powder was processed into a saline-soluble liquid ECM (ECM-SF) which was used to spike media given to cell lines of two types of breast cancer and

viable cells were counted using Trypan Blue exclusion. UBM-ECM-SF-spiked media was lung cancer and cervical cancer cell lines and cell viability was measured using an Alamar Blue assay.

5.2.2 Preparation of Non-Neoplastic Mammalian ECM

Porcine testicular ECM (ptECM) was isolated as previously described (173). Briefly, testes were decapsulated, sliced to 4 mm, and rinsed in type I water which was replaced every 20–60 min until water remained colorless. Material was then agitated in a 0.02% trypsin and 0.05% EGTA solution at 37°C for 2.5 hours, agitated in 0.075% sodium dodecyl sulfate (SDS) for 26 hours with one detergent change to 1% TX-100 at 24 hours. Material was washed in 0.1% peracetic acid and 4% ethanol for 2 hours and alternating PBS/water for 30 minutes four times before being lyophilized and comminuted through a 40-mesh sieve.

Porcine urinary bladder matrix (UBM-ECM) was isolated as previously described (174). Briefly, the basement membrane and lamina propria were mechanically isolated from the bladders. Material was further decellularized and disinfected by agitation in 0.1% peracetic acid with 4% ethanol before rinsing, lyophilization, and comminution through a 40-mesh sieve.

5.2.3 ECM Hydrogel Preparation

ECM powder as described above was solubilized by digestion with 1 mg/mL pepsin at pH 2.5 for 24 hours as previously described (175).

5.2.4 Liquid ECM Preparation

UBM-ECM powder as described above was tumbled in a Fisher Scientific tube revolver in normal saline at 10mg/mL for 24 hours at room temperature. ECM particles were pelleted via centrifugation at 100,000 rpm for 30 minutes and supernatant was decanted and passed through 100um cell strainers, then 0.22-micron sterile filters. Resulting liquid was concentrated 200-fold by volume in 10kDa cutoff columns (MilliporeSigma UFC901008, Darmstadt, Germany) and protein concentration was approximated using a Nanodrop A280 measurement to verify cutoff column filter integrity. Samples were stored at -20° C until use. UBM-ECM-SF was clear and colorless with viscosity comparable to water and protein content of approximately 25-30mg/mL as measured by A280 absorbance.



Figure 6 UBM-ECM-SF production method. Procedure for isolating and concentrating a salinesoluble fraction of ECM derived from porcine urinary bladder.

5.2.5 Cancer Panel Cell Culture

The following panel of human non-glioma cancer cell lines was obtained: SK-GT-4 and OE33 esophageal cancer cells (Sigma-Aldrich), MCF-7 and MDA-MB-231 breast cancer cells (ATCC), A549 lung cancer cells (ATCC), and HeLa cervical cancer cells (ATCC). A common growth media was used for all cells comprised of high glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

5.2.6 Cell Culture Experiments

Agarose (0.5% weight/volume) and ECM hydrogel substrates (ptECM and UBM-ECM, 6mg/mL) were cast into 24-well plates and allowed to set up at 37 °C overnight. 24,000 SK-GT-4 and OE33 cells in growth media were introduced into the wells and photographed at 1-, 3-, and 6-days post-plating (N=2).

24,000 MCF-7 and MDA-MB-231 cells were plated into 24-well plates and allowed to attach overnight. Media, media spiked with saline, or media spiked with volume-matched amounts of UBM-ECM-SF at 1, 2, and 3 mg/mL were added to the wells. After 24 hours viable cells were counted using Trypan Blue exclusion and normalized as a percentage of the media control (N=3, significance of p < 0.05 for oneway ANOVA).

24,000 A549 and HeLa cells were plated into 24-well plates and allowed to attach overnight. Media, media spiked with saline, or media spiked with volumematched amounts of UBM-ECM-SF at 1 and 3 mg/mL were added to the wells. After 24 hours cell viability was measured using the Alamar Blue Viability assay from Fisher Scientific according to manufacturer's instructions and normalized as a percentage of the media control (N=3, significance of p < 0.05 for one-way ANOVA).

5.3 Results

5.3.1 Esophageal Cancer Phenotype Modulated by ECM Hydrogel

The morphology and colony phenotype of two types of human esophageal adenocarcinoma cell lines, SK-GT-4 and OE33 was evaluated at several timepoints on substrates of agarose or two types of ECM hydrogel. SK-GT-4 and OE33 cells grown on agarose grew as expected with normal round, bright, white cell bodies at 24h growing and clustering into larger, round colonies at 3 and 6 days (Figure 7A, Figure 8A). SKGT-4 and OE33 cells grown on porcine testicular ECM presented round singly isolated, dull/dark cell bodies that decreased dramatically in number by 6 days (Figure 7B, Figure 8B). Many of these cells appeared to have condensed and fragmented nuclear material, and a large amount of small, vesicular bodies present nearby. None of them had grown into multicellular colonies as observed in the agar condition. SK-GT4 cells grown on 6 mg/mL porcine urinary bladder matrix-(UBM) ECM pepsin-digested hydrogel presented round, bright, white cell bodies at all timepoints and colonies were flatter, separated, and remained small (Figure 7C). OE33 cells grown on UBM-ECM presented dull/dark cell bodies at 3 and 6 days, with a dramatic decrease in cells present by 6 days (Figure 8C). Both cell types grown on UBM-ECM had many small, vesicular bodies present nearby at all timepoints.



Figure 7 SK-GT-4 esophageal adenocarcinoma cells. Cells were cultured on 0.5% w/v agarose, and 6mg/mL hydrogels of porcine testicular ECM and porcine UBM-ECM and imaged at 1, 3, and 6 days.

SK-GT-4 (esophageal adenocarcinoma) growth on various substrates



OE33 (esophageal adenocarcinoma) growth on various substrates 24 hrs 3 days 6 days

Figure 8 OE33 esophageal adenocarcinoma cells. Cells were cultured on 0.5% w/v agarose, and 6mg/mL hydrogels of porcine testicular ECM and porcine UBM-ECM and imaged at 1, 3, and 6 days.

5.3.2 Liquid ECM Decreases Breast Cancer Viability in a Dose-Dependent Manner

Viable cell count with Trypan Blue exclusion as a normalized percentage of cells grown in growth media control were recorded for slow growing MCF-7 and triplenegative MDA-MB-231 human breast cancer cell lines growing in media spiked with a saline control or increasing concentrations of saline-soluble fraction of urinary bladder matrix-ECM (UBM-ECM-SF) for 24 hours. One-way ANOVA of either dataset found no significant differences between media, saline, or 1 mg/mL UBM-ECM-SF groups (threshold at p < 0.05). However, there were significantly fewer MDA-MB-231 cells in the 2 mg/mL UBM-ECM-SF condition compared to media and saline controls (Figure 9B). Furthermore, there were significantly fewer cells in the 3 mg/mL UBM-ECM-SF condition than in all other conditions for both the MCF-7 and MDA-MB-231 datasets (Figure 9).



Figure 9 Cell counts MCF-7 and MDA-MB-231 breast cancer cells. Cells were cultured for 24 hours in media, a saline control, or media spiked with increasing concentrations of UBM-ECM-SF. N = 3, significance of p < 0.05 for one-way ANOVA

5.3.3 Liquid ECM Decreases Lung, Cervical Cancer Viability in a Dose-Dependent Manner

Relative fluorescent units (RFU) in an Alamar Blue assay normalized to cells grown in growth media were recorded for A549 human lung cancer and HeLa human cervical cancer cell lines growing in media spiked with a saline control or with 1 mg/mL or 3 mg/mL of saline-soluble fraction of urinary bladder matrix-ECM (UBM-ECM-SF) for 24 hours. One-way ANOVA found no significant differences between treatment groups of A549 cells, however the RFU for cells grown in 3 mg/mL UBM-ECM-SF seemed drastically decreased (p = 0.065, threshold at p < 0.05, Figure 10A). Additionally, HeLa cells grown in 3 mg/mL UBM-ECM-SF had significantly lower RFU compared to media and saline conditions (Figure 10B).



Figure 10 Alamar blue assay data for A549 lung cancer and HeLa cervical cancer cells. Cells were cultured for 24 hours in media, a saline control, or media spiked with 1 mg/mL or 3 mg/mL concentrations of UBM-ECM-SF. N = 3, significance of p < 0.05 for one-way ANOVA

5.4 Discussion

The results of these preliminary experiments support the general trend found in other studies, namely that ECM-based hydrogels and components thereof have some ability to modulate the behavior or malignancy of cancer cells.

The cell types present in this chapter, SK-GT-4, OE33, MCF-7, MDA-MB-231, A549, and HeLa are all of human origin, increasing the likelihood that their behavior would be similar to that of a human glioma cell line, which is the next line of inquiry. Any limitations that are intrinsic to the methodology of cell culture will be mirrored in the next set of glioma experiments which will also be carried out via cell culture. ECM is derived

from a natural source which introduces biological variability and requires multi-step manual processing which introduces sample variability. Multiple biological and technical replicates were included in all stages of experimentation to gather representative data.

Agarose gel is commonly used as a cell culture substrate in testing the ability of cells to proliferate in an anchorage-independent manner, a hallmark of cancer cells. Resistance to anoikis, cell death upon detachment from an ECM or other anchor, was observed in both the SK-GT-4 and OE33 cells as expected, and their colonies continued to grow in large, round bodies throughout day 6. However, neither cell type was able to thrive on the porcine testicular ECM (ptECM) hydrogel substrate, despite the presence of RGD sequences that could be used for attachment. Cell bodies appeared duller/darker than their healthy counterparts, with uniform roundness, appearing as single cells rather than growing in multicellular colonies, with possible nuclear condensation and fragmentation, and with lots of small vesicular debris on day 6, indicating cell death possibly by apoptosis (176). This coincides with the large number of vesicular debris (possible apoptosis bodies) seen in SK-GT-4 and OE33 cells grown on porcine urinary bladder matrix ECM (UBM) hydrogel at 1, 3, and 6 days, and the overall disappearance of cells at day 6 of the OE33 experiment. This is in line with a published report of UBM hydrogel increasing apoptosis in these same two cell lines (49). The flat appearance of colonies grown in UBM is likely due to cellular attachment to the hydrogel and lateral growth along that surface.

ptECM was included in the experiment opportunistically, and while it is interesting to note the dramatic amount of putative cell death induced on the SK-GT-4 and OE33 cell lines there were several reasons for not using this ECM type moving forward.

Having been isolated specifically for use in a separate research project it was in short supply (173). The isolation protocol for ptECM also includes several harsh detergents which, while it does include wash steps to remove these detergents, are nonetheless a lurking variable in explaining any cell death. UBM is created using a largely mechanical decellularization process and is devoid of this complicating factor. ptECM is not well characterized in the field as compared to UBM. ptECM does not have any counterpart commercial products, while UBM does, acting as a large regulatory hurdle in any downstream application of this work. ptECM was not used in any of the salient literature whose premises were being tested, while UBM was included in several of them. Pepsinsolubilized ECM hydrogels have a physical limit to their concentration and require increasing amounts of pepsin which can act as a confounding variable. Thick hydrogels with autofluorescent ECM molecules such as collagen and elastin complicate microscopic imaging and other assays (177). After testing several candidates, normal saline was chosen as the extraction buffer to reduce UBM powder into a liquid ECM and avoid these complications moving forward.

MCF-7 cells are known to be notoriously slow growing, with doubling times between 30-40 hours. MDA-MB-231 is triple negative for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. Despite these differences these cell types responded similarly to growth in media spiked with a salinesoluble fraction of UBM-ECM (UBM-ECM-SF) though with slight difference in sensitivity. While the A549 lung carcinoma cells did not show a statistically significant response, the results trended toward the same results as the MCF-7, MDA-MB-231, and HeLa cells as well, despite these cell types having many differences. This may suggest, as

does the body of anti-cancer ECM literature taken as a whole which spans a diversity of cancer cell types, that growth rate and the bulk of the mutation statuses are not key targeted features but that the targeted characteristic(s) are something more centrally intrinsic to the cancer phenotype.

The hallmarks of cancer, a compilation of facets that is common to all cancers, have been reimagined several times. Some of these lists claim all cancer cells resist cell death and create an abetting microenvironment, the tumor microenvironment (178, 179). Obviously resisting cell death is situationally dependent, and perhaps the protective tumor microenvironment may play an important role in that. After all, SK-GT-4 and OE33 cells were shown to have increased apoptosis in the presence of UBM hydrogel (49). The following chapter will more directly deal with the former feature and show that ECM can directly induce cell death in glioma cells. It is seemingly possible that replacing the latter feature with a normal, non-neoplastic microenvironment might directly modulate the other hallmarks of cancer. Other hallmark behaviors of cancer that could be contested by ECM treatment are immune modulation, since ECM is also known to modulate the immune system (124), invasion, since cancer cells have been shown to exhibit decreased invasion in porcine small intestinal submucosa-ECM hydrogel (48), and perhaps others.

5.5 Conclusion

A growing body of literature shows that non-neoplastic mammalian extracellular matrix (ECM) products and their components are able to modulate the phenotype and

progression of various types of cancer cells. Before extrapolating this conclusion and testing ECM for the rapeutic application in glioma, it is critical to carry out opportunistic preliminary experiments to experientially validate these claims in non-glioma cancer cells. Esophageal adenocarcinoma cell lines, SK-GT-4 and OE33, exhibited varying degrees of cell death with nuclear condensation and fragmentation and increased vesicular cell debris consistent with apoptosis when cultured on porcine testicular ECM and urinary bladder matrix ECM (UBM) hydrogels. Culturing breast cancer cells MCF-7 and MDA-MB-231 in media spiked with a saline-soluble fraction of UBM-ECM (UBM-ECM-SF) resulted in far fewer cells than the media control at 24 hours. A549 lung carcinoma and HeLa cervical cancer cells showed decreased fluorescence, associated with cell viability, when cultured in media spiked with UBM-ECM-SF. ECM biomaterials as laboratory research articles are known to have low cytotoxicity, thereby suggesting that these ECM materials directly modulated the proliferation and/or viability of multiple non-glioma cancer cell types, coinciding with findings from the body of literature in question. The results of this chapter support further experimentation testing the therapeutic potential of ECM materials for glioblastoma multiforme.

6.0 The Effect of Non-Neoplastic ECM on Primary Glioma Cells, Glioma Cell Lines, and Non-Neoplastic CNS Cells In Vitro

This chapter in part was adapted from the following manuscript: Murdock, Mark H., et al. "A liquid fraction of extracellular matrix inhibits glioma cell viability *in vitro* and *in vivo*." Oncotarget [Online], 13.1 (2022): 426-438. Web. 23 Feb. 2022

6.1 Introduction

Extracellular matrix (ECM) biomaterials have reportedly been used to modulate phenotype or mitigate several signs of malignancy in various types of cancer (46-50, 107, 111, 113). ECM biomaterials have also been shown to successfully promote repair and reconstruction in central nervous system (CNS) tissues, and several ECM products have received FDA approval for CNS indications (82-84). However, ECM biomaterials or components thereof have not yet been evaluated for application in the treatment of glioblastoma multiforme (GBM). ECM is composed primarily of the secreted products of resident tissue cells and acts as a reservoir for growth factors and other bioactive components (17). This microenvironment is a potent regulator of many aspects of cellular behavior including survival (16, 180, 181).

GBM is the most common type of malignant primary brain tumor. The incidence rate in the United States is 3.19 per 100,000 people and an estimated 10,000 new cases will be diagnosed in the upcoming year (182). One of the most lethal types of cancer, GBM has a five-year survival rate of just ~5%. While much has been learned

about the pathobiology of GBM the standard of care has not been significantly updated since 2005 when temozolomide and concomitant radiation therapy were shown to increase median survival by 2.5 months. Surprisingly, recent studies suggest that up to 60-75% of GBM patients derive no benefit from TMZ treatment and that 15-20% of TMZ-treated patients develop clinically significant toxicity (183). The poor prognosis combined with the limited survival benefits of treatment, along with the myriad of side effects which can greatly decrease quality of life has led some patients to electively refuse treatment altogether and let the disease run its natural course. The inability to effectively treat such a disease underscores the need for research into novel therapeutic approaches.

With downstream clinical application in mind, it is valuable to base the initial experiments of our study on patient-derived glioma cells. Primary human cells will afford more relevancy to biomedical research than transformed or animal cell lines. Thanks to the laboratory and graciousness of Dr. Nduka Amankulor, a specialist in the surgical treatment of complex brain tumors who at the time worked at the University of Pittsburgh Medical Center's Hillman Cancer Center, and Dr. Aparna Rao, a post-doctoral researcher in his lab at the time, our laboratory was supplied with three primary glioma cell cultures to be used in these experiments. These cells are not described in literature and were annotated as 031913, 101513, and 111913, coinciding with the dates that these were excised from patients. The last two identical numbers were dropped for shorthand reference during experiments. 0319 and 1015 cells were excised from high grade glioblastomas, and 1119 from a lower grade glioma. While gathering data from primary cells carried certain benefits it was also desirable to use established

glioma cell lines such as U-87 MG to increase the relevance to the glioma research community.

There are several choices to be made when contemplating a desirable form factor for an ECM-product used in the context of glioma treatment. Solid ECM, whether in sheet or powder form is undesirable. Gliadel® wafers are solid in form, consisting of the alkylating agent carmustine in a biodegradable polyanhydride copolymer known as polifeprosan. These wafers are known to spontaneously migrate after placement in the brain, damaging the soft surrounding tissue and causing acute hydroencephalus due to mechanical obstruction of cerebral spinal fluid pathways (184). Solid pieces of ECM may pose similar, unacceptable issues. Hydrogel forms of ECM have successfully been used in animal models of stroke to good effect (82, 83). However, as discussed in the previous chapter, for the in vitro analyses planned for this study hydrogels with increasing ECM content pose issues with imaging and certain types of assays. Additionally, injecting ECM hydrogel into the brain requires a volumetric void. This was accomplished in the aforementioned studies by displacing the necrotic soup of the stroke lesion with ECM, but for an animal model of GBM creating this volume would entail sophisticated equipment for uniform removal of tumor bulk to the borders in a small rodent model, which equipment and expertise our laboratory is lacking. Therefore, a rationale for creating and assessing a liquid form of ECM, which can be assessed with ease in vitro and injected intratumorally in vivo arises. So called "liquid ECM" is simply a liquid fraction derived from an ECM precursor and have been described as possessing many of the bioactive properties of their parent ECM (140). These fractions are a
cocktail of ECM components usually sharing a certain characteristic of extraction or isolation, such as solubility in a given solvent or similar molecular weight.

One form of liquid-ECM worthy of testing is a solution containing the matrixbound nanovesicles (MBV) of an ECM. MBV are a newly discovered component of the ECM. They were first identified by Huleihel et al. as nano-vesicles embedded within ECM scaffolds derived from porcine urinary bladder matrix, small intestinal submucosa, and dermis (18). Like exosomes, MBVs are nano-sized, lipid membrane bound vesicles with a size distribution from 10 to 200 nm. MBVs were isolated from acellular porcine tissues by enzymatic digestion followed by ultracentrifugation at increasing g-values. MBVs contain distinct profiles of microRNAs and proteins with some mutual cargo among the three ECM scaffolds evaluated. In vitro, MBVs can recapitulate some of the regenerative effects observed when cells are treated with ECM, thus highlighting the potential of MBVs as a key mechanism mediating the regenerative abilities of ECM scaffolds. For example, Huleihel et al. observed neurite outgrowth in neuroblastoma cells treated with MBVs and activation of macrophages toward a phenotype associated with a more regenerative, anti-inflammatory, constructive remodeling response. It was also desirable to determine whether any glioma-suppressive effects were due to the presence of MBV.

Although ECM biomaterials have consistently shown good biocompatibility with cells and tissue of the central nervous system (CNS) (81-84, 185), it is still desirable to characterize the effects of the liquid ECM form factor in non-neoplastic CNS cells. Additionally, although the effect of ECM biomaterials on macrophage activation states

have been extensively characterized (113, 145, 186-188), it is still desirable to observe the macrophage activation state elicited by the liquid ECM form factor.

The present chapter aims to characterize the effects of non-neoplastic mammalian ECM on primary glioma cells and glioma cell lines in vitro. Other objectives of interest are to identify a preferential source of ECM based on bioactivity and logistics for downstream application, to determine whether a liquid fraction of this ECM elicits the same effects as whole pepsin-solubilized ECM, to determine whether MBV play a role in any observed effects, to characterize the effects of liquid ECM on several non-neoplastic CNS cells, and to observe the activation state of macrophages exposed to liquid ECM.

6.2 Materials and Methods

6.2.1 Overview of Experimental Design

A panel of glioma cells, including patient-derived cells, was acquired to test, in vitro, the central hypothesis that non-neoplastic mammalian extracellular matrix (ECM) will have distinct anti-neoplastic effects on various models of glioblastoma multiforme (GBM). Glioma cells were exposed to media spiked with pepsin-solubilized dermal-ECM, SIS-ECM, and UBM-ECM and differentially affected cell confluence with UBM-ECM producing the most dramatic effects. Glioma cells were exposed to UBM-ECM-SFspiked media and viability, cell number, and apoptosis were measured. Matrix-bound nanovesicles (MBV) were isolated from UBM-ECM-SF and tested alongside MBVdepleted-UBM-ECM-SF for effects on glioma cell viability. Activation state of macrophages in the presence of UBM-ECM-SF was compared to M1-like and M2-like activation states.

6.2.2 Glioma Panel Acquisition, Culture, and Experimentation

Patient-derived glioma cells (designated as 0319, 1015, 1119) were graciously provided by the laboratory of Dr. Nduka Amankulor. These cells are not described in literature. Primary glioma cells were used before passage 20. Glioma cell lines (A172, T98G, U87MG, and C6) and non-neoplastic CNS cells (HCN2 and HMC3) were obtained from ATCC (Manassas, Virginia). All cells were cultured in high-glucose DMEM with 10% FBS and 1% pen/strep with incubation at 37°C in a 5% CO2 environment. For experiments involving ECM hydrogels, UBM-ECM-SF, and temozolomide, cells were plated at 12,000/well in 96 well plates and allowed to attach overnight before growth media spiked with the test article was introduced.



Figure 11 Representative images of primary glioma cell morphology after 24 hours of attachment

6.2.2.1 Primary Glioma Cell Characteristics

The mutation status of several target genes was characterized in the primary glioma cells by the Amankulor laboratory and characteristics were shared as follows. Representative pictures of cell morphology after 24 hours of attachment are included.

Excision date/cell ID	<u>Grade</u>	IDH1 mutation	EGFR mutation	MGMT mutation	<u>1p/19q</u> deletion
031913	HIGH	(-)	Unk.	Unk.	Unk.
(0319)					
101513	HIGH	(-)	(-)	(+)	(-)
(1015)					
111913	LOW	(+)	Amplified	(-)	(+)
(1119)					

Table 4 Primary Glioma Cell Mutation Statuses

6.2.3 MTT Assay

Cells were prepared as described in 6.2.2 and the Vybrant® MTT Cell

Proliferation Assay Kit was used as per manufacturer's instructions (Invitrogen).

6.2.4 Time-lapse Videography

125,000 cells of 0319, 1119, HFF, and HMC3 were plated into 12-well plates in duplicate and allowed to attach overnight. Growth media spiked with 3 mg/mL UBM-ECM-SF was added and time-lapse picture acquisition began within 20 minutes. For brightfield microscopy data pictures were taken every 20 minutes for 18 hours. For darkfield microscopy data pictures were taken every 10 minutes for 12 hours and growth media also contained 1.5 uM NucView 488 Caspase-3 Reagent (Biotium). Images were compiled in AxioVs40 (Carl Zeiss) to create time-lapse videos.

6.2.5 Flow Cytometry for Early and Late Apoptosis

Flow cytometry was performed using Annexin V/propidium iodide (PI) staining in six-well plates (N = 3). Live, nonpermeabilized cells were washed, trypsinized, and 200,000–1 million live cells/treatment were prepared for staining with the Annexin V Apoptosis Detection Kit (eBioscience) and with PI viability dye according to the manufacturer's instructions. Cellular debris were gated out, and the mean side scatter intensity was measured within 4 h at excitation 552 nm and 640 nm for PI and Annexin V-APC, respectively, using the BD FACSAria II.

6.2.6 Isolation of MBV

MBV were isolated from UBM-ECM as previously described (18). Briefly, after liquid-ECM was obtained from UBM-ECM as described in 5.2.4, supernatant was

subjected to successive centrifugations at 500g (10 min), 2500g (20 min), and 10,000g (30 min), repeated three times. Resulting supernatant was then centrifuged at 100,000g (Beckman Coulter Optima L-90K ultracentrifuge) at 4°C for 70 min. The 100,000g pellets were washed and suspended in 500 µl of PBS and passed through a 0.22-µm filter (Millipore). The supernatant after ultracentrifugation represented MBV-depleted UBM-ECM-SF and was retained for experimentation. MBV were confirmed to be present in the solution by nanoparticle tracking analysis with a NanoSight NS500 instrument (NanoSight NTA 2.3 Nanoparticle Tracking and Analysis Release Version Build 0025). Size distribution of MBVs was determined by measuring the rate of Brownian motion with a NanoSight LM10 system (NanoSight) equipped with fast video capture and particle-tracking software.

6.2.7 Rodent monocytes into BMDMs and mac activation tests

Murine bone marrow-derived macrophages were isolated as previously described (189). Briefly, bone marrow was harvested from 6- to 8-week-old C57bl/6 mice. Cells from the bone marrow were washed, plated at 1 x 10⁶ cells/mL, and given macrophage colony-stimulating factor with media every 48 hours for seven days to induce differentiation into macrophages. Macrophages were activated for 24 hours to produce various activation states with the following treatments: (1) 20 ng/mL interferon-g (IFNg) and 100 ng/mL lipopolysaccharide (LPS) (Affymetrix eBioscience, Santa Clara, CA; Sigma Aldrich), (2) 20 ng/mL interleukin (IL)-4 (Invitrogen), or (3) increasing concentrations of UBM-ECM-SF. After incubation at 37C, cells were washed with sterile PBS and fixed with 2% paraformaldehyde (PFA) for immunolabeling.

6.3 Results

6.3.1 Various ECM Hydrogels Differentially Modulate Primary Glioma Cell Confluence

Cell morphology and gross confluence of 1015 high grade primary glioma cells were evaluated at 24 hours after culture in media or media spiked with 300 ug/mL of pepsin-solubilized dermal-ECM, small intestinal submucosa (SIS)-ECM, or urinary bladder matrix (UBM)-ECM of porcine origin (Figure 12). Cells growing in media presented as normal, attached, and with spindle-shape appearance. Cells growing in the presence of dermal-ECM appeared to have no gross decrease in cell number compared to the media control and adopted an elongated, lattice-like morphology. Cells growing in the presence of SIS-ECM had an appreciable decrease in total number compared to the media control and remaining cells presented as normal with spindleshaped morphology. Cells growing in the presence of UBM-ECM were dramatically fewer in number compared to the media control, the remaining attached cells were difficult to identify due to scarcity and high presence of cellular debris.



Figure 12 1015 high grade glioma cells after exposure to various ECM types. Phase contrast microscopy images of cells were taken after 24 hours in media spiked with 300ug/mL of pepsin-solubilized dermal-ECM, small intestinal submucosa (SIS)-ECM, and urinary bladder matrix (UBM)-ECM and compared to a media control.

Gross confluence of 1119 low grade primary glioma cells was evaluated at 17, 64, and 120 hours after culture in media or media spiked with 300 ug/mL of pepsinsolubilized urinary bladder matrix (UBM)-ECM of porcine origin (Figure 13). Cells growing in media divided and filled the field of view until 100% confluence was reached at 64 hours, and a tightly packed over-confluence was seen at 120 hours, as expected. Cells growing in the presence of UBM-ECM do not reach 100% confluence at either 64 hours or 120 hours, and vacant tissue culture plastic was observable at these timepoints.



Figure 13 1119 low grade glioma cells after exposure to pepsin-solubilized UBM-ECM. Phase contrast microscopy images of cells were taken up to 120 hours in media spiked with 300ug/mL of pepsin-solubilized UBM-ECM and compared to a media control.

6.3.2 Various ECM-Saline-Soluble-Fraction Types Differentially Decrease Glioma but Not Microglia or Fibroblast Cell Viability

Cell viability was assessed via MTT assay and normalized to a media control for 0319 and 1015 high grade glioma cells, HMC3 (human fetal microglia line), and HFF (primary human foreskin fibroblasts) after 24 hours in the presence of media spiked with

3mg/mL saline-soluble fractions (SF) of dermal-ECM, SIS-ECM, or UBM-ECM (Figure 14). HMC3 and HFF cells showed slightly (up to 10%) increased absorbance values in all UBM-ECM-SF conditions compared to media. 0319 and 1015 cells showed a ~20% and ~10% reduction in absorbance in the presence of dermal-ECM-SF, a ~50% and ~30% absorbance reduction in the presence of SIS-ECM-SF, and an ~80% and ~70% absorbance reduction in the presence of UBM-ECM-SF, respectively.



Figure 14 Glioma and non-neoplastic cell survival after ECM-SF exposure. MTT assay comparing viability of non-neoplastic HMC3 and HFF cells to high grade glioma cells, 0319 and 1015 in 3mg/mL saline-soluble fractions of dermal-ECM, SIS-ECM, and UBM-ECM.

6.3.3 Various ECM-Saline-Soluble Fractions Have Distinct Protein Profiles

The protein profiles of dermal ECM-SF, SIS-ECM-SF, and UBM-ECM-SF were visualized via a silver stain containing a lane with protein molecular weight markers (Figure 16). Differences in the protein profile of each soluble fraction were apparent with dermal-ECM-SF exhibiting clusters of dark banding concentrated at ~70-170 kDa and ~14-25 kDa, SIS-ECM-SF showing narrow dark bands notable at >200 kDa, ~80 kDa, and ~14 kDa, and UBM-ECM-SF showing a narrow dark band at >200 kDa and clusters of dark banding throughout the rest of the lane.



Figure 15 Silver stain of saline-soluble fractions of dermal-ECM, SIS-ECM, and UBM-ECM biomaterials

6.3.4 The U-87 MG Cell Line Reacted Similarly to 0319 Primary Glioma Cells in the Presence of UBM-ECM-SF

U-87 MG cells, the glioma cell line used in the highest number of glioma research publications, reacted to media spiked with 3 mg/mL of UBM-ECM-SF in the same manner as 0319 primary glioma cells (Figure 16). In both populations there was a large reduction in viable cells visualized by a live/dead assay using calcein AM and propidium iodide, quantified to a ~64% reduction by cell count data normalized to media.



Figure 16 Live-dead assay of glioma cells exposed to UBM-ECM-SF. Calcein-AM and propidium iodide staining of 0319 high grade primary glioma cells and U-87 MG cells in the presence of 3 mg/mL UBM-ECM-SF with corresponding cell count data.

6.3.5 Glioma and CNS Cells Have Differing Sensitivities to UBM-ECM-SF

A panel of glioma and CNS cells were subjected to 1, 5, or 10 mg/mL UBM-ECM-SF and viability was assessed via calcein AM/propidium iodide staining (Figure 17). At 1mg/mL cell groups averaged dead-to-live ratios between 0.9- to 2.0-fold that of the media control (Figure 17). At 5mg/mL cell groups averaged ratios between 10- to 23fold, and at 10mg/mL cell groups averaged ratios between 15- and 37-fold compared to the media control. ANOVA indicated no differences within the 1mg/mL treatment group (P = 0.13) whereas at 5mg/mL the 1119 and A172 dead-to-live ratios were significantly higher than that of U87MG (P < 0.01). At 10mg/mL the 1015 and 1119 ratios were significantly higher than that of U87MG and the A172 dead-to-live ratio was significantly higher than those of non-neoplastic HCN-2 and HMC3 (P < 0.01).



Figure 17 UBM-ECM-SF effect on glioma panel. ANOVA with post hoc Tukey's Honest Significant Difference show no difference at 1mg/mL (P = 0.13), at 5mg/mL 1119 and A172 differ from U87MG (P < 0.01), and at 10mg/mL 1015, 1119, and A172 differ from U87MG, and A172 differs from HCN-2 and HMC3 (P < 0.01).

6.3.6 Non-Neoplastic and Glioma Cells Show Differential Sensitivity to UBM-ECM-SF But Not to Temozolomide at Increasing Concentrations

Cell viability in the presence of increasing concentrations of temozolomide or UBM-ECM-SF was assessed for non-neoplastic cells (HMC3 and HFF) and glioma cells (0319 and 1119) via MTT assay (Figure 18). For all cell types, viability decreased with increasing concentrations of temozolomide with no apparent differential sensitivity except a resistance at 100 uM seen in 1119 cells. For all cell types, viability decreased with increasing concentrations of UBM-ECM-SF except at 350 and 875 ug/mL HFF showed increased absorption and at concentrations higher than 875 ug/mL the glioma cells showed higher sensitivity than the non-neoplastic cells.



Figure 18 Glioma and non-neoplastic cell survival after exposure to TMZ or UBM-ECM-SF. MTT assay comparing viability of non-neoplastic HMC3 and HFF-3 cells to primary glioma cells, 0319 and 1119 in increasing concentrations of temozolomide or UBM-ECM-SF

6.3.7 Glioma Cells but Not Microglia or Fibroblasts Round Up and Bleb in Response to UBM-ECM-SF

Morphological response of non-neoplastic cells (HMC3 and HFF) and glioma cells (0319 and 1119) to 3 mg/mL UBM-ECM-SF was visualized through brightfield microscopy recorded with time-lapse videography (Figure 20, Figure 21). Throughout the 18-hour recording period HMC3 and HFF cells divided and appeared normal. Within the first 10 minutes of UBM-ECM-SF exposure some of the 0319 and 1119 cells,

however, were seen to round up and bleb into fragments. This behavior affected additional cells throughout the recording period. A greater number of 0319 cells were affected as compared to 1119 cells.



Figure 19 Microglia and fibroblasts after exposure to UBM-ECM-SF. Brightfield images of nonneoplastic microglia and fibroblasts in UBM-ECM-SF spiked media. Both cell types appeared to display normal levels of movement and proliferation in the presence of UBM-ECM-SF (time lapse pictures taken every 20 minutes).



Figure 20 0319 and 1119 glioma cells after exposure to UBM-ECM-SF. Brightfield images of neoplastic primary glioma cells in UBM-ECM-SF spiked media. Both cell types display a drastic change in morphology, becoming rounded and shriveling up within the first few hours of culture in the presence of UBM-ECM-SF (time lapse pictures taken every 20 minutes).

6.3.8 Glioma Cells but Not Microglia or Fibroblasts Contain Activated Caspase-3 in Response to UBM-ECM-SF

Caspase-3 activation of non-neoplastic cells (HMC3 and HFF) and glioma cells (0319 and 1119) to 3 mg/mL UBM-ECM-SF was visualized through darkfield

microscopy with NucView reagent signal being recorded with time-lapse videography (Figure 22). Throughout the 12-hour recording period HMC3 and HFF cells showed no caspase-3 activation. Within the first 10 minutes of UBM-ECM-SF exposure, caspase-3 activity was detected in the cultures of 0319 and 1119 cells. Increasing numbers of glioma cells gave off fluorescence throughout the recording period though a greater number of 0319 cells were affected as compared to 1119 cells. NucView reagent cleaved by active caspase-3 binds to DNA to fluoresce, and instances of nuclear fragmentation were observed.



Figure 21 Apoptosis of glioma cells after exposure to UBM-ECM-SF shown by NucView. Nonneoplastic HCM3 and HFF cells (top) and 0319 and 1119 primary glioma cells (bottom) at 12h cultured in 3mg/mL UBM-ECM-SF and 1.5uM NucView reagent. Fluorescent nuclei indicate binding of DNA to NucView cleavage products, effected by active caspase-3.

6.3.9 Flow Cytometry Confirmed UBM-ECM-SF-Induced Apoptosis in Glioma Cells

0319 primary glioma cells inoculated for 24 hours to media or media spiked with 0.5 and 3 mg/mL UBM-ECM-SF were passed through a flow cytometer which measured markers indicating viability as well as early and late apoptosis (Figure 23). Samples prepared from cells in the 3 ml/mL UBM-ECM-SF group could not be run through the flow cytometer due to the presence of too many vesicles, likely apoptotic bodies. The cells in the 0.05 mg/mL group had 49% viable cells compared to 70% in the media, with 3.1% and 14.6% of cells in early and late apoptosis compared to 0% and 3.4% in the media, respectively.



Figure 22 Apoptosis of glioma cells after exposure to UBM-ECM-SF shown by flow cytometry. Flow cytometry assessing early vs late apoptosis in 0319 high grade glioma cells in media or in the presence of 0.5 mg/mL UBM-ECM-SF. Wells containing 3 mg/mL could not be run in the flow cytometer due to the presence of too many vesicles, likely apoptotic bodies.

6.3.10 UBM-ECM-derived MBV Not Responsible Glioma Cell Death

Matrix-bound nanovesicles (MBV) were successfully isolated from UBM-ECM-SF (Figure 24). MBV, MBV-depleted UBM-ECM-SF, and UBM-ECM-SF were given to 1015

primary glioma cells and cell morphology was observed at 24 hours (Figure 25). MBVtreated 1015 cells were identical to those grown in media. MBV-depleted UBM-ECM-SF-treated and UBM-ECM-SF-treated glioma cells shared an apoptotic morphology.



Figure 23 Nanoparticle tracking analysis of MBV derived from UBM-ECM-SF.



Figure 24 MBV are not responsible for the anti-glioma effect of UBM-ECM-SF. 1015 high grade primary glioma cells cultured in UBM-ECM-SF compared to MBV derived from UBM-ECM and complementary MBV-depleted UBM-ECM-SF. Similar images were produced using 0319 and 1119 cells.

6.3.11 UBM-ECM-SF Induces a Distinct Macrophage Activation State

Rodent monocytes isolated from bone marrow were stimulated with MCSF to differentiate them into macrophages. Macrophages were stimulated with IFN-y and LPS, or IL-4 to induce classical M1-like and M2-like phenotypes. Macrophages were

stimulated with UBM-ECM-SF to assess phenotype (Figure 26). M1-like cells predominantly adopted the fried egg morphology and M2-like cells predominantly adopted the spindle-shaped morphology as previously described (190). Macrophages stimulated with UBM-ECM-SF adopted a morphology more similar to M1 than M2.

Additionally, increasing doses of UBM-ECM-SF-spiked media were presented to rat-derived primary bone marrow derived macrophages and classical markers of M1/M2 activation (iNOS and Arginase1) were labeled (Figure 27). Increasing doses results in increased expression of both iNOS and Arginase1, indicating an activation state with characteristics of both M1 and M2 phenotypes. Increasing amount of UBM-ECM-SF correlated with increased expression of iNOS and Arginase1 markers.



Figure 25 Bone marrow derived macrophages stimulated with classical M1/M2 inducers and UBM-ECM-SF. After 24 hours, classical fried egg and spindle morphologies are seen with IFN-γ /LPS and IL-4 stimulation, respectively. Saline control cells show morphology akin to MCSF-only treatment. UBM-ECM-SF treated macrophages adopt a mixture of densely populated fried egg and spindle shaped morphologies.



Figure 26 UBM-ECM-SF induces a mixed M1-M2 activation state in macrophages after 24h of treatment. Increasing doses of UBM-ECM-SF-spiked media were presented to rat-derived primary bone marrow derived macrophages and classical markers of M1/M2 activation were labeled. Increasing doses results in increased expression of both iNOS and Arginase1, indicating an activation state with characteristics of both M1 and M2 phenotypes.

6.3.12 Primary Astrocyte Cultures are Supported by the Presence of UBM-ECM-SF Compared to Media

Cell viability of rodent-derived primary astrocytes in the presence of media or media spiked with 3 mg/mL UBM-ECM-SF after 24 hours was visualized via live/dead stain containing calcein AM and propidium iodide and cultures were quantified via cell count (Figure 28). Live/dead staining showed very little cell death in either group and cell count revealed that there were significantly more astrocytes in the UBM-ECM-SFtreated group.



Figure 27 Rodent-derived primary astrocytes cultured with media and 3mg/mL UBM-ECM-SF

6.4 Discussion

The results presented in this chapter show that a saline-soluble fraction of ECM can decrease glioma cell viability in vitro, and that glioma cells are sensitive to the compositional differences between ECM environments of varying tissues (dermis vs small intestine vs urinary bladder). This suggests some active component, or cofactor, is present in varying degrees in these tissues and is retained throughout the decellularization process. An alternative scenario is that each ECM environment has a distinct mechanism of action altogether, though that seems less likely. Differences in cell response and wound healing have previously been observed when comparing ECM from small intestine to ECM from urinary bladder (188, 191). The silver stain clearly shows that ECM from each tissue contained many protein components and that the

types and amounts differed greatly between samples which is in agreement with previous proteomic studies regarding these materials (192-194).

It was important to determine whether these observations from primary cells could be duplicated with commonly used glioma cell lines, and what the response of non-neoplastic CNS cells would be. A preliminary live/dead assay using calcein AM/propidium iodide showed U-87 MG glioma cells acting similarly to 0319 primary glioma cells. An expanded panel including T98G and A172 glioma cell lines as well as 1015 and 1119 primary glioma cells revealed each glioma cell type is differentially sensitive to the UBM-ECM-SF environment. These differences are unsurprising given the diversity between cancer cells given the designation "glioblastoma multiforme". Each primary cell type and cell line was derived from a different patient and it is known that glioma cell lines possess differing mutations, proliferation rates, and gene expression levels that can affect how they respond to a given treatment or environment (195). The non-neoplastic cell lines showed significant cell death at the 10 mg/mL of UBM-ECM-SF, thereby indicating higher concentrations need not be tested in vitro. While ECM materials have traditionally been reported to have excellent biocompatibility, it seems UBM-ECM-SF contains one or more highly concentrated components that can damage non-neoplastic cells if administered in sufficient amounts. These components may or may not be the same that elicit the anti-cancer effect so attempting to remove them may or may not be a useful strategy in future studies. If solid or hydrogel ECM could be concentrated to the same degree as UBM-ECM-SF, similar cytotoxic results would likely be observed as virtually all test articles become cytotoxic at sufficiently high concentrations. While the glioma cells included both primary cells and cell lines, the

non-glioma cells used (HCN2 and HMC3) represent transformed immortalized cells that may not be representative of the cell population in patients.

Time lapse videography showed that glioma cells but not microglia or fibroblasts were induced by UBM-ECM-SF to undergo caspase-3 mediated apoptosis. This is somewhat surprising given that much of the literature regarding the effect of ECM on glioma cells describes a transformative effect, reverting the cancer cells back toward their pre-cancer behaviors. Bissel et al. showed that breast cancer cells respond to their microenvironment and can revert from their malignant phenotype (46). Hodde et al. found that porcine small intestinal submucosa ECM (SIS-ECM) did not hasten the formation of primary tumors and decreased the volume of recurring tumors after surgical resection (47). Hurst et al. found that high grade metastatic bladder cancer cells grown on SIS-ECM hydrogel decreased invasion and showed more organized growth than when grown on Matrigel or collagen (48). However, this observation of induced apoptosis in glioma cells is in line with the observations of Saldin et al. where UBM-ECM increased apoptosis in esophageal cancer cells (49). This may be due to the increased concentration of certain effector ECM components found in UBM-ECM-SF after the 200-fold reduction in volume. While NucView 488 is marketed as a caspase-3 specific substrate, it is based on a DEVD sequence which means it can also be cleaved by caspase-7. However, both caspase-3 and 7 are classified as executioner caspases, direct precursors to apoptosis.

Overall, these findings are consistent with the concept of tissue organization field theory which challenges the dogma of somatic mutation theory regarding the origin of cancer (40, 41). Somatic mutation theory is cell-centric, attributing the rise of cancer

solely to the accumulation of DNA damage and mutation in cells. In contrast, the TOFT is tissue-centric, ascribing the onset of cancer to aberrations in the microenvironment, of which the ECM is a major part. These concepts are not mutually exclusive. If a microenvironment is indeed sufficient to gradually nudge a cell toward cancer, perhaps a precisely engineered microenvironment can dissuade oncogenic behavior. This concept begets a potential alternative approach to conventional cancer therapeutics and may avoid the side effects of chemoradiation therapy.

The results from this chapter show that UBM-ECM-SF is the best material to continue with in the animal studies as it induced the most glioma cell death, and there are other reasons for continuing to use porcine urinary bladder. It is one of the most readily available and commonly used ECM biomaterials. The decellularization process to manufacture UBM-ECM is well established, relatively simple, and involves primarily mechanical removal of the tunica serosa, tunica muscularis externa, tunica submucosa, and most of the tunica muscularis mucosa to decrease cellularity (174). Harsh decellularization processes that rely upon chemical detergents tend to deplete growth factors, and these detergents often remain in the resulting bioscaffold, skewing experimental results. ECM biomaterials derived from porcine urinary bladder are commercially available (MatriStem, ACell, Columbia, Maryland), retain bioactivity after electron beam terminal sterilization, and have been used in a clinical trial involving esophageal adenocarcinoma patients wherein the implanted bioscaffold degraded within two weeks, promoting functional remodeling of the esophagi which have remained cancer-free to date (clinicaltrials.gov Identifier: NCT02396745).

The results from this chapter show that UBM-ECM-SF, in its liquid form, is sufficient to induce glioma cell death, and there are other reasons for using this liquid form in the upcoming animal experiments. Slivka et al. showed that UBM-ECM hydrogel can be centrifuged into "structural" and "soluble" fractions, which have differential effects in regulating macrophage behavior (145). A liquid form allows for the ECM components to be concentrated tens or hundreds of times, which is not possible with sheets, powders, or digested hydrogels. The liquid form allows for intratumoral injection without first resecting any tissue mass which may have future application in treatment of surgically inoperable tumors. Extraction of components from UBM-ECM using normal saline is logical due to its physiological relevance (interstitial fluid also having 0.9% NaCl). It is known that implanted solid forms of ECM and hydrogel formulations can recruit stem/progenitor cells and modulate macrophage responses within 24 hours and therefore, this length of time for the saline-extraction may capture many of the molecules responsible for these functional observations (83, 188). It is believed that host degradation of implanted ECM is essential for the release of bioactive components which account for positive remodeling effects (144, 196).

There are limitations for these experiments beyond those previously mentioned. Culturing cells on 2D tissue culture plastic with growth media containing BSA has limitations and introduces variables, but this approach represented proof of concept that evolved into and justified an in vivo pilot study in the next experiments.

6.5 Conclusion

Glioma cell cultures can be modulated by both ECM hydrogel and a salinesoluble fraction of UBM-ECM (UBM-ECM-SF). In terms of decreasing glioma cell viability in culture, UBM-ECM is more potent than SIS-ECM, which is more potent than dermis-ECM. Culturing primary glioma cells and glioma cell lines in the presence of UBM-ECM-SF resulted in caspase-3 mediated apoptosis, suggesting the presence of a direct antagonist against glioma cells. Flow cytometry confirmed increased early- and late-stage apoptosis in glioma cell cultures after treatment with UBM-ECM-SF. This is somewhat surprising given that much of the literature regarding the effect of ECM on glioma cells describes a transformative effect, reverting the cancer cells back toward their pre-cancer behaviors. However, this observation of induced apoptosis in glioma cells is in line with the observations of Saldin et al. where UBM-ECM increased apoptosis in esophageal cancer cells (49). The observations in this chapter support further testing in animal models, beginning with experiments to test the safety of UBM-ECM-SF injected intravenously and into the brain, then moving on toward testing within an animal model of glioblastoma multiforme.

7.0 The Effect of a Soluble Fraction of Non-Neoplastic ECM on Glioma Tumors In Vivo in Mouse and Rat Models

This chapter in part was adapted from the following manuscript: Murdock, Mark H., et al. "A liquid fraction of extracellular matrix inhibits glioma cell viability *in vitro* and *in vivo*." Oncotarget [Online], 13.1 (2022): 426-438. Web. 23 Feb. 2022

7.1 Introduction

While cell culture techniques can provide quick results that can answer relatively simple questions, they lack the complexity needed to robustly assess cancer therapeutics. After observing that components of non-neoplastic mammalian ECM decreased glioma cell viability in vitro, the next step was to assess this observation in vivo. Modeling cancer in animal models provides many of the physiological factors that are absent in traditional cell culture methods, such as vascularity, three-dimensionality, the presence of an immune system, and a level of biological variability between animals and tumors. Including each of these components is essential for developing a cancer therapeutic that may someday be used in patients.

While the in vitro experiments largely focused on human primary glioma cells to better answer how tumors in patients would respond to UBM-ECM-SF, these human cells would be of limited use in the animal models. Using human glioma cells would necessitate the use of immunocompromised animals, and it is valuable to have an immune system present in the experimental conditions since that better models any

immunocompetent patients benefitting from this research in the future. Furthermore, given the vast body of literature describing how macrophages are modulated by ECM, we anticipated that there may be an immune system component to how an animal would react to a glioma tumor injected with UBM-ECM-SF. The C6 glioma cell line is one of the most popularly cited among the glioma research community, especially in terms of testing agents for their tumoricidal activity (197). The C6 cell line was developed from Wistar-Furth rats in the 1960s and express many of the same genes that are characteristic of human gliomas, exhibit the same histological features as human GBM, and resembles human GBM immune infiltrates (197, 198). Furthermore, the C6 cell line was included in the glioma panel treated with UBM-ECM-SF and seems somewhat sensitive to its effects (see Figure 18).

The biocompatibility of ECM sheets, powders, and hydrogels have been well established, however concentrated saline-soluble fractions of ECM have not previously undergone such testing. While some ECM hydrogels are cast as form-filling gels before implantation, others are injected as liquids which then polymerize into gels upon reaching body temperature. For example, a cardiac-derived ECM formulation, VentriGel, is stored in lyophilized form and then rehydrated prior to intramyocardial injection where it becomes a porous, fibrous scaffold (199). Though similar, this is inherently different from injecting UBM-ECM-SF which will remain a liquid at body temperature and be free to immediately diffuse and permeate through the local tissue. In vivo safety studies regarding UBM-ECM-SF, especially in the brain, are crucial before beginning to evaluate any therapeutics benefits in glioma models.

After UBM-ECM-SF safety is established, a flank tumor model will give a quick indication of whether the in vitro observations of decreased glioma cell viability hold true in the in vivo setting. However, to model the condition of a glioma patient more closely, the final animal model should consist of intracranial tumors. Implanting C6 tumors into the frontal lobe will give rise to tumors that are less likely to cause rapid death or disturb motor function, thus allowing time to observe the effects of the intervention. A popular preclinical model for new drugs targeting GBM is to transplant C6 cells grown in vitro intracranially into Wistar rats (200, 201). This model can produce tumors displaying several of the histological characteristics of human GBM including neovasculature, parenchymal invasion, nuclear pleiomorphism, presence of hemorrhagic and necrotic areas, especially when using specifically the Wistar strain (200).

The objective of the present chapter is to evaluate the safety of injections of UBM-ECM-SF intravenously and directly into the brain, and then to characterize any therapeutic benefits intratumoral injections would have for both flank and intracranial tumor models. All animal experimentation mentioned in this chapter was regulated and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC).

7.2 Materials and Methods

7.2.1 Overview of Experimental Design

As saline-soluble ECM fractions had not been used in animals before, safety tests were first performed to ensure that UBM-ECM-SF was an appropriate material for in vivo testing. UBM-ECM-SF or saline was administered intravenously to Sprague-Dawley rats over a 10-week period and their temperature, weight, and hematology metrics were monitored and recorded throughout the experiment (N=5 per group). After sacrifice, organs were collected for histology, including lung, spleen, liver, heart, brain, and kidney. UBM-ECM-SF was injected intracranially into the right frontal lobes of Wistar rats and animals were sacrificed at 30 minutes and 24 hours. Brains were rapidly harvested, fixed, and stained with H&E to assess the acute cellular response. After UBM-ECM-SF was deemed safe for further testing, an intracranial glioma model was used. C6 cells were cultured and injected into the right frontal lobes of Wistar rats (N=12). Animals were randomly assigned to the UBM-ECM-SF treatment group, or the saline control group. MRIs were taken weekly until week 10 and tumor volume was calculated using DSI Studio software. UBM-ECM-SF or saline injections were given intratumorally at days 7, 14, and 21. Survival was monitored and recorded.

7.2.2 Animal Experimentation

All animal experimentation were performed in accordance with the regulations and approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh.

7.2.2.1 Intravenous safety study

Adult female Sprague-Dawley rats from Envigo (Indianapolis, Indiana) 250-300 g in weight were used for the systemic intravenous injection study following IACUC protocol 18011620. Animals were randomly assigned into treatment or control groups (N=5) and given either 120mg/kg of UBM-ECM-SF or the equivalent volume in normal saline every other week for a total of five injections through the tail vein. One week prior to this and every other week afterward for a total of six times, 500uL of blood was drawn via saphenous vein puncture into EDTA coated Sarstedt Microvette tubes (Sarstedt Inc 20.1341.102, Numbrecht, Germany). Complete blood count was performed by Marshfield Labs (Marshfield, WI) and red blood cell count, red blood cell distribution, hemoglobin, and hematocrit are reported. Weight and temperature for each animal was monitored and recorded weekly. Rat temperature was monitored using a small rodent infrared thermometer from Braintree Scientific, Inc (IR-B153, Braintree, Massachusetts). See Figure 28.


Figure 28 Experimental design for invtravenous injection safety study

7.2.2.2 Intracranial Injection Safety Study

UBM-ECM-SF was spiked with 1mg/mL 70 kda FITC dextran (Sigma Aldrich 46945, Cleveland, Ohio). Following IACUC protocol 18042257, adult male Wistar rats 250-300 g in weight (N=4) were anesthetized with ketamine/xylazine (90/15 mg/kg intraperitoneal injection) and placed in a stereotaxic frame with 1-3% isoflurane and oxygen flowing from a nose cone. Skin was shaved and a 5mm incision was made, allowing a burr hole to be drilled 2mm lateral to the midline on the right side and 1mm posterior to the bregma. A 500uL Hamilton syringe tipped with a 25-gauge needle and attached to a syringe pump (Harvard Apparatus PHD 2000, Holliston, Massachusetts) delivered FITC-ECM-SF at a rate of 1uL/minute for 10 minutes at a depth of 2mm below the brain surface. After a 5-minute equilibration period the syringe was slowly removed, the skin was sutured and glued, and the animal was recovered. Two animals were sacrificed at 30 minutes post injection, and two were sacrificed at 24 hours post injection. Sacrifice was achieved by CO2 asphyxiation followed by cervical dislocation, and brains were immediately harvested and placed in 10% neutral buffered formalin until further processing.

7.2.2.3 Flank Model

Recurrence/tumor growth rate model- 0319 high grade primary glioma cells were cultured, and 375,000 cells suspended in PBS were injected into left and right flanks of athymic nude mice following IACUC protocol 17029801 (N=2). Tumors were allowed to develop for 28 days, after which tumor bulk was surgically resected. Left flanks of animals received a 300uL Matrigel injection (Fisher Scientific, CB-40234), right flanks of animals received a 300uL injection of 30mg/mL UBM-ECM-SF. After 7 days flanks were photographed and ranked subjectively for size of recurrent tumor growth.

Intratumor injection/survival model- 0319 high grade primary glioma cells were cultured, and 375,000 cells suspended in PBS were injected into left and right flanks of athymic nude mice following IACUC protocol 17029801 (N=6). Animals were randomly assigned to the UBM-ECM-SF treatment group, or PBS control group. Tumors were allowed to develop for 28 days, after which PBS or UBM-ECM-SF injections were administered intratumorally each week for three weeks. Survival was monitored, and animals were sacrificed according to IACUC guidelines if their tumor burden became too large. Survival curves were analyzed for differences using the Log-rank, Mantel-Cox test method.

7.2.2.4 Intracranial GBM Model

Adult male Wistar rats 250-300 g in weight were used for the intracranial glioma model following IACUC protocol 21049151. Animals were randomly assigned into control or treatment groups (N=6 each). C6 glioma cell suspensions at a density of 1 x

10⁶ cells per 10uL in PBS were prepared fresh from mid-confluent cultures to maximize viability upon inoculation. C6 inoculum was administered via a stereotaxic frame and syringe pump as described above. At 7 days post operation and every week thereafter until day 70 animals were subjected to MRI. At days 7, 14, and 21 post inoculation animals received 10uL of either normal saline or 30mg/mL UBM-ECM-SF at the same stereotaxic coordinates and flow rate as the tumor inoculation. At 70 days animals were sacrificed by CO2 asphyxiation and cervical dislocation.

7.2.2.5 MRI Analysis

Rats were anesthetized via a nose cone with 1.5-2.5% isoflurane and O2 IACUC protocol 21049151. The rats were positioned on an animal bed with a stereotaxic head holder and placed in the scanner. A rectal temperature probe was used for monitoring and maintenance of temperature at 37.0 ± 0.5 °C using a warm air heating system. Respiration was monitored (SA Instruments, Stonybrook, New York). MRI was performed using a 7T/30-cm AVIII spectrometer (Bruker Biospin, Billerica, Massachusetts) equipped with a 12 cm gradient set and using an 86 mm quadrature RF transmit volume coil, a 2-channel receive surface RF coil and Paravision 6.0.1. A T2weighted RARE sequence was used to visualize the glioma in both axial and coronal orientations, with the following parameters (axial): repetition time (TR)/echo time (TE) = 5000/80 ms, field of view (FOV) = 35×35 mm, acquisition matrix = 192×192 , 25 slices with a slice thickness of 1 mm, 2 averages, and a RARE factor = 8. The coronal images used the same parameters except 13 slices were used. Tumor volumes were calculated by manual segmentation in a blinded fashion using DSI Studio software (http://dsistudio.labsolver.org/).

7.2.2.6 Statistical Analysis

Data statistical analysis was performed using GraphPad Prism 7.03 software. Log-rank, Mantel-Cox test method was used for survival curves in flank study. Mantel-Haenszel approach for hazard ratio and confidence interval of Kaplan-Meier survival curve was used for intracranial glioma study. Student's t-test was used to compare groups of two for intracranial glioma volume comparison. One-way ANOVA and Tukey's honest significant difference were used to find differences in groups of more than two. For t-test and ANOVA, P-values lower than 0.05 were considered significant. Appropriate numerical data are shown as mean ± standard deviation.

7.3 Results

7.3.1 Intravenous UBM-ECM-SF Injection Did Not Alter Hematology Metrics, Weight, Temperature, or Multi-Organ Histology

Systemically injected UBM-ECM-SF did not alter hematology metrics. There were no significant differences in weight (P = 0.8) or temperature (P = 0.18) between the UBM-ECM-SF and saline-treated groups (Figure 30A, B). The UBM-ECM-SF group did gain more weight per animal on average (15.6 grams) than the saline group (4.6 grams), but the difference was not significant (P = 0.08). Hematology metrics for the two groups were normal with respect to red blood cell count (Figure 30C, P = 0.46), red blood cell distribution (Figure 30D, P = 0.20), hematocrit (Figure 30E, P = 0.32), and

hemoglobin levels (Figure 30F, P = 0.30). H&E staining of lung, spleen, liver, heart, brain, and kidney appeared normal for both groups.



Figure 29 Systemic UBM-ECM-SF safety study. No differences were found in weight (A) or temperature (B) between the UBM-ECM-SF and saline-treated groups. Hematology metrics for the two groups were normal with respect to red blood cell count (C), red blood cell distribution (D), hematocrilt (E), and hemoglobin levels (F).

7.3.2 Intracranial UBM-ECM-SF Injection Did Not Induce an Acute Mononuclear Response

UBM-ECM-SF was injected intracranially to determine the cellular response at 30 minutes and 24 hours. H&E staining indicated that direct UBM-ECM-SF injection did not include a mononuclear response. No cellular infiltration was observed in response to the injection at 30 minutes (Figure 31A) or 24 hours (Figure 31B). No indications of necrosis, pyknosis, or apoptosis were observed. Yellow dotted line indicates the midline of the brain.



Figure 30 Acute cellular response to UBM-ECM-SF injections. No cellular infiltration was observed in response to the injection at 30 minutes (A) or 24 hours (B).

7.3.3 Intracranial FITC-Dextran-Spiked UBM-ECM-SF Injection Produced Signal up to 24 Hours

UBM-ECM-SF mixed with FITC-dextran was injected intracranially to identify injection site and approximate possible diffusion rate of some components. FICT signal

at 30 minutes occupied about 22 times more area than at 24 hours and displayed a gradually fading periphery compared to the sharply disappearing edge of the 24-hour signal (Figure 32A, B). Yellow dotted line indicates the midline of the brain.



Figure 31 Diffusion and retention of FITC-Dextran-spiked UBM-ECM-SF injection. FITC signal at 30 minutes occupied ~22 times more area than at 24 hours and displayed a gradually fading periphery compared to the sharply disappearing edge of the 24-hour signal (A, B). Yellow dotted line indicates the midline of the brain.

7.3.4 Intratumoral UBM-ECM-SF Injection Intervention Reduces Tumor Mass of Resected Subcutaneous Glioma Tumor

0319 cells were injected into athymic nude mouse flanks and allowed to grow for 28 days. Resulting tumor masses were resected and the areas were injected with either UBM-ECM-SF or Matrigel. After 7 days the resection sites were visually compared, and sites treated with UBM-ECM-SF had minimal volumetric tumor regrowth compared to the dramatic recurrence at the Matrigel injection sites (see Figure 33). No measurements were taken, or statistical tests employed due to the objectively large difference in result.



Figure 32 Subcutaneous glioma tumor model in athymic nude mouse flank. 0319 high grade glioma cells were injected into each flank, allowed to grow into tumors for 28 days, underwent resection, suturing, and injection with either Matrigel or UBM—ECM-SF, and recurrent bulk was photographed after 7 days. N=2.

7.3.5 Intratumoral UBM-ECM-SF Injection Intervention Increases Lifespan in a Subcutaneous Tumor Model of Glioma

0319 cells were injected into athymic nude mouse flanks and allowed to grow for 28 days. UBM-ECM-SF or PBS was injected into the resulting tumors weekly for three weeks and survival was monitored and recorded. Median survival times of mice receiving UBM-ECM-SF was 24 days compared to 11 days for the control group (P = 0.03).



Figure 33 Subcutaneous glioma tumor model in athymic nude mouse flank 0319 high grade glioma cells were injected into each flank, allowed to grow into tumors for 28 days, then injected with either phosphate buffered saline or UBM-ECM-SF weekly for three weeks. Animals died naturally or were euthanized when conditions met IACUC guidelines and survival time was recorded. N=3.

7.3.6 Intratumoral UBM-ECM-SF Injection Intervention Increased Lifespan Which Correlated with Lower Tumor Volume in an Intracranial GBM Model

A graphical overview of the C6-Wistar glioblastoma multiforme model shows C6 cells being injected intracranially on day 0, MRI imaging weekly thereafter, UBM-ECM-

SF or saline vehicle control interventions being injected at days 7, 14, and 21 post C6 inoculation, and sacrifice at day 70 (Figure 35).



Figure 34 C6-Wistar glioblastoma multiforme model overview. The 70-day experimental outline including inoculation of Wistar rats with C6 cells, three weekly injections of UBM-ECM-SF or saline, and weekly MRI scans until day 70.

Survival was recorded and represented as survival curves (Figure 36A). Of the six saline-treated animals, three died naturally at days 18, 21, and 30 and three were sacrificed at days 24, 25, and 30 in accordance with IACUC tumor burden guidelines. Three UBM-ECM-SF treated animals were sacrificed at days 22, 24, and 32 in accordance with IACUC tumor burden guidelines. The three remaining UBM-ECM-SF treated animals survived until day 70 and presented as bright, alert, and responsive before electively being taken off protocol. UBM-ECM-SF increased median survival from 24.5 to 51 days and the hazard ratio for death was 0.22 (95% CI, 0.05 to 0.97). Survival curves were compared using both the Gehan-Breslow-Wilcoxon, which places more weight on early time points, and the Mantel-Cox method, which gives equal weight to all timepoints, resulting in P = 0.086 and P = 0.046, respectively

The saline-treated rats had an average tumor volume of 349 mm³ at time of death compared to 90 mm³ for the UBM-ECM-SF treated animals. The three UBM -SF

treated rats that survived to day 70 had decreasing tumor volumes over time (Figure 36B).



Figure 35 C6-Wistar survival and tumor volume. (A) The survival curves of UBM-ECM-SF vs saline-treated animals (N=6). No significant difference, P = 0.08. (B) The average tumor volumes of UBM-ECM-SF vs saline-treated animals over 70 days. Error bars indicate standard deviation.

Representative MRI images show tumor characteristics over time (Figure 37). The six saline-treated animals which died by day 30 had large solid tumors which grew rapidly (Figure 37A). The three UBM -SF treated animals which died by day 32 had smaller, slower growing tumors (Figure 37B). The three UBM -SF treated animals which survived to day 70 had smaller, slower growing tumors which resolved into a necrotic core (approximately 1.7 mm3) by day 70 (Figure 37C).



Figure 36 C6-Wistar model MRIs. Representative MRI images of primary tumor at days 14, 21, and 28 of (A) a saline-treated rat and (B) a UBM-ECM-SF treated rat that died in week 4, and (C) weekly images through day 70 of a UBM-ECM-SF treated rat that was electively sacrificed at day 70. Tumor appears to have resolved into a small necrotic core with no recurrence of tumor mass. Tumor border outlined in a red dotted line.

7.4 Discussion

UBM-ECM-SF did not elicit an acute (24 hours) cellular response in rat brain tissue, nor have deleterious effects on weight gain, temperature, or hematology metrics when administered intravenously (10 weeks). It is not surprising that UBM-ECM-SF did not elicit an acute cellular response in the rat brain. UBM-ECM hydrogels have been used intracranially for stroke studies without ill effect and indeed have exhibited positive tissue remodeling within the central nervous system (81, 83). Encouragingly, studies have also shown UBM-ECM hydrogels to recruit doublecortin-positive neural progenitor cells, support neuron viability, and stimulate axon growth (82, 84, 202, 203). SIS-ECM (Durasis, Cook Biotech, West Lafayette, Indiana) has been used as a dural substitute where it is claimed to remodel into native tissue. Other ECM-based materials made from pericardium or dermis are also commercially available (204). Intravenous injection was conducted to determine the safety of this ECM formulation and allowed for future potential investigation of a systemic approach for cancer, whether prophylactic or therapeutic. Future studies will address the mechanism by which the body processes and metabolizes ECM components, the salient species that are permeable through the blood-brain-barrier, the effect upon the immune system, and toxicological studies of specific molecules.

The FITC signal attached to 70kda dextran did not specifically track the diffusion and retention of any particular component within the UBM-ECM-SF and was meant to provide a limited approximation of the level of diffusion at early time points and where to search for a cellular response to the UBM-ECM-SF. In the next chapter we learn that the core matrisome of UBM-ECM-SF contained components as small as Von

Willebrand factor (500 – 20,000 kDa) and as large as versican (protein core of 360 kDa), supporting speculation that some of the UBM-ECM-SF components were also retained up to 24 hours. This is also supported by previous work showing that ECM components from hydrogels injected into stroke cavities in rat brains were retained at 24 hours and up to at least 12 weeks (82, 84, 205). The spatial diffusion observed in the FITC signal after 24 hours provided the rationale for using 30 mg/mL UBM-ECM-SF in the animal model as we expected the UBM-ECM-SF to diffuse over time and wanted to maintain a sufficiently high concentration throughout the tumors to see an anti-glioma effect.

In a pre-study sample size calculation with continuous endpoints, two independent samples determined a minimum of N = 6 for the control and treatment groups where α and β were set to 0.05 and 0.8 respectively and control and treatment group means were 150 mm³ and 75 mm³ with a standard deviation of 45 mm³. Control volume mean was informed by the day 28 timepoint of a similar C6 glioma model, treatment mean was conservatively informed by in vitro studies, and standard deviation was set conservatively to allow for biologic variability (206). Due to the unexpected result of half of the treatment animals succumbing to their tumor burden, biological variability was higher than anticipated, resulting in a significant difference between the survival curves being found by the Mantel-Cox method (P = 0.045) but not the Gehan-Breslow-Wilcoxon method (0.086). ECM is derived from a natural source and requires manual processing which results in sample variability. Multiple biological replicates were included in all stages of experimentation to gather representative data. Wistar rats are outbred, which gives more confidence that the data parallels a genetically diverse

human population than if a fully inbred strain with homozygous autosomal genes were used. However, this biologically variability also gives less uniform results which likely played a role in the standard deviation being large and the treatment populations having both 3-week and 10-week survivors. Survival curves include animals that died directly from the glioma burden as well as animals that had to be sacrificed in accordance with IACUC tumor burden guidelines.

It seems likely that in addition to the direct anti-glioma effects of UBM-ECM-SF observed in vitro there are indirect anti-glioma effects attained through modulation of the immune system. Direct antagonization was seen in vitro, and the reduction in tumor growth rate in the athymic nude mice was done in the presence of the innate, not adaptive, immune system, on a short timescale. It seems unlikely that the UBM-ECM-SF could continue exerting direct anti-glioma effects in the immunocompetent C6-Wistar model throughout ten weeks when UBM-ECM-SF injections only occurred on days 7, 14, and 21. It is known that non-neoplastic ECM modulates macrophages away from an M1 activation state toward an M2-like state. Tumor associated macrophages, known as TAMs and perhaps better thought of as M(tumor activated), create an immunosuppressive microenvironment and secrete cytokines that activate antiapoptotic programs in cancer cells (207). Microenvironmental conditioning provided by UBM-ECM-SF may modulate TAMs away from their tumor-supportive role toward an M(ECM activated) state and allowing anti-tumor efficacy. This mechanism would explain the observation of gradually diminishing tumor mass.

The results presented in this chapter show that upon intratumoral injection, UBM-ECM-SF can decrease glioma tumor volume in vivo, correlating with longer survival.

These results support the findings of other ECM-based studies and are in line with TOFT; that is, that non-neoplastic mammalian ECM contains bioactive cues that can antagonize cancer cells and their in vivo progression (48-50, 107, 111, 112).

While these results are encouraging, rodent models to not represent human disease with high fidelity and these preclinical studies require further investigation before a first-in-man study. The glioma in this model is applied externally, rather than arising within the brain from natural causes. Glioma carcinogenesis is not fully understood, and this model does not reflect the complicated set of circumstances that contribute to spontaneous glioma formation. However, there does seem to be a trend in the literature, and these results that non-neoplastic mammalian ECM contains some component, or combination of components, which can directly, and perhaps indirectly, antagonize cancer cells in vitro and in vivo. Proteomic analysis of UBM-ECM-SF is a first step toward identifying those components.

7.5 Conclusion

Intravenously injected UBM-ECM-SF did not alter the hematology metrics, weight, or temperature of rats compared to the saline control group. Intracranially injected UBM-ECM-SF did not elicit an acute mononuclear response in the rat brain. Thus, UBM-ECM-SF was deemed a safe material for continued use in animal models. In the tumor flank model immunodeficient mice were used and the UBM-ECM-SF treated flanks regrew smaller tumors than the Matrigel treated flanks, indicating that a component in the UBM-ECM-SF can directly antagonize glioma tumor growth in vivo

without the adaptive immune system. In the C6-Wistar rat model of glioma, animals given intratumoral UBM-ECM-SF injections had increased median survival from 24.5 to 51 days and the hazard ratio for death was 0.22 (95% Cl, 0.05 to 0.97). Survival curves assessed with the Mantel-Cox method indicated significantly higher survival in the UBM-ECM-SF treatment group (P = 0.046). The saline-treated rats had an average tumor volume of 349 mm³ at time of death compared to 90 mm³ for the UBM-ECM-SF treated animals. Unpaired t-test analysis showed significant differences between the tumor volumes of the two groups (P = 0.0057). The therapeutic benefits bestowed by UBM-ECM-SF support further investigation for an ECM-based glioma therapy, including a search for specific therapeutic molecules contained within the ECM.

8.0 Proteomic Composition of Urinary Bladder, UBM-ECM, and UBM-ECM-SF

This chapter in part was adapted from the following manuscript: Murdock, Mark H., et al. "A liquid fraction of extracellular matrix inhibits glioma cell viability *in vitro* and *in vivo*." Oncotarget [Online], 13.1 (2022): 426-438. Web. 23 Feb. 2022

8.1 Introduciton

The in vitro and in vivo experiments showed that ECM components have potential for clinical application in treating glioma. While the saline-soluble fraction of the UBM-ECM is sufficient to confer a therapeutic benefit, the determinant components of this milieu have yet to be identified. It is known that UBM-ECM contains multiple bioactive growth factors and other proteins (208). A comparison of protein species between the saline-soluble fraction of the porcine urinary bladder matrix (UBM-ECM-SF), the parent ECM, and the native tissue is desirable.

The isolation of ECM from native porcine bladder involves a selective reduction in composition and elimination of cellular debris. The further processing of UBM-ECM to UBM-ECM-SF represents an enrichment of any saline-soluble species, excluding saline-insoluble macromolecules. Previous evaluations of UBM composition show that the material is comprised mainly of collagens, proteoglycans, growth factors sequestered into the ECM, and cytokines (174, 209, 210). The objective of this chapter was to compare the proteomic composition of the native bladder, UBM-ECM, and UBM-ECM-SF as a first step toward identifying potential molecules of interest. In doing so the complement of ECM-associated factors could be separated from the "core matrisome", providing further insight into the complex interactions between ECM components (17). It is not necessarily the logical conclusion of this effort to isolate the effector molecules and discard the remainder. We are reminded that ECM is a synergistic and complex microenvironment, akin to an ecosystem, and outside that ecosystem a protein species may behave differently or lose its beneficial properties altogether.

The objective of the present chapter is to compare the proteome of the UBM-ECM-SF to its parent ECM, and native tissue of origin, and identify key differences that would shape future efforts to identify specific components of interest. This work was completed in collaboration with the proteomic specialists in the laboratory of Dr. Kirk Hansen at the University of Colorado.

8.2 Materials and Methods

8.2.1 Overview of Experimental Design

Samples of native porcine bladder, UBM-ECM, and UBM-ECM-SF in biological triplicate were sent to the Hansen laboratory for proteomic analysis preparation. Technical triplicates were analyzed using liquid chromatography tandem mass spectrometry and protein identification was performed with the Proteome Discovery Software package. Groups of protein species from each material were sorted by functional categorization, compared via principal component analysis, organized via hierarchical clustering, and expressed as heat maps to identify differences between samples and highlight species enriched in UBM-ECM-SF.

8.2.2 Mass Spectrometry

Three biological replicates of lyophilized, comminuted native bladder, UBM-ECM, and UBM-ECM-SF were prepared as described above. Protein was extracted from milled samples as previously described (211). Briefly, bladder and ECM samples were homogenized with a bead beater in three rounds of a CHAPS/high salt buffer followed by successive extractions with guanidine-HCI, and hydroxylamine-HCI. Approximately 30 µg of protein from each fraction and 30 µg of protein from UBM-ECM-SF was then trypsin digested using a filter-aided sample preparation (FASP) approach and C18 cleanup. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was conducted as previously described (211). Briefly, samples were analyzed by in triplicate by a nano-UHPLC-MS/MS on an Easy-nLC1200 coupled with an orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific, Waltham, Massachusetts) mass spectrometer operating in positive ion mode. Approximately 3 ug of total protein was directly loaded onto an in-house packed 100 µm i.d. x 250 mm fused silica column packed with CORTECS C18 resin (2.7 µm). Samples were run at 250 nL/min over a 90 min linear gradient from 4%-28% acetonitrile in 0.1% formic acid. MS data acquisition was identical to those previously described (211). Protein identification and label free quantification was done with Proteome Discoverer Software package (v2.4). Database searching was done using Mascot (v 2.5) with peptide spectral matches mapped

against the Uniprot *Sus Scrofa* database. Mass tolerances were set to +/- 10 ppm for parent ions, and +/- 25 ppm for fragment ions. Mixed C-terminal N (hydroxylamine specificity) and trypsin were used as cleavage rules, allowing for 2 missed cleavages. Asn and Gln deamidation, Met oxidation, Pro hydroxylation, Gln to Pyro-Glu were set as variable modification with Cys carbamidomethylation set as a fixed modification. False discovery rates were calculated using Percolator (<u>http://percolator.ms/</u>) with peptide and protein identifications requiring an FDR of less than 1%

8.2.3 Statistical Analysis

Heat maps plotted the relative abundance of protein species along -2- to 2-fold change. Volcano plots used cutoff criteria of P = 0.1 and increased presence by at least a 2-fold ratio.

8.3 Results

8.3.1 Functional Categorization of Tissue, Source ECM, and Liquid ECM Proteomes

Native porcine bladder, UBM-ECM, and UBM-ECM-SF were subjected to global and targeted mass spectrometry analysis. A total of 2,562 distinct protein species (N=3 biological and technical replicates) were identified. Of these proteins, bladder contained 2,373 protein species, UBM-ECM contained 2,010 species, and UBM-ECM-SF contained 1,017 species. Proteins were sorted into functional categories for all proteins, all proteins excluding cellular & secreted proteins, proteins comprising the matrisome, and the fifty most abundant proteins comprising the core matrisome (Figure 38).



Figure 37 Proteomic results categorized by functional. Functional categories across all 2,562 protein species found between bladder, UBM-ECM, and UBM-ECM-SF; sorting by functional categories reveals the majority to be Cellular & Secreted, or Fibrillar Collagen (A). Functional categories except the Cellular & Secreted category (B). Functional categories in matrisome proteins only (C). Functional categories in core matrisome proteins only (D).

8.3.2 Principal Component Analysis Confirms Distinct Profiles of UBM-ECM-SF, UBM-ECM, and Native Tissue

All proteins and core matrisome proteins from bladder, UBM-ECM, and UBM-ECM-SF were shown to be members of distinct compositional groups via principal component analysis (Figure 39). Bladder biological replicates were shown to have the least amount of variability with the core matrisome in UBM-ECM also having low variability.



Figure 38 PCA of bladder, UBM, and UBM-ECM-SF. Principal component analysis of protein composition for each sample including all proteins (A), the matrisome only (B), and the core matrisome (C) representing relative biocompositions

8.3.3 Hierarchical Clustering and Heat Maps Show Differential Presence of Protein Species

Hierarchical clustering of all proteins showed biological replicates within each source material to be most related to each other, as represented by the brackets on the

periphery of the heatmaps. Global analysis showed UBM-ECM and UBM-ECM-SF to be the most related groups (Figure 40A), however among the 50 most differentially expressed UBM-ECM and bladder were more similar (Figure 39B), and among the matrisome and core matrisome bladder and UBM-ECM -SF were most related (Figure 41). There were many instances of differential presence of certain proteins between the biological replicates in each source material (bladder, UBM-ECM, or UBM-ECM -SF), though the biological replicates within each group are most related to each other than to replicates in other groups (Figure 40, Figure 41). ANOVA analysis identified the top fifty core ECM protein showing a difference between the source materials (Figure 40B).



Figure 39 Hierarchical clustering and global gene expression heatmaps of biological samples. Brackets of hierarchical clustering relating sample similarity can be seen along the top edge of each heat map (A,B). 2,562 distinct protein species were identified: 2,373 in bladder, 2,010 in UBM-ECM, and 1,017 in UBM-ECM-SF and expressed as a heat map (A) and the top 50 differentially expressed proteins identified by ANOVA were mapped (B).



Figure 40 Hierarchical clustering and targeted gene expression heatmaps of biological samples. Native bladder proteins are differentially retained in processed UBM-ECM and UBM-ECM -SF in both the matrisome (A) and core matrisome (B).The hierarchical clustering between samples does not change, however, with bladder and UBM-ECM -SF being more related (top brackets).

The top fifty differentially expressed core matrisome protein species are given in Table 5 for reference. Measurements are in arbitrary units. Coefficient of variation (%CV) represents all three biological replicates within that material type. Intensity of green indicates lowness of %CV. Blue, yellow, and red bars represent relative magnitude of presence of protein species.

Core Matrisome									
Brotein	GENE	ECM Annotation		Bladder		UBM-ECM		UBM-ECM-SF	
Protein	GENE	Functional	Matrisome	Average	%CV	Average	%CV	Average	%CV
Agrin	AGRN	Basement Membrane	ECM Glycoproteins	1.14E+06	65.99%	6.73E+05	115.24%	3.62E+07	114.96%
Collagen alpha-2(IV) chain	COL4A2	Basement Membrane	Collagens	2.39E+08	17.95%	1.14E+08	11.52%	9.35E+07	40.66%
Alpha-3 type IV collagen	COL4A3	Basement Membrane	Collagens	6.93E+06	37.93%	7.60E+04			
Basement membrane-specific heparan sulfate proteoglycan core	HSPG2	Basement Membrane	Proteoglycans	1.32E+09	17.59%	1.39E+08	21.72%	2.47E+08	20.24%
Laminin subunit alpha-3	LAMA3	Basement Membrane	ECM Glycoproteins	1.12E+06	43.97%	3.10E+06	45.77%	4.16E+06	74.76%
Laminin subunit alpha-4	LAMA4	Basement Membrane	ECM Glycoproteins	1.50E+09	15.22%	1.88E+07	66.19%	4.90E+07	27.23%
Laminin subunit alpha-5	LAMA5	Basement Membrane	ECM Glycoproteins	1.63E+08	27.07%	2.97E+07	47.68%	2.42E+07	53.35%
Maltoporin 1	LAMB1	Basement Membrane	ECM Glycoproteins	1.19E+08	15.85%	9.33E+06	81.95%	5.08E+07	23.56%
Maltoporin 2	LAMB2	Basement Membrane	ECM Glycoproteins	5.70E+08	28.57%	7.46E+07	27.14%	3.76E+07	31.33%
Laminin subunit gamma-1	LAMC1	Basement Membrane	ECM Glycoproteins	6.33E+08	20.38%	3.59E+07	44.43%	1.21E+08	15.86%
Nidogen-1	NID1	Basement Membrane	ECM Glycoproteins	2.85E+08	12.46%	1.73E+07	24.39%	2.81E+07	78.85%
Nidogen-2	NID2	Basement Membrane	ECM Glycoproteins	1.80E+08	20.43%	1.51E+07	18.35%	1.88E+07	65.57%
Collagen alpha-1(XII) chain	COL12A1	FACIT Collagen	Collagens	1.13E+08	21.05%	1.16E+08	33.80%	9.13E+07	15.80%
Collagen alpha-1(XIV) chain	COL14A1	FACIT Collagen	Collagens	1.89E+09	21.02%	5.07E+08	51.02%	4.46E+08	12.41%
Collagen alpha-2(IX) chain	COL9A2	FACIT Collagen	Collagens					7.37E+04	33.19%
Procollagen alpha 1(V)	COL5A1	Fibrillar Collagen	Collagens	7.22E+08	28.52%	1.68E+09	12.66%	5.08E+07	37.25%
Procollagen alpha 2(V)	COL5A2	Fibrillar Collagen	Collagens	8.21E+08	16.21%	2.56E+09	18.40%	1.35E+08	46.55%
Fibulin-5	FBLN5	Matricellular	ECM Glycoproteins	7.54E+07	23.58%	1.69E+07	14.61%	7.55E+05	59.11%
Periostin	POSTN	Matricellular	ECM Glycoproteins	1.02E+08	12.07%	3.07E+07	48.81%	6.74E+06	22.01%
Thrombospondin-1	THBS1	Matricellular	ECM Glycoproteins	5.01E+05		3.08E+05		2.66E+06	53.79%
Tenascin XB	TNXB	Matricellular	ECM Glycoproteins	8.44E+07	1.79%	2.78E+07	35.24%	2.41E+08	17.47%
Aggrecan core protein	ACAN	Structural ECM	Proteoglycans	6.36E+05	21.43%				
Chondroadherin-like protein	CHADL	Structural ECM	Proteoglycans			3.05E+05	67.35%		
Collagen alpha-1(XVIII) chain	COL18A1	Structural ECM	Collagens	1.65E+08	17.71%	1.85E+07	47.63%	6.12E+07	12.27%
Collagen type VI alpha 6 chain	COL6A6	Structural ECM	Collagens	2.80E+07	37.62%	3.86E+06	51.76%		
EGF-containing fibulin-like extracellular matrix protein 2	EFEMP2	Structural ECM	ECM Glycoproteins	2.34E+07	12.96%	3.69E+06			
EMILIN-1	EMILIN1	Structural ECM	ECM Glycoproteins	3.15E+08	27.49%	4.91E+07	82.16%	2.89E+08	36.60%
EMILIN-2	EMILIN2	Structural ECM	ECM Glycoproteins	4.98E+06	31.29%	3.00E+06	64.76%	2.57E+07	72.25%
Fibulin-1	FBLN1	Structural ECM	ECM Glycoproteins	1.54E+08	4.05%	2.29E+07	53.68%	5.64E+07	43.21%
Fibulin-2	FBLN2	Structural ECM	ECM Glycoproteins	2.67E+06	13.74%	8.01E+05	114.52%	1.16E+07	32.54%
Fibrillin-1	FBN1	Structural ECM	ECM Glycoproteins	2.13E+08	5.69%	1.75E+08	51.63%	1.65E+09	18.14%
Hemicentin-2	HMCN2	Structural ECM	ECM Glycoproteins	1.97E+05	25.19%			1.21E+06	37.11%
Latent-transforming growth factor beta-binding protein 1	LTBP1	Structural ECM	ECM Glycoproteins	7.37E+05	36.56%	1.75E+06	113.94%	6.42E+07	21.21%
Latent-transforming growth factor beta-binding protein 4	LTBP4	Structural ECM	ECM Glycoproteins	1.90E+07	34.58%	6.05E+05	31.56%	1.23E+07	38.17%
Microfibrillar-associated protein 2	MFAP2	Structural ECM	ECM Glycoproteins	1.95E+07	44.37%	9.77E+05	28.16%	2.08E+07	112.38%
Microfibrillar-associated protein 5	MFAP5	Structural ECM	ECM Glycoproteins	3.61E+07	32.38%	2.62E+06	50.26%		
Spondin-1	SPON1	Structural ECM	ECM Glycoproteins	7.27E+06	37.10%	6.89E+05	17.92%	5.14E+06	111.14%
Versican	VCAN	Structural ECM	Proteoglycans	9.21E+06	51.74%	1.27E+06	25.14%	5.86E+07	105.94%
Vitronectin	VTN	Structural ECM	ECM Glycoproteins	2.12E+07	24.84%	6.32E+06	16.25%	2.64E+07	94.70%
Extracellular matrix protein 1	ECM1	ECM affiliated	ECM Glycoproteins	1.04E+07	7.28%	6.71E+06	28.47%	1.23E+07	14.58%
Target of Nesh-SH3	ABI3BP	Secreted	ECM Glycoproteins	1.07E+07	29.21%	2.98E+05		2.10E+06	73.58%
Fibrinogen beta chain	FGB	Secreted	ECM Glycoproteins	3.44E+08	17.06%	1.13E+08	18.72%	2.16E+07	29.66%
Insulin-like growth factor binding protein 7 splice variant IGFBP7-	IGFBP7	Secreted	ECM Glycoproteins	2.50E+05	53.89%	1.39E+05		1.10E+07	35.00%
Leucine-rich alpha-2-glycoprotein	LRG1	Secreted	ECM Glycoproteins	5.06E+05	48.01%	8.43E+04			
Procollagen C-endopeptidase enhancer 1	PCOLCE	Secreted	ECM Glycoproteins	4.53E+07	5.79%	2.46E+06	65.81%		
Somatomedin-B and thrombospondin type-1 domain-containing	SBSPON	Secreted	ECM Glycoproteins	8.48E+07	33.22%	2.64E+06	135.20%		
Transforming growth factor-beta-induced protein ig-h3	TGFBI	Secreted	ECM Glycoproteins	1.26E+08	12.85%	4.42E+07	12.50%	1.41E+06	68.96%
Tubulointerstitial nephritis antigen-like	TINAGL1	Secreted	ECM Glycoproteins	1.07E+08	11.05%	7.24E+06	4.81%	6.06E+05	79.98%
von Willebrand factor	VWF	Secreted	ECM Glycoproteins	6.75E+06	52.48%	1.41E+07	123.52%	1.52E+08	35.48%
Profilin	PFN1	Cellular & Secreted	Cellular & Secreted	1.22E+09	15.21%	4.58E+07	84.68%	4.90E+06	105.95%

Table 5 Differential Core Matrisome Species

8.3.4 Volcano Plots Show Enrichment of UBM-ECM-SF Proteins from Parent ECM

A volcano plot comparing the UBM-ECM-SF to UBM-ECM proteome showed 618

proteins enriched in the UBM-ECM-SF above a 2-fold change and below a P value of

0.1. 528 proteins present in the UBM-ECM were identified with the same inclusion criteria (Figure 42A). In the core matrisome, UBM-ECM -SF contained five proteins above a 2-fold change and below a P value of 0.1 compared to UBM-ECM, while UBM-ECM contained 51 proteins (Figure 42B, Table 5).



Figure 41 Volcano plot of UBM-ECM-SF compared to ECM. (A) 618 protein species were enriched two-fold or more in the UBM-ECM-SF while 528 protein species remained two-fold or higher in the parent ECM. (B) For the core matrisome, five protein species were enriched in the UBM-ECM-SF while 51 protein species remained two-fold or higher in the parent ECM.

8.4 Discussion

Although the molecular mechanism(s) by which UBM-ECM-SF mitigates cancer cell growth is beyond the scope of the present study, the proteomic analysis conducted herein does shed light on possible protein effector molecules. Of the 2,562 protein

species identified in the native bladder, UBM-ECM, and UBM-ECM-SF, 1,017 were contained in the UBM-ECM-SF. The volcano plot showed UBM-ECM-SF contained only five proteins enriched more than two-fold from the parent ECM which combined have a mixed effect on glioma. The expression levels of COL5A1 and COL5A2 are significantly correlated with glioma progression stage (212). FBLN5 is downregulated in stage III and IV glioma and has been shown to inhibit glioma cell proliferation and invasion (213). High TGFBI expression promotes proliferation and migration of glioma cells (214). TINAGL1 is largely unstudied in the context of glioma but suppresses triple-negative breast cancer progression and metastasis (215). Although these proteins were highlighted by the volcano plot, their role in driving the observed effects is unknown.

As with all proteomic analyses the list of identified protein species is not totally comprehensive as the dark proteome could not be represented in the results.

8.5 Conclusion

Mass spectrometry analysis identified 2,562 proteins in the UBM-ECM-SF, the parent UBM-ECM, and the porcine bladder tissue of origin. The majority were classified as cellular or secreted proteins, or as fibrillar collagen. Within the core matrisome, the functional categories were limited to ECM regulators, structural ECM components, matricellular components, FACIT collagen, and basement membrane proteins. Principal component analysis confirmed that each material had a distinct proteomic profile and hierarchical clustering showed biological replicates within each source material were most related to each other. Global analysis showed UBM-ECM and UBM-ECM-SF to be

the most related groups, however among the 50 most differentially expressed proteins UBM-ECM and bladder were more similar, and among the matrisome and core matrisome bladder and UBM-ECM-SF were most similar. Volcano plots showed 618 proteins enriched at least two-fold in UBM-ECM-SF compared to UBM-ECM, with five of them belonging to the core matrisome (COL5A1, COL5A2, FBLN5, TGFBI, TINAGL1). The proteomic analysis herein lays a foundation for further investigation which could lead to the identification of specific effector molecules of therapeutic importance.

9.0 Final Remarks

This chapter in part was adapted from the following manuscript: Murdock, Mark H., et al. "A liquid fraction of extracellular matrix inhibits glioma cell viability *in vitro* and *in vivo*." Oncotarget [Online], 13.1 (2022): 426-438. Web. 23 Feb. 2022

9.1 Synopsis

The present work investigated the effects of a saline-soluble fraction of porcine urinary bladder ECM (UBM-ECM-SF) upon glioma cells in vitro and in vivo. Viability at 24 hours in the presence of 1, 5, or 10 mg/mL UBM-ECM-SF was evaluated in three primary glioma cell types (0319, 1015, 1119), four glioma cell lines (A172, T98G, U87MG, C6), and two CNS cell lines (HCN-2, HMC3). Viability decreased in a dose dependent manner among all cell types at 5 mg/mL and 10 mg/mL. Cells showed differing sensitivities to UBM-ECM-SF with U87MG, HCN-2, and HCM3 being least sensitive. The safety of UBM-ECM-SF (120 mg/kg) was evaluated by bi-weekly intravenous injections for 10 weeks in Sprague-Dawley rats. Biosafety outcome measures included weight, temperature, complete blood count, and analysis of multiorgan histology. No abnormalities were observed compared to a saline control group. Tumor volume and survival time were measured over 10 weeks in a C6 Wistar rat glioma model in which intratumoral injections were administered at weeks 2, 3, and 4 (N=6). All saline treated animals and half of UBM-ECM-SF treated animals died by week 4 with average tumor volume of 349 mm³ at time of death for the control group

and 90 mm³ for the UBM-ECM-SF treatment group. Half of the UBM-ECM-SF treated animals survived until week 10 with tumor volumes decreasing to 0 mm³. UBM-ECM-SF increased median survival from 24.5 to 51 days (hazard ratio for death 0.22; 95% Cl, 0.05 to 0.97). Mass spectrometry analysis identified 2,562 protein species in the profiles of UBM-ECM-SF, the parent UBM-ECM, and the bladder tissue of origin. Principal component analysis showed each profile to be distinct. The UBM-ECM and UBM-ECM-SF samples had more biological variability within their groups than the bladder samples. Volcano plots showed that several components were enriched twofold or more in the UBM-ECM-SF.

9.2 Conclusions

The results support the findings of other ECM-based studies and the TOFT; that is, that non-neoplastic mammalian ECM contains bioactive cues that can antagonize cancer cells and their in vivo progression (48-50, 107, 111, 112). Specifically, it was found that saline-soluble fractions of UBM-ECM decrease glioma cell viability via caspase-3 mediated apoptosis, and intratumoral injection of UBM-ECM-SF can decrease glioma tumor volume in vivo, correlating with longer survival.

Clinical intervention with liquid fractions of non-neoplastic ECM may lead to smaller tumor volume and increased lifespan in patients with GBM. Fractionated ECM therapy may represent an alternative or complementary approach to conventional GBM therapy. Further study of the role of individual ECM proteins and the molecular mechanism of action is warranted.

9.3 Summary of Major Findings and Future Directions

The central hypothesis of this dissertation was that non-neoplastic mammalian extracellular matrix (ECM) would have distinct anti-neoplastic effects on various models of glioblastoma multiforme (GBM). A corollary to this hypothesis was that delivery of ECM components would bestow a clear therapeutic benefit in a preclinical model of GBM. It was shown that saline-soluble fractions of porcine urinary bladder matrix (UBM-ECM-SF) induced apoptosis in glioma cells in vitro, and decreased tumor volume and increased longevity in GBM models in vivo. Further research may help translate these findings into a clinically viable product.

<u>Major finding 1</u>: UBM-ECM-SF antagonizes the viability of primary glioma cells and in glioma cell lines in vitro through the induction of caspase-3 mediated apoptosis.

Future directions 1: Future experiments should further characterize the mechanism of apoptosis induction in glioma cells. While the caspase-indicating substrate used, NucView 488, is marketed as a caspase-3 specific substrate, it is based on a DEVD sequence meaning it can also be cleaved by caspase-7. While both caspase-3 and caspase-7 are both classified as executioner caspases and have many similarities, they do occupy slightly different roles in the apoptosis mechanism and have differential activity toward multiple substrates (216). One important observation made in Chapter 5 is that MCF-7 breast cancer cells are sensitive to UBM-ECM-SF and do not express caspase-3 as a result of a functional deletion in the CASP3 gene (217). This

may indicate that caspase-7 plays a dominant role in the observed effects but requires more study to confirm.

It was observed that different glioma cell types had differing sensitivities to UBM-ECM-SF which may reflect differences in the presence or abundance of surface receptors, cellular metabolism, or other features. Discovering the salient features that lead to differential sensitivity would help to identify the effector molecules within the UBM-ECM-SF and thus further refine the decellularization/isolation process. Testing a larger panel of glioma cells may reveal glioma cells that are largely resistant or immune to UBM-ECM-SF which would serve as an important comparison. The contribution of specific ECM components or combinations of components remains one of the most crucial pieces of information for future work to determine. Further fractionation by size or charge could determine key components or reveal that a complex combination of components is necessary.

<u>Major finding 2</u>: UBM-ECM-SF decreases glioma tumor size and correlates with significantly higher lifespan in models of GBM.

Future directions 2: This is perhaps the most exciting finding from the study as it is more clinically relevant than the in vitro work and shows a significant therapeutic benefit conferred by the ECM-based treatment. While the statistics employed were sufficient to show significance, the N number should be increased in future experiments to distinguish between what seemed to be two populations within the treatment group: a subpopulation that had decreased tumor size but still died, and a subpopulation whose

tumors shrank into a small necrotic core and were bright, alert, and responsive at the end of the 70-day experiment. With the N=6 from the study, each individual glioma tumor seemed distinct in terms of the exact location the primary tumor grew, despite the stereotaxic injection coordinates being identical. Additional animals could reveal if a specific pattern in tumor location correlates with the two treatment outcomes. Further MRI analysis could show whether any secondary tumors played a role. Histological analysis could characterize any differences in vascularization or level of necrosis within the tumors.

Future studies should determine whether any tumor cells survive in animal subjects whose tumors shrink into necrotic cores. This could be done by using in vivo imaging systems (IVIS) which could visually track luciferase-transfected glioma cells upon luciferin injection. The extent to which the immune system interacts with glioma tumors injected with UBM-ECM-SF should also be elucidated in future experiments. One important observation made in Chapter 7 was that subcutaneous glioma tumors in athymic nude mice, mice which lack an adaptive immune system, were sensitive to UBM-ECM-SF, perhaps highlighting a role of the innate immune system which can be interrogated by depleting macrophages with clodronate-encapsulated liposomes.

In contrast to intratumoral injections, UBM-ECM-SF could be injected intravenously into animals with either subcutaneous or intracranial glioma tumors to determine whether UBM-ECM-SF components in the bloodstream can influence tumors through their vascularization, whether any ECM components can make it past the blood brain barrier to have an effect, or whether intravenous injection alters the immune
system phenotype in any way that has an indirect effect on tumor growth. This might in turn provide novel treatment strategies for "liquid tumors" like leukemia and lymphoma.

9.4 Funding Sources

Research reported in this work was primarily supported by the National Center for Advancing Translational Sciences of the National Institutes of Health (NIH) under Award Number TL1TR001858 (MHM). The content is solely the responsibility of the author and does not necessarily represent the official views of the NIH. Additional support for this work was provided by sponsored research agreements from CR Bard/Becton Dickinson, ECM Therapeutics, Inc, the Commonwealth of Pennsylvania, and the Badylak Laboratory. This project also used the resources of the UPMC Hillman Cancer Center In Vivo Imaging Facility that is supported in part by NIH award P30-CA-047904.

9.5 Acknowledgements

After this long journey there are so many people to thank. To those not on this short list, you know who you are, and I am so grateful for your help. A sincere thank you to all the members of the Badylak lab and staff who have created a stimulating, energetic, and rewarding environment to do research in. A big thank you to my dissertation committee for your valuable feedback and support. A special thank you to Dr. Wendy Mars for having answers to all my questions and for chairing my committee. A massive thank you to my advisor Dr. Stephen Badylak for your encouragement, mentorship, and many examples of excellence. A solemn thank you to Dr. George Hussey for sharing your wisdom and perspective. A warm thank you to my parents, other family, and friends who encouraged me from afar. A heartfelt thank you to my wife Jana for brightening the last leg of this journey.

I am grateful for the method development assistance of Aimon Iftikhar and Jordan Chang regarding the intracranial model, the technical assistance of Lori Walton for histology, the imaging assistance of Kelsey Hall, the IACUC and drug/chemical expertise of Scott Johnson, the flow cytometry assistance from Lynda Guzik, the proteomic data and analysis from Ryan Hill and Kirk Hansen, the MRI assistance from Lesley Foley and Kevin Hitchens, the DLAR personnel at the Hillman Cancer Center, the DLAR personnel at the Bridgeside Point II animal facility, the IACUC personnel at the University of Pittsburgh, and many other who contributed directly or indirectly to this work. Finally, I am thankful to all the administrators, fellow students, faculty, and staff in the Badylak laboratory who contributed to such an excellent and welcoming learning environment over the years.

Appendix A Proteomic Data

Complete list of protein species identified in the UBM-ECM-SF, ordered from highest to lowest abundance:

Collagen alpha-1(I) chain, Tropomyosin alpha-1 chain, Collagen alpha-2(I) chain, Collagen alpha-1(III) chain, Myosin light polypeptide 6, Vimentin, Desmin, Tropomyosin alpha-4 chain, Filamin A, alpha,Keratin, type I cytoskeletal 14,Tropomyosin beta chain,Prelamin-A/C,Calmodulin-2,Fibrillin-1, Myosin regulatory light polypeptide 9, Keratin, type II cytoskeletal 8, Thioredoxin, Tropomyosin alpha-3 chain, Histone H2B, Decorin, Galectin, Neuroblast differentiation-associated protein AHNAK, Beta actin, Serum albumin, Keratin 7, Collagen alpha-1(XIV) chain, Caveolae-associated protein 1,Lumican,Myosin-11,Cadherin-13,Nucleolin,Alpha-1B-glycoprotein,Fibronectin,EMILIN-1,Actin alpha 2,Collagen type VI alpha 2 chain,IgG heavy chain,Collagen alpha-3(VI) chain,Histone H2A,Basement membrane-specific heparan sulfate proteoglycan core protein, Adiponectin, Transferrin, Vinculin, Tenascin XB,Protein S100,Histidine-rich glycoprotein,Osteoglycin/mimecan,Hyaluronan and proteoglycan link protein 1, Histone H3, Calumenin, Plectin, Keratin, type I cytoskeletal 18, Lamin-B2, Myosin light chain kinase, smooth muscle, Annexin A2, Transgelin, Protein disulfide-isomerase, Endoplasmic reticulum chaperone BiP,Integrin beta-1,von Willebrand factor,Ribosome binding protein 1,Lamin-B1,Collagen alpha-4(IV) chain, Transglutaminase 2, Procollagen alpha 2(V), Talin-1, Laminin subunit gamma-1, Collagen alpha-1(II) chain. Acidic leucine-rich nuclear phosphoprotein 32 family member A. Protein SETSIP, Polyubiquitin-C, N-myosin-9, Cytoskeleton-associated protein 4, Integrin alpha-5, Cofilin-1, 26S proteasome non-ATPase regulatory subunit 1,14-3-3zeta, Heat shock protein beta-1, Collagen alpha-2(IV) chain, Tubulin beta chain, Filamin-C, Collagen alpha-1(XII) chain, Synemin, Histone H3.3, Protein disulfideisomerase, Nucleophosmin, Neural cell adhesion molecule 1, Peroxiredoxin-1, 10 kDa heat shock protein, mitochondrial, ATP synthase subunit delta, mitochondrial, Type VI collagen alpha-1 chain, 14-3-3 sigma protein, Nuclear mitotic apparatus protein 1, Dermatopontin, Calreticulin, Keratin, type I cytoskeletal 10,Reticulocalbin-1,Latent-transforming growth factor beta-binding protein 1,Reticulocalbin-3,Eukaryotic

translation initiation factor 5A,Collagen alpha-1(XVIII) chain,Procollagen galactosyltransferase 2,Heat shock protein HSP 90-alpha,Versican,Spectrin, alpha, non-erythrocytic 1,Fibulin-1,Keratin 1,Clusterin,Charged multivesicular body protein 4b,Peroxiredoxin-4,Brain acid soluble protein 1 homolog,Procollagen alpha 1(V),Maltoporin 1,Beta-2-glycoprotein 1,Sialic acid synthase,Laminin subunit alpha-4,Biglycan,C4b-binding protein alpha chain,Filamin-B,Nucleobindin-1,Protein canopy homolog 2,Liprin-beta-1,Four and a half LIM domains protein 1,CD44 antigen,Collagen alpha-1(XV) chain,Spectrin beta chain,60S acidic ribosomal protein P2,Inter-alpha-trypsin inhibitor heavy chain H4,Serine/arginine-rich-splicing factor 1,Calreticulin-3,Caldesmon,Fibrinogen gamma chain,Suppression of tumorigenicity 13,Destrin,Heat shock cognate 71 kDa protein,Creatine kinase B-type,Hemoglobin subunit beta,Protein S100,Annexin,Endoplasmin,Heterogeneous nuclear ribonucleoprotein K,Maltoporin 2,Alpha-fetoprotein,Neuroplastin,Immunoglobulin J chain,SPARC,Microtubule-associated protein RP/EB family member 2,PDZ and LIM domain protein 7,Hemopexin,Peroxiredoxin-2,Fibrinogen alpha chain,Elongation factor 1-alpha,Histone H1.0,ATP synthase subunit alpha,Sarcolemmal membrane-associated protein,14-3-3 protein theta,Protein disulfide-isomerase,Saposin-B-

Val, Tetranectin, Calnexin, Thymosin beta-4, Nidogen-1, Syndecan, Gelsolin, EH domain-containing protein 2,60 kDa heat shock protein, mitochondrial, Vitronectin, UV excision repair protein RAD23 homolog B, EMILIN-2, Ras GTPase-activating-like protein IQGAP1, Myotrophin, Laminin subunit alpha-5, Heterogeneous nuclear ribonucleoprotein D0, Nestin, Hemoglobin subunit alpha, Chondroitin sulfate proteoglycan 4, Histone cluster 1 H1 family member e, Alpha-2-HS-glycoprotein, U6 snRNA-associated Sm-like protein LSm8, Thy-1 membrane glycoprotein, V-type proton ATPase catalytic subunit A, Rootletin, Annexin, Transitional endoplasmic reticulum ATPase, Fibrinogen beta chain, Complement factor H, Smoothelin, Keratin, type II cytoskeletal 1b, Cysteine and glycine-rich protein 1, Collagen, type IV, alpha 1, Microfibrillar-associated protein 2, Dynactin subunit 1, Placenta-specific protein 9, Serpin H1, N-acetyl-D-glucosamine kinase, Phosphatidylethanolamine-binding protein 1, N(G), N(G)-dimethylarginine dimethylaminohydrolase 2, Glutathione S-transferase alpha-1, Caveolae-associated protein 2, Alternative protein USO1, Heat shock 70 kDa protein 1B, Regulator of calcineurin 2, Nidogen-2, Glucosidase 2 subunit beta, Antithrombin-III, Pyruvate kinase, Proteasome activator complex subunit 1, S-phase kinase-associated protein 1, Early endosome antigen 1, Lysosome-associated membrane glycoprotein 1, Alpha-actinin-

1,Cystatin-B,Glutathione peroxidase,Membrane cofactor protein,CD59 glycoprotein,Dual specificity protein phosphatase 3, ELAV-like protein, Mesencephalic astrocyte-derived neurotrophic factor, Elongin C,Collagen alpha-5(VI) chain,Interleukin enhancer binding factor 3, 90kDa,Sorcin,Serine/arginine-rich splicing factor 7, Elongation factor 1-delta, Protein canopy homolog 4, Superoxide dismutase 1, KN motif and ankyrin repeat domain-containing protein 2,14-3-3 protein epsilon,Collagen alpha-5(IV) chain, Heterogeneous nuclear ribonucleoprotein C, DnaJ homolog subfamily A member 2. Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex. mitochondrial, Talin-2, Caprin-1, Dynactin subunit 2, Fructose-bisphosphate aldolase, Ezrin, Troponin C, slow skeletal and cardiac muscles, Olfactomedin-like protein 3, Protein S100-A10, Heat shock protein beta-6,Cathepsin D,A-kinase anchor protein 12,SFRS6,Latent-transforming growth factor beta-binding protein 4,Extracellular matrix protein 1,Thymosin beta-10,Nucleoprotein TPR,Golgin B1,Tether containing UBX domain for GLUT4, Keratin, type II cuticular Hb5, Prolow-density lipoprotein receptor-related protein 1, Peptidyl-prolyl cis-trans isomerase A, Spermatogenesis-associated protein 21, Olfactomedin-like protein 1,AE binding protein,Utrophin,Integrin, alpha V,Acyl carrier protein,Fibulin-2,Stress-induced phosphoprotein 1, Heterogeneous nuclear ribonucleoprotein A2/B1, Glyceraldehyde-3-phosphate dehydrogenase, Calponin-1,14-3-3 protein gamma, Actinin, alpha 4, Profilin, Band 4.1-like protein 2, NSFL1 cofactor p47, Insulin-like growth factor binding protein 7 splice variant IGFBP7-745, Tubulin beta chain, Protein S100-A14, Dystroglycan 1, DBF4-type zinc finger-containing protein 2, Collagen type VII alpha 1 chain, Elongation factor 1-beta, Fibromodulin, Protein phosphatase 1G, Protein disulfide isomerase P5, Disks large homolog 1, Prolargin, MHC class II antigen, Ribonuclease inhibitor, Plasminogen, Apolipoprotein R, Hexokinase-4, Reversion-inducing cysteine-rich protein with Kazal motifs,NADH dehydrogenase,Collectin sub-family member 12,Peptidyl-prolyl cis-trans isomerase, Nucleobindin-2, Periplakin, Synaptopodin-2, Tryptase, Prothrombin, Prefoldin subunit 4, Rab GTPase-binding effector protein 2, Calcineurin subunit B type 1, Complement C3, Tyrosine 3monooxygenase/tryptophan 5-monooxygenase activation protein, beta, Protein S100-A13, Peptidylprolyl isomerase, Microtubule-associated protein RP/EB family member 1, Serine/arginine-rich splicing factor 2, Calpastatin, Lipoma-preferred partner, HIV Tat-specific factor 1, Gamma-interferon-inducible-lysosomal thiol reductase, Selenium-binding protein 1, Heterogeneous nuclear ribonucleoprotein U, Barrier-to-

autointegration factor, Haptoglobin, Tumor protein D54, Poly(rC)-binding protein 1, Ribosomal protein L19, Complement component C7, Nascent polypeptide-associated complex subunit alpha, muscle-specific form, Apolipoprotein A-1,60S ribosomal protein L35, Peptidase inhibitor 16, Complement decay-accelerating factor, Protein S100-A11, Enolase 1,, Ubiquitous tropomodulin U-Tmod, Complement component C8G, Rhoassociated protein kinase 2, Tenascin, Scaffold attachment factor B1, Lysosome membrane protein 2, Moesin, LIM and cysteine-rich domains protein 1, Platelet endothelial cell adhesion molecule, Palladin, Ectonucleotide pyrophosphatase/phosphodiesterase family member 1, Collagen alpha-1(XVI) chain, Myristoylated alanine-rich C-kinase substrate, Periostin, Perilipin, Endoplasmic reticulum resident protein 29, Basal cell adhesion molecule, CDC37 cell division cycle 37 protein, Transthyretin, Tcomplex protein 1 subunit delta, Frataxin, mitochondrial, Catenin alpha-1, Transcription factor A, Keratin, type I cytoskeletal 15, Keratin, type II cytoskeletal 2 epidermal, Transforming acidic coiled-coil-containing protein 1,Src substrate cortactin,Kinesin-like protein,Paralemmin-1,Centrin-2,Protein phosphatase 1 regulatory subunit 12B, Echinoderm microtubule-associated protein-like 4, Keratin, type II cytoskeletal 75, Protein bicaudal D homolog 2, Protein lin-7 homolog, Cofilin-2, Translocon-associated protein subunit alpha, Ubiquitin carboxyl-terminal hydrolase, Ribonuclease 4, Glycogenin-1, Myoferlin, Complement factor B,Lectin, galactoside-binding, soluble, 3 binding protein,Fibrillin-2,Alpha-1-antichymotrypsin 2,CD109 antigen, Charged multivesicular body protein 4a, Clathrin light chain, Thioredoxin domain-containing protein 5, Macrophage-capping protein, Microtubule-associated protein, Diablo homolog,

mitochondrial,Cytochrome b5,Tensin-1,Reticulon,Thioredoxin-dependent peroxide reductase, mitochondrial,T-complex protein 1 subunit epsilon,T-complex protein 1 subunit zeta,Inter-alpha-trypsin inhibitor heavy chain H1,Kinectin,Spondin-1,IgA heavy chian constant region,Ubiquitin-conjugating enzyme E2 H,Protein Hook homolog 3,Guanine nucleotide-binding protein subunit beta-4,Isoaspartyl peptidase/L-asparaginase,Caveolae-associated protein 3,High mobility group protein B1,Voltagedependent calcium channel alpha-2 delta subunit,Small ubiquitin-related modifier,Myocardial zonula adherens protein,Profilin,Prefoldin subunit 6,Eukaryotic translation initiation factor 2 subunit 1,Leucinerich repeat flightless-interacting protein 2,SH3 domain-binding glutamic acid-rich-like protein,Plateletactivating factor acetylhydrolase IB subunit alpha,High density lipoprotein binding protein,Basigin,ATP synthase subunit beta,Kininogen-1,Sorbin and SH3 domain-containing protein 1,Serine/arginine-rich

splicing factor 10 isoform 2,40S ribosomal protein S3,Heterogeneous nuclear ribonucleoprotein A/B,RNA binding motif protein 25,40S ribosomal protein S12,Brain Y-box binding protein 1,MHC class II antigen, Transketolase, Paraspeckle component 1, p53-induced death domain-containing protein 1, Myosin phosphatase Rho interacting protein, Golgi membrane protein 1, Rho-related GTP-binding protein RhoC,Platelet-activating factor acetylhydrolase IB subunit beta,Complement component C9, Apolipoprotein D, Protein kinase C and casein kinase substrate in neurons protein 2, GTP-binding protein 1.Heat shock 70 kDa protein 4.Acidic leucine-rich nuclear phosphoprotein 32 family member E,Cystatin C,Cell growth regulator with EF hand domain protein 1,Laminin subunit alpha-3,Inhibitor of nuclear factor kappa-B kinase-interacting protein,40S ribosomal protein S8,Small glutamine-rich tetratricopeptide repeat-containing protein alpha, DJ-1 protein, BAG family molecular chaperone regulator 2,26S proteasome non-ATPase regulatory subunit 9,Bifunctional glutamate/proline--tRNA ligase,CD5 antigen-like,ELKS/Rab6-interacting/CAST family member 1,Plasmalemma vesicle-associated protein, Calpain small subunit 1, RNA-binding protein Raly, Mitochondrial complement component 1 Q subcomponent-binding protein, Protein LZIC, NADH dehydrogenase, Pre-B-cell leukemia homeobox interacting protein 1, Sepiapterin reductase, Elongin-B, Sodium/potassium-transporting ATPase subunit alpha, Hepatoma-derived growth factor-related protein 3, Cell surface glycoprotein MUC18, Selenoprotein F,Small nuclear ribonucleoprotein F,Vitamin D-binding protein,Ran-specific GTPase-activating protein, Amine oxidase, Charged multivesicular body protein 5, Aminoacyl tRNA synthase complexinteracting multifunctional protein 1, RL23A_FRIAG 60S ribosomal protein L23A, Amyloid-beta A4 protein,L-lactate dehydrogenase B chain,Membrane-associated progesterone receptor component 1,Beta-2-microglobulin,Nuclear distribution protein nudE homolog 1,40S ribosomal protein S19, Nucleosome assembly protein 1-like 1, Serine/threonine-protein kinaseR1, Proliferating cell nuclear antigen, Heterogeneous nuclear ribonucleoprotein H1, Protein phosphatase 1F, Nexilin, PRKC apoptosis WT1 regulator protein, Dystrophin, Prostaglandin F2 receptor negative regulator, 40S ribosomal protein S6,Adenylyl cyclase-associated protein,TRIO and F-actin-binding protein,C1 inhibitor,Glutathione reductase, Acetyltransferase component of pyruvate dehydrogenase complex, Carboxylic ester hydrolase, Sorbin and SH3 domain-containing protein 2, Keratin, type II cytoskeletal 5, BUB3-interacting and GLEBS motif-containing protein ZNF207, Hypoxia up-regulated protein 1, Protein AHNAK2, Coatomer

subunit zeta-1,Alpha-2-macroglobulin receptor-associated protein,B-cell receptor-associated protein 31,CAP-Gly domain-containing linker protein 1,Cathepsin B,Dihydropyrimidinase-like 2,Subtilisin-like protease 1, Azurocidin, GRIP1-associated protein 1, Limbic system-associated membrane protein, Acyl-CoA-binding protein, Annexin, Multimerin-2, Insulin-like growth factor 2 receptor, Sushi repeat-containing protein SRPX,CD166 antigen,T-complex protein 1 subunit theta,Heterogeneous nuclear ribonucleoprotein F,Vacuolar protein sorting-associated protein 35,Inactive tyrosine-protein kinase 7,Rho-associated protein kinase,Immunoglobulin superfamily containing leucine-rich repeat protein,Protein S100,Tubulin beta chain, Creatine kinase M-type, Eukaryotic translation initiation factor 3 subunit C, Adipogenesis regulatory factor, 39S ribosomal protein L12, mitochondrial, Microtubule-associated protein 1 light chain 3 beta, Endosialin, Thioredoxin-like protein 1, Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit, Eukaryotic translation initiation factor 1A, Y-chromosomal, Thrombospondin-1, RNAbinding protein with multiple splicing, Cytochrome b-c1 complex subunit 7, Peptidylprolyl isomerase, 14-3-3 protein eta,40S ribosomal protein S20,Clathrin heavy chain,Testin,Annexin A4,40S ribosomal protein S15,U5 small nuclear ribonucleoprotein 200 kDa helicase, Major vault protein, 26S proteasome regulatory subunit 8, Serglycin, Proteasome subunit beta, Prohibitin, Golgi-associated plant pathogenesis-related protein 1,LIM and senescent cell antigen-like-containing domain protein,Heat shock protein beta-7,D-3phosphoglycerate dehydrogenase, Heterogeneous nuclear ribonucleoprotein M, Glutathione peroxidase 1, Heterogeneous nuclear ribonucleoprotein R tv2, F-actin-capping protein subunit alpha, Hepatomaderived growth factor, EGF-containing fibulin-like extracellular matrix protein 1, Single-stranded DNAbinding protein, RNA-binding protein FUS, Splicing factor 3B subunit 4,60S ribosomal protein L22, U6 snRNA-associated Sm-like protein LSm3,RNA-binding protein 8A,26S proteasome regulatory subunit 6A, Calpain-2 catalytic subunit, RNA-binding protein EWS, Protein canopy homolog 3, Glycerol-3-phosphate dehydrogenase, Poly(rC)-binding protein 2, CD99 molecule, ATPase ASNA1, Adenylate kinase isoenzyme 1, Pigment epithelium-derived factor, Tubulin beta chain, RNA transcription, translation and transport factor protein,SH3 domain-binding glutamic acid-rich-like protein,40S ribosomal protein S3a,Tyrosine-protein phosphatase non-receptor type, Heterogeneous nuclear ribonucleoprotein A3, Integrin-linked protein kinase, Eukaryotic translation initiation factor 3 subunit B, SAP domain containing ribonucleoprotein,Radixin,Succinate--CoA ligase,Electron transfer flavoprotein subunit beta,Microtubule-

associated protein RP/EB family member 3,Poly,Larval cuticle protein 1,Transgelin,Histone H1x,Target of Nesh-SH3,Peptidylprolyl isomerase,C-Jun-amino-terminal kinase-interacting protein 4,Galectin,Protein Niban 1,Syndecan-4,Proteasome 26S subunit non-ATPase 4,Migration and invasion enhancer 1,SH3 and PX domain-containing protein 2B,Erythrocyte band 7 integral membrane protein isoform a,Eukaryotic translation initiation factor 4A isoform 2,Transaldolase,Tubulin polymerization-promoting protein family member 3,40S ribosomal protein S14,Adducin 1,Polypyrimidine tract-binding protein 1,TRA2B,Stress-70 protein, mitochondrial,Glial fibrillary acidic protein,THO complex subunit 4,Chromobox protein homolog 1,Spliceosome RNA helicase DDX39B,Ras-related protein R-Ras2,Eukaryotic translation initiation factor 3 subunit J,Myocilin,ATP synthase subunit d, mitochondrial,Heterogeneous nuclear ribonucleoprotein H3,F-actin-capping protein subunit beta,Growth factor receptor bound protein 2,Coronin,Lactoylglutathione lyase,L-xylulose reductase,Y-box-binding protein 3,Glycoprotein,Ras

GTPase-activating-like protein IQGAP2, RAP1B, member of RAS oncogene family, Protein SEC13 homolog, Proteasome subunit alpha type, Fetuin-B, BCL2-associated athanogene 3, Rab GTPase-binding effector protein 1, Matrilin-2, Zyxin, Heat shock-related 70 kDa protein 2, Prostaglandin E synthase 3, Receptor protein-tyrosine kinase, Multiple coagulation factor deficiency protein 2,Granulin,Aspartyl/asparaginyl beta-hydroxylase,Na(+)/H(+) exchange regulatory cofactor NHE-RF,Dimethylaniline monooxygenase,Small nuclear ribonucleoprotein Sm D3,Calcium-binding protein A9, Sorting nexin-2, Phenol hydroxylase P0 protein, Peptidylprolyl isomerase, Apolipoprotein A-IV,SFRS4,Syntaxin-7,Programmed cell death protein 5,Syntaxin-4,Chaperonin containing TCP1, subunit 2,EH domain-containing protein 1,60S ribosomal protein L6,Sorting nexin,Fructose-bisphosphate aldolase, EF-hand domain-containing protein D2, MHC class I antigen, Procollagen alpha 3(V), NADHcytochrome b5 reductase 3, Vesicle-associated membrane protein-associated protein A, Pleckstrin homology-like domain family B member 2,NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial, Ras-related protein Rab-23, Eukaryotic translation initiation factor 6, High mobility group protein B2,Alpha-1-antichymotrypsin,Serine/arginine-rich splicing factor 3,CD2-associated protein,SPARC-like protein 1,Inter-alpha-trypsin inhibitor heavy chain H3,Triosephosphate isomerase, ATP-binding cassette sub-family A member 2, Intercellular adhesion molecule 1,Serine/threonine-protein kinase PRP4 homolog,Emerin,Transforming growth factor-beta-induced

protein ig-h3, Protein S100, Microtubule-associated protein 1B, Enoyl-CoA hydratase, mitochondrial, Guanine nucleotide-binding protein subunit gamma, PDZ and LIM domain protein 3, Elongation factor 2, Small calcium-binding mitochondrial carrier 1, Mitochondrial fission 1 protein, Phosphoglycerate mutase, Programmed cell death protein 4, Voltage-dependent anion-selective channel protein 2, Echinoderm microtubule-associated protein-like 1, FACT complex subunit SSRP1, Transcription intermediary factor 1-beta, Ubiguitin carboxyl-terminal hydrolase isozyme L1, Synaptophysin-like protein 1, Serine/threonine-protein kinase Nek9, Golgi resident protein GCP60, Sad1 and UNC84 domain containing 2, Ectonucleoside triphosphate diphosphohydrolase 1, Serine/threonineprotein phosphatase 2A 55 kDa regulatory subunit B, Myosin light chain 6B, Isocitrate dehydrogenase 1, Exocyst complex component 1, Receptor of-activated protein C kinase 1, PDZ and LIM domain protein 1, DnaJ homolog subfamily A member 1, Formin-binding protein 1, Cytoplasmic FMR1-interacting protein 2,Coatomer subunit gamma,Osteoclast-stimulating factor 1,Protein phosphatase 1 regulatory subunit 12C,Beta-2-syntrophin,Hemicentin-2,40S ribosomal protein S27,40S ribosomal protein S28,26S proteasome regulatory subunit 7, Phosducin-like protein 3, Tubulin--tyrosine ligase-like protein 12, Programmed cell death 6-interacting protein, Translationally-controlled tumor protein, Protein-Lisoaspartate(D-aspartate) O-methyltransferase,Coatomer subunit gamma,Progesterone receptor membrane component 2, Folate binding protein, Microtubule cross-linking factor 1, Prefoldin subunit 2, Proteasome subunit alpha type, Scavenger receptor class A member 5, ERO1-like protein alpha, Aminopeptidase, RILP-like protein 1, Cathepsin Z, V-type proton ATPase subunit F, T-complex protein 1 subunit gamma, UMP-CMP kinase, D-dopachrome decarboxylase, Cytochrome c1, heme protein, mitochondrial,MHC class II antigen,Caveolin-1,Succinate dehydrogenase,Lysosome-associated membrane glycoprotein 2,Intracellular hyaluronan-binding protein 4,tRNA-splicing ligase RtcB homolog, Multiprotein bridging factor 1, cGMP-dependent protein kinase, Splicing factor U2AF 35 kDa subunit, Coronin, Cysteine and glycine-rich protein 2, Leucine-rich repeat flightless-interacting protein 1, Probable ATP-dependent RNA helicase DDX17,40S ribosomal protein SA, Profilin protein, U2 small nuclear ribonucleoprotein A', Protocadherin-16, Ovarian and testicular apolipoprotein N, Charged multivesicular body protein 3, Serum amyloid P-component, Astrocytic phosphoprotein PEA-15, Cytosolic non-specific dipeptidase, Glutamine amidotransferase-like class 1 domain-containing protein 3A,

mitochondrial, Alpha-1-antitrypsin, Heat shock protein HSP 90-beta, Phosphatidate cytidylyltransferase, Rho GDP dissociation inhibitor alpha, Methylosome protein 50, Solute carrier family 8, Histone-binding protein RBBP4, Chromobox protein homolog 3, Molybdopterin molybdenumtransferase, Cdc42-interacting protein 4,ATP synthase subunit e, mitochondrial, Splicing factor 3A subunit 3, Alpha-mannosidase, Enhancer of rudimentary homolog,Cactin,Histone H2A,Coronin,Clathrin light chain,Low molecular weight phosphotyrosine protein phosphatase, von Willebrand A domain containing protein, Fermitin family homolog 2, Integrin alpha-7, Stomatin-like protein 3, Malate dehydrogenase. Coiled-coil domain-containing protein 6,ADP-ribosylation factor 3,Peroxiredoxin,Flavin reductase,Envoplakin,SL1278 acyltransferase Chp1,T-complex protein 1 subunit alpha,Thiosulfate:glutathione sulfurtransferase,Mitochondrial tRNA methylthiotransferase CDK5RAP1,Neutral alpha-glucosidase AB,Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform, Endophilin-A2, Importin-7, Adhesion G protein-coupled receptor E5, Vinexin, LEM domain-containing protein 2, Putative RNA-binding protein Luc7-like 1, Malate dehydrogenase, cytoplasmic,Cytoplasmic dynein 1 intermediate chain 2,Eukaryotic translation initiation factor 4H, Heterogeneous nuclear ribonucleoprotein D-like, Galactokinase, Heat shock protein 105 kDa, E3 ubiquitin-protein ligase HUWE1, Tubulin alpha-1A chain, Arginine--tRNA ligase, cytoplasmic, Selenoprotein M, Eukaryotic translation initiation factor 3 subunit A, Cytochrome b5 type B, Actin-related protein 2/3 complex subunit, Tight junction protein ZO-1, Integrin alpha-6, Prostaglandin G/H synthase 1, DnaJ homolog subfamily B member 1, Epidermal fatty acid-binding protein, Syntaxin-12, NADH dehydrogenase, DnaJ homolog subfamily B member 4, Apolipoprotein C-III, Ubiquitin-fold modifier 1, Glutathione peroxidase, 28 kDa heat- and acid-stable phosphoprotein, Four and a half LIM domains protein 2, Coagulation factor XII, Proteasome subunit alpha type, Guanine nucleotide-binding protein subunit gamma, TROVE domain family, member 2, Proteasome subunit alpha type, PDZ and LIM domain protein 5, Immunity-related GTPase family Q protein, Peripherin, Protein BRICK1, Solute carrier family 25,KH domain-containing, RNA-binding, signal transduction-associated protein 1,Myc box-dependentinteracting protein 1,Septin 2,NADH dehydrogenase,Heterogeneous nuclear ribonucleoprotein Q,Phosphoglycerate kinase,3-hydroxyisobutyrate dehydrogenase,Fibulin-5,Tropomodulin-1,LSM7 homolog, U6 small nuclear RNA and mRNA degradation associated, DnaJ homolog subfamily B member 11, Heat shock 70 kDa protein 12B, Peptidylprolyl isomerase, Calcium/calmodulin-dependent protein

kinase type II subunit delta, Nucleolar MIF4G domain-containing protein 1, Protein AMBP, MICOS complex subunit,45 kDa calcium-binding protein,Laminin subunit gamma-2,Mitochondrial import inner membrane translocase subunit Tim8 A, Squamous cell carcinoma antigen recognized by T-cells 3, Focal adhesion kinase 1, Prostaglandin-H2 D-isomerase, Golgin subfamily A member 3, Isocitrate dehydrogenase, 60S ribosomal protein L29, Proteasome subunit alpha type, Mitogen-activated protein kinase, Cytochrome c oxidase subunit 4 isoform 1, mitochondrial, SNARE-associated protein Snapin, Chloride intracellular channel protein 1,U4/U6.U5 tri-snRNP-associated protein 1,Heparin cofactor 2,Methyl-CpG binding protein 2, Fatty acid synthase, Fibrinogen-like protein 2, Pyrroline-5-carboxylate reductase, LIM and SH3 domain protein 1, Annexin, Heterogeneous nuclear ribonucleoprotein U-like protein 2, Arf-GAP with coiledcoil, ANK repeat and PH domain-containing protein 2, Peptidylprolyl isomerase, Charged multivesicular body protein 6, Integrin alpha-1, Neuropilin, Galectin-4, DNA topoisomerase I, Transmembrane emp24 domain-containing protein 7, ER lipid raft-associated 2 isoform 2, Ankyrin repeat and MYND domaincontaining protein 2, Calponin, Importin-5, Inositol-1-monophosphatase, ATP-binding cassette sub-family F member 1, Tubulointerstitial nephritis antigen-like, Inter-alpha-trypsin inhibitor heavy chain H5, AH receptorinteracting protein, Cell cycle and apoptosis regulator protein 2, ADP-ribosylation factor 4,2',3'-cyclicnucleotide 3'-phosphodiesterase, Complement factor I, Epidermal growth factor receptor kinase substrate 8-like protein 2,COP9 signalosome complex subunit 4,Tyrosine--tRNA ligase,Rho guanine nucleotide exchange factor 17, Rab GDP dissociation inhibitor, Eukaryotic translation initiation factor 3 subunit G,NADH dehydrogenase, Transcription initiation factor IIA subunit 1, Neuronal growth regulator 1, Collagen type XXI alpha 1, Phosphoglucomutase 1, Ribosomal protein L12, Sarcoplasmic/endoplasmic reticulum calcium ATPase 2, Guanylate-binding protein 1, Protein-lysine 6-oxidase, Plexin-B2, Heat shock protein beta-8, Tubulin-specific chaperone D, Protein transport protein SEC31 homolog A, Tetratricopeptide repeat protein 1, Ras-related protein Rab-35, Armadillo repeat-containing protein 6, Rab GDP dissociation inhibitor, Leiomodin-1, cAMP-dependent protein kinase type II-alpha regulatory subunit, Alpha-2macroglobulin, Mitochondrial aldehyde dehydrogenase 2,3-hydroxyacyl-CoA dehydrogenase type-2, Methylenetetrahydrofolate dehydrogenase, Glutaredoxin-related protein 5, mitochondrial, Apolipoprotein B-100, Actin-related protein 2/3 complex subunit, Ubiquitin-conjugating enzyme E2L 3, Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha, Uncharacterized protein C1orf198, Complement C1q subcomponent

subunit A,ATP synthase-coupling factor 6, mitochondrial,DnaJ homolog subfamily A member 3, mitochondrial, Sorting nexin, Afamin, Steroidogenic factor 1, cAMP-dependent protein kinase type II-beta regulatory subunit, Phosphoglucomutase-like protein 5, CDGSH iron sulfur domain 1, Thioredoxin-related transmembrane protein 1, Endonuclease domain-containing 1 protein, Peptide-methionine, 26S proteasome regulatory subunit 6B,6-phosphogluconate dehydrogenase, decarboxylating,V-type proton ATPase subunit G, Guanine nucleotide-binding protein subunit alpha-13, Rac-like GTP-binding protein RAC1,Leukocyte surface antigen CD47,Plasma serine protease inhibitor,Rho guanine nucleotide exchange factor 2, Tripeptidyl-peptidase 2, Alpha-parvin, Inter-alpha-trypsin inhibitor heavy chain H2, GNAS complex locus, Density-regulated protein, RNA binding protein fox-1 homolog, Mx1 protein, Cartilageassociated protein, Protein phosphatase 1B magnesium-dependent beta isoform, Alpha-2antiplasmin, Anion exchange protein, Casein kinase II subunit beta, Kinesin-like protein, Leukocyte elastase inhibitor, Ciliary neurotrophic factor receptor subunit alpha, Optineurin, Desmoplakin, Elongation factor Tu, Citrate synthase, mitochondrial, Dihydrolipoyl dehydrogenase, mitochondrial, Beta-catenin, Splicing factor, proline- and glutamine-rich, GTP-binding protein SAR1a, Serine/threonine-protein phosphatase, Histone-lysine N-methyltransferase SETD7, V-crk avian sarcoma virus CT10 oncogenelike,Sorting nexin-3,Protein kinase C and casein kinase II substrate protein 3,Coatomer subunit delta, Signal transducer and activator of transcription, Cadherin-1, Secreted phosphoprotein 24, Mitochondrial import inner membrane translocase subunit Tim13, Dynein light chain roadblock, Tetraspanin, Coiled-coil and C2 domain-containing protein 1A, Dolichyldiphosphooligosaccharide--protein glycosyltransferase subunit 1, Elastin, Nucleosome assembly protein 1like 4,Aldehyde dehydrogenase,Protein FAM98A,E3 ubiquitin-protein ligase RNF114,40S ribosomal protein S13,UBX domain-containing protein 1,Ribosomal protein L18,BTB/POZ domain-containing protein KCTD16, Aminopeptidase, ATP-dependent RNA helicase A, Small nuclear ribonucleoprotein E, Cullinassociated and neddylation-dissociated 1, Ferritin, Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5,Beta-1-syntrophin,60S ribosomal protein L14,Signal transducer and activator of transcription, Protein archease, Far upstream element-binding protein 2, Small nuclear ribonucleoproteinassociated protein, Laminin subunit alpha-2, Ribulose-phosphate 3-epimerase, DNA-directed RNA polymerase II subunit RPB4, ATP-dependent, Keratin, type II cytoskeletal 78, Periplasmic nitrate

reductase,cAMP-dependent protein kinase type I-alpha regulatory subunit,Ras-related protein Rab-10,Calcium-regulated heat-stable protein 1,Peroxiredoxin-like 2A,Ras-related protein Rab-14,Glycogenin-1,LOC100125121 protein,Ubiquilin-2,Neurocalcin-delta,Ryanodine receptor 1,Sulfhydryl oxidase,Water stress-inducible protein Rab21,Collagen alpha-2(IX) chain,TELO2-interacting protein 1 homolog

Appendix B Miscellaneous

Journal Articles

Kessler, D., Kallenbach, M., Diezel, C., Rothe, E., **Murdock, M**., & Baldwin, I. T. (2015). How scent and nectar influence floral antagonists and mutualists. *Elife*, *4*, e07641.

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He, J., Fandino, R. A., Halitschke, R., Luck, K., Köllner, T. G., **Murdock, M. H.**, ... & Schuman, M. C. (2019). An unbiased approach elucidates variation in (S)-(+)-linalool, a context-specific mediator of a tri-trophic interaction in wild tobacco. *Proceedings of the National Academy of Sciences*, *116*(29), 14651-14660.

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Murdock, M. H., Hussey, G.S., Chang, J. T., Hill, R. C., Nascari, D. G., Rao, A. V. Hansen, K. C., Foley, L. M., Hitchens, T. K., Amankulor, N. M., Badylak, S. F. A Liquid Fraction of Extracellular Matrix Inhibits Glioma Cell Viability In Vitro and In Vivo. *Oncotarget* 13 (2022): 426.

Book Chapter

Scarritt, M., **Murdock, M.**, & Badylak, S. F. (2019). Biologic scaffolds composed of extracellular matrix for regenerative medicine. In *Principles of Regenerative Medicine* (pp. 613-626). Academic Press.

Patent Filed

Badylak, Stephen Francis, George S. Hussey, Lindsey Tamiko Saldin, and **Mark Hikaru Murdock.** "Extracellular matrix (ecm) hydrogel and soluble fraction thereof for the treatment of cancer." U.S. Patent Application 16/490,056, filed March 5, 2020.

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