GRANZYME B MEDIATES CLEAVAGE OF THROMBOSPONDIN-2: IMPACT ON KERATINOCYTE VIABILITY AND ADHESION

by

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Abstract

The epidermis, consisting of keratinocytes, is the first defense against the exterior environment of the skin. Integral to maintaining the homeostasis of skin function by aiding in thermoregulation, fluid retention, and pathogen defense, the extracellular matrix composition aids in regulating keratinocyte migration after injury. Impaired healing processes can progress into chronic non-healing wounds, leading to infection and mortality. Granzyme B (GzmB) is a serine protease, significantly elevated in chronic non-healing wounds. GzmB impairment of wound healing is attributed to its ability to sustain its proteolytic activity and downstream inflammation, inhibiting wound repair and tissue remodeling following injury. GzmB is classically known for its intracellular role in cytotoxic lymphocyte-mediated apoptosis. Recent evidence demonstrates that extracellular GzmB has the ability to cleave a variety of important extracellular proteins. Thrombospondin-2 (TSP-2) is an extracellular glycoprotein secreted by a variety of cells known to be involved in cell-cell and cell-matrix interactions. TSP-2 has been reported to impair viability, adhesion, and migration in endothelial cells and squamous carcinoma cells. Although extensive studies have been done with TSP-2, literature investigating the role of TSP-2 in the skin has largely been neglected. I hypothesized that GzmB cleaves TSP-2, and GzmB cleavage of TSP-2 mediates extracellular matrix disorganization, altered keratinocyte viability, and reduced adhesion. To investigate this hypothesis purposed two aims. In the first aim, cell-free protease assays were utilized to determine whether TSP-2 is a proteolytic substrate of GzmB. In the second aim, the impact of TSP-2-mediated cleavage was assessed using cultured human keratinocytes. TSP-2 was cleaved by GzmB in a time- and dose-dependent manner. Further, while GzmB-mediated TSP-2 cleavage did not affect cell viability, it

did reduce cellular adhesion in a dose-dependent manner. In summary, TSP-2 is a proteolytic substrate of GzmB and such cleavage interferes with keratinocyte adhesion.

Lay Summary

Wound healing impacts everyone, from scraping a knee to getting a bedsore. The top layer of skin that is affected first is composed of keratinocytes, which move from the edges of the wounds to form a new barrier between the tissue and the outside environment. Proteins in the skin environment influence the behaviors of the keratinocytes. The influence of one such protein, thrombospondin-2, has not yet been studied in the top layers of the skin. This project focuses on the influence of thrombospondin-2 on keratinocytes. Additionally, the consequences of a degrading protein impact on thrombospondin-2 was investigated. This study provides information towards effective wound healing therapeutics.

Preface

All experimentation, analysis, figures, and writing were done by Anna-Catharina Wilhelm, under the direction of Dr. David J. Granville.

Dr. Matthew Zeglinski assisted in experimental design to address the specific aims of the study hypothesis.

Proofreading was done by Dr. Granville, Dr. Zeglinski, Thomas Wilhelm, and Marie-Therese Wilhelm.

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List of Abbreviations

ADAM: A disintegrin and metalloproteinase

C20: Compound 20

CTL: Cytotoxic T lymphocytes

dATP: Deoxyadenosine triphosphate

DEJ: Dermal epidermal junction

DMEM: Dulbecco’s Modified Eagle Medium

DPBS: Dulbecco's phosphate-buffered saline

ECM: Extracellular matrix

EDA: Extra domain A

FBS: Fetal bovine serum

FN: Fibronectin

HaCaT: Human adult low calcium high temperature

GzmA: Granzyme A

GzmB: Granzyme B

GzmH: Granzyme H

GzmK: Granzyme K

GzmM: Granzyme M

IL: Interleukin

MMP: Matrix metalloproteinase

MTOC: Microtubule-organizing center

NK: Natural killer

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PI-9: Protease inhibitor 9

PVDF: Polyvinylidene fluoride

RGD: Arginine-glycine-aspartate

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulfate

TAILS: Terminal amine isotopic labeling of substrates

tBid: Truncated bid

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline – tween 20

TIMP: Tissue inhibitors of matrix metalloproteinases

TSP: Thrombospondin

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

vWF: von Willebrand factor

WT: Wild-type

ZO: Zonula occludens

Acknowledgements

First, I would like to acknowledge the experience of graduate school as without such experiences, I would not have learned what resilience truly means. Thank you, Dr. Granville, for your patience and challenge, for giving me the opportunity to grow scientific knowledge, fostering an environment to developing mental strength and academic escalation. Thank you to my committee for pushing me to think critically about my own work. Thank you to my teachers, postdocs, the incredible scientists of the lab. I would like to extend special gratitude to Dr. Matthew Zeglinski and Dr. Lara Utsch Mendes Gouveia for mentoring me during this process. They have taught me more about science and dedication than I can say. Thank you to all other members of my lab past and present: Dr. Jenny Chik, Stephanie Santacruz, Cameron Oram, Dr. Valerio Russo, Dr. Hongyan Zhao, Dr. Chris Turner, Dr. Sho Hiroyasu, Dr. Karen Jung, Katlyn Richardson, and Dr. Keerit Tauh. Additionally, the wonderful community at ICORD, full of friendly, welcoming, and intelligent individuals.

Thank you to the incredible community that I have built around me. The friends that I have made here in Vancouver are part of my story. For always picking me up and showing me what I have done so far and that I need to keep going. Specifically, my cheerleaders, Tseday Zewdu Tegegn, Simon Teskey, Dr.Ward Plunet, Andrea Lee, Amanda Lee, Jessie McDougall, and Jon Baker.

Lastly, my family's support these last few years has kept me on my feet, for their unconditional love, and undocumented time. Special thanks are owed to my parents, Thomas and Birgit Wilhelm, who have supported me throughout my years of education, both emotionally and financially. Thank you to my sister, Marie-Thérèse Wilhelm, for making me laugh and keep my head up. I want to thank my grandparents who all showered me with love growing up; all have

passed from various pathologies that are researched in this department. As their memories serve me, even as a child, I believed that one day I will work to help patients and those that suffer from illness.

Dedication

I want to dedicate this thesis to those in pain. Whether those are going through wound care treatment, have a rare chronic illness, a sports injury, or having a difficult time. May the scientific community bring more light to solutions that may serve the purpose of bettering society.

Chapter 1: Introduction

Wound healing is an important, underappreciated aspect of health, with 1-2% of people of developed countries are estimated to experience chronic wounds healing during their lifetime[1]. Wounds can impact the layers of the skin and take place in sequential phases. Many factors go into wound healing, one of which is granzyme B, an extracellular matrix-degrading enzyme and thrombospondin-2, a less-explored protein matrix protein in wound healing. Given that both proteins may be found in the skin, to examine the interaction between the two could shed light on the complex ways in which chronic wounds form.

1.1 Anatomy of the Skin

The skin provides a barrier to the external environment. As the largest organ in the body, it accounts for 15% of an adult's body weight[2]. The skin protects the body's internal environment from the external one while also acting as a mediator of sensation, protection from infection, trans epidermal homeostasis, and thermoregulation[3]. The skin is composed of three main layers: the epidermis, the dermis, and the subcutaneous (Figure 1).

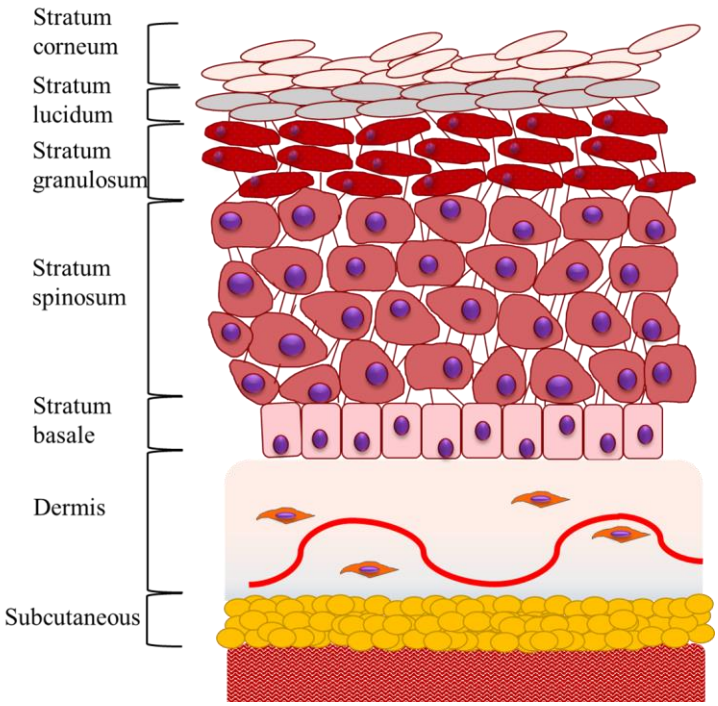

Figure 1. Different layers of the skin. The epidermis is the outermost layer of the skin and is made up of 4-5 layers consisting mainly of keratinocytes. Small amounts of langerhans cells, merkel cells, and melanocytes are also found in the epidermis (not pictured). Below, the dermis is a cushioned layer of the body consists of dense irregular connective tissue, vasculature, and different cell types such as fibroblasts. The third layer, the subcutaneous, attaches the skin to the underlying muscle and bone.
Note: Image created based on Betzalel et al. 2017[131].

1.1.1 Layers of the skin

Epidermis. The epidermis is the outermost layer of the skin made of keratinized, stratified squamous epithelium. This layer is primarily composed of keratinocytes (~80%), but also contains Langerhans cells, melanocytes, and Merkel cells[2]. Keratinocytes sit at the basement membrane. As they differentiate and become less metabolically active, keratinocytes migrate from the basal layer to the top layers of the epidermis[2].

Shown in Figure 1, stratum corneum is the outermost layer of the epidermis composed of 12 to 16 layers of corneocytes (keratinocytes without a nucleus)[2]. This layer of corneocytes is replenished by cells from the deeper layers of the epidermis. The corneocytes are surrounded by a lipid matrix in a brick and mortar type-structure. The next layer, the stratum lucidum, presents

in very thick skin in areas such as the palms of the hands and soles of the feet. In the stratum granulosum layer, keratinocytes are flattened, possess thicker membranes preventing water loss and a barrier to pathogens[3]. At this stage, keratinocytes ultimately transition into cornified cells through squamous differentiation[4]. The stratum spinosum contains desmosomes providing linkages between the keratinocytes[3]. Keratocytes produce keratin intermediate filaments providing structural support[3]. The stratum spinosum layer is marked by keratin intermediate filaments K1 and K10 indicating early differentiation[3]. Within the sea of keratinocytes, antigen-presenting cells, also known as Langerhans cells, scavenge for pathogens[3]. The deepest layer of the skin just above the dermis is the stratum basal, connected to the dermis by the basement membrane. It is made up of multiple layers of keratinocytes undergoing rapid cell division[3]. These replicative keratinocytes are marked by K5 and K14 keratin intermediate filaments[3]. Though to a lesser extent than keratinocytes, stratum basal also contains melanocytes and Merkel cells[5]. Melanocytes produce melanin giving the skin its pigment, and Merkel cells associate with nerve endings forming the Merkel disc which acts as a sensory receptor to touch[2]. There are no blood vessels in the epidermis, thus nutrients are delivered to the cells by diffusion from the capillaries at the dermal-epidermal junction.

Dermis. At the transition from the epidermis to the dermis, an area known as the basement membrane allows for the passage of molecules and cells. The dermis provides the skin with its structural strength, elasticity and fixability. The main components of the dermis include collagen and elastin, aiding in the elasticity and strength of the skin[6]. The dermis itself consists of two layers of connective tissue: the papillary dermis and the deeper reticular dermis. The papillary dermis consists of loose connective tissue while the reticular layer consists of more of the dense collagen fibers[1,2] Connective tissue in this region mostly consists of collagen I, collagen III, and

elastin fiber produced by fibroblasts[2,3,7]. This region of the skin is highly vascularized, providing oxygen and nutrients. Cell types present in this region include macrophages, mast cells, and most abundantly, fibroblasts[2].

Subcutaneous layer. The subcutaneous layer is the third layer below the dermis. It attaches the skin to the underlying muscle and bone consisting of loose connective tissue and elastin[8]. Cells found in this layer include fibrocytes, macrophages, and adipocytes[9]. This tissue acts in energy storage, cushioning, and cutaneous immunity[9].

1.1.2 Skin Extracellular Matrix

The extracellular matrix (ECM) is an interstitial network of proteins that provides structural integrity and an anchoring substrate to hold cells together to form tissues. This scaffold provides an environment for biochemical processes and signaling such as in regulating cell attachment to the matrix, cell migration, and cell shape[6]. The ECM is a dynamic structure remodeled by enzymes and proteins that may undergo post-translational modifications[6].

The most abundant components of the ECM are collagen, elastin, fibronectin (FN) and laminin. They aid in buffering, hydrating, binding components, and sustaining structural integrity[6]. Collagen is the most abundant fibrous protein found in the skin. Over 28 types of collagen can be found in the skin, but collagen type I and III are the most abundant[6]. Collagen provides a large component that contributes to tensile strength, cell adhesion, migration, and direct tissue development[6]. Less abundant, but equally important fibrous proteins are elastin and FN. Elastin provides stretch to the skin, and FN aids in organization as well as cell-matrix functions such as cellular attachment[6]. Adhesive proteins are also a major component of the ECM and provide a variety of roles, including holding the matrix components and cells together to form tissues[6,10]. For example, the basal lamina, which is part of the basement membrane on

which epithelial cells adhere, largely consists of collagens and laminin. Laminin is a large adhesive glycoprotein that binds to cell-surface receptors and other ECM components that influence cellular functions such as attachment, differentiation, and migration[11].

Growth factors also bind to and regulate the ECM. Such growth factors include platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and transforming growth factor-beta 1 (TGF-β1)[12]. Growth factors allow for rapid communication within the ECM matching that of the rapid intracellular signaling, crucial in facilitating tissue repair after damage[13]. Lastly, the ECM contains matricellular proteins that have diverse functions that are not primarily structural, such as thrombospondins (TSPs), tenascin, and osteopontin[12,14]. Although participating as multifunctional molecules, proteoglycans and growth factors play an integral role in the integrity of the ECM.

1.2 Acute Wound Healing

Cutaneous wound healing consists of four continuous and overlapping phases: hemostasis, inflammation, granulation tissue formation, and remodeling (Figure 2).

The first phase, hemostasis, begins with coagulation and the formation of the fibrin clot, creating an initial protective barrier to the exterior environment[10,15]. Upon wounding of the skin, the epidermal barrier has been damaged. Immediately, the blood vessels of the skin contract through a process known as vasoconstriction, stopping the loss of blood and forming a blood clot[15]. Thereafter, capillaries undergo vasodilation allowing for platelets to enter the wound and aggregate while filling the area with growth factors and proteins such as TSP, FN, fibrinogen, von Willebrand factor (vWF), TGF-α and TGF-β[15,16]. Signals, from injured cells and platelets that are activated by encountering collagen, cause clotting cascades to stop bleeding[15]. The fibrin containing blood clot encompasses a provisional wound matrix, forming a framework for the

migration and proliferation of cells involved in wound healing, such as leukocytes, endothelial cells, keratinocytes and fibroblasts[10,15].

Overlapping with hemostasis, the inflammation phase ensues as neutrophils are rapidly recruited to the area. Neutrophils are the first leukocytes to come into the wound area to induce phagocytosis and secrete inflammatory mediators. Monocytes accumulate within 24 h, mediated in part by IL-8 released from neutrophils, and differentiate into macrophages[17]. Macrophages phagocytose and clear wound debris while also secreting key cytokines, such as TGF-α, TGF-β, basic FGF, PDGF, VEGF, and IL-1, to aid in the synthesis of ECM[15,17].

The proliferative and migratory phases are marked by the formation of a new skin barrier. In this stage, the protective barrier reforms through restoring the skin surface through the re-epithelization, restoration of granulation tissue, and repair of the vasculature. The proliferative phase is characterized by re-epithelization as keratinocytes migrate over the wound bed to form a new top layer of the skin, and the granulation tissue is repaired by fibroblast deposition of new collagen and ECM[16]. Re-epithelialization begins quickly within 24 h post-injury[16]. Loosened from intracellular desmosomes (by collagenase and elastase), detached resident basal keratinocytes shuffle from the wound edge, and epithelial stem cells (from hair follicles and sweat glands) initiated by the presence of multiple cytokines and growth factors (EGF-1, IGF-1) begin to migrate underneath the fibrin clot[16,18]. As they migrate, keratinocyte loses cell-cell and cell-matrix attachment and proliferation are inhibited[16]. Keratinocyte-mediated interactions with integrin receptors help mediate migration[16]. When this migration stops, and keratinocytes reattach to the basement membrane and proliferation recommences as the keratinocytes resume differentiation, allowing for stratification of the epidermis[16]. Similar to keratinocyte migration, endothelial cells migrate on a gradient of angiogenic factors like VEGF, as they control the status

of angiogenesis in the granulation tissue. Concurrently, fibroblasts function to repair the dermis by synthesizing collagen III. They deposit new collagen III, divide and proliferate, and organize the collagen by aligning the fibers to reduce the scarring. This allows for increased tensile strength of the wound. In addition to producing the structural collagen III, fibroblasts contribute the skin renewal by the production of new growth factors and ECM proteins[16].

Remodeling of the skin tissue is consistently active during the entire repair process. Collagen III is digested by collagenases and fibroblasts, being replaced with collagen I. This replacement matures the granulation tissue restoring tensile strength to the wound and continues for an extended period of time after the wound closure[18,19].

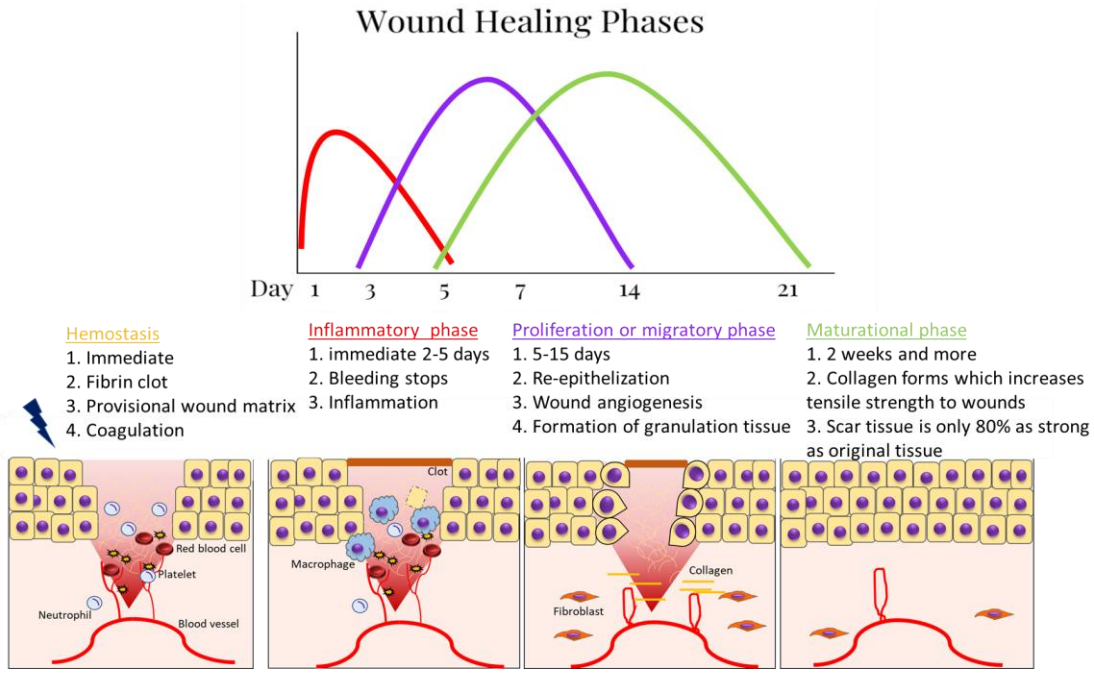

Figure 2. The four main overlapping phases of wound healing: Hemostasis is the initial wound healing phase; coagulation begins along with the formation of a fibrin clot and provisional matrix. Concurrently, the inflammation phase begins with the infiltration of immune cells, such as neutrophils and macrophages. In the proliferation and migration phase, fibroblasts move into the wound area, new granulation tissue formation, and re-epithelialization. The final phase, maturation, is marked by ECM maturation and increased tensile strength of the newly formed tissue.

1.3 Chronic Wound Healing

Though costs of wound care are mostly undocumented, it is estimated that treatment of diabetic cutaneous wounds costs patients up to $50,000 USD, and the healthcare system $25 billion USD per year in the US[20,21]. In the US, about 6.5 million people are in need of annual wound care, and this number is on the rise[20]. Chronic wound care accounts for a large proportion of these healthcare costs estimated to be 2-4 percent of high development index countries health care costs[22]. Additionally, chronic cutaneous wounds are seen more often in elderly, obese, and diabetic populations[1]. Moreover, patients are left with pain and cosmetic scarring, leaving them with additional distress[20].

Chronic wound healing is defined as an inadequate procession through the 'normal' healing stages typically defined by resolution within about 3 months after injury (Figure 2)[23]. Chronic wounds are commonly classified into vascular ulcers, diabetic ulcers, and pressure ulcers. These wounds are often plagued by persistent inflammation and infection due to a lack of wound closure, hindering progression into the proliferation and tissue remodeling stages [24].

Chronic wound tissues are largely defined by excessive infiltration by neutrophils[25]. Local macrophages remain in their pro-inflammatory stage as M1 macrophages, inhibiting the progression into M2 macrophages past the inflammatory phase[1]. Though their presence is normal, their continued presence in much larger quantities than in acute wounds are thought to be detrimental to wound repair. Neutrophils and macrophages are sources of proteases and as their presence persists, production of these proteases increases. The increased level of proteases leads to degradation of wound regulators like growth factors VEGF and PDGF[25]. One of the contributors to the extensive continuation of the inflammation is excessive neutrophil infiltration,

which leads to the production of reactive oxygen species (ROS), as well as pro-inflammatory cytokines and protease[20]. The increase of ROS and pro-inflammatory cytokines increase protease production, such as matrix metalloproteinases (MMP)s and granzymes, that degrade the existing ECM and growth factors[20]. ROS and pro-inflammatory cytokines, such as IL-1β and TNF-α, not only increase proteases such as MMPs and granzymes but also decrease growth factors and tissue inhibitors of MMPs (TIMP)[25–27]. The balance between pro-inflammatory regulators and inhibitors is tipped towards pro-inflammation, exacerbating ECM breakdown[25,28]. This imbalance augments degradation of the ECM, impairs cell migration, and reduces fibroblast proliferation and collagen synthesis[25]. At the same time, fibroblasts do not produce adequate ECM proteins, consequently creating an inopportune environment for normal wound closure. Growth factors aid in cellular recruitment for wound closure[17] such that deficiency of growth factors may lead to wound persistence. There is also an increased presence of proteases such as elastase, MMPs and granzymes[15,29,30]. MMP-8 degrades growth factors such as PDGF and TGF-β, which aid in cell recruitment to the wound, and this is suggested to contribute to chronic wound persistence.[28,31] The cleavage of ECM components can contribute to the promotion of inflammation. Keratinocytes remain hyperproliferative, while fibroblasts and keratinocytes are exhibiting inhibited migratory behavior in chronic wound healing.[20] Chronic wounds display dysregulated ECM environments including the degradation of proliferation and migration proteins FN and tenascin, alongside the increase in proteases like MMPs and granzyme B[25,32]. Specifically, extracellular protease granzyme B appears elevated in chronic skin diseases[33]. Proteases such as GzmB, largely contribute to the reoccurring cycle of ECM degradation impeding cell survival consequently resulting in chronically impaired wound healing[20,32]. Figure 3 below outlines the distinction between acute and chronic wound healing.

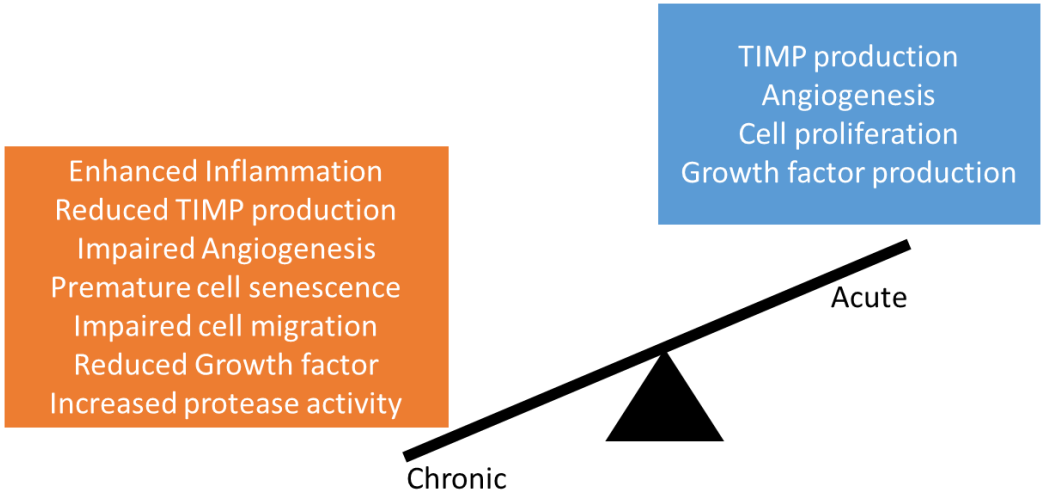

Figure 3. Balance between acute vs. chronic wound healing. Wound healing is a balance between the components of the environment that aid in the resolution of new tissue formation consisting of proteases, ECM proteins, immune cells, and skin cells.

1.4 Granzymes

Granzymes, a family of serine proteases, were originally investigated in the granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and found to protect against cellular transformation and viral infection[32,34]. There are 5 granzymes found in humans (A, B, H, K, and M; see Table 1) and 11 in mice (A, B, C, D, E, F, G, K, L, M, and N)[34,35] Of these serine proteases, granzymes A and B have been the most studied due to their higher abundance[36]. Some granzymes genes such as GzmA and GzmB are found in chromosomal clusters. GzmA cluster on chromosome 5 includes GzmA and GzmK, GzmB cluster is found on chromosome 14 and contains GzmB and GzmH, while GzmM is located on chromosome 19[34]. The genetic organization of the proteases is similar in that their transcripts are composed of 5 exons[34]. The catalytic activity amongst granzymes depends on the conserved amino acid – histidine, aspartic acid and serine at their active site[34]. All the granzymes are produced as zymogens, and are processed at the time of their packaging into the cytotoxic granule in the Golgi apparatus[34].

Granzymes become enzymatically active once the N-terminus is removed by constitutively expressed lysosomal enzyme cathepsin C[34,35]. Granzymes are stored inside acidic granules and bind to a dense serglycin scaffold, thus providing an environment that keeps the proteases proteolytically inactive[27,35].

GzmA is the only granzyme found as a homodimer, and with a molecular mass of 65 kDa, it is the largest of the granzymes[34]. As a tryptase, GzmA cleaves after basic residues arginine and lysine[37]. The types of cells that most abundantly express GzmA are $CD8^+$ CTLs and NK cells[37]. GzmA is also known to cleave ECM proteins allowing for NK and CTL to move throughout the body's tissues[34]. GzmA is understood to act in the induction of cellular death, extracellular effects (inflammation), and cleavage of extracellular proteins (collagen IV and FN)[34,38]. Inflammation is augmented by GzmA cleavage of IL-1β and activating macrophages. GzmA cleavage of the thrombin receptor is able to induce the release of cytokines such as IL-6 and IL-8 from monocytoid cells[34,36,38].

First discovered from lymphokine-activated killer cells (LAK cell), GzmK, a tryptase, also cleaves after basic residues arginine and lysine[37]. GzmK is expressed by activated M1 macrophages, CD4+ CTL, CD3+, CD56+, NKT cells, and γδ T cells[39,40]. The protease is acutely elevated in response to viral infection, allergic asthma, pneumonia, sepsis, and endotoxemia[40–42]. A study by Turner and colleagues determined that GzmK is produced and secreted by activated macrophages, augmenting inflammation and impeding epithelialization[40].

The lesser studied human granzymes are GzmH and GzmM. As a chymase, GzmH cleaves after tyrosine and phenylalanine and is produced by CTLs and NK cells.[34] GzmH can induce target cell death through DNA fragmentation and cleavage of pro-apoptotic protein, Bid, mediating mitochondrial damage[39]. Additionally, GzmH may play a role in antiviral defense.

Recently, Tang and colleagues discovered that GzmH aids in the clearance of the hepatitis B virus through the cleavage of hepatitis B virus X protein[43]. GzmM cleaves after methionine or leucine[34]. Cellular sources of GzmM include NK cells, NK T cells, and γδ T cells[39]. Extracellularly, GzmM is suggested to have a coagulative regulatory role as it cleaves pro-coagulative vWF[44,45]. GzmM has also been linked to inflammation. GzmM-deficient mice exhibited reduced levels of serum IL-1α, IL-1β, TNF, and IFN-γ resulting in a reduction in lethal endotoxicosis[46]. Elevated levels of GzmM have also been detected in patients with meningococcal sepsis[45].

Table 1. Five Human Granzymes.

	Size (kDa)	Cleavage Preference	Source	Pathologies	Reference
GzmA	65 (Dimer)	arginine and lysine	CD8+ CTLs and NK cells	Inflammation	[27,34,47]
GzmB	32	aspartic acid	NK, CTL, CD4+ cell, mast, T reg, SMC, chondrocytes, keratinocyte, dendritic cells, type 2 pneumocytes, granulosa cells, syncytial trophoblasts, myeloid cells	Inflammation, atherosclerosis, aneurysm, rheumatoid arthritis, transplant rejection, discoid lupus, drug eruption, atopic dermatitis, impaired burn wound, autoimmune blistering, and photoaging	[32,34]
GzmH	32	tyrosine and phenylalanine	CTLs and NK	Clearance of the hepatitis B virus through cleavage of the hepatitis B virus X protein	[34,43]
GzmK	28	arginine and lysine	M1 macrophages, CD4+ cytotoxic T cells, CD3+ CD56+ NK T cells, and γδ T cells	viral infection, allergic asthma, pneumonia, sepsis, and endotoxemia	[34,40]
GzmM	30	methionine or leucine	NK cells, NK T cells, and γδ T cells	meningococcal sepsis	[34,45]

1.4.1 Granzyme B

GzmB is traditionally known as a CTL-associated serine protease, with a molecular weight of 32 kDa[34]. *GzmB* is 3500 base pairs long, contains 5 exons and 4 introns, and has been localized to chromosome 14[48]. GzmB is now recognized to be expressed by numerous other cell types, both immune and non-immune: CD4+ cell, mast, basophils, macrophages, plasmacytoid dendritic cells, regulatory T cells, smooth muscle cells (SMC), chondrocytes, keratinocyte, type 2 pneumocytes, keratinocyte, granulosa cells, and syncytial trophoblasts[27].

1.4.2 Mechanism of Granzyme B-Mediated Apoptosis

GzmB is traditionally known for its role in facilitating intracellular apoptosis. GzmB granules contain a signal sequence that is directed towards the endoplasmic reticulum. The signal peptide is cut off, thereby producing N-terminals containing zymogen GzmB. Subsequently, in the Golgi, zymogen GzmB is tagged with a mannose-6-phosphate receptor to be used to direct it to the acidic lytic granule[49]. Removal of the N-terminal by cathepsin activates GzmB[34,35]. The activated granule is stored in a scaffold of serglycan[27]. This scaffold confines GzmB to an acidic pH within the granule, minimizing its proteolytic activity[27,34]. The enzyme becomes highly active at the neutral pH of 7.5[34]. The GzmB-containing granule is released into the immunological synapse of the CTL after lytic granules polarization by the microtubule-organizing center. The granules fuse with the presynaptic membrane and release perforin and granzymes into the synaptic cleft[27]. After secretion, perforin forms the basis for transmembrane pores into the target cell which allows for granzymes to move into the target cell[34].

Upon internalization, GzmB initiates target cell apoptosis through the proteolysis of cytosolic and nuclear substrates. GzmB has several intracellular substrates including inhibitor of caspase-activated DNase (ICAD), several caspases (-3,-7, and -8 *in vivo*), lamin B, and the pro-

apoptotic Bcl-2 member Bid[50]. GzmB induces apoptosis through both caspase-dependent and -independent mechanisms. Direct cleavage of procaspase-3 leads to caspase-3 activation, which ICAD to caspase-activated DNAse that then translocates to the nucleus to trigger DNA fragmentation and apoptosis[27,35]. The caspase-independent mechanism by which GzmB induces apoptosis occurs by means of proteolytic cleavage of Bid into granzyme-truncated Bid (gtBid). Thereafter, cytochrome c is released from the mitochondria and activates the apoptosome via deoxyadenosine triphosphate (dATP) binding; this complex formation leads to downstream caspase activation and cell death[27]. Figure 4 illustrates the GzmB mediated apoptotic pathways.

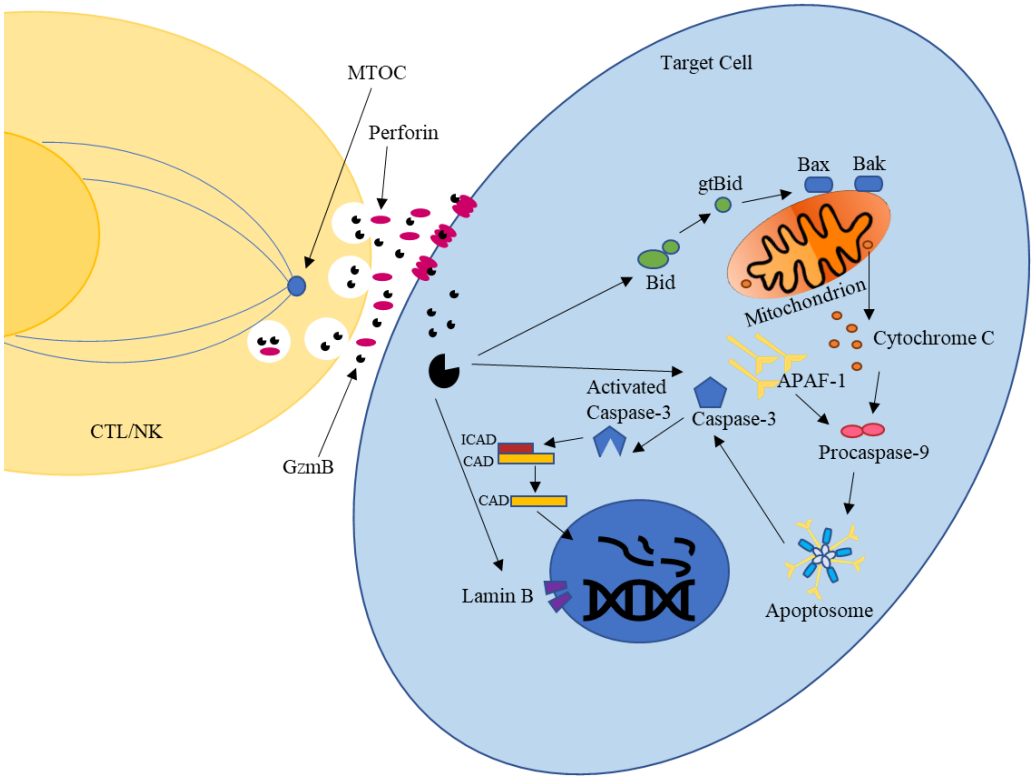

Figure 4. GzmB Facilitated Apoptotic Pathway. As GzmB is internalized into the target cell through perforin, caspase-dependent pathway is activated via the direct cleavage of caspase-3 inducing apoptosis. Separately, GzmB can also activate the mitochondrial apoptotic pathway through the cleavage of Bid into gtBid inducing the release of cytochrome c and binding to dATP, APAF-1 and procaspase-9 for apoptosome formation allowing for caspase activation resulting in apoptosis.
Note: Image created based on Boivin et al. 2009[27].

1.4.3 Granzyme B - Extracellular Role

During CTL or NK -mediated apoptosis, a large proportion of GzmB is not internalized by the target cell and accumulates in the ECM where it proteolytically cleaves multiple components of the ECM[51,52]. A summary of extracellular GzmB substrates are listed in Table 2.

GzmB retains its activity in bodily fluids and research has revealed several pathologic consequences of such activity. GzmB has multiple extracellular substrates including decorin, FN, VE-cadherins, vitronectin, and laminin. Degradation of such proteins by GzmB plays a plethora of roles including disorganization of the ECM, inflammation, impaired cell adhesion, cell migration, and anoikis[32].

The influence of GzmB on inflammation has been linked to cytokine processing as well as cleavage of substrates that impact subsequent inflammatory signaling. GzmB influences cytokine processing by cleaving IL-18 from inactive to active form and cleavage of IL-α to produce a more potent version of this proinflammatory cytokine[53,54]. Furthermore, GzmB cleavage of ECM proteins produces indirect effects on inflammation. For instance, cleavage of FN stimulates the migration of monocytes, which in turn influences the promotion of TNF-α production and proinflammatory Toll-like receptors (TLR)[51]. Fragments of FN trigger downstream TLR-4 activation of macrophages which then induces MMP-9 production[51,55]. Thereafter, MMP-9 can free proteoglycans, decorin and biglycan from the ECM, sending signals for increased proinflammatory cytokine production[51].

Cell integrins and growth factors are important in facilitating the migration of skin cells in wound healing. *In vitro* studies of GzmB show that it's cleavage of decorin, biglycan, and

betaglycan released active TGF-β, promoting dysregulated would healing and fibrosis[51,56]. In conditions of fibrosis marked by dysregulated wound repair, GzmB is elevated, reducing levels of decorin. As GzmB cleaves, FN and decorin, MMP expression and collagen degradation intensify. Fragments of FN and vitronectin have also been noted in chronic wounds[57].

Weakened wound repair is marked by dysregulated hemostasis and increased vascular permeability[58]. Wound repair processes are impaired by cleavage of matrix proteins such as vitronectin, laminin, FN, and vWF. Hendel et al. (2014) have shown that GzmB-mediated FN cleavage releases VEGF and promotes vascular permeability[59]. The pro-hemostatic protein vWF is also cleaved by GzmB, preventing platelet aggregation[30]. An additional consequence of GzmB in the ECM is the induction of cell detachment-mediated cell death, referred to as anoikis[30,60]. A study done with GzmB-treated SMC showed cells undergo anoikis after treatment, resulting in FAK-phosphorylation reduction and cleavage of FN[60]. Vitronectin is a substrate of GzmB, which may act in disrupting migration and proliferation of keratinocytes by interrupting its ability to interact with integrin-binding sites via the cell adhesion motif, RDG, as well interacting with insulin growth factor–1[51,52,61]. When laminin is cleaved in its cell binding region by GzmB, the migratory capacity of keratinocytes and fibroblasts is impaired[30,61]. GzmB has the potential to change the remodeling of the ECM and tissue repair via the cleavage of ECM proteins and inducing endothelial and SMC anoikis[51,60,61]. Knowing the role that GzmB has in these processes, GzmB is said to be involved in the various phases of wound healing (summarized in Figure 2)[32].

Table 2. Validated substrates of GzmB.

Substrate	Biological consequence of cleavage	Reference
Acetylcholine receptor	Fragmentation produces neoantigens	62
Aggrecan	Impairs cartilage integrity	63
Biglycan	Release of active TGF-β and disorganize collagen fiber assembly	56
Cartilage Proteoglycans	Impairs cartilage integrity	64
Collagen VII	Increased DEJ separation	65
Collagen XVII	Increased DEJ separation	65
Decorin	Releases active TGF-β1, collagen disorganization, and disruption of fibrillogenesis	56
FGFR1	Inhibits prostate cell proliferation and survival signaling	66
Fibrinogen (matrix form)	Anti-thrombotic actions: Inhibits platelet adhesion and spreading Thrombus growth	61
Fibronectin	Anoikis, VEGF release, impaired adhesion and migration	59,61
Laminin	Impaired cell adhesion and spreading	61
Neuronal glutamate receptor	Produces an autoantigenic fragment	67
Notch1	Inhibiting viral infection and or tumour cell activities	66
Plasmin	Decreases angiogenesis	68
Plasminogen	Produces anti-angiogenic angiostatin	68
Smooth muscle cell matrix	Anoikis and impaired cell adhesion	60
Soluble β-glycan	Increases active TGF-β1 secretion	56
VE-cadherin	Impaired cell-cell junctions, endothelial permeability	69
Vitronectin	Anoikis and impairs adhesion and migration	61
Von Willebrand Factor	Delays thrombosis	70
ZO-1	Disruption of cell-cell junctions	71
α6/β4 integrin	Increased dermal epidermal junction separation	65

1.4.4 Granzyme B in Wound Healing

An emerging and growing field of interest is the role of GzmB in chronic wound healing. Several pathological conditions have higher levels of GzmB such as photoaging, atherosclerosis, chronic obstructive pulmonary disease, aneurysm, rheumatoid arthritis, and diabetic wounds, suggesting it is an important factor in chronic diseases[30,64,72,73].

GzmB has been shown to be important in skin aging. Using an apolipoprotein E-knockout model of skin ageing, GzmB deficiency was found to attenuate the appearance of skin ageing by preventing decorin cleavage and the loss of collagen organization[52]. Subsequently, Parkinson et al (2015) investigated the effects of GzmB in chronic UV-induced ageing (photoaging)[73], and found chronic UV exposure led to elevated GzmB in the skin. This led to cleavage of ECM proteins FN and decorin and increased collagen-degrading matrix metalloproteinase-1 (MMP-1)[73]. GzmB-deficient mice were protected from the loss of collagen density and FN degradation in the UV-irradiated skin, demonstrating the importance of GzmB in this process[73].

Several *in vitro* and *in vivo* studies have investigated the role(s) of GzmB in impaired wound healing in various tissues and found that GzmB cleaves ECM proteins highly involved in this process. GzmB cleaves angiogenic factor plasmin into angiostatin, a pro-hemostatic regulatory molecule[68]. GzmB also cleaves the clotting factor vWF which interferes with the normal process of clotting via delaying platelet aggregation through inhibition of adhesion and spreading of platelets[70]. In a model of diabetic wound healing, Hsu et al. (2014) showed

increased levels of GzmB contributed to the pathogenesis of diabetic wound repair via cleavage of substrate FN[74]. When GzmB inhibitor, SerpinA3N, was applied to these diabetic wounds, wound closure was significantly improved in addition to preventing the cleavage of FN[74]. Together, these data suggest that GzmB presence contributes to the pathology of wounds via cleavage of ECM proteins like vWF and FN.

GzmB also interferes with wound closure and re-epithelization. GzmB interferes with epidermal growth factor receptors, resulting in inhibition of the keratinocyte migratory function[75]. Shen et al.(2018), tested a novel small-molecule GzmB inhibitor, VTI-1002 on mice for 30 days following thermal injury and found GzmB's inhibition resulted in an accelerated re-epithelization, increases in tensile strength, increased decorin levels, and improved overall collagen organization in the tissue compared to vehicle controls[76].

Full-thickness chronic wounds and skin blistering diseases also demonstrate ECM impairment by GzmB cleavage. Following a full-thickness wound the basement membrane is disrupted, extinguishing the contact between keratinocytes and the protein-rich barrier. Instead, contact between the cells and the basement membrane zone triggers signaling for cells to modify, migrate and form the new wound closure. Both FN and laminin, key facilitators of this signaling cascade, are cleaved by GzmB [61]. GzmB also disrupts basement membrane integrity through the cleavage of key cell junction proteins E-cadherin and zonula occludens (ZO)-1 [71]. Russo et al. (2018) demonstrated that GzmB is elevated in autoimmune blistering diseases and cleaves several dermal-epidermal junction proteins, cleaves key attachment proteins α6/β4 integrin, collagen VII, and collagen XVII, thus breaking apart important connective proteins between these layers of the skin[65].

GzmB is clearly an important factor in the transition of a wound from acute to chronic. Its role in skin aging and wound healing (including wound closer and re-epithelialization) demonstrates the potent effects this protease can have, and how difficult it is to stop its detrimental activity in the ECM. Understanding how GzmB can be inhibited, and the effects of such inhibition, is an important step towards the prevention of chronic wounds.

1.4.5 Granzyme B Inhibitors

Several inhibitors of GzmB have been identified, however there is currently only one known human endogenous inhibitor of GzmB. Protease inhibitor-9 (PI-9), also known as SerpinB9, is the only known endogenous intracellular inhibitor of GzmB. PI-9 has been shown to inhibit GzmB/perforin-induced apoptosis[77]. It is highly specific to GzmB in that it does not inhibit 8 of the caspases nor does it protect against Fas-mediated apoptosis[78]. A second inhibitor though only found in mice is SerpinA3N, also known to inhibit enzymes chymotrypsin, trypsin, cathepsin G and elastase. First found in cultured mouse Sertoli cells, Serpin A3N has been shown to inhibit both human and mouse GzmB[79]. However, serpinA3N is exclusive to mice and is not found in humans. Thirdly, compound 20 (C20) is a GzmB-specific competitive inhibitor, and its structure proved crucial to the selectivity and cellular efficacy of this compound in inhibiting GzmB but not the caspases[80]. Inhibition of GzmB has proven difficult, with only one endogenous intracellular inhibitor and no extracellular inhibitors currently identified.

Endogenous extracellular inhibitors for GzmB are currently lacking, and research is ongoing for the synthesis of such a product. Recently a topical extracellular GzmB inhibitor, VTI-1002, has been developed and has shown to be highly potent and specific to GzmB *in vitro* cleavage assays and an *in vivo* model of impaired healing and scarring[76]. This study has provided

the first topical inhibitor of GzmB that prevented cleavage of decorin and FN but may also provide benefit in preventing cleavage of other proteins found in the ECM such as thrombospondin-2.

1.5 Thrombospondin-2

1.5.1 Structure of TSP-2

Thrombospondin-2 (TSP-2) is a matricellular glycoprotein mainly produced by fibroblasts, SMC, and macrophages whose action is not well understood[81,82]. TSP-2 is found to be expressed in connective tissues including blood vessels, ligaments, dermis, as well as expression by basal epidermal keratinocytes theorized to contribute to aid preventing epidermal vascularization[83,84]. It is secreted as a 450 kDa, homotrimer molecule, made of three identical 145-kDa polypeptide chains linked by disulfide bonds[81,82,85–87]. TSP-2 is part of a family of five oligomeric extracellular, calcium-binding glycoproteins, and which belong to two subgroups, A and B. Subgroup A consists of TSP-1 and TSP-2, both are trimers that have a high degree of similarity; 82% in type 2 and 3 repeats of the C-terminal region[88]. Subgroup B consists of pentameric thrombospondin-3, -4, and -5[89]. Subgroup B is not as well understood as subgroup A, but it is known to have some structural differences such as an extra type 2 repeat and no procollagen[90,91].

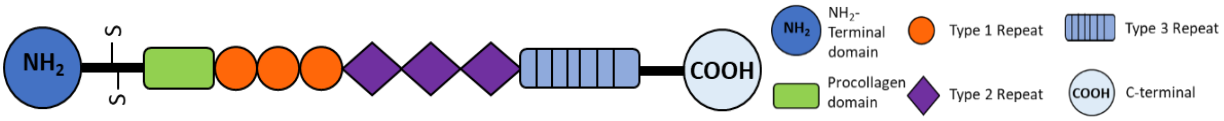

Figure 5. Structure of Thrombospondin-2. TSP-2 is a glycoprotein consisting of an amino-terminal domain, type 2 and type 3 repeat sequence and a carboxy-terminal domain. These domains have different functions. The NH2-terminal domain stimulates angiogenic response via β1 integrins. Type 1 repeats contain anti-angiogenic sequences that interacts with heparan sulfated glycoconjugates, fibronectin, TGF-β, receptor CD36, and inhibit tumor progression. The type 3 repeats contain the adhesive RGD sequence that interacts with α5β1 and αvβ3 integrins. Lastly, C-terminal domain interacts with CD47.
Note: Image created based on Jeong et al 2015[92]**.**

TSP-2 is composed of six functional domain structures and these interact in many different manners with the ECM (Figure 5). For instance, domains type 1 and 3 repeats stimulate angiogenic response via α3β1 and other integrins, and the C-terminal interacts with cell signaling markers such as CD47[93,94]. Specifically, TSP-2 binds to many different ECM proteins, cellular receptors, and proteases: decorin, FN, heparin sulfate proteoglycans, CD36, CD47, integrins, VLDL, and FGF2[87,95,96]. The N-terminal domain consists of 200 amino acids that have high-affinity binding to proteoglycans heparin and heparan sulfate[95,97]. The vWF procollagen region is a region that is specific to subgroup A and its function is yet to be defined. Type I repeats, also known as the thrombospondin structural homology repeats (TSRs), are a trademark of TSP-1 and -2[98,99]. Studies have suggested that this region regulates cell adhesion via its integrin interactions. Numerous functions of TSPs have been linked to the TSR region known to bind to β1 integrins, CD36, TGFβ[95]. For example, TSRs are said to play a role in the anti-angiogenic function unique to the subgroup A of the thrombospondins, contributing to inhibition of tumor angiogenesis and growth[100]. The TSRs are said to inhibit tumor progression by TGF-β dependent and independent mechanisms[101]. This was demonstrated when tumors treated with the TSRs had a 3-fold increase

in apoptosis[102]. After the TSR are the three type 2 repeats, also referred to as epidermal growth factor (EGF)-like repeats, and type 3 repeats. The type 3 repeats consist of thirteen aspartate-rich calcium-binding regions[85,99]. Each of these repeats contains 23-38 amino acids with a high content of aspartic acid residues[90]. The type 3 repeats contain an RGD sequence, a cellular adhesion sequence, that interacts with α5β1 and αvβ3 integrins[90]. Exposure of this sequence has been described to be important to make TSP-1 very adhesive to endothelial cells[88]. The C-terminal domain interacts with CD47[103]. Lastly, the C-terminal region of the protein has been indicated to play a role in cytoskeletal organization[104].The different components of TSP-2 clearly play different and important roles in ECM interactions. TSP-2, through the different domains of its structure, is able to interact with many cellular receptors in the ECM[105].

1.5.2 Functions of TSP-2

TSP-2 has multiple roles in mediating the ECM both in endothelial and epithelial cells. These functions have generally been found using knockout studies in mice *in vivo* and *in vitro* studies. TSP-2 has been proposed to have a role(s) in wound healing, angiogenesis and matrix organization that remains poorly understood. The major focus thus far has been on the TSP-2 role in ECM remodeling in influencing fibroblasts and MMPs, but TSP-2 has also been noted for its importance in cellular functions such as cell adhesion, spreading, migration, proliferation, and apoptosis[87,103,105].

1.5.2.1 Adhesion

TSP-2 appears to have predominantly inhibitory effects on cell adhesion, but evidence from TSP-1 suggests this action may be cell-dependent. TSP-2 action has generally been studied in endothelial cells where it inhibits adherence of endothelial cells. Although TSP-2 is evident in

the skin, its action here is not well understood, however, TSP-1 promotes keratinocyte cell adherence.

TSP-2 interacts with various cell receptors that function in cell adhesion in the endothelium. TSP-2 interacts with endothelial cell β_1 integrins such as $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$[106], as well as with proteoglycan adhesion receptors and low-density lipoprotein receptor-related protein (LRP)[84,88,107]. It is suggested that variability that is observed in such studies may be dependent on the receptors and regions, such as with heparin sulfate proteoglycans and $\alpha_v\beta_3$ integrin, of TSP-2 that interact with the cells[97]. An example, bovine aortic endothelial cells displayed anti-adhesive phenotypes when they came in contact with TSP-2 as a result of TSP-2 heparin-binding domain located in the N-terminal region[97]. Additionally, inhibiting TSP-2 gene expression in human aortic smooth muscle cells, resulting in improved smooth muscle cell attachment[108].

The effects of TSP-2 on cell adhesion in epithelial regions are far less clear but suggest there are potential cell-adherence promoting effects. For example, dermal fibroblasts from TSP-2- null mice display a lack of attachment and spreading as well as increased levels of MMP-2[84]. It has been reported that TSP-2-null fibroblast phenotype is influenced by TSP-2, as they display defective cellular adhesion to proteins such as vitronectin, collagen I, and TSP-2[109,110]. Furthermore, though the effects of TSP-2 on cell adhesion have not been investigated directly on keratinocytes, it was demonstrated that primary keratinocytes use TSP-1 as an attachment factor and accordingly promote keratinocyte attachment and spreading[111]. TSP-1 coated dishes demonstrated to facilitate attachment of keratinocytes overtime, but on the contrary, after chymotrypsin fragmentation, this effect was not observed[111]. These attachment studies suggest that TSP-2 attachment may be a cell specific function.

1.5.2.2 Cell Proliferation

TSP-2 has been investigated for its role in cell proliferation. TSP-2 blocks cell cycle progression in the presence of multiple growth factors such as bFGF, EGF, IGF-1, VEGF[86]. Additionally, very low-density lipid receptor (VLDLR) on endothelial cells binds TSP and this inhibits cell cycle progression[112].

1.5.2.3 Apoptosis

The current understanding of TSP-2 in apoptosis is limited, but existing literature does provide insight into its potential effects in inducing apoptosis. The induction of apoptosis is suggested to be mediated by the binding of TSP-1/TSP-2 to CD36 (an apoptosis-related cell receptor)[113,114]. TSP-2 appears to bind to CD36 by the TSRs (just like TSP-1) in a concentration-dependent manner, providing evidence that the induction of apoptosis is mediated by the binding of TSP-2, via the TSRs, to CD36[113,114]. Other potential avenues TSP-2 impacts apoptosis could include activation of JNK and/or up-regulation of the Fas/FasL receptor and ligand pair, similar to TSP-1[91]. Supporting this, Armstrong et al. (2002) showed that TSP-2 was able to inhibit the growth of human microvascular endothelial cells (HMVECs) as well as impair viability of these cells in the presence of bFGF, IGF-1 and EGF[86]. TSP-2 was able to induce caspase activation in the absence of growth factors, and caspase inhibitors did not prevent TSP-2 induced cell cycle arrest[86]. This suggests that TSP-2 inhibition of cell cycle progression is independent of those leading to cell death and is yet to be shown in other cell types.

1.5.2.4 TSP-2 in wound healing

Multiple TSP-2 - null *in vivo* models demonstrate that TSP-2 has a role in ECM organization. TSP-2 is highly expressed during tissue remodeling in the context of wound healing, carcinogenesis, ischemia, and inflammation[93]. Studies using TSP-2 - null mice suggest

that TSP-2 may inhibit angiogenesis and modulate ECM remodeling[115]. Kyriakides et al. (2002) observed that TSP-2-null mice exhibited accelerated wound healing, which was accompanied by disorganized collagen fibers, thickened epidermis, and accelerated re-epithelialization[115]. The higher amount of MMPs may be a contributing factor to the disorganization of collagen fibers. Cutaneous wounds in TSP-2-null mice exhibit increased levels of MMP-2 and -9, as well as increased levels of VEGF and TIMP1/2[93,116]. It is believed that MMPs are capable of releasing ECM bound VEGF, which is also observed in TSP-2-null wounds[93,117,118].

Defining specific effects of biologically appropriate levels of TSP-2 has been said to be a hurdle due to that extraction of enough TSP-2 from tissues has been difficult[84]. TSP-2 proteolytic degradation has been studied with a few proteases to date.

1.5.3 Thrombospondin-2 as Cleavage Substrate

TSP-2 has been noted to be present in the skin and TSP-2-null wound healing models have displayed faster wound closure though not without displaying disorganized tissue and increased MMPs. GzmB is elevated in chronic wound healing and known for its role in dysregulation in the ECM including the proteolysis of ECM proteins. So far, TSP-2 cleavage has been demonstrated by a variety of other proteases including trypsin, ADAMS1, MMP-2, and MMP-9[72,107,119]. Chen and colleagues demonstrated early evidence of TSP-2 cleavage by trypsin. TSP fragments were used to demonstrate that EC adhered to the fragments containing type 3 repeats, kindling adhesion to integrin $\alpha_3\beta_{1,}$ a property being conserved in their cellular adhesion motif arginine-glycine-aspartate (RGD) sequence[88]. Fragments of TSP-2 via ADAMTS1 cleavage, a protease upregulated in inflammation, mediates the release of polypeptides from the trimeric structure of both TSP-1 and -2 generating a pool of antiangiogenic fragments from matrix-bound thrombospondin. This cleavage generates two fragments of 42 and 30 kDa, but

does not fully cleave the full-length protein[119]. This fragmentation is hypothesized to facilitate modulation of TSPs functions when TSPs and/or bioactive domains are released from being matrix-bound and released into the ECM[119]. Insights into fragmentation of TSP-2 by gelatinases, MMP-2 and MMP-9, were given in a study of quantitative proteomics by incorporating whole protein labelling with terminal amine isotopic labelling[120]. It was recently discovered that higher levels of TSP-2 and fragments are detected in vitreous samples with proliferative diabetic retinopathy and control patients without diabetes[121]. Although our understanding of the relevance of TSP-2 fragments into chronic diseases is expanding, much remains unknown. Specific effects of TSP-2 fragmentations from these proteases are yet to be defined and particularly whether potential GzmB proteolysis may modulate TSP-2 function.

1.6 Rational and hypothesis

In wound healing, keratinocytes migrate from the wound edges underneath the eschar tissue to form a barrier with the external environment. The composition of this matrix is important in mediating cellular behavior. Proteases, such as GzmB, can alter tissue structure and function that can impair wound healing.

Anti-angiogenic functions of the thrombospondins have been well documented in the literature, yet their role in the skin matrix and wound healing is still being defined. Interactions between TSP-2 and the ECM impact on cellular functions are context-dependent. This has made documenting TSP-2's specific role in wound healing a challenge for investigators. As matricellular proteins, TSPs are known to be involved in cell-cell and cell-matrix interactions and may play a role in regulating cell proliferation, migration, viability, and differentiation. In

1985, Wight et al. demonstrated that there was TSP in the basement membrane of the epidermis and that TSP-1 has the ability to facilitate adhesion and migration in primary keratinocytes[111,122,123]. Although extensive studies were conducted with TSP-1, the literature investigating the role of TSP-2 is scarce. The objective of this study is to investigate the role of **TSP-2** on keratinocytes adhesion and viability. Furthermore, this study will determine how GzmB **cleavage of TSP-2** will alter the effects of these functions. This study will generate information relevant to the goal of providing understanding of their function in cutaneous wound healing.

General Hypothesis:

GzmB cleaves TSP-2, and GzmB-mediated TSP-2 cleavage induces a loss in keratinocyte viability and/or adhesion.

To address this hypothesis, my specific aims were:

1. To determine whether TSP-2 is cleaved by GzmB.
2. To assess whether TSP-2 affects keratinocyte viability and/or adhesion.

Chapter 2: Materials & Methods

2.1 Reagents

Human recombinant thrombospondin-2 (TSP-2), reconstituted in phosphate-buffered saline (PBS), was obtained from R&D systems (Minneapolis, MN). Human GzmB was obtained from Emerald Bioscience (Bainbridge Island, WA). Compound 20, dissolved in dimethyl sulfoxide (DMSO), was obtained from UBC Centre for Drug Research and Development (Vancouver, BC)[124]. Antibodies against TSP-2 and GAPDH were obtained from R&D systems and Cell Signaling (Danvers, MA), respectively. Fibronectin, DMSO, the methyl-thiazolyl diphenyl-tetrazolium bromide (MTT) assay reagent, staurosporine, Dulbecco's phosphate-buffered saline (DPBS), and the Brilliant Blue R concentrate Coomassie stain were obtained from Sigma-Aldrich (St. Louis, MO). Tween 20 was obtained from Fisher Scientific (Waltham, MN). Pierce bicinchoninic acid (BCA) protein assay kit and trypsin-EDTA (under Gibco) was obtained from Thermo Scientific (Rockford, IL). SDS loading buffer was made in the lab[125]; Cell Culture 6 and 96 well plates were obtained from Sarstedt Inc. (Montreal, Canada).

2.2 Cleavage Prediction

GrabCas Program was obtained from the Saarland University Faculty of Medicine, Department of Human Genetics. The software is available at the following web address: https://www.uniklinikum-saarland.de/en/einrichtungen/university_departments_of_theoretical_and_clinical_medicine/human_genetics/human_genetics/software/. The TSP-2 amino acid sequence (P35442), obtained from the UniProt website database, was entered into the input-form along with a cutoff score of 1. Fragment sizes were calculated using the proposed cleavage motifs and converted to kDa.

Motif locations were also compared to their location in the TSP-2 domains represented on the UniProt website database.

2.3 Biochemical Cleavage Assays

2.3.1 Dose Response

Recombinant human TSP-2 (1 µg) was incubated with GzmB (1, 10, 100 nM) in 50 nM Tris pH 7.5 buffer, with a total reaction volume of 30 µL per closed 1.5 mL Eppendorf tube for 2 h at 37℃. FN (1 µg), was used as a positive control for GzmB activity and was incubated with and without GzmB (100 nM) for 18 h in a water bath at 37℃. To stop the reactions 5 µL of 6X SDS loading buffer was added to each of the reaction tubes. To fully denature the substrate, each reaction tube was then heated for 5 min at 95℃. The samples were then run on a 10% SDS-PAGE for 15 min at 90 V then 1 h 18 min at 120 V. The gel was stained with Brilliant Blue R Coomassie stain for 1 h. To destain, the gel was placed on a shaker in Coomassie destaining solution comprised of 45% methanol (vol/vol) and 10% acetic acid (vol/vol)) for 18 h. The gel was scanned using a LI-COR Odyssey imaging system (LI-COR Biotechnology, Lincoln, NE). The experiment was repeated twice.

2.3.2 Inhibition Cleavage Assay

Recombinant human TSP-2 (1 µg) was incubated with GzmB (10 nM) in 50 nM Tris pH 7.5 buffer, with a total reaction volume of 30 µL. FN (1 µg), used as a positive control for GzmB activity, was incubated with and without GzmB (10 and 100 nM). Compound 20 (C20) at 50 µM concentration was incubated with GzmB for 30 min at room temperature (RT) before the addition of each substrate (TSP-2 or FN). All conditions were incubated at RT for 30 min prior to the addition of the substrates. All cleavage reactions were incubated at 37℃ in a water bath for

2 h. After the incubation period, the procedure was kept the same as described above. The experiment was repeated twice.

2.3.3 Cleavage Assay (Time course)

For the cleavage time course, the reactions were carried out, stopped, and analyzed by SDS-PAGE as above. Prior to the addition of each substrate, GzmB was preincubated at RT for 30 min, after which the cleavage reactions were then incubated at 37℃ for time points of 0.5, 1, 2, 4, 10, and 24 h at 37℃. FN (1 µg) was used as a positive control for GzmB activity and was incubated with GzmB (10 and 100 nM) for 24 h. After the incubation period, the procedure was kept the same as described above. The experiment was repeated twice.

2.4 Cells

HaCaT (Human Adult low Calcium high Temperature) cells were grown and maintained in Dulbecco's Modified Eagle's media (DMEM, Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco) and 1% (vol/vol) penicillin/streptomycin (P/S) (Gibco) and kept in an incubator humidified chamber with 5% CO_2 at 37°C, unless indicated otherwise.

2.5 Western Blot

HaCaT cells ($3x10^5$ cells) were seeded into 6-cm cell culture dishes. The next day, the cells were washed, and the media exchanged with serum-containing and serum-free media to three representative wells each. HaCaT cells were harvested after 2 days. Culture supernatant for each well was collected and adherent HaCaT cells were lysed with Laemmli lysis buffer (recipe obtained from bio-rad[126]) containing protease inhibitor (Sigma). Lysates were incubated on ice for 15 min followed by sonication. Sonication was done using Qsonica (Newtown,CT) by placing the clean probe at 20 kHz in the sample tube for 2 x 10 sec. The probe was cleaned between each sample with deionized water. Cell debris was pelleted by centrifugation at

18,000×g for 20 min at 4 °C. The cell lysates (supernatants) were transferred to new tubes and their protein concentration determined by BCA. Cell lysate (10 µg) was separated by 10% SDS-PAGE at 90 V for 15 min, and 120 V for 80 min. Thereafter, proteins were transferred to 0.45 µm polyvinylidene fluoride (PVDF) membrane at 300 mA for 75 min on ice. PVDF membrane was washed three times in TBS-Tween (50 mM Tris-Cl; 150 mM NaCl pH 7.5, 0.1% tween-20) and blocked for 1 h at RT in 5% skim milk in TBS-Tween. The blot was then washed three times in TBS-T and incubated overnight at 4 °C in polyclonal anti-TSP-2 antibody (1:2000; R&D) and GAPDH (Cell Signaling). The next day, the blot was washed three times and incubated for 1 h with secondary donkey-anti-goat horseradish peroxidase antibody (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA) at RT. Lastly, the blot was visualized using enhanced chemiluminescence substrate (Thermo Fisher) on the Licor Odyssey Fc system using at 700 nm for 30 sec and chemiluminescence channel for 10 min.

2.6 Viability Assay

HaCaT cells were seeded in a 96-well plate at a cell density of $1x10^4$ cells/well and grown to 60-70% confluence. At this point, the media was replaced with 200 µL of fresh medium with the following conditions for 24 h: Untreated (with 200 µL of serum-containing media and serum-free media), TSP-2 (0.2, 1, 5 µg/mL), staurosporine (1µL), and vehicle controls: 5% PBS (10 µL), Tris 50 mM pH 7.5 (30 µL), and 0.5% DMSO (1 µL) for 24 hours. For GzmB-containing viability experiments, conditions were treated as follows:

Recombinant human TSP-2 (1 µg) was incubated with GzmB (1, 10, 100 nM) in 50 nM Tris pH 7.5 buffer, with a total reaction volume of 30 µL per Eppendorf tube for 2 h at 37℃. C20 (50 µM) was added to all GzmB-containing reactions and incubated for 15 min to inhibit any remaining active GzmB activity prior to the addition of the fragments to the HaCaT cells. Media

was replaced with 200 µL of fresh medium under the following conditions for 24 h: Untreated (with serum and without serum), TSP-2 (5 µg/mL), GzmB-fragmented TSP-2 (1, 10, 100 nM), GzmB (100 nM), C20 (50 µM), staurosporine (1µL), and vehicle controls: 5% PBS (10 µL), Tris (30 µL), and 0.5% DMSO (1 µL) in 200 µL serum-free media for 24 h.

Cell viability was determined using 20 µL of MTT. The MTT solution was added to the cells and incubated for 3 h at 37℃, at which time the tetrazolium is reduced to formazan crystals. After incubation, the media was removed, and the formazan crystals were dissolved in 1:1 ethanol to DMSO. Colorimetric detection was done on Tecan Infinite M1000 Pro plate reader at 570 nm. The amount of color produced is directly proportional to the number of viable cells on the plate. Four independent experiments were done.

2.7 Adhesion Assay

For TSP-2 dosed treatment, a clear, flat-bottom 96-well plate was coated with 200 µL of various concentrations of TSP-2 (0.2, 1, 5 µg/mL), wrapped in parafilm, and incubated overnight at 4°C. In triplicate, wells were coated with either 5% BSA or FN, which served as negative controls and positive controls, respectively. For the GzmB-treated TSP-2 matrix assay, a clear, flat-bottom 96-well plate was coated with 200 µL TSP-2 (5 µg/mL), wrapped in parafilm, and incubated overnight at 4°C. In triplicate, wells were coated with either 5% bovine serum albumin (BSA) or FN which served as negative controls and positive controls, respectively. Each well was washed once with DPBS. Tris or 50 µL of GzmB (1,10, 100 nM), which was preincubated for 30 min prior, was added to wells containing TSP-2 and was incubated for 2 h at 37°C.

Thereafter, each well was washed once with DPBS, and non-specific protein binding sites were blocked by incubating the wells for 1 h with 5% BSA in DPBS, followed by two rinses with DPBS. HaCaT cells were then washed with DPBS, then harvested with Trypsin-EDTA (1.5

mL) and centrifuged down. The cells were washed again with only DPBS to remove any remaining FBS-containing media, centrifuged again, and resuspended in DMEM serum-free media. HaCaT cells were seeded at $1x10^4$ cells per well, pretreated according to the concentrations of TSP-2 stated earlier in this document. HaCaT cells were then incubated for 24 h at 37°C with 5% CO_2. After incubation, unbound HaCaT cells were removed by removing the media and washing each well 2 times with DPBS with a multichannel pipette. Images were taken with EVOS FL microscope (Life Technologies) of four similar areas of top/bottom left/right areas in each well, at 10X magnification. Images of the cells were quantified in ImageJ by manual cell count for each of the four representative areas per well. Four independent experiments were done.

2.8 Statistical Analyses

All data were analyzed with one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test. Data are represented as mean ± Standard Deviation (SD). P-values are represented on graphs, and $p < 0.05$ was considered significant in all experiments. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA).

Chapter 3: Results

3.1 TSP-2 is a Predicted GzmB Substrate

To predict potential GzmB cleavage sites in the TSP-2 protein sequence, an initial screening of TSP-2 using the bioinformatic tool GrabCas, was performed (Figure 6). Five different motifs were identified as potential cleavage loci. The remaining fragments were also investigated to determine whether there could be further fragmentation of the remaining protein. Protein fragments were determined to be potentially cleaved further into smaller fragments (Figure 7).

Cut Profile (amino acid position)	Fragment Size (kDa)	Domain	Output Score
VSND↓NQ (300)	33	Interchain	9.27
IDTD↓NN (791)	87	Type-3 4	10.77
VDND↓LV (852)	93	Type-3 6	6.178
IDDD↓GH (867)	95	Type-3 6	2.851
VGFD↓EF (1003)	110	C-Terminal	3.434

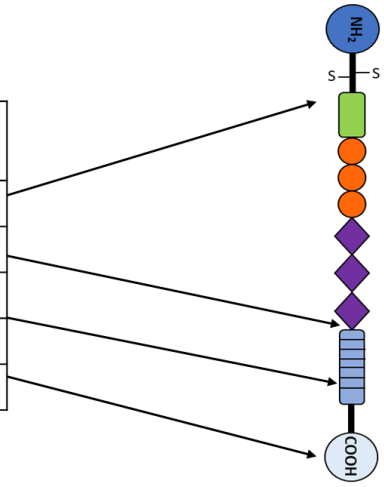

Figure 6. Predicted GzmB Cleavage of TSP-2. GraBCas program used to generate predictive Granzyme B cleavage of TSP-2 fragment cut profile and fragment size. Five motifs were generated, and the arrows indicate the predicted cleavage location.

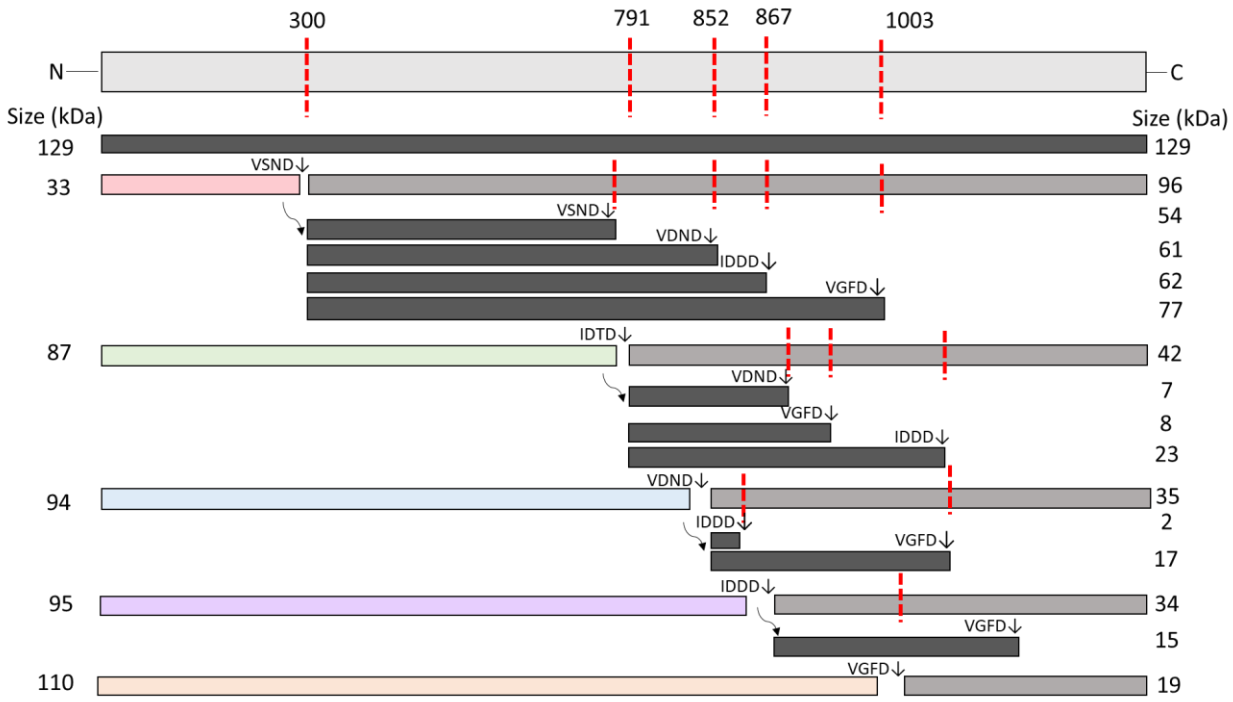

Figure 7. TSP-2 is predicted to be further fragmented by GzmB. Predicted TSP-2 fragments are also expected to be further fragmented by GzmB via the GrabCas bioinformatic tool. Remaining fragments of the originally predicted TSP-2 fragments were examined to determine if additional cleavages could occur with the remaining TSP-2 protein length. Cleavage prediction demonstrated that further fragmentation could take place and result in smaller fragment sizes.

3.2 GzmB cleaves TSP-2 *in vitro*

In order to confirm that TSP-2 is a substrate of GzmB, TSP-2 was incubated with GzmB at various concentrations. Proteolysis of TSP-2 was dose-dependent as increasing concentration of GzmB resulted in increased fragmentation of TSP-2 (Figure 8). The highest concentration of GzmB (100 nM) resulted in the full cleavage of TSP-2.

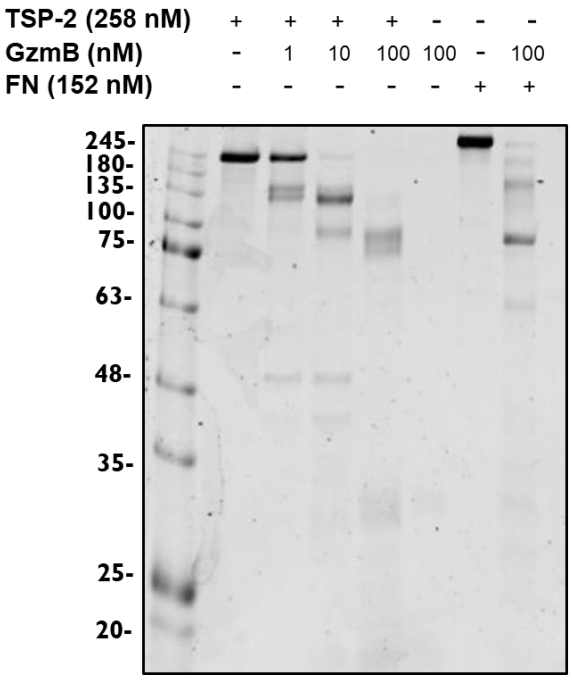

Figure 8. GzmB cleavage of TSP-2 occurs in a dosage-dependent manner. To test whether TSP-2 is a substrate of GzmB, recombinant human TSP-2 was exposed to various concentrations of GzmB (1, 10, 100 nM) at 37℃ for 2 h *in vitro*. Fibronectin (FN) was used as a positive control for GzmB activity, was incubated with GzmB (100 nM) for 18 h at 37℃. Reactions were stopped with 6X SDS loading buffer and heated denaturation at 95℃ for 5 min. The conditions were run in SDS-PAGE and stained by Coomassie Blue.

GzmB was pre-inhibited using a highly specific GzmB inhibitor, C20, to confirm that TSP-2 fragmentation was the result of GzmB catalytic activity and exclude the possibility that TSP-2 was fragmented by other proteases. GzmB treated with C20 reduced TSP-2 fragmentation and confirmed that GzmB was responsible for producing the observed TSP-2 fragments (Figure 9). DMSO, the C20 vehicle, did not affect cleavage.

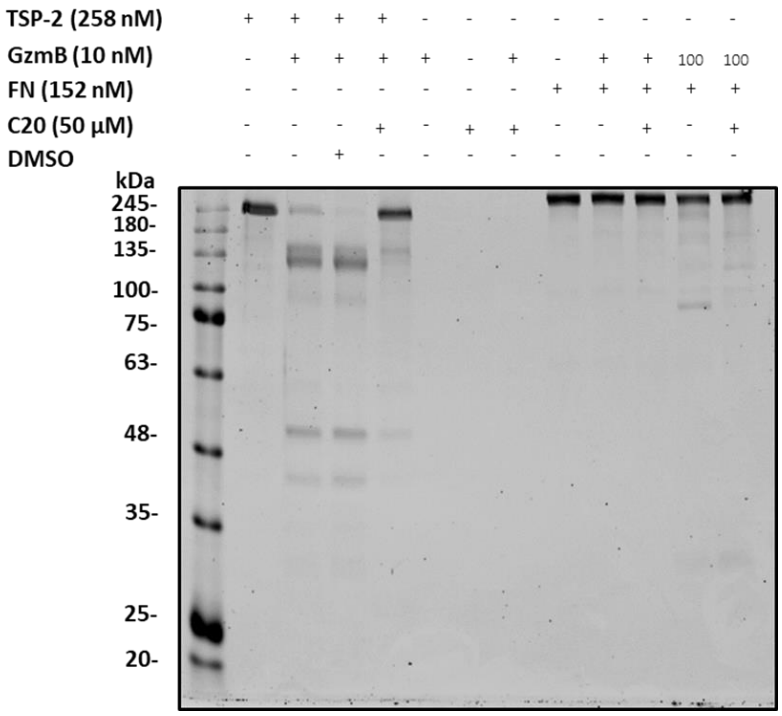

Figure 9. Compound 20 Inhibits GzmB Cleavage of TSP-2. Compound 20 (C20) was used to inhibit TSP-2 cleavage by GzmB. C20 was pre-incubated with GzmB for 30 min at RT before addition of each substrate, TSP-2 and fibronectin (FN). As positive control for GzmB activity, FN was incubated with GzmB (10 nM and 100 nM) for 2 h at 37℃. Reactions were stopped with 6X SDS loading buffer and heated denaturation at 95℃ for 5 min. The conditions were run in SDS-polyacrylamide gel electrophoresis and stained by Coomassie Blue.

To assess whether TSP-2 fragmentation changes over time, a time course was performed (Figure 10). GzmB-mediated cleavage of TSP-2, from its full-length form, occurred rapidly as fragmentation was observed as early as 0.5-h incubation with GzmB (10 nM). After 2 h, the full-length band became faint suggesting that fragmentation of TSP-2 by GzmB increased over time. The pattern of TSP-2 fragmentation at 2 h was similar to the dose-response and inhibition assay (Figures 8 and 9). Full cleavage of TSP-2 with 10 nM GzmB was observed after 4 h of incubation. At later time intervals, the higher molecular weight fragments were further processed while a lower fragment around the 90 kDa became more visible. Two smaller bands around 50 kDa and 45 kDa also persisted through all time points but became fainter at later time points.

These results suggest original TSP-2 fragments undergo further processing by GzmB over time resulting in smaller cleavage fragments. The cleavage pattern of TSP-2 treated with 10 nM GzmB remained consistent at 2 h throughout all experiments. Compared to TSP-2, cleavage of FN, a positive control for GzmB activity, cleavage was only observed after longer incubation periods (18-24 h) and the higher concentration of GzmB (100 nM). This suggests that TSP-2 is a preferred GzmB substrate compared to FN.

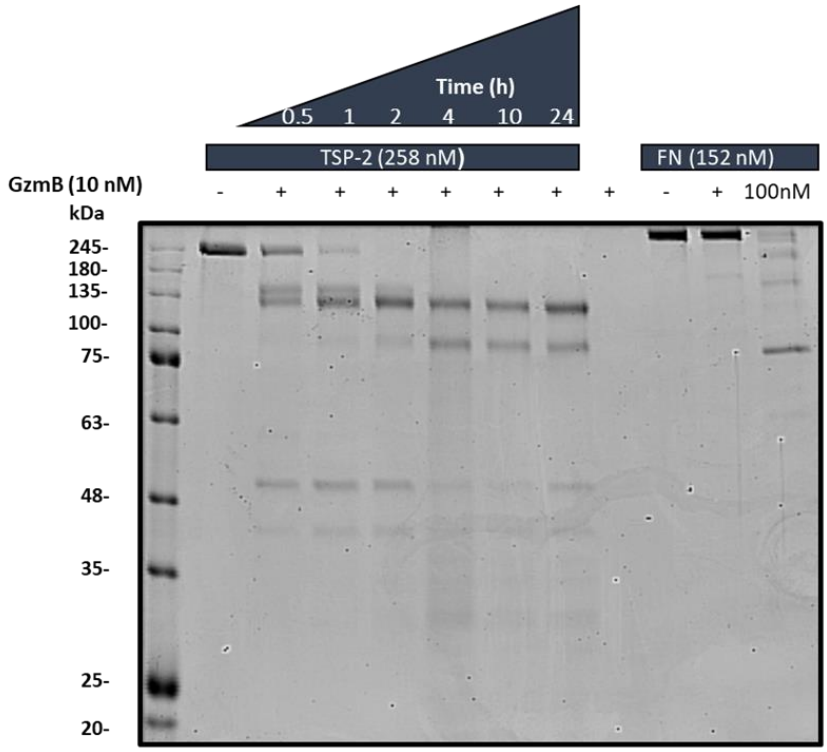

Figure 10. GzmB (10 nM) cleavage of TSP-2 shows time dependence. To test whether GzmB fragments TSP-2 overtime, GzmB was incubated with TSP-2 for 0.5, 1, 2, 4, 10, 24 h. GzmB was preincubated for 30 min at RT before adding each of the substrates, TSP-2 and fibronectin (FN). Recombinant human TSP-2 was incubated with GzmB at 37℃ for 2 h *in vitro*. FN was incubated with GzmB (10 nM and 100 nM) for 24 h at 37℃ as a positive control for GzmB activity. Reactions were stopped with 6X SDS loading buffer and heated denaturation at 95℃ for 5 min. The conditions were run in SDS-polyacrylamide gel electrophoresis and stained by Coomassie Blue.

3.3 HaCaT Cells Do Not Express TSP-2

To address the addition of exogenous TSP-2 in the subsequent *in vitro* experiments, Western blot was carried out to address whether HaCaT cells may be a cellular source of TSP-2 protein secretion (Figure 11). No TSP-2 was detected in HaCaT cells supernatant and cell lysate. Some TSP-2 was detected in human dermal fibroblast lysate, used as a positive control for TSP-2 with Western blot.

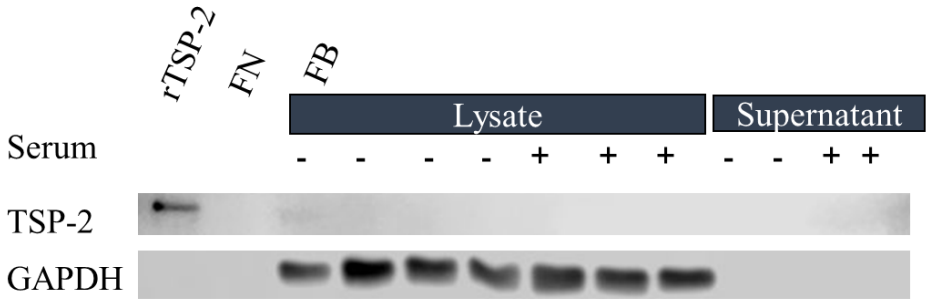

Figure 11. HaCaT cells may not express TSP-2. HaCaT cells were grown to confluence and maintained in serum-containing and -free media for 24 h. Cell lysates and supernatants were removed from the wells, samples were separated on SDS-PAGE and visualized by Western blotting. Recombinant thrombospondin-2 (TSP-2) and fibronectin (FN) serve as positive and negative controls respectively. Fibroblast (FB) lysate serves as an additional positive control.

3.4 Thrombspondin-2 Reduces HaCaT Cell Viability

TSP-2 has been previously observed to decrease the viability of endothelial cells[86]. To address whether TSP-2 treatment influences HaCaT cell viability, the MTT viability assay was used to measure the loss of mitochondrial activity. There was a significant reduction (p=0.019) in cell viability at the highest concentration of TSP-2 treatment after 24 h (Figure 12). There was no significant difference in viability between 1 and 0.2 µg/mL TSP-2 treatments. Cells treated with staurosporine, a positive apoptosis control resulted in a significant reduction in cell viability compared to untreated HaCaT cells in serum-free media.

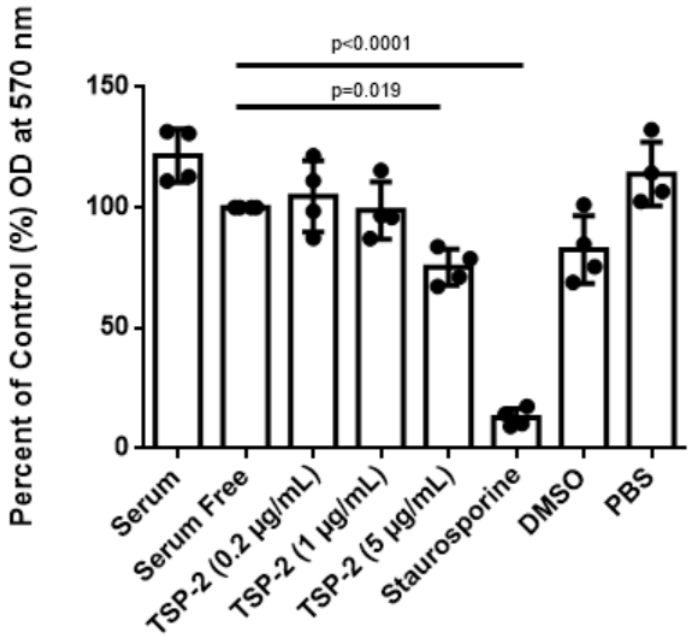

Figure 12. TSP-2 Decreases HaCaT Cell Viability after 24h Treatment.
HaCaT cells showed a significant reduction in viability after 24 h with treatment with full-length TSP-2 compared to untreated cells. Comparison was made to cells in serum-free media. All conditions were incubated with serum-free media, except for HaCaT cells incubated in serum-containing media condition. DMSO was vehicle for Staurosporine and PBS is a control for TSP-2; n=4 independent replicates, data presented as mean ± SD.

3.5 GzmB-Mediated TSP-2 Fragmentation Does Not Show Significant Improvement in HaCaT Cell Viability

To test whether TSP-2 GzmB-mediated fragmentation influences HaCaT cell viability, HaCaT cell viability was assessed using an MTT assay using different concentrations of GzmB (1, 10, 100 nM). While full-length TSP-2 (5 µg/mL) did show a reduction in viability after 24 h ($p=0.0243$), no significant difference in viability was observed with the GzmB fragmented TSP-2 treatments (Figure 13). There appeared to be an increasing trend in viability with increasing GzmB+TSP-2, though not significant. Cells treated with staurosporine exhibited a significant reduction in cell viability compared with untreated HaCaT cells in serum-free media.

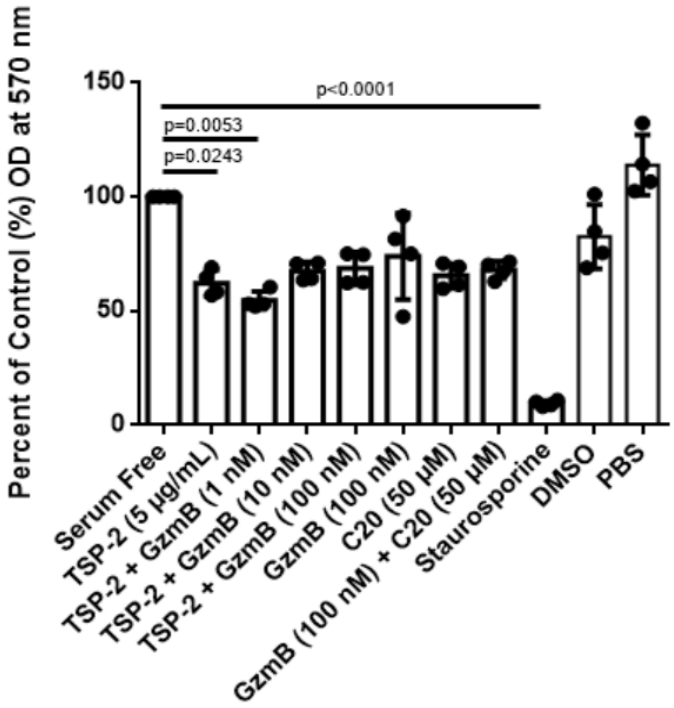

Figure 13. GzmB-mediated TSP-2 fragmentation does not change HaCaT Cell Viability after 24h. HaCaT cells did not show an improvement of viability with treatment with TSP-2 cleavage fragments compared to untreated cells. Inhibitor C20 was added to all wells to inhibit active GzmB. DMSO was vehicle for Staurosporine and C20. PBS was a vehicle control for TSP-2; n=4 independent replicates, data presented as mean ± SD.

3.6 HaCaT Cells Adhere to Full-Length Thrombospondin-2

TSP-2 has been reported to inhibit endothelial cell adhesion[97]. On the contrary, a study by Varani et al. (1988) demonstrated that TSP-1, a protein of the same family, had an adhesive effect on primary keratinocytes[111]. In the present study, it was determined HaCaT cells show a similar adhesive effect to TSP-2 coated matrix. As the concentration of TSP-2 increased adhesion also increased. HaCaT cells showed the more adherence at higher TSP-2 concentrations of 5 µg/mL and 1 µg/mL (p=0.0003 and p=0.0246 respectively). BSA served as a negative control for adhesion, while FN was a positive control.

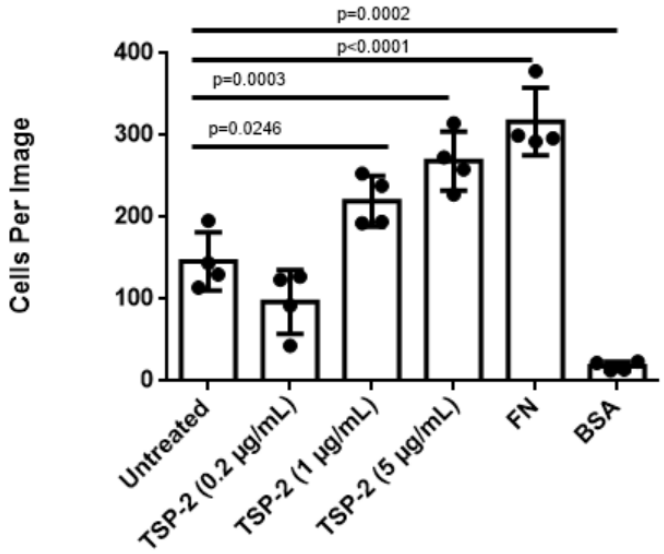

Figure 14. HaCaT cells Adhere to TSP-2.
96-well plate was pretreated with various concentrations of TSP-2 and HaCaT cells were allowed to adhere for 24 h. HaCaT cells showed greater adherence to TSP-2 compared to untreated wells. Fibronectin (FN) and BSA serve as positive and negative control respectively, and images were acquired at 10x magnification; n=4 independent replicates, data presented as mean ± SD.

3.7 GzmB Fragmentation of TSP-2 Decreases HaCaT Cell Adhesion

To explore the impact of GzmB-mediated cleavage of TSP-2 on HaCaT cell adhesion, TSP-2 (5 µg/mL) coated wells were treated with increasing concentrations of GzmB (1, 10, 100 nM) for 2 h. GzmB attenuated TSP-2-augmented HaCaT cell adhesion in a dose-dependent manner (Figure 15). While 1 nM GzmB, demonstrated no significant difference compared to vehicle alone, both 10 nM and 100 nM of GzmB significantly impaired TSP-2-augmented adhesion. Non-specific adherence of cells to plastic within the wells was blocked since a negligible number of cells adhere to wells that were coated with 5% BSA.

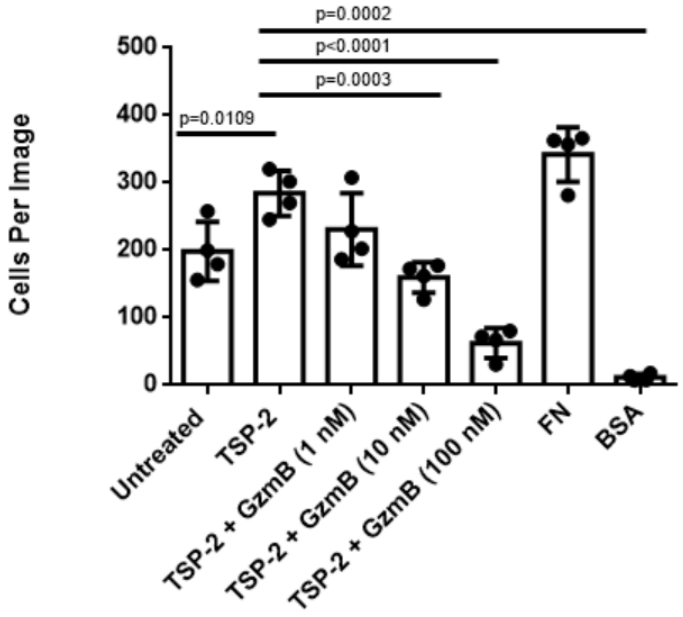

Figure 15. GzmB Decreases HaCaT Cell Attachment to TSP-2. TSP-2 pretreated 96-well plate was incubated with various concentrations of GzmB (1, 10, 100 nM) for 2 h. HaCaT cells were allowed to adhere for 24h. HaCaT cells demonstrated a dose-dependent reduction in adhesion to GzmB cleaved TSP-2 matrix. Fibronectin (FN) and BSA serve as positive and negative control respectively and images at 10x; n=4 independent replicates, data presented as mean ± SD.

Chapter 4: Discussion

TSP-2, a matricellular protein present during wound healing, is expressed in the proliferation and remodeling phases[127]. Though it is a potent antiangiogenic protein, its role in influencing skin matrix and keratinocytes is yet to be characterized. In the present study the impact of TSP-2 and GzmB-generated TSP-2 fragments have on keratinocyte adhesion and viability were explored. TSP-2 was cleaved by GzmB in a time- and dose-dependent manner. TSP-2 demonstrated reduced viability of HaCaT cells, while TSP-2 fragments did not alter result. HaCaT cells adhered to TSP-2, and GzmB-mediated cleavage of TSP-2 resulted in reduced HaCaT cell adhesion. This *in vitro* study provides some insight into understanding of how TSP-2 and GzmB fragmentation of TSP-2 influence keratinocytes in the epidermis.

This research sought to investigate whether the cleavage pattern is altered over time or with different GzmB concentrations. Moreover, it sought to confirm that TSP-2 is a substrate of GzmB using the inhibitor C20. GzmB-mediated cleavage of TSP-2 was prevented through this inhibitor (Figure 9). C20 is a reversible inhibitor of GzmB, which could account for the remaining cleavage band in the inhibition condition. Previous studies using C20 revealed results similar to observations in Figure 9. Specifically, Parkinson et al. (2015) and Hendel et al. (2014) showed that full-length FN remains after C20 treatment, but residual GzmB-cleaved substrate bands persist[73,128]. GzmB-mediated TSP-2 fragments differ from previously reported proteolytic cleavage by ADAMS1 and trypsin[88,119]. For example, Lee et al. (2006) demonstrated that ADAMS1 cleavage generated two fragments of 42 and 30 kDa, but not all full length TSP-2 was cleaved, as some intact protein remained[119]. Chen et al. (1994) alternatively reported that trypsin cleaved to 142, 123, 110, 95, and 38 kDa fragments[88]. Fragments produced from GzmB cleavage

of TSP-2 can be observed around 135, 130, 90, 50, and 45 kDa (Figures 8-10). These differences in cleavage patterns are important in indicating that GzmB produces fragments of TSP-2 that have not been extensively studied before and may impact cells *in vitro* and *in vivo* differently than previously noted with other proteases.

Demonstrated in the cleavage assays, TSP-2 exhibited greater sensitivity to GzmB-mediated proteolysis compared to FN. After only 0.5-h, TSP-2 was cleaved by GzmB in the time course assay (Figure 10). By observing the cleavage pattern of FN at the same time intervals in the 2-hour inhibition assay and more prolonged incubation of 24 hours, FN did not exhibit cleavage at 10 nM of GzmB, whereas 100 nM of GzmB did cleave FN. Interestingly, results from Hendel et al. (2013), reported similar findings with respect to the fact that higher levels of GzmB are required to cleave FN[128]. As such, TSP-2 may be a preferred substrate for GzmB.

Through further predicted fragmentation with GrabCas, smaller fragments from the initially predicted cleavage fragments could be generated. This can be done with remaining fragments containing aspartic acid after the original cleavage fragmentation, as pictured in Figure 7. Based on the predicted fragments, it can be inferred that the fragments generated by GzmB cleavage may still have aspartic acid in the P1' site, allowing for further preferential cleavage of those fragments. In the time course cleavage assay, it was observed that the presence of lower molecular weight fragments at 50 and 45 kDa became fainter over extended periods of time, indicating further degradation of the TSP-2 protein fragments (Figure 10). It is unlikely that increased fragmentation observed overtime was due to degradation of the protein since the full-length control protein was incubated for the same time duration as all the controls. Currently, it is unknown precisely where GzmB cleaves TSP-2. However, GzmB preferentially cleaves after

aspartic acid residues. Future research using proteomic analysis, such as with Edman sequencing, could determine the precise location of these cleavages.

The direct influence of TSP-2 and GzmB cleavage of TSP-2 on wound healing requires further elucidation. TSP-2 is mainly produced by FB and SMC[81], but is also produced by macrophages[82] at low levels in normal skin, however levels peak around day 10 of wound healing[81,109]. The expression of TSP-2 is increased in aged mice and in delayed wound healing[127], with peak levels of TSP-2 delayed from day 10 to day 14[81]. Similar to TSP-2, GzmB is elevated with diabetic wounds and age, and also contributes to delayed wound healing, disorganization of the ECM[73], and re-epithelialization[76]. Noted by Kyriakides et al. (1999), TSP-2 null mice showed accelerated re-epithelization[115], showing that TSP-2 should be considered an important factor alongside GzmB in this process. Nonetheless, mice wounds heal primarily by contraction whereas human wounds heal by epithelialization[129], meaning the role of TSP-2 in human wound re-epithelization requires additional investigation.

The present study sought to investigate the direct role that TSP-2 has on keratinocytes, but it is unknown whether keratinocyte cell models, HaCaT cells, produce TSP-2. Therefore, through Western blot, HaCaT cell lysate and culture supernatant were probed for TSP-2 protein expression. This aimed to eliminate the possibility of an innate TSP-2 matrix produced by the HaCaT cells that may influence experimental results of the addition of exogenous TSP-2. Though in this study it was found that TSP-2 may not be produced by HaCaT cells, it cannot be excluded that keratinocytes interact with the layers below the basement membrane when the skin is damaged. TSP-2 is produced by fibroblasts, SMC and macrophages which reside in the layers below the epidermis. When the skin integrity is compromised, keratinocytes will come into contact with the dermis, which is rich in fibroblasts and macrophages that both produce TSP-

2[81,82]. For the purposes of this study, the lack of production of TSP-2 by HaCaT cells provided a model to measure the direct impact that TSP-2 and GzmB mediated TSP-2 fragments would have on keratinocytes. Interestingly, TSP-2 demonstrated a significant reduction in HaCaT cell viability. A study completed by Armstrong and colleagues (2002) using human microvascular endothelial cells (HMVEC), observed that TSP-2 increased caspase activity and impaired viability in these cells as well[86]. In the present study, possible differences could result from variances in cells in the wells via pipetting or cell count errors. Using the fold change, the trend in the reduction in viability is still visible without losing the information between independent experiments. TSP-2 decreases the viability of HaCaT cells, but the reversal of this effect was not observed with the cleavage of TSP-2. Though the MTT assay is a quick method, this method is limited in that it measures mitochondrial dehydrogenase activity within the attached cells, while non-adherent cells are removed when the tetrazolium crystals are dissolved, and the absorbance is read. Thus, any cells that may have died were removed and unaccounted for. In other words, there may be some missing information about the cells that detach during the TSP-2 and TSP-2 fragment treatment. In order to address this limitation, different viability assay types would need to be used, such as flow cytometry to probe the adhered cells and non-adhered cells for viability and cell cycle markers.

Cell adhesion is notoriously complex in the TSP family. There are many domains binding with various cellular receptors and cell types. According to a study by Varani et al. (1988), TSP-1 attachment properties appeared to be located in the non-heparin binding domain [111]. When the intact TSP-1 was cleaved using chymotryptic digestion, it appeared to uphold its attachment property to human keratinocytes, suggesting that the N-terminal of TSP may not be important in

the attachment property of the keratinocytes. Furthermore, after further degradation TSP-1 fragments observably had a similar effect on keratinocytes as in this present study, as smaller fragments appeared to reduce their attachment capability. In the present research study, GzmB cleaved TSP-2 matrix resulted in a similar observation, but it is difficult to determine which specific fragment or domain would influence HaCaT cell functions. What can be inferred, however, is that fragmentation of the protein by GzmB does change the role of the protein in the cellular function. With the fragmentation of TSP-2, the adhesive property observed in Figure 14 was inhibited with the GzmB-mediated cleavage. Fragmentation of the full-length protein may produce anti-adhesive proteins. Fragmentation of TSP-2 may create a twofold effect, possibly aiding in accelerated re-epithelization or inhibiting complete anchoring of keratinocytes to TSP-2. Understanding the direct impact of the fragments generated by GzmB-mediated cleavage strikingly reveals a decrease in adhesion with increasing GzmB concentrations. Cleavage of TSP-2 with the higher concentrations of GzmB produced smaller fragments of TSP-2 and revealed a lack of full-length TSP-2 (Figure 8). This finding suggests that the adhesive property observed with full-length TSP-2 is altered within a domain cleaved by GzmB. Additionally, full-length TSP-2, as seen in Figure 8, may still be present in the 1 nM condition meaning that after full cleavage of TSP-2, this results in the anti-adhesion property.

Chapter 5: Conclusion and Future Directions

This study provides a basis of how TSP-2 impacts keratinocyte function, and how GzmB may impair such activities. Future studies should focus on the influence of TSP-2 and GzmB-generated TSP-2 fragments on keratinocyte cell signaling pathways. Furthermore, using primary human keratinocytes as a model of the epidermis may provide a more physiological understanding of how TSP-2 and GzmB-TSP-2 fragmentation influence their keratinocyte behavior and adhesion. The summary of the findings of this study can be reviewed in Figure 16.

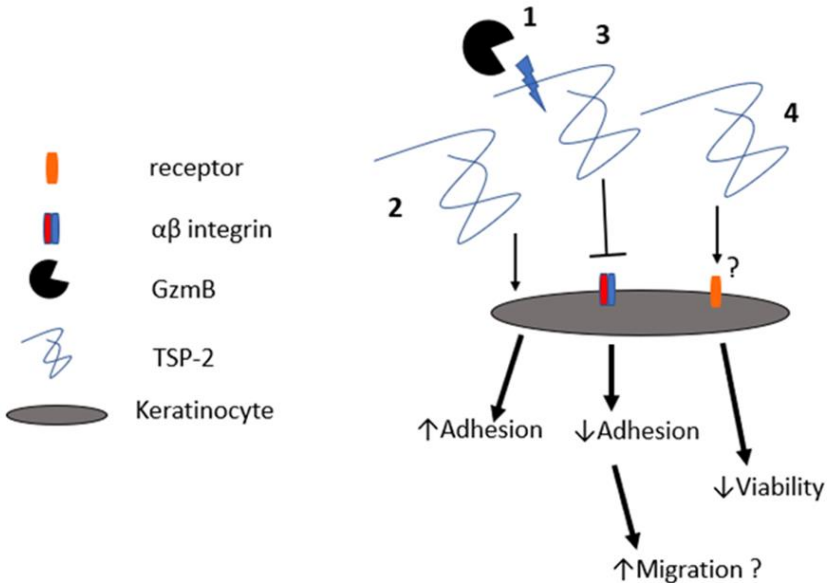

Figure 16. Summary of Findings and Possible Mechanisms (1) GzmB cleaves TSP-2. (2) TSP-2 aids in keratinocyte adhesion. (3) GzmB reduced keratinocyte adhesion by cleaving TSP-2 matrix. Loss of adhesion of keratinocytes may aid in increased migration, which may lead to scarring during wound healing. (4) HaCaTs demonstrated a reduction in viability, while GzmB cleavage thereby showed slight increase in viability. Proposed mechanisms for this loss of viability may be a result of apoptotic mechanisms or a reduction in cell cycle progression. Mechanisms behind these experimental results may be avenues for future investigations.

In vitro studies should assess cell adhesion to TSP-2 and further investigate its role in cytoskeletal organization, similar to that which has been done with TSP-1. Furthermore, research can be directed to study signaling pathways that are altered with this fragmentation. It could also

be interesting to investigate GzmB in squamous cell carcinoma after UV treatment (a major contributor to this type of cancer) and how the aggravation of chronic inflammation by GzmB-mediated cleavage of angiogenic factors (TSPs) and how this may influence tumor growth and metastasis. Investigations could be done on TSP-2 and GzmB expression differences after UV treatment. TSP-1 has been seen to decrease with UV treatment in the literature, but this has yet to be done with TSP-2[130]. In a study by Parkinson et al. (2015), GzmB is known to be expressed by keratinocytes after UV treatment[73]. This could indicate that UV and GzmB could have additional implications in this regard when TSP-2 is fragmented, and its matrix and cell interactions are disturbed.

This research demonstrated a new extracellular target of GzmB. GzmB cleaves TSP-2 in a dose-dependent manner resulting in different fragmentation patterns, including lower molecular weight fragments with the higher dosage. Furthermore, TSP-2 is highly sensitive to GzmB proteolysis. TSP-2 reduces HaCaT cell viability, while fragmentation does not significantly change this effect. TSP-2 results in HaCaT cell adhesion; however, TSP-2 fragmentation demonstrated a dose-dependent reversal of its adhesive effect. Studies of TSP-2 direct effects on keratinocytes have never been done before, thus this project aimed to set foundations on which further wound healing studies can be explored.

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