The Pennsylvania State University
The Graduate School
College of Medicine

COMPETITIVE BINDING OF CONNEXIN 43 AND SMADS TO MICROTUBULES:

CHANGES IN THE SYNTHESIS OF COLLAGEN AND A PROTEASE INHIBITOR

A Thesis in
Anatomy
by
Christopher H. Schank

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The thesis of Christopher H. Schank was reviewed and approved* by the following:

H. Paul Ehrlich  
Professor of Surgery  
Thesis Adviser

Patricia J. McLaughlin  
Professor of Neural and Behavioral Sciences  
Director, Graduate Program in Anatomy

Henry J. Donaue  
Michael and Myrtle Baker Professor

Christopher Niyibizi  
Associate Professor of Orthopaedics and Rehabilitation

*Signatures are on file in the Graduate School.
Abstract

TGF-β1 promotes fibrosis through the Smad signaling pathways within the cell’s cytoplasm. When Smad2 and Smad3 (Smad2/3), members of a family of intracellular effector proteins, are phosphorylated, they bind to Smad4 and that protein complex enters the nucleus where they induce the expression of a number of proteins (procollagen and tissue inhibitor of metalloproteinases-1 (TIMP-1) being examples). Gap junctional intercellular communications (GJIC) between fibroblasts requires the coupling of hemichannel structures, the connexon, which is made up of six connexin 43 (Cx43) proteins located on the surface of adjacent cells. The transport of Cx43 from its site of synthesis to the plasma membrane can involve direct attachment to cytoskeleton microtubules. The Cx43-microtubule binding site is shared with Smad2/3. The attachment of Cx43 to microtubules releases Smad2/3 into the cytosol compartment, where it can participate in Smad signaling pathway. However, if Smad2/3 are retained on microtubules, the Smad signaling pathway is disrupted preventing their dissociation into the cytosol pool making them unavailable for phosphorylation, binding to Smad4 and entering the nucleus, interfering with TGFβ1 promotion of procollagen and TIMP-1 synthesis. The tubulin binding motif (TBM), located within C-terminus of Cx43, is the specific tubulin binding sequence for Cx43 to microtubules. The binding of TBM peptide to microtubules obstructs the binding of Cx43 and the release of Smad2/3 into the cytosol compartment of the fibroblast. The inclusion of TBM peptide with cultured human dermal fibroblasts showed a change in the localization of Smad2/3 from the cytosol and nuclear compartments to the insoluble microsomal compartment, where microtubules accumulate. The competitive binding of TBM peptide to microtubules reduced the synthesis of procollagen and TIMP-1. In addition, there was a reduction in GJIC between fibroblasts treated with TMB peptide compared to saline treated control cells. The failure to bind Cx43 and the release of Smad2/3 from
microtubules both inhibited the synthesis of proteins involved in fibrosis and GJIC. The binding of Cx43 to microtubules and the release of Smad2/3 demonstrated a relationship between GJIC and Smad signaling pathway. The possibility exists that controlling GJIC by limiting Cx43 accumulation at the plasma membrane may be a novel approach for the control of fibrosis.
# TABLE OF CONTENTS

List of Figures ........................................................................................................... vii
Abbreviations .............................................................................................................. ix
Acknowledgments ...................................................................................................... xiii

Chapter 1. INTRODUCTION ......................................................................................... 1
  1.1: Connexin Proteins and Gap Junction Intercellular Communications .............. 1
  1.2: Microtubule Polymerization Dynamics ......................................................... 6
  1.3: Trafficking and Delivery of Cx43 to the Plasma Membrane ......................... 8
  1.4: TGF-β1 Signaling through Smad Proteins .................................................. 10
  1.5: Disruption of Target Gene Expression: Tissue Inhibitor of Metalloproteinase-1 and Collagen Synthesis ................................................................. 13
  1.6: Research Limitations .................................................................................... 15

Chapter 2. OBJECTIVES ............................................................................................. 18

Chapter 3. MATERIALS AND METHODS ................................................................. 20
  3.1: Cell Culture .................................................................................................... 20
  3.2: Standard Procedure for Nuclei Isolation ..................................................... 20
  3.3: Standard Procedure for Microtubule Isolation ............................................. 21
  3.4: Gap Junction Dye Coupling Assay ............................................................... 22
  3.5: Dot Blot Analysis ............................................................................................ 23
  3.6: Western Blot Analysis .................................................................................... 24
  3.7: Statistical Analysis ........................................................................................ 24

Chapter 4. RESULTS .................................................................................................. 25
  4.1: Nuclei Isolation ............................................................................................. 25
  4.2: Microtubule Isolation .................................................................................... 26
4.3: Gap Junction Dye Coupling Assay ................................................................. 27
4.4: Collagen III Production ................................................................................. 28
4.5: Tissue Inhibitor of Metalloproteinase-1 Production ................................. 29
4.6: Smad 2/3 Location ....................................................................................... 30

Chapter 5. DISCUSSION ......................................................................................... 32
5.1: Nuclei and Microtubule Isolation ................................................................. 32
5.2: Scrape Loading ........................................................................................... 33
5.3: Collagen III Production ............................................................................... 34
5.4: Tissue Inhibitor of Metalloproteinase-1 Synthesis .................................... 35
5.5: Smad 2/3 Location ....................................................................................... 36
5.6: Effectiveness of the Tubulin Binding Motif Peptide ................................. 37

References ............................................................................................................. 39
List of Figures

**Figure 1.** Model of gap junction intercellular communications..........................1

**Figure 2.** Image of two apposed connexin43 proteins in a one-sixth cut from a gap junction shown above.................................................................2

**Figure 3:** Human dermal fibroblast Cx43 amino acid sequence........................5

**Figure 4.** Model of the structure of a microtubule..............................................8

**Figure 5.** Trafficking and assembly route of gap junctions through the Golgi apparatus..9

**Figure 6.** Activation of the Smad signaling pathway........................................12

**Figure 7.** Interaction of Cx43 under control and TBM peptide conditions...........19

**Figure 8.** Flow diagram illustrating various stages of cellular separation..............21

**Figure 9:** DAPI stained blue nuclei of human dermal fibroblasts.........................25

**Figure 10:** Western blot of fibroblasts showing α-tubulin density.......................26

**Figure 11:** Influence of peptide on Lucifer yellow uptake .................................27

**Figure 12:** Dot Blot of human dermal fibroblasts (HF) showing density of collagen III production at 4hr and 24 hours.................................................................28
Figure 13: Western Blot of human dermal fibroblasts showing density of TIMP-1........29

Figure 14: Western blot showing the density of Smad2/3 from different centrifugation fractions of a human dermal fibroblast.................................................................31
### Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
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<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin 43</td>
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<tr>
<td>C-terminal</td>
<td>Carboxy Terminal</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common Mediator Smads</td>
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<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>COL-III</td>
<td>Type III Collagen</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>°</td>
<td>Degree</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
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<td>Abbreviation</td>
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<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap Junction Intercellular Communication</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>I-Smads</td>
<td>Inhibitory Smads</td>
</tr>
<tr>
<td>IgG1</td>
<td>Immunoglobulin G-1</td>
</tr>
<tr>
<td>k</td>
<td>Informational Abbreviation for One Thousand</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
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<tr>
<td>MAPs</td>
<td>Microtubule Associated Proteins</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messanger Ribonucleic Acid</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitter</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MH</td>
<td>Mad-homology</td>
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<td>x</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino Terminal</td>
</tr>
<tr>
<td>NBBS</td>
<td>New Born Bovine Serum</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD95/disc large/ZO-1 Homology Domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>P value</td>
<td>Probability Value</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor Activated Smad</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad Anchor for Receptor Activation</td>
</tr>
<tr>
<td>Src2</td>
<td>Src Homology Domain 2</td>
</tr>
<tr>
<td>Src3</td>
<td>Src Homology Domain 3</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor-β1</td>
</tr>
<tr>
<td>TBM</td>
<td>Tubulin Binding Motif</td>
</tr>
<tr>
<td>TBRI</td>
<td>TGF-β1 Type I Receptor</td>
</tr>
<tr>
<td>TBRII</td>
<td>TGF-β1 Type II Receptor</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue Inhibitor of Metalloproteinase-1</td>
</tr>
<tr>
<td>TES</td>
<td>N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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Chapter 1. Introduction

1.1: Connexin Proteins and Gap Junction Intercellular Communications

The process of intercellular co-operation, gap junction intercellular communications (GJIC), is a prevalent feature found among cells in tissues (Evans and Boitano, 2001; Laird, 2005; Shaw et al., 2007). When open, as seen in figure 1, these intercellular communication channels connect the cytosolic contents between coupled cells as well as providing a fundamental mechanism used to ensure cellular homeostasis (Dai et al., 2007; Evans and Boitano, 2001; Feldman et al., 1997; Giepmans et al., 2001) GJICs directly exchange secondary messengers and other small molecules, having a molecular weight of less than 1000 Da, such as sugars, amino acids and oxygen (Kardami et al., 2007). Intercellular communications are extremely important since their loss is immediately evident by the appearance of various complications such as lethal arrhythmic beating of the heart (Evans and Boitano, 2001).

![Figure 1. Model of gap junction intercellular communications.](image-url)

**Figure 1. Model of gap junction intercellular communications.** Connexin43 subunits of each plasma membrane oligomerize into homomeric connexons and connect to form a gap junction. Reproduced from Evans and Martin, 2002: Permission to use figure granted by Informa Healthcare.
Gap junctions are composed of connexin proteins (Cx), which are from a family of 20 different proteins (Kardami et al., 2007). Each connexon complex or hemichannel is composed of six connexin proteins anchored in the plasma membrane. As seen in figure 2, all connexin proteins exist in a similar organizational pattern, composed of four transmembrane, two extracellular and three cytosolic subdomains with both the N-terminal as well as the carboxy-terminal tail representing the most variable portion of the molecule (Evans and Martin, 2002; Laird, 1991).

Figure 2. Image of two apposed connexin43 proteins in a one-sixth cut from a gap junction shown above. Four transmembrane domains (shown as barrels), two extracellular loops, one intracellular loop, N-terminal and C-terminal tails are shown. The C-terminus contains the binding sequence for tubulin. Reproduced from Evans and Martin, 2002: Permission to use figure granted by Informa Healthcare.
Most of the connexins are phosphoproteins, containing multiple phosphorylation sites on their carboxy-terminal tail domain (Kardami et al., 2007). The lateral clustering of gap junctions in the plasma membrane is a dynamic process and many studies express the requirement of extracellular matrix proteins, such as cadherins, integrin α3β1, and laminin 5, in establishing the cell to cell attachment (Evans and Martin, 2002). Hemichannels accumulate at the cell periphery and form densely packed gap junction plaques. These gap junction plaques form a functional gap junction channel when hemichannels from adjacent cells come together. When the hemichannels reach the center of the plaque, they will become internalized for degradation as annular gap junctions (Laing et al., 1997; Laing and Beyer, 1995; Lauf et al., 2002; Leithe and Rivedal, 2004; Musil et al., 2000; Qin et al., 2003; VanSlyke and Musil, 2002; VanSlyke et al., 2000).

Cx43 is the most common and extensively studied connexin (Follandier et al., 2008). Connexin-43 possesses at least 13 phosphorylation sites, which can be a target for several different signal pathways (Banes et al., 2000; Feldman et al., 1997; Kardami et al., 2007). Hemichannels from adjacent cells form a pore across the intercellular space (Kardami et al., 2007). Because connexins have relatively short half lives, 1.5-3.5 hours for Cx43, they must be continuously synthesized, transported to the cell surface, and assembled into a plaque (Laird, 2005; Laird et al., 1991; Paulson et al., 2000). GJIC are regulated by the constant trafficking of Cx along microtubules and assembly of hemichannels within the plasma membrane. The regulation of GJIC by the connexins enables tissues to modulate gap junctions quickly both in a positive and negative manner as a response to the environment (Paulson et al., 2000; Shaw et al., 2007).

Figure 3 is a model of the amino acids composing Cx43 which identifies the modular protein interaction domains and consensus sequences. Each transmembrane domain of
the connexin participates in the oligomerization into a hemichannels. The two disulphide linked extracellular loops are critical for the docking of two hemichannels to form a gap junctional pore (Krutovskikh and Yamasaki, 2000). The C-terminus not only contains the tubulin binding motif unique for tubulin but also Src homology 2 (SH2), Src Homology 3 (SH3), and the PSD95/disc large/ZO1-1 homology domain (PDZ) which facilitate the interaction of Cx43 with accessory proteins to disrupt gap junctions by phosphorylating two specific tyrosine residues Tyr247 and Tyr265. The phosphorylation of Tyr265 in Cx43 forms a docking site for the SH2 domain which facilitates SH3 domain binding to aliphatic proline residues. The docking of SH2 to the Cx43 Tyr265 domain increases the affinity for Tyr247 phosphorylation leading to channel closure (Giepmans, 2004). PDZ domains determine binding specificity by binding the C-terminal residues to target proteins for cytoskeletal anchorage and the sequestration of transcription factors (Giepmans 2003).
Figure 3: Human dermal fibroblast Cx43 amino acid sequence. A complete overview of the protein interaction domains and amino acid sequences that is characteristic of connexin 43. Connexins are composed of four transmembrane, two extracellular and three cytosolic subdomains with a carboxy-terminal tail, which represents the most variable portion of the molecule. The C-terminal tail includes the binding motif of connexin 43 directly to microtubules. Reproduced from Giepmans (2004): Permission to use figure granted by Oxford University Press and Ben N.G. Giepmans.
1.2: Microtubule Polymerization Dynamics

One cytoskeletal structure aiding in the formulation of gap junctions is microtubules. Cytoskeletal microtubules are elongated, non-branching and rigid hollow tubes composed of alpha and beta tubulin that have the ability to release tubulin at one end and add tubulin at the other end. Alpha tubulin has a bound molecule of GTP and never hydrolyzes. Beta tubulin can exist with GTP or GDP bound. Under certain conditions, the β-tubulin can hydrolyze its bound GTP to GDP and P_i, subsequently releasing the P_i in order to exchange the GDP for GTP (figure 4a). The α- and β-tubulin monomers that make up the heterodimer subunit of microtubules are ≈ 50% identical at the amino acid level and each has a molecular mass of about 55kDa (Burns, 1991). Within the microtubule, a tubulin heterodimer will be arranged into linear protofilaments which associate laterally to form a 25nm hollow, cylindrical polymer (Desai and Michison, 1997). Microtubules are polar structures formed by the head to tail association of α- and β- heterodimers (Amos & Klug, 1974). Due to polarity, the development of microtubules at the different ends is a consequence of different polymerization rates; the faster growing end is the plus end and the slower growing end is the minus end, in a process called dynamic instability (Allen and Borisy, 1974; Mimori-Kiyosue et al., 2005; Ross and Pawlina, 2006). During polymerization, both α- and β-tubulin dimmers will be bound to GTP. The GTP bound to α-tubulin is stable. However, the GTP bound to β-tubulin may be hydrolyzed to GDP shortly after assembly. The kinetics of GTP versus GDP bound tubulin is different. GDP bound tubulin is prone to depolymerization while GTP bound tubulin is prone to polymerization. Therefore, a GDP bound tubulin is spontaneously released. Due to the polarity of microtubule polymerization (addition at the GTP bound positive end), a cap of GTP bound tubulin will prevent disassembly. When hydrolysis catches up to the GTP cap, a rapid
depolymerization takes place switching the microtubule dynamics from growth to shrinkage, in a process is known as catastrophe. GTP bound tubulin is once again added to the microtubule. This process, known as rescue, establishes a new cap and prevents further shrinkage (figure 4b; Akhmanova and Hoogenraad, 2005; Chausovsky et al., 2002).

The αβ heterodimer of each protofilament is oriented with the β-tubulin monomer directed toward the plus end of the microtubule while the α-tubulin monomer is exposed at the minus end of the microtubule (Desai and Michinson, 1997). The protofilaments orient in what is called a 3-start helical formation due to the fact that if the path of adjacent monomers is followed for one complete helical turn, you end up three monomers above where you started. Furthermore, three of these parallel helices must be initiated in order for the entire surface of the microtubule lattice to be covered. This formation occurs due to lateral associations of α and β monomers in which the α and β monomers of one protofilament will laterally associate with the α and β monomers of an adjacent protofilament (B-type lattice formation). Originally, microtubules were thought to have an A-type lattice formation, in which lateral associations between protofilaments in which the α monomer of one protofilament interacts with a β monomer of an adjacent protofilaments. Since that time, ultrastructural analysis has established that the correct lattice structure is the B-type lattice with a seam (Kikkawa et al., 1994; Mandelkow et al., 1986; Song and Mandelkow, 1993). The neighboring monomer within a 3-start helical B-type lattice formation are both either α or β except at the seams. The seam will express a discontinuity where each 3-start helical path will revert from α to β or vice versa (Fan et al., 1996). One of the methods of Cx43 trafficking is characterized by the binding of Cx43 to the β-tubulin subunits at its tubulin binding motif for transport to the plasma
membrane. This topic will be discussed in detail during the trafficking and delivery of Cx43 to the plasma membrane.

1.3: Trafficking and Delivery of Cx43 to the Plasma Membrane

There are two pathways that play a role in the dynamic trafficking and delivery of Cx to the plasma membrane. As seen in figure 5, one method includes a secretory

Figure 4. Model of the structure of a microtubule. (a) The head to tail interactions between α and β dimmers to form a linear protofilament. Thirteen linear protofilaments will associate laterally to form a 25nm diameter hollow, cylindrical polymer. (b) The effects of bound GTP or GDP in microtubule polymerization and depolymerization. Reproduced from Conde and Cáceres, 2009: Permission to use figure granted by Nature Publishing Group.
pathway involving the transport of Cx43 through the Golgi apparatus toward the periphery. This method requires the intracellular assembly of Cx into connexons for successful insertion into the plasma membrane.

![Diagram of gap junctions through the Golgi apparatus]

**Figure 5. Trafficking and assembly route of gap junctions through the Golgi apparatus.** Cx43 proteins are inserted into the endoplasmic reticulum, where they will oligomerize into connexons and then trafficked by the Golgi to the plasma membrane. Reproduced from Evans and Martin, 2002: Permission to use figure granted by Informa Healthcare

However, it has been shown that the predominant secretory pathway for Cx43 trafficking is contingent upon the direct interaction of Cx43 with tubulin (Giepmans et al., 2001; Lauf et al., 2002; Martin et al., 2001). Cx43 binds directly to tubulin by the amino acid motif found in the C-terminus (\textsuperscript{234} KGKDRVKGK \textsuperscript{243}; the binding domains typically found in tubulin are underlined) resulting in the release of Smad2/3 into the cytosol while Cx43 remains bound to the microtubules for delivery to the plasma membrane. After the microtubule mediated delivery under control conditions, connexin43 will insert in the plasma membrane and subsequently oligomerize into a hemichannels. This leads to a
intriguing question that will require further research. Does tubulin have its greatest affinity for unphosphorylated Smad2/3 when it is protein free?

1.4: TGF-β1 Signaling through Smad Proteins

TGF-β1 is part of a family of proteins involved in immune suppression, angiogenesis, apoptosis, cell growth, and fibrosis (Brown et al., 2007). The TGF-β1 signal transduction involves two transmembrane serine/threonine protein kinase receptors, TGF-β1 type I (TβRI) and TGF-β1 type II (TβRII). The signal cascade begins when TGF-β1 binds to a TβRII, a constitutively active kinase receptor, activating it. TβRII will subsequently recruit and phosphorylate the glycine-serine rich domain of TβRI forming a heterotetrameric complex resulting in the activation of TβRI kinase activity. (Wieser et al., 1995; Yamashita et al., 1994). Activated TβRI will interact and phosphorylate a variety of proteins (including Smad proteins) stimulating multiple signaling pathways. Following ligand activation, the signaling cascade from TβRI to the nucleus occurs by phosphorylation of Smad proteins (Verrecchia and Mauviel, 2002).

Smad proteins, part of a family of eight mammalian proteins that are divided into three functional groups: receptor activated Smads (R-Smads), common mediator Smads (C-Smads), and inhibitory Smads (I-Smads), are the intracellular signaling effectors that shuttle continuously between the cytoplasm and the nucleus. Smad2/3, as well as Cx43, binds to β-tubulin (Dai et al., 2007). The binding of Cx43 to β-tubulin through its C-terminal tail causes Smad2/3 release causing a pooling of Smad2/3 within the cytosol (Refer to Figure 7a & b: Dai et al., 2006; Dong et al., 2000; Giepmans et al., 2001; Nicolas et al., 2004; Zhu et al., 2004). Type I receptors will recognize and phosphorylate
the ligand-specific region of the pooled Smads. Smads are recruited to the activated TβRI by SARA (Smad anchor for receptor activation) proteins. Smad proteins downstream of TGF-β1 [Smad2 (52kDa) and Smad 3 (48kDa)] are characterized by two Mad-homology (MH) domains, each forming a globular structure separated by a linker region (Miyazono, 2000). The N-terminal MH1 domain is involved in nuclear import, cytoplasmic anchoring, DNA binding, and regulation of transcription activity while the C-terminal MH2 domain is involved in protein binding. The MH2 domain is conserved among all Smads and is regulates Smad oligomerization, cytoplasmic anchoring, and transcription of target genes. Smad 3 is unique because it has a transactivation domain in the linker region. This transactivation domain has functional roles in ubiquitination and transcriptional activation (Moustakas et al., 2001; Prokova et al., 2005). The phosphorylation of Smads by TβRI occurs on two serine residues on the C-terminus of the MH2 domain (Derynck and Zhang, 2003). Once phosphorylated by the TβRI, Smads form a complex with Smad 4. The Smad2/3-Smad4 complex is then translocated into the nucleus by the cytoplasmic protein, importin. Inside the nucleus, the complex will serve as transcription factors binding DNA either directly or forming an association with other DNA binding proteins to regulate target gene expression, including collagen synthesis and TIMP-1 (Macias-Silva et al., 1996; Miyazono, 2000). A summary of the TGF-β1 signaling through Smad protein cascade can be seen in figure 6.
Figure 6. Activation of the Smad signaling pathway. TGF-β1 begins the signaling pathway through the TGF-β type I (TβRI) and TGF-β type 2 (TβRII) transmembrane protein kinase receptors. TβII binds to the TGF-β ligand, recruits TβRI and forms a hetero-tetrameric complex. TβRII phosphorylates TβRI, which will initiate the Smad signaling pathway by phosphorylating Smad2/3. Smad 2/3 are presented to TβRI by adaptor proteins, SARA. Activated Smad2/3 will form a complex with Smad4 and translocate to the nucleus to modulate transcription of target genes. Reproduced from Brown et al., 2006: Permission to use figure granted by John Wiley and Sons.
1.5: Disruption of Target Gene Expression: Tissue Inhibitor of Metalloproteinase-1 and Collagen Synthesis

The turnover of extracellular matrix is an integral aspect for embryonic development, wound repair, and tissue remodeling. Turnover of the extracellular matrix is the responsibility of a class of proteinases known as matrix metalloproteinases (MMPs) a family of zinc dependent endopeptidases which are responsible for the degradation of the major components of the matrix. These MMPs are also referred to as matrixins (Brew et al., 2000; Mannello and Gazzanelli, 2001). In order for the cell to maintain a balance of deposition and degradation of the extracellular matrix, the activities of the matrixins are controlled by a combination of zymogen activation and inhibition by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMP-1). TIMP-1 is a 28kDa sialoglycoprotein that functions to inhibit the action of MMPs and stimulate cell proliferation (Mannello and Gazzanelli, 2001; Ritter et al., 1999). Research by Wang et al., showed that the mRNA for MMP and TIMP-1 are transported to the trans-Golgi network though endosomes where they will assemble inside vesicles (Wang et al., 2004).

Collagens are a family of fibrous proteins, found in all multicellular animals, which are synthesized by connective tissue cells. Typical collagen molecules have a characteristic feature as being a long and stiff triple stranded helical (Alberts et al., 2008). The focus of these experiments will be on type III collagen due to the fact that the triad of $\alpha_1$[III] chains composing a type III collagen helix require the Smad signaling pathway.

Collagen fibrillogenesis is a multistep mechanism that involves both intracellular and extracellular assembly reactions regulated by fibroblasts (Birk et al., 1989; Trelstad,
Collagen is synthesized as a larger precursor protein, procollagen. Within the endoplasmic reticulum, procollagen is hydroxylated at select proline and lysine residues, followed by a glycosylation at select hydroxysine residues before being assembled into a triple helix. The triple helix structure eaves the ER compartment and enters the Golgi compartment where it will be packaged into vesicles for secretion. Once secreted, the procollagen will undergo cleavage of propetides and assemble into a fibril (Alberts et al., 2008). A failure of collagen production leads to a lack of connective tissue deposition in granulation tissue.
1.6: Research Limitations

The research performed deals with a novel concept that the tubulin binding motif (TBM) peptide from Cx43 effectively disrupts the Smad signaling pathway. When the TBM peptide, an amino acid sequence from the Cx43 C-terminus, binds to tubulin, the TBM peptide blocks Cx43 binding. Prevention of Cx43 binding to tubulin has several effects. The failure of Cx43 binding to microtubules disrupts Cx43 localization at the plasma membrane reducing available Cx43 for GJIC formation. The accumulation of Smad 2/3 on microtubules reduces unphosphorylated Smad2/3 in the cytosol. The absence of unphosphorylated Smad 2/3 in the cytosol reduces Smad signaling causing decreases in transcription of type III collagen and TIMP-1. However, there may be an alternative explanation for GJIC inhibition and the synthesis of TIMP-1 and type III collagen. These alternative explanations lead to the questioning of whether or not the usage of microtubule and GJIC inhibitors will have similar effects upon the Smad signaling pathway.

Alternative Gap Junction Intercellular Communication Inhibitors

Gap junction proteins emerge at the membrane periphery as specialized locations that invite the development and exploitation of chemicals and drugs to influence intercellular communications (Moyer et al., 2004). Even though there is universality in the hemichannels of the GJIC, there are few reagents that are known to block the communication in an acceptable manner (Evans and Boitano, 2001). Gap junction channels are well insulated from the extracellular space preventing direct channel modulation (Paulson et al., 2000). There have been a few classic inhibitors, such as octanol, heptanol, anesthetic halothane, and oleamide that have been shown to limit GJIC by dissolving lipids within the membrane thus changing the membranes
fluidity ultimately leading to membrane channel closure (Feldman et al., 1997). The specificity of such compounds is being debated due to the belief that they will also modify lipophilic compounds other than just the GJIC channels. Other drugs such as oleic and arachidonic acids will act on GJIC indirectly by depleting the calcium storages modifying phospholipase C activities (Schmilinsky-Fluir et al., 1997). Recently, there has been an alternate approach to GJIC inhibition which included attempts to devise and apply more specific antibodies and antibody fragments to the GJIC. These antibodies and antibody fragments block electrical resistance and dye transfer leading to compaction problems (Becker et al., 1995). Furthermore, data indicates that connexin mimetic peptides disrupt GJIC assembly/disassembly by simulating the extracellular loop sequences, which cover up the binding sites for the coupling between hemichannels and interference with the interactive physiological processes. This mechanism will disrupt the linkage of hemichannels and prevent GJIC (Evans and Boitano, 2001).

**Microtubule and Smad Interference**

As previously noted, microtubules are key components to the cytoskeleton of eukaryotic cells as well as an integral facet for cellular function due to their role in Smad signaling. Pharmaceutical drugs have been shown to interfere and disrupt the assembly of microtubules (Dai et al., 2007; Usui et al., 2001). Drugs such as colchicine, colcemid and nocadazol inhibit the polymerization of microtubules by binding directly to the tubulin. The disruption of microtubules with colchicines, colcemid, and nocodazole increases the dissociation of Smad2/3 from microtubules leading to the enhancement of Smad2/3 phosphorylation and activity leading to the prevention of tubulin addition at the plus end of the developing microtubule (Dong et al., 2000). Other drugs such as vinblastine and vincristine will amass tubulin thus leading to microtubule
depolymerization (Usui et al., 2001). Additionally, Smads can interact with filamin. Filamin is an intracellular signaling protein that crosslinks actin, and is associated with Smad proteins, including the TGF-β1/activin receptor-regulated Smad2. Cells that are filamin deficiencies or have defective filamin expression have impaired TGF-β1 signaling leading to a lack of Smad2/3 phosphorylation due to impaired receptor-induced serine phosphorylation of Smad2/3 (Sasaki et al., 2001).
Chapter 2: Objectives

As seen in figure 7a, under control conditions Smad2/3 are bound to microtubules. The binding of Cx43 directly to tubulin by a amino acid motif found on the C-terminus (KGVKDRVKGK) results in the release of Smad2/3 into the cytosol while Cx43 remains bound to the microtubules for delivery to the plasma membrane (Figure 7b: Dai et al., 2007; Giepmans et al., 2001; Martinez et al., 2003). The pooling of unphosphorylated Smad2/3 in the cytosol makes them available for the Smad signaling pathway. Three specific aims have been developed for investigation. Specific aims #1- Demonstrate the effectiveness of the TBM peptide in disrupting gap junction intercellular communications. Specific aims #2- Demonstrate the effectiveness of the TBM peptide in decreasing type III collagen and TIMP-1 synthesis. Specific aims #3- Demonstrate the effectiveness of the TBM peptide in disrupting Smad2/3 localization (nucleus vs. cytoplasm). As seen in figure 7c, d, it is hypothesized that the tubulin binding motif (TBM) peptide effectively disrupts the Smad signaling pathway, including the effects upon GJIC, Smad2/3 location, TIMP-1, and type III procollagen. When bound to the tubulin binding sequence localized on the Cx43 C-terminus, the TBM peptide blocks Cx43 binding. Prevention of Cx43 binding to tubulin will have several effects which include: the disruption of Cx43 trafficking to the plasma membrane reducing available Cx43 for GJIC formation, and Smad2/3 accumulation on microtubules, which subsequently reduces free Smad2/3 in the cytosol. The lack of Smad 2/3 in the cytosol will reduce Smad signaling causing decreases in the transcription of type III collagen and TIMP-1.
Figure 7. Interaction of Cx43 under control and TBM peptide conditions. (a) A model indicating the binding interaction on microtubules (indicated in green) between Smad 2/3 and Cx43. Under control conditions, Smad2/3 is bound to the microtubules. (b) Indicates that the binding of Cx43 to the tubulin binding motif releases Smad2/3 from the microtubule. (c) Indicates that the TBM peptide binds to β-tubulin by the exact tubulin binding motif as seen on the C-terminal tail of Cx43. (d) Indicates that under TBM peptide treated conditions, the TBM peptide binds to the tubulin binding motif of Cx43 preventing Cx43 binding and the release of Smad2/3. Image courtesy of Ashley Pistorio
Chapter 3. Materials and Methods

3.1: Cell Culture

Human dermal fibroblasts derived from neonatal foreskin were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% new born bovine serum (NBBS) and 15µg/mL of gentamicin. Cells are trypsinized to get into suspension, pelleted in a clinical centrifuged, re-suspended in DMEM to deactivate trypsin, and then subjected to buffers and centrifugation steps to extract nuclei or microtubules.

3.2: Standard Procedure for Nuclei Isolation

A method for nuclei isolation was designed to test the effectiveness of the tubulin binding motif peptide in regulating Smad 2/3 location and decreasing TIMP-1 production. Buffers for nuclei extraction are designated as follows: Buffer A, contains 10mM HEPES (pH 7.9 at 4°C), 1.5mM MgCl₂, 10mM KCl; Buffer B, contains 0.3M HEPES (7.9 pH at 4°C), 1.4M KCl and 0.03M MgCl₂. Human dermal fibroblasts harvested from cell culture were centrifuged at room temperature at 2000 rpm in an IEC clinical centrifuge using a 2/5 head at 25°C. Pelleted cells were re-suspended in 5 volumes of PBS at 4°C and collected by centrifugation at 2000 rpm for 10 minutes at 25°C (IEC clinical centrifuge with a 2/5 head). Fibroblasts were then suspended in 5 packed cell pellet volumes of chilled buffer B and allowed to stand for 10 minutes on ice. Cells were collected by centrifugation and re-suspended in two packed cell pellet volumes of buffer A. Suspended cells were subsequently lysed by 10 strokes with a B pestle type Dounce homogenizer. To pellet the nuclei, the homogenate was centrifuged for 10 min at 2000 rpm at 25°C (IEC clinical centrifuge with 2/5 head) as seen in figure 8. Pelleted nuclei were checked microscopically to identify pelleted nuclear fraction. The nuclei smear was
allowed to dry before staining with DAPI (Invitrogen Corporation, Carlsbad, CA) for 5 minutes. Nuclei stained slides were visualized with a fluorescent microscope.

3.3: Standard Procedure for Microtubule Isolation

The microtubule isolation buffer was composed of 0.5% Triton X 100, 5mM TES (pH 7.5 at 4°C) 0.12M NaCl, and 2mM MgCl₂. Fibroblasts were harvested from cell culture by centrifugation at room temperature at 2000 rpm in an IEC clinical centrifuge. Pelleted cells were suspended in 5 volumes of PBS at 4°C and collected by centrifugation. Fibroblasts were then suspended in 5 packed cell pellet volumes of chilled microtubule buffer and allowed to stand for 10 minutes on ice. Microtubules were collected by centrifugation of cell homogenate in a high speed centrifuge for 30 minutes.
at 25,000g using a Beckman Coulter Optima L-90K Ultracentrifuge with a SW 55 Ti head. The resulting pellet, microsomal pellet, was re-suspended into 5 packed cell pellet volumes of the microtubule buffer and centrifuged at 90,000g for 2 hours (Beckman Coulter Optima L-90K Ultracentrifuge with a SW 55 Ti head). The resulting pellet was the microtubule fraction containing organelles (refer to Figure 8).

3.4: Gap Junction Dye Coupling Assay

The effectiveness of a tubulin binding motif (TBM) peptide that acted as a competitive inhibitor of connexin 43 was evaluated by scrape-loading technique to determine its efficiency in disrupting gap junction intercellular communications (El-Fouly et al., 1987; McKarns and Doolittle, 1992). Human dermal fibroblasts approaching confluence in 35mm dishes had their media removed and rinsed three times in PBS. Fibroblasts were bathed in 2mL of dye solution, consisting of 10mg/mL Lucifer yellow and 10mg/mL rhodamine dextran (Molecular Probes, Eugene, OR) in PBS. The dye covered fibroblasts were scratched with a commercial glasscutter and allowed to sit in the dark for 2 minutes, at which time the dye was removed and the cells were rinsed 3 times with PBS. The cells were fixed in buffered 4% paraformaldehyde (PFA). The scratch lines were visualized under a Zeiss Axiovert 135 fluorescent microscope. Lucifer yellow and rhodamine dextran accumulated within the scraped injured cells but only those injured cells that were coupled by GJIC passed the Lucifer yellow dye to coupled cells. The rhodamine dextran did not pass into uninjured coupled cells due to its size being too large to pass through gap junctions. The coupling index was determined by counting the red and yellow stained cells within eight randomly selected fields. The ratio of the number of yellow cells to the number of red cells is the coupling index. Data for
the coupling index of 2 or greater indicated that coupling took place. If the coupling index had unity then no coupling took place (Pepper et al., 1989).

3.5: Dot Blot Analysis

A modified procedure of Dot Blot analysis as described by Dot Blot apparatus was used to assess levels of released soluble type III collagen from cultured fibroblasts employing the Bio-Rad Dot Blot apparatus (Bio-Rad, Hercules, CA). Confluent fibroblast media collected from 6.0 cm dishes were used for Dot Blot analysis after they had reached confluence. The medium was removed, the fibroblasts rinsed in PBS, and then 5ml of serum-free-DMEM supplemented with 50 µg/ml of ascorbic acid 2-phosphate sequimagnesium, vitamin C and 10 µg/ml β-aminoproprionitrile fumarate (Sigma Chemical Co.) were added. The purpose of the vitamin C was to optimize the levels of collagen synthesis. After the aforementioned steps were completed, the fibroblasts were incubated for 4h and 24 h, followed by the collection of the media and addition of 1µl/mL of concentrated HCl to acidify the medium. Acidifying the medium inhibits proteinases that cleave collagen thus promoting collagen solubility. The collected media was stored at 4°C until analysis.

Protein blotted on nitrocellulose membrane was subjected to immune-blot detection for native type III collagen using mouse monoclonal IgG1 clone COL-III (Sigma Chemical Co.). COL-III antibody is designed specifically for native type III collagen and will not detect denatured collagen α chains, limiting the Dot Blot analysis for collagen III. The nitrocellulose membrane was incubated in 5% dried milk solution overnight to block non-specific protein binding sites. Following that step the membrane was further incubated with a peroxidase-conjugated antibody directed toward mouse IgG1 (Jackson Immuno Research Laboratories, West Grove, PA). Super Signal West Dura
Chemiluminescence detection system (Pierce Biotechnologies, Rockland, Ill) was used to detect the antibody antigen complex on the membrane following the manufacturer’s provided instructions.

3.6: Western Blot Analysis

Western blot analysis quantified levels of TIMP, Smad2/3 proteins in the nucleus, cytosol, and microtubule fractions for determining the influence of the TBM peptide upon treated cells. Cell samples were sonicated in lysis buffer. 20µl of the nuclear, cytosolic, and microsomal fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Transfer of the proteins from the SDS-PAGE gel to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, Mass.) occurred by the use of an additional electrophoresis step. The membranes underwent sequential incubations with primary antibodies directed toward TIMP (Rabbit Anti-TIMP-1; Sigma Chemical Co.), Smad2/3 (Mouse Anti-Smad2/3; BD Laboratories), and microtubules (Mouse Monoclonal Anti-α-Tubulin; Sigma Chemical Co.). The membrane was washed, and then incubated with peroxidase-tagged anti-immunoglobulin G-directed antibody. Detection of the proteins on the membrane utilized the Super Signal West Dura Chemiluminescence detection system (Pierce Biotechnologies, Rockland, Ill.) following their provided manufacturer’s instructions.

3.7: Statistical Analysis

Values were expressed as mean ± SD. The Student t test was used to evaluate the statistical differences between groups: a P value of <.05 was considered significant.
Chapter 4. Results

Experiments were performed to test the effectiveness of the TBM peptide in disrupting the Smad signaling pathway. It was necessary to develop and confirm the effectiveness of the centrifugation techniques for nuclei and microtubules isolation from human dermal fibroblasts in order to determine the effects of the TBM peptide on the Smad signaling pathway.

4.1: Nuclei Isolation

The use of 4',6-diamidino-2-phenylindole (DAPI), a blue-fluorescent nucleic acid stain, stain confirmed the effectiveness of the nuclei isolation technique. 10 µl of the cell lysate, nuclei fraction, and supernatant were smeared on a slide and allowed to dry prior to DAPI staining. As seen in figure 9, nuclei were found in the cell lysate and nuclei samples but were absent from the supernatant fraction.

![Figure 9: DAPI stained blue nuclei of human dermal fibroblasts.](image)

Cell membranes of human dermal fibroblasts were disrupted by buffers and subjected to centrifugation. The cells were stained with DAPI at the lysate (A), pellet (B), and supernatant (C) stages to visualize the ability to isolate nuclei. Images were captured at 10x.
4.2: Microtubule Isolation

The effectiveness of the microtubule isolation procedure was confirmed. 20 µl of the cell lysate, microsomal fraction, and the cytosolic fraction were subjected to SDS-PAGE electrophoresis and Western blot technique. As seen in figure 10, results indicated the presence of α-tubulin within the cell lysate and microsomal fractions. Densitometry was used to quantify the levels of α-tubulin in each of the samples. Densitometry of the cell lysate and microsomal samples indicated a near identical density for α-tubulin while the densitometry of the cytosolic fraction showed a faint trace for α-tubulin. Results indicate that the procedure for isolating microtubules is effective. The experiment was repeated. The uses of known proteins for each fraction were run as a control as a means of qualitatively assess the concentration of proteins loaded for each sample. The samples used were Lamin A/C, a nuclear protein, α-tubulin, tubulin monomer found in microtubules, and GAPDH, a cytosolic protein. Results showed a positive result for each control protein.

**Control Proteins**

<table>
<thead>
<tr>
<th>Control Proteins</th>
<th>Lamin A/C</th>
<th>α-tubulin</th>
<th>GAPDH</th>
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<td><img src="image5.png" alt="Image" /></td>
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<tr>
<td>Cytosolic Fraction</td>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
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</tbody>
</table>

**Figure 10: Western blot of fibroblasts showing α-tubulin density.** Western blot analysis techniques were used to determine the concentration of α-tubulin contained within the cell lysate, microsomal and cytosolic centrifugation fractions. The use of known proteins (Lamin A/C for the nuclei fraction, α-tubulin for the microsomal fraction, and GAPDH for the cytosolic fraction) were used to qualitatively assess that equal concentrations of protein were loaded for each sample. Equal volumes of 20µl were added into each well.
4.3: Gap Junction Dye Coupling Assay

A modified procedure of the scrape-loading/dye transfer as described by El-Fouly et al. (1987) was used to assess the effectiveness of a peptide that acted as a competitive inhibitor of connexin 43 in disrupting gap junction intercellular communications. As seen in figure 11, the uncoupling action of the TBM peptide on GJIC between human dermal fibroblasts was confirmed. Dye localization within cells was recorded from 8 randomly selected fields. The coupling index, the ratio of Rhodamine dextran to Lucifer yellow fluorescence (± the standard deviation) at the scrape line following 5 minute exposure, from three 35mm control dishes showed a coupling index of 3.3 ± 0.18 (P≤0.0001). The three 35mm TBM peptide treated dishes showed a coupling index of 1.45 ± 0.04 (P≤ 0.0001). A coupling index of less than 2 indicates gap junction uncoupling.

![Scrape Loading Graph](image)

**Figure 11: Influence of peptide on Lucifer yellow uptake.** (A) Human dermal fibroblast monolayer exposed to TBM peptide for 6 hours. Dye uptake was assessed by determining the coupling index, the ratio of Rhodamine dextran to Lucifer yellow fluorescence surrounding the scrape line after 5 minute exposure. Eight random fields were analyzed for both control and TBM peptide groups. The data is expressed as a ratio of the Rhodamine dextran to Lucifer yellow passages ± the standard deviation. (B) Scrape loading images from control and TBM Peptide treatments.
4.4: Collagen III Production

Harvested media from dermal fibroblasts at confluence incubated for 4 h and 24 h with 10 µl/mL Vitamin C, 5 µl/mL TGF-β1, and 10 µl/mL TBM peptide was analyzed by Dot Blot analysis. Results from 10 µl media from each treatment (Fibroblasts + Vitamin C [control], Fibroblasts + Vitamin C + TGF-β1, and Fibroblasts + Vitamin C + TGF-β1 + TBM Peptide; at 4 and 24 hours) indicated that the TBM peptide was effective in decreasing type III collagen production. As seen in figure 12, densitometry analysis of the results showed that the addition of TGF-β1 to a fibroblast and vitamin C in monolayer doubled type III collagen production at 4h and tripled the production over 24h. The addition of the TBM peptide at a volume of 10 µl/mL to the fibroblast cultures decreased type III collagen production to a level near or below the control levels at both 4h and 24h. This experiment was repeated.

HF+ Vit. C (4 hrs)

HF+Vit. C+ TGF-β (4h)

HF+ Vit. C+ TGF-β + Peptide (4 hrs)

HF+ Vit. C (24 hrs)

HF+Vit. C+ TGF-β (24 hours)

HF+ Vit. C+ TGF-β + Peptide (2 4 hrs)

Figure 12. Dot Blot of human dermal fibroblasts (HF) showing density of collagen III production at 4hr and 24 hours. Collagen III production for a known collagen III stimulator, TGF-β1, versus the addition of TBM peptide was compared at 4hrs and 24 hrs by dot blot analysis. TGF-β1 increased levels of collagen III production while use of peptide decreased the level both at 4 hrs and 24 hrs. Equal volumes of 10 µl were added for each sample.
4.5: Tissue Inhibitor of Metalloproteinase-1 Production

The TBM peptide was effective in decreasing the levels of TIMP-1 production. 20 µl of the nuclei, microsomal, and cytosolic fractions from the control and TBM peptide samples were subjected to SDS-PAGE electrophoresis and Western blot analysis. Results, seen in figure 13, indicated that TIMP-1 was present in the control microsomal fraction, suggesting that the control fibroblasts underwent normal signaling pathways leading to TIMP-1 production. The TBM peptide samples all lacked a measurable density for TIMP-1 production. This result suggests that the TBM peptide effectively decreased the level of TIMP-1 production to an immeasurable concentration. As a control, each sample was run with a known protein found in each fraction. The uses of known proteins for each fraction were run as a control as a means of qualitatively assess the concentration of proteins loaded for each sample. The samples used were Lamin A/C, a nuclear protein, α-tubulin, tubulin monomer found in microtubules, and GAPDH, a cytosolic protein. Results showed a positive result for each control protein.

### Control Proteins

<table>
<thead>
<tr>
<th>GAPDH</th>
<th>α-Tubulin</th>
<th>Lamin A/C</th>
<th>GAPDH</th>
<th>α-Tubulin</th>
<th>Lamin A/C</th>
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</tr>
<tr>
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<td>TBM Microsomal</td>
<td>TBM Nucleus</td>
<td>Control Cytosol</td>
<td>Control Microsomal</td>
<td>Control Nucleus</td>
</tr>
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</table>

**Figure 13:** Western Blot of human dermal fibroblasts showing density of TIMP-1. Density of TIMP-1 production was determined by Western Blot analysis of control and peptide nucleus, cytosolic sample, and microsomal samples. TIMP-1 expression was evident in the control microsomal sample while control nuclei and cytosolic samples showed a negative result from TIMP-1. Tubulin binding motif peptide treated samples lacked TIMP-1 density. The use of known proteins (Lamin A/C for the nuclei fraction, α-tubulin for the microsomal fraction, and GAPDH for the cytosolic fraction) were used to qualitatively assess that equal concentrations of protein were loaded for each sample. Equal volumes of 20µl were added into each well.
4.6: Smad 2/3 location

Treating human dermal fibroblasts with 10 µl/mL of TBM peptide for 6 h proved effective for increasing Smad 2/3 concentrations in the cytoplasm. 20 µl of the nuclei, microsomal, and cytosolic fractions from both control and TBM peptide treated sample were subjected to SDS-PAGE electrophoresis and Western blot analysis to determine the location of Smad 2/3 (Smad 2: 52kDa; Smad 3: 48kDa) in the control versus TBM peptide treated fibroblasts. Results of the control samples showed a dense concentration of Smads in the nuclei fraction. As seen in figure 14, fibroblasts treated with the TBM peptide showed measurable densities for Smad 2/3 in both the nuclei and microsomal fractions. Both the control and TBM peptide treated fibroblasts lacked Smads in the cytosolic fraction. The uses of known proteins for each fraction were run as a control as a means of qualitatively assess the concentration of proteins loaded for each sample. The samples used were Lamin A/C, a nuclear protein, α-tubulin, tubulin monomer found in microtubules, and GAPDH, a cytosolic protein. Results showed a positive result for each control protein.
Figure 14: Western blot showing the density of Smad2/3 from different centrifugation fractions of a human dermal fibroblast. Human dermal fibroblasts were subjected to buffers and centrifugations to determine the density of Smad2/3 after treatment with a peptide for 6 hours. Control data had a Smad2/3 located only within the nucleus. Treatment of fibroblasts with the TBM peptide showed a density of Smad2/3 remaining within the microsomal fraction. The use of known proteins (Lamin A/C for the nuclei fraction, α-tubulin for the microsomal fraction, and GAPDH for the cytosolic fraction) were used to determine the correct isolation of each fraction as well as a qualitative measurement of protein loading.
Chapter 5. Discussion

5.1: Nuclei & Microtubule Isolation

It was necessary to develop and confirm the effectiveness of the centrifugation techniques for nuclei and microtubules isolation from human dermal fibroblasts in order to determine the effects of the TBM peptide on the Smad signaling pathway. Nuclei isolation was confirmed with DAPI staining. The binding of DAPI to nucleic acids produces a blue fluorescence without cytoplasmic labeling (Invitrogen Corporation, Carlsbad, California). Data showed nuclei in human dermal fibroblasts in the cell lysate and nuclei fractions, while the supernatant fraction lacked nuclei. The presence of nuclei only within the fraction designated as the “nuclei fraction” confirmed the isolation technique was efficient for nuclei isolation.

Microtubule isolation was confirmed by Western blot analysis of the cell lysate, microsomal and cytosolic centrifugation fractions. A positive result for the known proteins located in each fraction, Lamin A/C (a nuclear protein), α-tubulin (tubulin monomer found in microtubules), and GAPDH (a cytosolic protein), were used to qualitatively assess the concentration of proteins loaded for each sample. Densitometry indicated that near identical concentrations of protein were run per sample. Therefore, the α-tubulin result was due to the use of buffers and various centrifugation techniques and not a loading error. Results showed that the use of anti-α-tubulin antibody had a positive result for α-tubulin within the cell lysate and microsomal samples indicating the presence of tubulin. The cytosolic fraction showed a slight density of α-tubulin. This result was due to the presence of a pool of soluble tubulin monomers that are location within the cytosolic fraction. The positive result for α-tubulin in the cell lysate samples confirmed that there were microtubules present in human dermal fibroblasts prior to performing the
microtubule isolation techniques. The presence of the majority of α-tubulin in the microsomal sample and a minimal amount in the cytosolic fraction confirmed that the extraction procedure for microtubules was an efficient method for microtubule isolation.

5.2: Scrape Loading

A modified procedure of the scrape-loading/dye transfer as described by El-Fouly et al. (1987) was used to assess the effectiveness of the TBM peptide as a GJIC uncoupler by preventing Cx43 binding to tubulin. Prevention of Cx43 binding will disrupt the transport of Cx43 along microtubules to the plasma membrane thus disrupting GJIC. The results obtained using the scrape-loading and dye transfer technique were consistent with previous studies that compared control vs. GJIC uncouplers (McKarns and Doolittle, 1992). The effectiveness of the TBM peptide as a GJIC uncoupler was confirmed. The coupling index, the ratio of Rhodamine dextran to Lucifer yellow fluorescence at the scrape line after 5 minute exposure, for three 35mm control dishes showed a coupling index of 3.3 ± 0.18 (P≤0.0001). The three 35mm peptide treated dishes showed a coupling index of 1.45 ± 0.04 (P≤ 0.0001). A coupling index of less than 2 for the TBM peptide treated samples indicated that the TBM Peptide was an effective uncoupler of GJIC between human dermal fibroblasts. Disruption of GJIC by the addition of the TBM peptide was consistent with results from known GJIC uncouplers (Lee and Rhee, 2007). Data supported the findings by Giepmans et al., 2001, which stated the predominant secretory pathway of Cx43 for plaque formation was contingent upon the direct interaction of Cx43 with microtubules. However, the presence of a minimal amount of dye passage, despite the addition of the TBM peptide, confirmed the research of Martin et al., 2001 which suggested that there are multiple pathways in the trafficking and assembly of connexin 43 into gap junctions. The dynamic trafficking and
delivery of Cx43 to the plasma membrane included a minor secretory pathway that involved the transport of Cx43 through the Golgi apparatus toward the periphery. This pathway might account for the persistence of minimal levels of dye passage. Scrape loading and dye transfer provides a quick and cost effective way to study the disruption of gap junction intercellular communications by potential inhibitors in monolayer cultured cells.

5.3: Collagen III Production

The purpose of utilizing the dot blot technique was to study the effectiveness of a peptide in suppressing type III collagen production in human dermal fibroblasts by disrupting the Smad signaling pathway. Vitamin C was essential for the synthesis of collagen and equal volumes of 10µl/mL were used in each treatment. Results showed that fibroblasts treated with TGF-β1 had stimulated type III collagen synthesis at both 4h and 24h. The results agree with research from Inagaki et al., 2005 and Ghosh et al., 2008 which state that the synthesis of type III collagen was stimulated by TGF-β1. However, the TBM peptide and TGF-β1 treated samples showed an inhibitory effect on type III collagen synthesis. The decrease in type III collagen synthesis suggested that the TBM peptide was effective in the disruption of the Smad signaling pathway. Under control conditions, Smad2/3 were bound to microtubules. The binding of Cx43 directly to tubulin by the amino acid motif found on the C-terminus (KGVKDRVKGK) resulted in the release of Smad2/3 into the cytosol while Cx43 remained bound to the microtubules for delivery to the plasma membrane. The pooled Smad2/3 in the cytosol was available for the Smad signaling pathway. It is believed, although the exact mechanism is unknown, that the competitive binding between Cx43 and the TBM peptide, for the amino acid sequence unique to Cx43 on tubulin, disrupted the ability of
Cx43 to bind to tubulin. The prevention of Cx43 binding to tubulin prevented Smad 2/3 release and disrupted the Smad signaling pathway. The disruption of this pathway prevented the transcription of target genes which included type III procollagen. Further studies will be necessary to determine if the decrease in type III collagen was due to the failure of Cx43 binding to microtubules and subsequent Smad2/3 release or if the disruption of GJIC by the TBM peptide has an effect on the optimization of fibroblast type III collagen synthesis as seen by the research of Ehrlich et al., 2006 which showed that GJIC optimized the synthesis of type I collagen synthesis.

5.4: Tissue Inhibitor of Metalloproteinase-1 Synthesis

The purpose of utilizing the Western blot technique specific for anti-TIMP-1 was to study the effectiveness of the TBM peptide in disruption of the Smad signaling pathway resulting in the prevention of TIMP-1 synthesis. A positive result for the known proteins located in each fraction, Lamin A/C (a nuclear protein), α-tubulin (tubulin monomer found in microtubules), and GAPDH (a cytosolic protein), were used to qualitatively assess the concentration of proteins loaded for each sample. Densitometry indicated that near identical concentrations of protein were run per sample. Therefore, the TIMP-1 result was due to the effects on synthesis by the TBM peptide and not a loading error. TIMP-1 synthesis is increased by the Smad signaling pathway. The mRNA encoding TIMP-1 is exported from the nucleus into the cytosol where TIMP-1 will be formed within the trans-Golgi network. Results showed banding for anti-TIMP-1 at 29kDa for the control microsomal sample, while the rest of the samples lacked TIMP-1 densities. This result showed that under control conditions, TIMP-1 synthesis occurred but treating fibroblasts with the TBM peptide was effective for preventing TIMP-1 synthesis. Furthermore, the microsomal sample was the sample of interest since that
The sample contains the structures, microtubules and organelles including the trans-Golgi network, utilized by TIMP-1 for transport. Ritter et al., 1999 used an enhanced green fluorescent protein and TIMP-1 to show that the transport of TIMP-1 involved the unidirectional movement along microtubule tracks through the cytoplasm to the trans-Golgi network. The lack of TIMP-1 density for control and TBM peptide nuclei samples was expected since TIMP-1, in its sialoglycoprotein form, is not present within the nucleus. The lack of TIMP-1 density for the cytosolic sample was expected since that sample doesn’t contain any structures utilized by TIMP-1 for transport. Additional studies are required to determine if the decrease in TIMP-1 synthesis had an additional effect on the decrease in type III collagen. TIMP-1 is produced to act as the inhibitor to matrix metalloproteinases (MMPs), a family of zinc dependent endopeptidases which are responsible for the degradation of the major components of the matrix. The lack of TIMP-1 production allows the MMPs to essentially degrade type III collagen without being regulated. This raises the question, was the reduction of type III collagen synthesis due to the interference of the Smad signaling pathway by the TBM peptide or, in actuality, is the decrease of type III collagen a combined result of the decreased synthesis from the disruption of the Smad signaling pathway, as well as, degradation of preexisting type III collagen due to unregulated MMP-1 activity?

5.5: Smad 2/3 Location

The purpose of utilizing the Western blot technique was to study the effectiveness of a TBM peptide in preventing Smad2/3 release from tubulin. A positive result for the known proteins located in each fraction, Lamin A/C (a nuclear protein), α-tubulin (tubulin monomer found in microtubules), and GAPDH (a cytosolic protein), were used to qualitatively assess the concentration of proteins loaded for each sample.
Densitometry indicated that near identical concentrations of protein were run per sample. Therefore, the Smad2/3 result was due to the effects on location by the TBM peptide and not a loading error. Smad proteins are intracellular signaling effectors of the TGF-β1 family that are continuously being shuttled between the microtubules and nucleus for transcription purposes. Results showed that human dermal fibroblasts treated with TGF-β1 had Smad 2/3 density only in the nuclei fraction. This result is in agreement with research done by Dong et al., 2006 which stated that TGF-β1 induced interactions will trigger pooled cytosolic Smad2/3 to enter the nucleus. Data from the TGF-β1 treated samples indicate that Smad2/3 was released from tubulin and entered the nucleus for transcription of target genes. The lack of Smad2/3 density of the microsomal sample showed that, under control conditions, Smad2/3 do not remain attached to microtubules. Results from fibroblasts treated with the TBM peptide had a positive result for Smad2/3 both in the nucleus, having the greatest density, as well as the microsomal sample. Density of Smad2/3 in the microsomal fraction showed that the TBM peptide was effective in preventing Smad2/3 release from tubulin. Although the exact mechanism is unknown, it is believed that the failure of Smad2/3 release was due to the prevention of Cx43 binding to tubulin by the TBM peptide. This theory is indicated by the positive result for Smads in the microsomal sample with less density within the nuclei sample.

5.6: Effectiveness of the Tubulin Binding Motif Peptide

The results indicate that the TBM peptide was effective in disrupting the Smad signaling pathway and confirmed the hypothesis. By disrupting the Smad signaling pathway, the TBM peptide effectively decreased levels of type III collagen, as well as TIMP-1 production. Furthermore, an uncoupling of GJIC was shown due to the prevention of Cx43 binding to tubulin and transport by microtubules to the cell periphery.
The process of Cx43 binding to tubulin was disrupted by the TBM peptide. Moreover, due to the failure of Cx43 binding to tubulin because of the TBM peptide, the release of Smad2/3 was prevented. Therefore, Smad2/3 levels increased in the microsomal fraction (being bound to microtubules) and were not available within the cytosol for phosphorylation thus disrupting the Smad signaling pathway.
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