# MMP9 AND WNT SIGNALING IN PERITONEAL ANGIOGENESIS

# THE ROLE OF MMP9 AND WNT SIGNALING IN PERITONEAL ANGIOGENESIS

By

Manreet Padwal, Hons. B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

DOCTOR OF PHILOSOPHY (Ph.D.) (2017) McMaster University

Department of Medical Sciences Hamilton, Ontario

TITLE: The role of MMP9 and WNT Signaling in Peritoneal Angiogenesis

AUTHOR: Manreet Padwal (H. B.Sc. McMaster University)

SUPERVISOR: Dr. Peter Margetts

SUPERVISORY COMMITTEE: Dr. Judith West-Mays
Dr. Darren Bridgewater

PAGES: 230

## ABSTRACT

Patients on peritoneal dialysis (PD) are reliant on the peritoneum to provide a semi-permeable barrier to allow for dialysis (solute clearance), salt and water removal (ultrafiltration). PD patients are at risk of developing peritoneal fibrosis and angiogenesis which can lead to a decline in peritoneal membrane function. Specifically, PD patients develop increased solute transport and decreased osmotic conductance leading to ultrafiltration failure. Peritoneal angiogenesis is the leading factor that results in augmented peritoneal membrane solute transport which is associated with worse outcomes – increased risk of mortality and PD technique failure. Transforming growth factor beta (TGFB) is one of the primary cytokines involved in inducing epithelial to mesenchymal transition (EMT) and fibrosis. We hypothesize that PD leads to injury of the epithelial lining of the peritoneum – the mesothelial cells. These cells undergo a transition process and transitioned mesothelium are a source for angiogenic and fibrogenic growth factors.

Matrix Metalloproteinase (MMP) 9 is an angiogeneic factor and has been observed to correlate with increased expression of vascular endothelial growth factor (VEGF). MMP9 has the ability to cleave and activate membrane bound factors such as E-cadherin and b-catenin respectively. There is substantial evidence that the canonical WNT/b-catenin pathway is active during fibrosis, and angiogenesis in different biological contexts. Thus, we investigated the role of MMP9 and WNT signaling in peritoneal angiogenesis. Limited evidence exists

describing the role of noncanonical WNT signaling but some reports suggest that non-canonical WNT signaling inhibits WNT/b-catenin signaling. Non-canonical WNT5A has differential effects based on receptor context and has been shown to block WNT/b-catenin signaling in the presence of Receptor Tyrosine Kinase Like Orphan Receptor 2 (Ror2). *The overall hypothesis of this PhD thesis is that MMP9 and WNT signaling play a key role in inducing peritoneal angiogenesis and are associated with changes in peritoneal membrane function. We expect WNT5A and Ror2 to protect against peritoneal membrane injury.*

From the overnight effluent of stable PD patients, we cultured mesothelial cells and assayed these for expression of MMP and WNT related genes. MMP9 and WNT1 gene expression were observed to be strongly correlated with peritoneal membrane solute transport in patients on PD. WNT2 mRNA was also positively correlated with peritoneal solute transport. We overexpressed MMP9 in the mouse peritoneum to demonstrate its role in angiogenesis and confirmed these findings using MMP9 -/- mice. In addition to this, we have shown a novel mechanism by which MMP9 induces angiogenesis by E-cadherin cleavage and b-catenin mediated signaling. The observed cross-talk between MMP9 and b-catenin prompted investigation of the activation of canonical WNT/b-catenin signaling in development of peritoneal membrane injury. In an experimental model of TGFB induced pertioneal injury, we confirmed the activation of WNT/b-catenin signaling. In addition to this we, we blocked the WNT pathway and observed that WNT/b-catenin signaling is required to induce peritoneal

angiogenesis. WNT5A mRNA was downregulated during TGFB induced injury suggesting a more protective role. Furthermore, several studies have demonstrated its ability to antagonize the WNT/b-catenin signaling pathway. We demonstrated that WNT5A protected against angiogenesis by blocking the canonical WNT pathway. WNT5A is thought to antagonize the WNT/b-catenin signaling pathway by signaling through receptor Ror2. In cell culture, we overexpressed TGFB and blocked Ror2. This resulted in elevated levels of VEGF and fibronectin suggesting that Ror2 is involved in mediating protection. Therefore, Ror2 possesses the ability to regulate VEGF and may be a potential candidate by which WNT5A mediates its protective effects.

In conclusion, our findings identified MMP9 and WNT1 as potential biomarkers of increased peritoneal solute transport in patients that are on PD. We have also found a novel mechanism by which MMP9 interacts with b-catenin to induce peritoneal angiogenesis and have provided a first look at WNT/b-catenin signaling in peritoneal angiogenesis. Lastly, we have shown WNT5A to protect against peritoneal angiogenesis. Taken together, our findings are not only significant to the realm of PD research but hold wide applicability to research in the biomedical sciences.

**ACKNOWLEDGEMENTS**

I first wish to express my sincere thanks to my supervisor and mentor, Dr. Peter Margetts for giving me this opportunity and for the tremendous amount of support throughout my PhD. It has been an absolute honor to work with you and words cannot express my gratitude toward you over the last five years. You've exemplified what it means to be a dedicated and successful scientist, but above all a wonderful human being. Thank you for fostering such an encouraging learning environment which taught me how to take risks and lose my fear of being wrong. It was with your unwavering support, guidance and continued faith in me that I was able to achieve my greatest potential.

Thank you to my supervisory committee, Dr. Judith West-Mays and Dr. Darren Bridgewater, for their extraordinary support and guidance. Thank you for sharing your perspectives and always providing insightful suggestions and advice in regards to both research and life.

Thank you to all of those who have supported me along this journey – colleagues, family and friends. Thank you to my parents for their unconditional love and support – look, I really did become a doctor. Thank you to my brother and sister-in-law, Sunny Padwal and Pooja Thareja, for all the support from miles away. Thank you to my sister, Harleen Padwal, for always believing in me – you make me feel invincible. To my friend, Supreet Randhawa, you have truly been the calm in this storm. Thank you for always listening and knowing exactly what to say. Thank you for the endless encouragement from my friends, Jasmeen Mangat and Bina Hare – your enthusiasm for research has seriously made me feel like a groundbreaking scientist. And to my Bowman Family, Tharshika Sugumaran, Davneet Poonia and Ruban Lingam, thank you for all of the laughter, especially during the most desperate of times.

Thank you to the members of the Margetts' lab, past and present, Limin Liu, Imad Siddique, Katelynn Tang, Doug Miller and Ayesha Ghayur. It was a pleasure working with you. Thank you to the St. Joseph's crew who has really become family over the years (in no specific order) – Safaa Naiel, Hassan Al-Saleh, Diane Ojo, Richard Van Krieken, Chiko Shimbori, Chandak Upugupta, Ehab Ayaub, Manpreet Sehmbi, Victor Tat, Vincent Lu, Nazihah Bakhtyar, Ozair Banoor, Kiho Son, Kimia Sorouri, JP Oliviera, Celeste Bouchard, and Neel Mehta (I hope I've included everyone). Thank you for keeping me sane throughout graduate school.

Finally, my mother always reminds me to thank God.

## PREFACE

This is a "sandwich" style thesis. Chapter 1 is a general introduction and Chapter 2 outlines the hypothesis and aims. Chapter 3 is a review paper that I have published during my PhD and supplements the introduction with more detail on the origin of the myofibroblast and development of peritoneal membrane injury. Chapters 4 - 6 contribute to the body of this thesis where Chapter 4 has been published and Chapters 5 and 6 will be submitted to peer-reviewed journals for publication. Each chapter includes a preface that outlines the significance of each study to the overall thesis and the contribution to each of the works. Chapter 7 is a critical analysis of major findings highlighting the overall conclusions, significant impacts and potential limitations.

# TABLE OF CONTENTS

**LIST OF FIGURES AND TABLES**

**CHAPTER 1**



**CHAPTER3**



**CHAPTER 4**



**CHAPTER 5**

**CHAPTER 6**



**CHAPTER 7**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ad | Adenovirus |
| AdDL | Control adenovirus |
| ANGPT | Angiopoietin |
| a-SMA | Alpha Smooth Muscle Actin |
| BMP | Bone Morphogenetic Protein |
| CREBBP | Cyclic AMP Response Element Binding Protein |
| CKD | Chronic Kidney Disease |
| COL1A | Collagen Type1 |
| PD | Peritoneal Dialysis |
| DKK-1 | Dickkopf WNT signaling pathway inhibitor 1 |
| D/P Creatinine | Dialysate to Plasma Creatinine |
| D/D0 Glucose | 4h:initial glucose ratio |
| DVL | Dishevelled Segment Polarity Proteins |
| E-Cadherin | Epithelial Cadherin |
| ECM | Extracellular Matrix |
| EPS | Encapsulated Peritoneal Scelrosis |
| EMT | Epithelial to Mesenchymal Transition |
| ESRD | End Stage Renal Disease |
| FN | Fibronectin |
| FSP1 | Fibroblast Specific Protein 1 |
| FZD | G protein coupled receptors, Class F Frizzled |
| FITC | Fluorescein isothiocyanate |
| GFP | Green Fluorescent Protein |
| GSK3B | Glycogen Synthase Kinase 3 Beta |
| GTP | Guanine Triphosphate |
| HD | Hemodialysis |
| IF | Immunofluorescence |
| IHC | Immuno Histochemistry |
| IL | Interleukin |
| IP | Intraperitoneal |
| LRP | Low Density Lipoprotein Receptor Related Protein |
| MMP | Matrix Metalloproteinase |
| PCP | Planar Cell Polarity |
| PET | Peritoneal Equilibrium Test |
| Pfu | Plaque Forming Units |
| PBS | Phosphate-Buffered Saline |
| PDGFB | Platelet Derived Growth Factor B |
| P4H | Prolyl 4-hydroxylase subunit alpha 1 |
| qRT-PCR | Quantitative Real-Time Polymerase Chain Reaction |
| RFP | Red Fluorescent Protein |
| RHO | Rho Family of GTPases |
| Ror2 | Receptor Tyrosine Kinase Like Orphan Receptor 2 |

| | |
|---|---|
| RTK | Receptor Tyrosine Kinase |
| RYK | Receptor-like Tyrosine Kinase |
| SMAD | Mothers Against decapentaplegic |
| SFRP | Secreted Frizzled-Related Proteins |
| SiRNA | Small-interfering RNA |
| SNAIL1 | Snail Family Transcriptional Repressor 1 |
| TCF | T-Cell Factor |
| LEF | Lymphoid Enhancer Binding Factor |
| TGFB | Transforming Growth Factor beta |
| TIMP | TIMP Metalloproteinase Inhibitor |
| TWIST | TWIST family bHLH Transcription Factor |
| VEGF | Vascular Endothelial Growth Factor |
| WIF | Wnt Inhibitory Factor Protein |
| WNT | Wnt family |
| WT1 | Wilms' Tumor 1 |
| UUO | Unilateral Ureteral Obstruction |
| γGT | γ-glutamyl transferase promoter |

# CHAPTER 1: INTRODUCTION

## 1.1 Peritoneal dialysis

### *1.1.1 Mechanism and Clinical Relevance*

Patients suffering from chronic kidney disease or end-stage renal disease suffer from a loss of kidney function and must eventually undergo renal replacement therapy [1]. Peritoneal dialysis (PD) or hemodialysis (HD) are two dialysis options that are available to patients as a substitute for renal function [2]. Peritoneal dialysis is used by almost 200,000 patients worldwide accounting for 10% of the dialysis population [1]. PD uses the peritoneal membrane to filter the blood and can be completed at home resulting in a better quality of life for patients [2].

The peritoneum is a highly vascularized semipermeable membrane and can therefore facilitate the exchange of fluids and dissolved substances [1]. In this treatment, a catheter is permanently and surgically introduced into the peritoneal cavity. The peritoneal dialysis solution is transferred into the abdomen. This transferred fluid is referred to as a dwell (Figure 1.1 A-B). Dialysis fluid contains an osmotic agent, usually a high percentage of glucose, which generates a concentration gradient to drive the diffusion process [1]. As the dwell sits within the peritoneal cavity, water, solutes and waste products are filtered via the processes of diffusion and ultrafiltration (Figure 1.1 C-D). After several hours following the exchange, the waste fluid is removed and the process can be repeated with fresh PD fluid (Figure 1.1E) [1]. This regimen may be completed

manually (continuous ambulatory peritoneal dialysis) or it can be completed automatically over several cycles (automated peritoneal dialysis) [3].

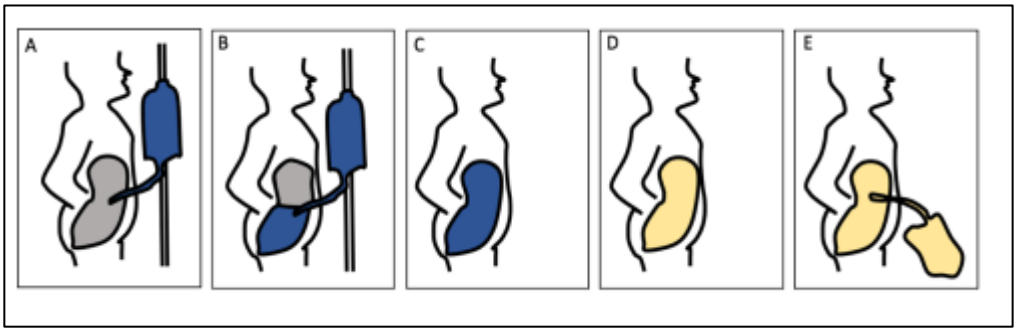



**Figure 1.1 Animation summarizing the process of peritoneal dialysis -** The peritoneum is a blood-vessel rich membrane lining the inside of the abdomen. The nature of the peritoneum makes it ideal for dialysis. A) A catheter is surgically inserted into the abdomen of the patient. B) Dialysis fluid is introduced into the peritoneal cavity of the patient. C) Dialysis fluid consists of an osmotic agent (usually glucose) which generates a gradient to drive exchanges from the plasma to the dialysate. Through the process of diffusion and ultrafiltration, wastes, solutes and water flow from the plasma into the dialysate. D) The exchanges have been completed and the dialysate is now made up of waste solution. E) Waste dialysate is drained at the end of the dwell (adapted from https://www.niddk.nih.gov/health-information/kidney-disease).

### *1.1.2 Peritoneal membrane structure and function*

The peritoneum is a blood vessel rich semipermeable membrane that lines the entire abdominal and pelvic cavity [1,4,5]. Normally, the peritoneum consists of three distinct layers, a superficial mesothelial layer, a basement membrane, and a thin submesothelial zone (Figure 1.2). The mesothelial layer is composed of a continuous monolayer of squamous-like epithelial cells called mesothelial cells [5]. The mesothelial layer acts as a barrier regulating peritoneal permeability [5]. The monolayer of mesothelial cells is supported by the basal lamina composed of

type IV collagen and laminin [4]. Underlying the superficial mesothelial layer is the submesothelium which is a matrix of collagen, fibronectin, fibroblasts, adipose, blood vessels and lymphatics [6]. The deepest layer is comprised of loose adipose connective tissue and a network of capillaries [1] (Figure 1.2 B). The physiological function of the mesothelium involves offering protection against physical damage and foreign pathogens and providing a frictionless interface for free movement of organs and tissues [5]. Under physiological settings, the peritoneum does not regulate the movement of water and is impervious to solutes [7].

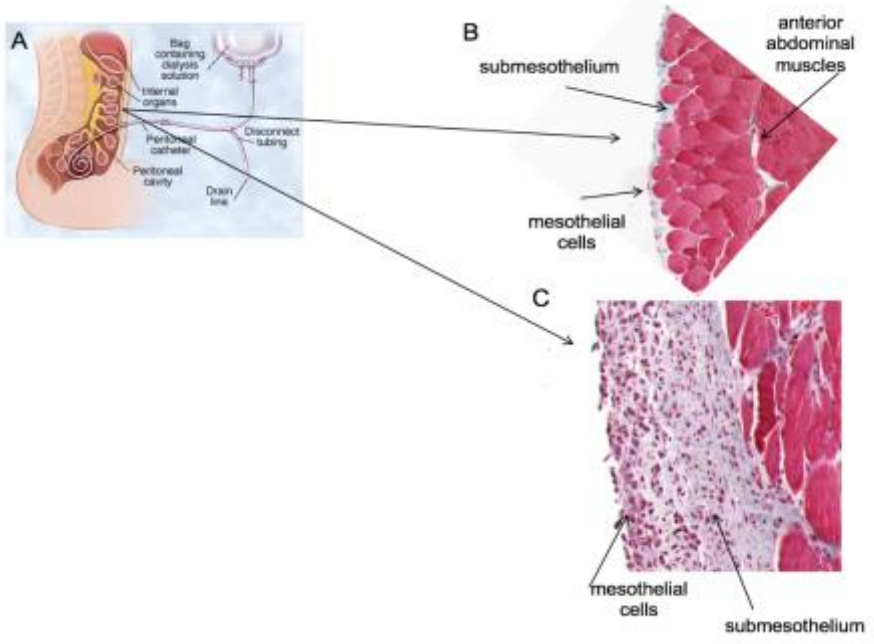



**Figure 1.2 The Peritoneal Membrane -** A) The peritoneal cavity used for peritoneal dialysis (adapted from http://www.mayoclinic.org/health/medical/IM00801) B) Normal rat parietal peritoneum taken from the anterior abdominal wall shows the superficial mesothelial cells and a thin submesothelial zone. C) Fourteen days after exposure to AdTGFB1, the peritoneal membrane demonstrates marked submesothelial thickening, angiogenesis, and cellular proliferation.

## 1.2 Peritoneal Membrane Injury

The primary difficulty with peritoneal dialysis involves protecting and preserving the membrane integrity. Injury to the peritoneum can occur due to the bioincompatibility of the dialysis fluids as a result of glucose breakdown products or hyperglycemic or acidic nature of the solutions [8]. Long-term exposure to hyperglycemic PD solutions induces a change in peritoneal membrane structure and function in patients on PD [9,10]. The development of more biocompatible PD fluid is an area of intense research and over the years dialysate composition has improved to some extent [11]. The uremic status of the patient has also been implicated in peritoneal membrane injury [12].

### *1.2.1 Peritoneal Fibrosis and Angiogenesis*

Continuous and repeated insults result in structural changes in the peritoneum including injury to the mesothelium and loss of mesothelial cells from the monolayer [12,13]. A subset of these mesothelial cells undergo epithelial to mesenchymal transition (EMT) resulting in the appearance of the myofibroblast which induces submesothelial fibrosis and angiogenesis (Figure 1.2C and Figure 1.3) [12,13]. Furthermore, the recruitment of inflammatory cells and induction of reactive oxygen species is also observed. The myofibroblast is thought to be the main effector cell involved in the development of peritoneal membrane injury [12,13]. This cell appears in the submesothelium and produces fibrotic factors depositing components of the extracellular matrix (ECM) resulting in submesothelial thickening (Figure 1.3). The extracellular matrix is a dynamic

structure comprised of fibronectin, collagens, and proteoglycans [14]. Biopsy samples taken from both humans [15] [6] and animals [16] have demonstrated changes in membrane architecture including thickening of the submesothelial zone accompanied by deposition of extracellular matrix.

Peritoneal angiogenesis is another modification observed in long term PD patients [6]. The increase in density of peritoneal microvasculature with time spent on dialysis was observed in human peritoneal biopsy samples [9,17]. In peritoneal dialysis, the exchange of solutes is regulated by changes in the peritoneal vasculature. Peritoneal membrane function is associated with modifications in the permeability of individual blood vessels and expansion of total vascular surface of the peritoneum (Figure 1.3) [18,19]. The process of angiogenesis involves the formation of new blood vessels from existing vessels. The endothelial cells may sprout to form new branches or existing vessels may split to form new ones. Angiogenesis is regulated by the microenvironment surrounding the extracellular matrix [20]. Vascular endothelial growth factor (VEGF) is an angiogenic cytokine which has been identified as a major contributor to expansion of the vasculature observed in peritoneal dialysis. VEGF participates in bone formation, wound healing and development in normal physiologies. However it also has a role in pathogenesis of cancer and fibrosis [21]. VEGF is a potent mitogen and chemoattractant for vascular endothelial cells. It is produced by a variety of cell types including mesothelial cells and myofibroblasts. VEGF also increases vascular permeability by increasing cellular

fenestrations within endothelial cells [22]. VEGF has been identified in biopsy samples taken from long-term peritoneal dialysis patients [17]. In chronic peritoneal dialysis patients, levels of VEGF in peritoneal effluent have been associated with peritoneal solute transport [23].

*1.2.2 Altered Solute Transport and Ultrafiltration*

The structural alterations observed during peritoneal fibrosis are associated with changes in peritoneal membrane function and therefore overall dialytic capacity. The standard method of assessing peritoneal membrane transport characteristics is using a peritoneal equilibrium test (PET) [24]. This semi-quantitative test yields the rate of exchange of solutes between the peritoneal capillary blood and the dialysate by generating a ratio of creatinine concentrations in dialysate and plasma (D/P creatinine) at a given time [24]. The D/P ratio of creatinine clearance is commonly used as a measure of solute equilibration. The D/P glucose ratio cannot be used as glucose is absorbed by the blood from the dialysate. Thus, the 4-hour dialysate/initial dialysate ratio of glucose (D/D0 Glucose) is used in place of this. The net ultrafiltration which is the difference between the volume instilled and volume drained, can also be calculated [25]. This gives a general measure of the ultrafiltration capacity of the peritoneal membrane.

The structural changes in submesothelial thickness and blood vessel density observed in biopsies taken from long term PD patients [6] seem to occur around the same time a decline in membrane function was observed. Therefore,

the structural changes that are seen in patients on peritoneal dialysis are likely associated with changes in peritoneal membrane function. This is supported by a longitudinal observational study where modifications in peritoneal membrane function was observed in 547 incident PD patients [26]. These patients exhibited an increase in solute transport and decline in ultrafiltration with increased time spent on PD over a period of 84 months on treatment [26]. A meta-analysis examining the relationship between peritoneal membrane function and mortality demonstrated an increased risk of mortality is correlated with patient solute transport status. High solute transporters have a 77.3% higher risk of mortality than low solute transporters [27].

Peritoneal solute transport is theoretically proportional to vascular surface area [28]. Vascular surface area is determined by the density of blood vessels and the surface that is in contact with PD fluid. Therefore, a greater density of blood vessels has been correlated with high transport status in patients (Figure 1.3) [10,26]. Glucose is most commonly used as the osmotic agent in PD fluid and is therefore inversely proportional to solute transport. High transporters tend to rapidly reabsorb glucose resulting in the loss of the ultrafiltration gradient and poor clinical outcomes [29]. However, the underlying mechanism of this process is not entirely understood and require further investigation.

## 1.3 Pathophysiology

### *1.3.1 Acute Wound Repair*

The changes in peritoneal membrane architecture is suggested to occur as a consequence of dysregulated wound healing response due to repeated injury by incompatible PD solutions [13]. Wound healing is a common physiological response to restore normal tissue structure with minimal loss of function [30]. The repair of internal organs and the skin involves an overlapping sequence of events including coagulation, inflammation, formulation of granulation tissue and resolution of the scar [30]. The first phase of wound healing begins immediately after injury and this includes vascular constriction followed by platelet activation. Platelets begin to adhere to the vessel wall initiating the coagulation cascade [31]. Platelets release growth factors and chemokines reinforcing the hemostatic plug and recruiting macrophages and fibroblasts to the site of injury [32]. As inflammatory cells approach the site of injury, the vessels begin to vasodilate to facilitate a greater infiltration of cells. Neutrophils and macrophages work to eliminate bacteria and cellular debris [31]. As the proliferative phase takes over, epithelial cells begin to migrate at the wound's edge. As inflammatory cells subside, the fibroblast emerges and begins to deposit large amounts of ECM including collagen III, fibronectin, fibrin and hyaluronic acid during scar formation [30]. Formation of granulation tissue requires a network of blood vessels to supply necessary oxygen and nutrients [30]. Progression to the proliferative phase results in macrophage secretion of transforming growth factor

beta (TGFB) inducing fibroblasts to differentiate into myofibroblasts to prepare for contraction of the wound [31]. Over time these cells contract reducing the size of the wound. Following contraction of the wound, the preliminary extracellular matrix is modified into normal functioning tissue. Collagenases and metalloproteinases secreted by granulocytes, macrophages and fibroblasts mediate the remodeling of disorganized immature matrix. As collagen I replaces the provisional collagen, myofibroblasts begin the process of wound contraction [33,34]. Wound contraction is terminated with apoptosis of fibroblasts and myofibroblasts [33,34].

*1.3.2 The Myofibroblast*

The final stage of the normal wound healing response in adults involves resolving the scar and restoring normal function of the tissue. However, the normal wound repair may become dysregulated as a result of continuous exposure to injury such as with bioincompatible PD fluids [13]. Severe injury can develop into tissue fibrosis and eventual organ failure [35]. The state of fibrosis is characterized by an excessive deposition of extracellular matrix and the main effector cell associated with the pathogenesis of all fibrotic diseases is the myofibroblast [36]. Resident cells at the site of the damaged tissue and inflammatory cells begin producing cytokines as a part of the wound healing response. The myofibroblast migrates to the site of injury where it becomes stimulated by the cytokine TGFB and high mechanical stress [36]. The myofibroblast shares features with fibroblast cells, however the change in

mechanical stress that occurs during wound healing results in the development of contractile stress fibers unique to the protomyofibroblast [37]. In response to large amounts of TGFB and high mechanical stress, the protomyofibroblast transitions into a more differentiated state. It begins to express alpha smooth muscle actin (a-SMA) which contributes to generating a greater force of contraction during acute wound repair. Normally, the myofibroblast undergoes apoptosis at the end of wound repair [37,38], however during fibrosis the myofibroblast is still present and continues to deposit large amounts of collagen and fibronectin [39].

The peritoneal myofibroblast was first identified in biopsies taken from 40 patients exposed to PD and its presence was associated with increased levels of collagen and vascularization [9,40]. The myofibroblast has long since been observed to induce thickening the submesothelial zone of the peritoneal membrane in patients on long term PD as well as in mouse models of peritoneal fibrosis [41]. The origin of the myofibroblast remains contentious, however different hypothesis propose it may arise from resident fibroblasts [38], perivascular cells [42], bone marrow derived cells [43], endothelial cells [44] and epithelial cells via epithelial to mesenchymal transition [45].

*1.3.3 Transforming Growth Factor Beta*

During peritoneal dialysis, bioincompatible PD solutions and episodes of peritonitis result in an increase in inflammation stimulating the production of profibrotic cytokines such as TGFB [13]. TGFB belongs to the transforming growth factor superfamily and has three different isoforms, TGFB1, 2, 3 with

similar biological properties [14]. TGFB is a multifunctional cytokine which controls cellular growth and differentiation but has also been implicated in the development of fibrosis in different organ systems. TGFB is released from platelets and latent forms of TGFB bound to the extracellular matrix can be activated following injury. The major role of TGFB during acute wound healing involves activating these cells to begin depositing extracellular matrix [14]. TGFB plays a fundamental role in each step of the normal wound healing cascade and administration of TGFB can replicate these events in normal tissue [46].

The TGFB1 isoform is associated with fibrosis and its signaling occurs via transmembrane serine-threonine kinases referred to as type I and II receptors. This pathway has several roles including regulating the synthesis and deposition of extracellular matrix [14,47]. Active TGFB acts through a canonical signaling pathway by binding to TGFB receptor II resulting in the recruitment and activation of TGFB receptor I. Active TGFB receptor I phosphorylates SMAD2 and SMAD3 complexing with SMAD4. This complex of proteins translocates to the nucleus to upregulate transcription of a-SMA, collagen 1 and other fibrosis related genes. This results in the activation of the myofibroblast and deposition of extracellular matrix [47].

The signal of TGFB is terminated following normal wound repair, however sustained production of TGFB can result in tissue fibrosis and this has been noted in the kidney, skin, lung and liver [48]. In the context of peritoneal membrane injury, elevated levels of TGFB in peritoneal effluent were established

early on [49,50]. The first in vitro studies of peritoneal mesothelial cells demonstrated that TGFB mRNA was heightened in these cells in response to increasing glucose concentrations [51,52]. The high concentrations of glucose in PD solutions stimulates mesothelial cells to secrete TGFB and also induce an increase in both TGFB receptor I and receptor II in peritoneal mesothelial cells. [53,54].

Since then it has been well established that TGFB induces peritoneal fibrosis and vascularization (Figure 1.3) [55]. TGFB stimulates fibroblasts to produce collagen I and III and induces production of extracellular matrix in the rat peritoneum and this is comparable to what occurs in patients on long-term PD [55]. TGFB also induced angiogenesis in the rat peritoneum and has been observed to promote production of VEGF. Elevated levels of VEGF were observed in response to TGFB in different cell types including fibroblasts and mesothelial cells [55,56]. Animals treated with adenovirus expressing TGFB also exhibited modifications in peritoneal membrane function including a significant increase in albumin clearance and a marked decrease in ultrafiltration. These changes in structure and function recapitulated what is observed in patients on peritoneal dialysis [55].

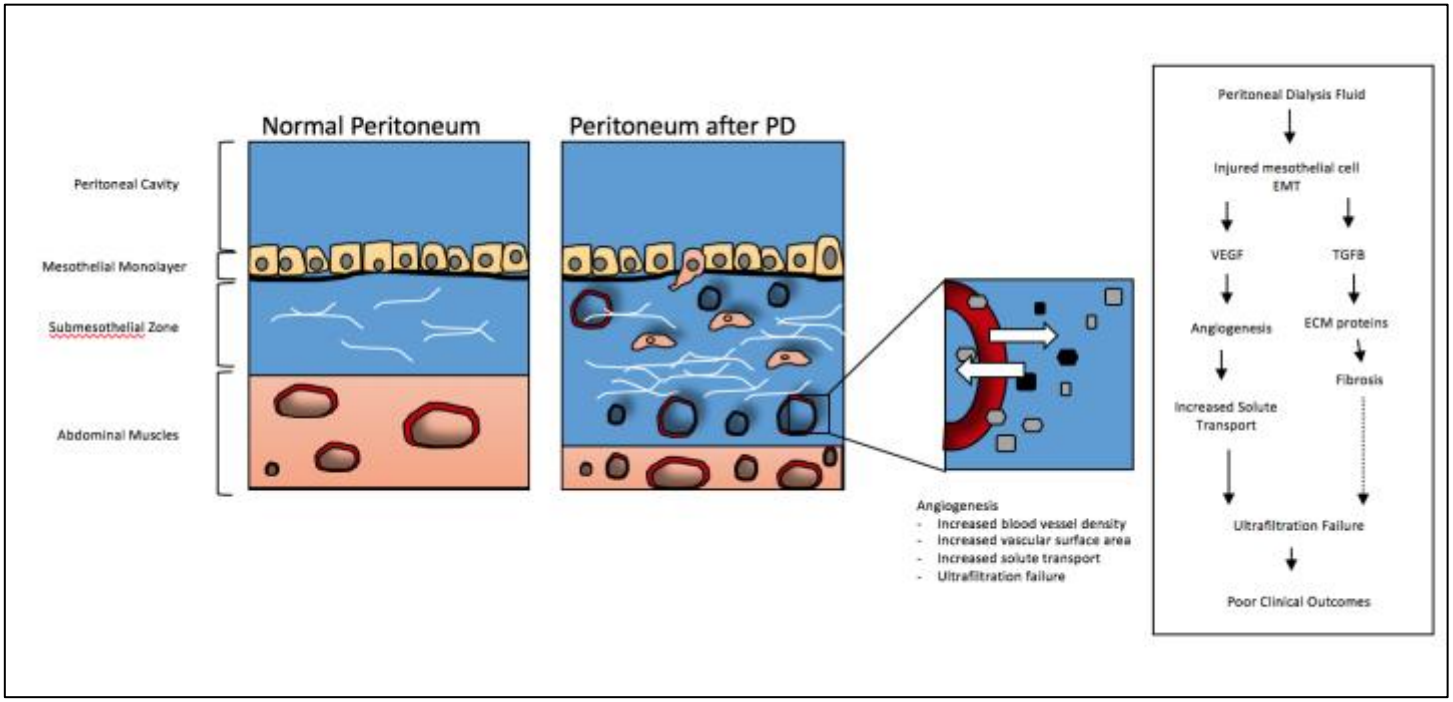



**Figure 1.3 The development of peritoneal membrane injury** - The mechanism behind peritoneal membrane injury. Bioincompatible dialysis solutions and infection results in injury to the mesothelium. Injured mesothelial cells undergo EMT and produce large amounts of TGFB. The myofibroblast secretes fibrotic and angiogenic factors resulting in fibrosis and angiogenesis. Angiogenesis causes the peritoneal membrane to become more leaky so there is an increase in solute transport and decline in membrane function. Peritoneal solute transport is theoretically proportional to vascular surface area. This includes the density of blood vessels and the surface that is in contact with PD fluid. Therefore, a greater density of blood vessels has been correlated with high transport status in patients. Glucose is most commonly used as the osmotic agent in PD fluid and is therefore inversely proportional to solute transport. High transporters tend to rapidly reabsorb glucose resulting in the loss of the ultrafiltration gradient and poor clinical outcomes.

### *1.3.4 Epithelial to Mesenchymal Transition*

In progressive fibrosis, the origin of the myofibroblast remains undetermined. Accumulating evidence has demonstrated epithelial to mesenchymal transition to be one of the major sources of the myofibroblast and fibrogenesis [45]. EMT has been well described in fibrotic disease of the kidney and liver in which TGFB has been established to be the prototypical initiator of this phenomenon [57]. EMT can be divided into two major stages, one being the cellular transition from an epithelial to a mesenchymal phenotype and the second being cellular invasion where the cell migrates into the interstitial layers (Figure 1.4) [45].

In the peritoneum, mesothelial cells are tightly connected with their neighbouring cells via intercellular adhesion molecules such as E-cadherin. On the basal side they interact with the basement membrane, while the apical side faces the peritoneal cavity [58]. During cellular transition, the epithelial layer loses cell adhesion and polarity and begins to rearrange the cytoskeleton. One of the main changes that is observed during EMT is the loss of E-cadherin expression which is a cell adhesion protein [45]. The Snail family transcriptional repressor 1 (SNAIL1) has been identified as an inhibitor of E-cadherin and a mediator of epithelial to mesenchymal transition [59–61]. The transitioning cells have increased expression of a-SMA and acquire migratory behaviour. During the second stage of EMT, the basement membrane is degraded and the myofibroblast

can migrate away from the epithelial cell layer into the interstitial layers and secrete components the extracellular matrix (Figure 1.4) [62].

Initially, the proliferating peritoneal fibroblasts residing in the stroma were thought to be the primary cell type associated with peritoneal fibrosis [63]. Evidence of EMT as a source of the myofibroblast was first explored in a landmark paper in 2003 by Yanez-Mo and colleagues [58]. Mesothelial cells taken from patients on peritoneal dialysis had marked changes in cell morphology and the appearance of non-epithelial cells was correlated with time spent on PD. Non-epithelioid cells also had significantly lower levels of E-cadherin protein with greater levels of vimentin, a mesenchymal marker. Cells derived from the omentum acquired migratory capacity in response to a mechanical stimulus. These cells were more fibroblast like in appearance. Mesothelial cells treated with TGFB expressed decreased levels of E-cadherin. The phenotypic changes associated with cells taken from patient peritoneal effluent were correlated with increased SNAIL1 and decreased E-cadherin gene expression as the cell became more mesenchymal like. Furthermore, analysis of peritoneal biopsy specimens revealed evidence of EMT including the loss of the mesothelial cell monolayer and presence of elongated mesothelial cells in fibrotic tissue. Collectively these findings characterized the role of mesothelial cells in the process of EMT and progression of peritoneal fibrosis [58].

Since then, evidence has accumulated in support of EMT as a source of the myofibroblasts and a mediator of peritoneal membrane injury (Figure 1.3). We

have also observed EMT after adenoviral delivery of TGFB to the rat peritoneum resulting in an increase of a-SMA positive cells in the submesothelial tissue [55]. In support of this, immunohistochemical examination of human peritoneal biopsies confirmed expression of mesothelial markers in fibroblast like cells embedded in the submesothelial zone. These myofibroblasts co-expressed a-SMA and cytokeratin and were found in the upper component of the submesothelial tissue [64]. Using a similar method, we further examined EMT in our lab after adenoviral delivery of TGFB to the rodent peritoneum. Gene expression analysis revealed an increase in a-SMA, SNAIL1 MMP2 and type 1 collagen in response to AdTGFB. Dual labeled cells co-expressing a-SMA and cytokeratin were observed in the mesothelial layer and a-SMA positive cells were observed underlying this layer. Progressively, cells expressing both epithelial and myofibroblast characteristics could be observed in the submesothelial zone and disruption of the basement structure provided evidence of cell migration into the interstitium [41]. Follow up studies using chronic infusion model also resulted in mesothelial cells that invaded the submesothelial zone. These cells co-expressed cytokeratin and a-SMA actin suggesting EMT takes place in the mouse peritoneum in response to peritoneal dialysis fluid [65].

Aroeira and colleagues examined the link between EMT and peritoneal membrane function. Presence of fibroblast-like mesothelial cells in PD effluents correlated with high peritoneal membrane solute transport [66]. In support of this, analysis of mesothelial cells from patients on peritoneal dialysis for a two year

period of time showed a transition in morphology and this was associated with high solute transport [67]. Collectively, these studies demonstrate that mesothelial cells undergo transition and migrate into the submesothelial zone as myofibroblasts producing extracellular matrix and vascular growth factors (Figure 1.3).

The phenomenon of epithelial to mesenchymal transition has been recently challenged creating much debate surrounding the mesothelial as the initiator of injury. Recent genetic fate mapping studies demonstrate that the myofibroblast do not originate from injured mesothelial cells rather they appear to come from residing submesothelial fibroblasts [68]. In support of this, lineage tracing studies of kidney fibrosis reveal perivascular cells to be the main source of the myofibroblast, questioning existing evidence of EMT as a major source [69]. We have also described a non-invasive form of EMT where the mesothelial cell undergoes in situ cellular transition without invading into the submesothelial tissue. Addition of PDGFB to the peritoneum resulted in cellular transition without mobilization of the mesothelial cells. Mesothelial cells co-expressed epithelial and mesenchymal markers but remained as a single monolayer of cells [70].

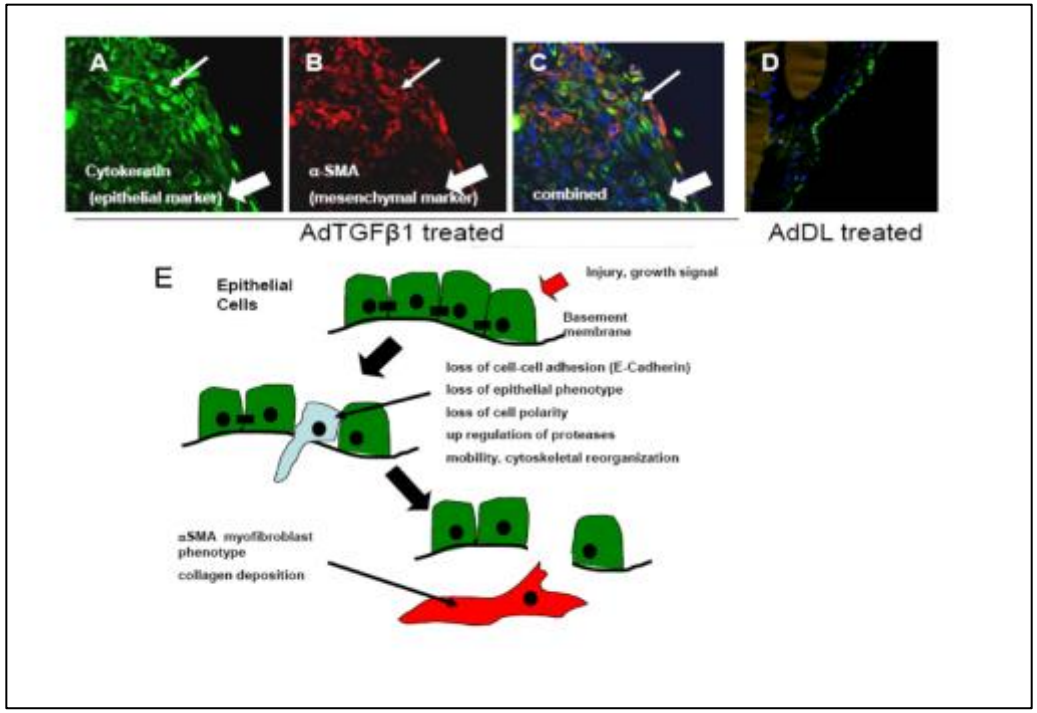



**Figure 1.4 The process of Epithelial to Mesenchymal Transition-** The mechanism behind peritoneal membrane injury. Bioincompatible dialysis solutions and infection results in injury to the mesothelium. Injured mesothelial cells produce large amounts of TGFB. We hypothesize that TGFB contributes to cellular transition of these mesothelial cells to become more myofibroblast like. The myofibroblast secretes fibrotic and angiogenic factors resulting in fibrosis and angiogenesis. Angiogenesis causes the peritoneal membrane to become more leaky so there is an increase in solute transport and decline in membrane function. Peritoneal solute transport is theoretically proportional to vascular surface area. This includes the density of blood vessels and the surface that is in contact with PD fluid. Therefore, a greater density of blood vessels has been correlated with high transport status in patients. Glucose is most commonly used as the osmotic agent in PD fluid and is therefore inversely proportional to solute transport. High transporters tend to rapidly reabsorb glucose resulting in the loss of the ultrafiltration gradient and poor clinical outcomes.

## 1.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidase enzymes involved in remodeling of the extracellular matrix in normal physiological processes such as embryogenesis, angiogenesis, cell migration and wound repair [71]. The MMP family consist of 24 vertebrae MMPs with 23 human homologues. In addition to this they appear to be involved in reproductive processes and bone development [72]. MMPs also modulate the processing of several molecules such as precursors to growth factors, receptors and other proteinases [71]. Physiological levels of MMPs usually appear low and become elevated during wound repair or diseases such as development of fibrosis and angiogenesis [72]. MMP2 and MMP9 are secreted gelatinases involved in degrading collagen IV, a component of the basement membrane [73]. MMP9 has been observed to regulate angiogenesis during embryonic development [74]. During wound healing, MMP9 has been shown to coordinate epithelial wound repair and aid in resolution [75] and wound contraction [76]. MMP9 has also been reported to regulate cell migration of inflammatory cells [77].

### *1.4.1 The role of MMP9 in EMT and fibrosis*

Aberrant expression of these MMP2 and 9 has been implicated in the process of EMT playing a crucial role in facilitating migration and cellular invasion [78]. The increased expression of MMP2 and MMP9 has been observed to be associated with metastasis in ovarian cancer and breast cancer [79–83]. In vitro matrigel assays have also been useful in clearly demonstrating that MMP2

and MMP9 can degrade the basement membrane reinforcing their roles in cellular invasion [84]. MMP2 is also sufficient to induce invasive EMT in vitro and in vivo [85,86]. There is still some conflicting evidence on the role of MMPs in inducing both cellular transition and invasion. Some studies suggest MMPs may induce EMT without invasion but further investigation is required [87].

Traditionally, MMPs were thought to prevent fibrosis due to their ability to degrade ECM. However, accumulating evidence demonstrates the pro-fibrotic nature of MMPs and most of these studies have been linked to EMT. High levels of MMP9 have been observed in cardiac, lens and renal fibrosis [88] [89]. Elevated levels of MMPs including MMP9 have also been observed in human and experimental idiopathic pulmonary fibrosis. However, conflicting findings suggest MMP9 may induce or inhibit fibrosis in the lungs and this may be dependent on the effector cell present, the local inhibitor levels and availability of target substrates [90].

In functional studies of MMP9, macrophages secreting MMP9 induced full EMT in tubular epithelial cells resulting in the development of renal fibrosis [91]. MMP9 mediates EMT in these cells by cleaving type IV collagen and disrupting tubular cell membrane structure [92]. Studies that have inhibited MMP9 expression have resulted in decreased EMT and preservation of tubular basement membrane integrity [93][94]. Studies of renal fibrosis have indicated the reciprocal relationship between TGFB and MMP9 in inducing EMT and fibrosis [91,95,96].

MMPs have been somewhat studied in peritoneal membrane injury. MMP2 concentrations in peritoneal effluent are strongly correlated with measures of solute transport [97–99] and MMP9 is associated with infections peritonitis [97,100]. We have demonstrated some insight into the role of MMP2 and 9 in the first description of the non-invasive EMT phenotype in peritoneal fibrosis [70]. Overexpression of TGFB in the mouse peritoneum resulted in a full EMT phenotype where injured mesothelial cells transitioned into a-SMA expressing myofibroblast like cells and invaded into the submesothelial tissue. These animals demonstrated augmented levels of MMP2 and 9 in response to TGFB. Overexpression of PDGFB in the mouse peritoneum, resulted in cellular transition, however invasion into the underlying submesothelial layers was not observed. This non-invasive EMT phenotype was attributed to a lack of MMP2 and 9 activity [70].

*1.4.2 The role of MMP9 in angiogenesis*

The process of angiogenesis involves formation of new blood vessels by migration of endothelial cells and proteolytic remodeling of the matrix [101]. MMPs have the ability to mobilize components of the ECM allowing for migration of endothelial cells and many studies have confirmed their expression in these cells [102]. Additionally, MMPs can release proangiogenic signaling molecules stored in the ECM [103]. Little evidence exists describing the contribution of MMP9 to peritoneal angiogenesis. However, MMP9 has been implicated in the expansion of vasculature in normal physiological processes such

as embryogenesis and also in conditions such as tumor growth, wound healing and ischemia [102,104–107]. Some evidence demonstrates that MMP9 induces blood vessel formation through the proteolytic degradation of the basement membrane of blood vessels. Cleavage of collagen IV of the basement membrane mediated endothelial cell growth and migration [102]. However, accumulating evidence is suggesting that MMP2 and 9 do not play as large of a role in invading the basement membrane, but are more involved in regulating the bioavailability of angiogenic factors [108]. For instance, in studies related to pancreatic tumors, MMP9 has been shown to be a component of the angiogenic switch contributing to the release of matrix bound VEGF [109–111]. Moreover, MMP9 can mobilize other ECM bound factors such as TGFB and FGF2 initiating proliferation and migration of endothelial cells during tumorigenesis [108]. Furthermore, the role of MMP9 in remodeling the extracellular matrix can result in exposing sites that act as signals for migration and angiogenesis [108]. MMP9 may also contribute to the release and differentiation of endothelial precursor stem cells from the bone marrow [112]. Thus MMP9 has been suggested to promote angiogenesis through a variety of different mechanisms but more evidence is required to pinpoint its exact role.

*1.4.3 MMP9 regulates cell adhesion protein E-cadherin*

In addition to the role of MMP9 in remodeling of the extracellular matrix, MMP9 can also regulate the bioavailability of cell adhesion molecule epithelial cadherin (E-cadherin) [113]. E-cadherin a transmembrane protein part of the

cadherin superfamily. E-cadherin enables the homotypic adhesion of cells to facilitate the organization of tissue which occurs during embryo development. Cadherins are connected to the actin cytoskeleton via catenins forming a complex referred to as the adherens junction [114]. During the process of cellular transition and invasion, E-cadherin has been observed to be downregulated [115,116]. E-cadherin also modulates cell differentiation by acting as a cell signaling receptors [117].

MMP9 has been observed to downregulate E-cadherin during the process of EMT and fibrosis as well as tumorigenesis. The decrease in E-cadherin levels has been attributed to evidence of ectodomain shedding via proteolytic processing. This results in release of a truncated soluble E-cadherin fragment which can participate in paracrine and autocrine signaling in the extracellular environment [118]. Loss of E-cadherin may also occur via upregulation of E-cadherin repressors such as in the process of EMT. Several studies of tumor metastasis have demonstrated an imbalance between the MMP9 and E-cadherin [119–121]. Studies in ovarian carcinoma cells, demonstrate that MMP9 induced ectodomain shedding of E-cadherin [122]. Blocking MMP9 resulted in reduction of soluble E-cadherin [113] and decreased cellular migration and invasion [123]. Cleavage of 120kDa E-cadherin produces an extracellular 80kDa fragment and an intracellullar 38kDa fragment [113,118].

Studies of fibrosis provide some insight into the proteolytic role of MMP9 in fragmentation of E-cadherin. Fragmented E-cadherin was produced in response

to MMP9 and this resulted in Slug induced EMT in kidney tubular epithelial cells. TGFB alone also increased levels of fragmented E-cadherin. Inhibiting MMP activity during TGFB induced EMT resulted in increased levels of E-cadherin [124]. In lens fibrosis, MMP2 and 9 expression is increased in rodent lenses exposed to TGFB and a 72kDa E-cadherin fragment was also observed. Inhibition of MMP2 and 9 suppressed fragmentation of E-cadherin and reduced cataract formation [125].

*1.4.4 Cleavage of E-cadherin releases b-catenin*

The cleavage of E-cadherin by MMP9 results in the release of b-catenin from the adherens junction. The adherens junction connects the plasma membranes of two adjacent cells and is comprised of the cytoplasmic domain of E-cadherin which is directly bound to b-catenin. b-catenin is bound to a-catenin which attaches to the actin filaments allowing for anchoring to the cytoskeleton [126]. In addition to regulating cell adhesion, b-catenin is also the main signaling mediator of the WNT pathway involved in controlling cellular proliferation and differentiation. Therefore, the adherens junction sequesters b-catenin at the plasma membrane preventing it from translocating to the nucleus. However, if E-cadherin is cleaved, b-catenin is released to the cytoplasm can potentially translocate to the nucleus to upregulate transcription of target genes [118]. The loss of E-cadherin has been established as one of the main determinants of TGFB induced EMT and fibrosis. TGFB has also been involved in increasing b-catenin concentrations in the nucleus of tubular cells [127,128].

Therefore, the loss of E-cadherin and the upregulation of b-catenin are two major events observed in the development of fibrosis. Decreased expression of E-cadherin and translocation of b-catenin to the cytoplasm were both observed in patients with glomerulophepritis and diabetic and chronic allograft nephropathies [96,124,129,130]. Similar results come from patients with fibrosis in the lung and liver further suggesting the disassembly of the adherens junction during fibrogenesis [131–133]. Furthermore, Orsulic et al. demonstrate the ability of E-cadherin to sequester b-catenin at the cell membrane. The loss of E-cadherin results in nuclear transport of b-catenin and its association with transcription factor LEF in vitro [134]. In normal kidney epithelial cells, TGFB appears to mediate the disassociation of b-catenin from the adherens junction and stabilizes b-catenin in the cytoplasm [127,128,135]. b-catenin upregulates essential genes involved in EMT. During EMT, E-cadherin levels decrease resulting in the accumulation of b-catenin in the cytoplasm [124]. Therefore, the interceullular adherens junction indirectly regulates WNT signaling [124].

## 1.5 Canonical WNT/b-catenin Signaling

The WNT signaling pathway is an evolutionarily conserved pathway originally identified in drosophila. The human genome consists of 19 WNT genes that are involved in regulating cellular proliferation and differentiation and cell polarity during development and homeostasis. There are 10 frizzled (FZD) proteins that have been observed to act as receptors for WNT ligands and 2 co-receptor proteins termed lipoprotein receptor proteins (LRP) 5 & 6 [136]. This

signaling pathway is divided into the canonical WNT pathway, which is the b-catenin dependent pathway and the non-canonical pathway which is independent of b-catenin signaling. The non-canonical pathway can be further subdivided into the planar cell polarity pathway and the WNT/calcium pathway [137]. The effect of a specific WNT to activate b-catenin or non-canonical signaling is dependent on its receptor complement [138][139]. The FZD/LRP5/6 co-receptor model suggests that interaction of WNT with FZD and co-receptor LRP5/6 will activate the b-catenin pathway [140]. Some evidence suggest that LRP6 antagonizes the non-canonical WNT pathway [141]. Other WNT receptors such as RYK and Ror2 block WNT/b-catenin signaling [139,142]. Studies have attempted to assign the WNT ligands to canonical or non-canonical signaling ligands, however many of the WNT proteins have been observed to have overlapping roles. Nonetheless, WNT1, 3A, 8 and 8B are thought to be associated with canonical WNT signaling and WNT4, 5a and 11 are a component of noncanonical WNT signaling in most settings [142].

In the absence of canonical WNT signaling, b-catenin is degraded by complex of proteins including glycogen synthase kinase 3B (GSK3B). GSK3B is responsible for the phosphorylation of b-catenin which mark it for ubiquitination and degradation. In the absence of b-catenin, lymphocyte enhancer binding factor 1 (LEF1) and T cell factor (TCF) inhibit the transcription of WNT genes (Figure 1.5A) [136,143]. WNT binds to its frizzled receptor and co-receptors LRP5/6 to activate WNT/b-catenin signaling. The binding of WNT to frizzled results in the

activation of dishevelled (DVL) and the phosphorylation and inhibition of GSK3B. Disruption of the destruction complex allows for the accumulation of b-catenin in the cytoplasm and translocation to the nucleus. b-catenin binds to LEF1 and TCF to reduce their repressor activity to initiate the transcription of target genes (Figure 1.5B) [136].

The WNT/b-catenin signaling pathway is antagonized by a number of secreted protein families including dickkopf WNT signaling pathway inhibitor (DKK-1), secreted frizzled-related proteins (SFRP) and WNT inhibitory protein (WIF). SFRPs bind to WNT and frizzled to antagonize both canonical and non-canonical WNT signaling. [144]. DKK-1 binds to LRP5/6 to antagonize WNT/b-catenin signaling [136].

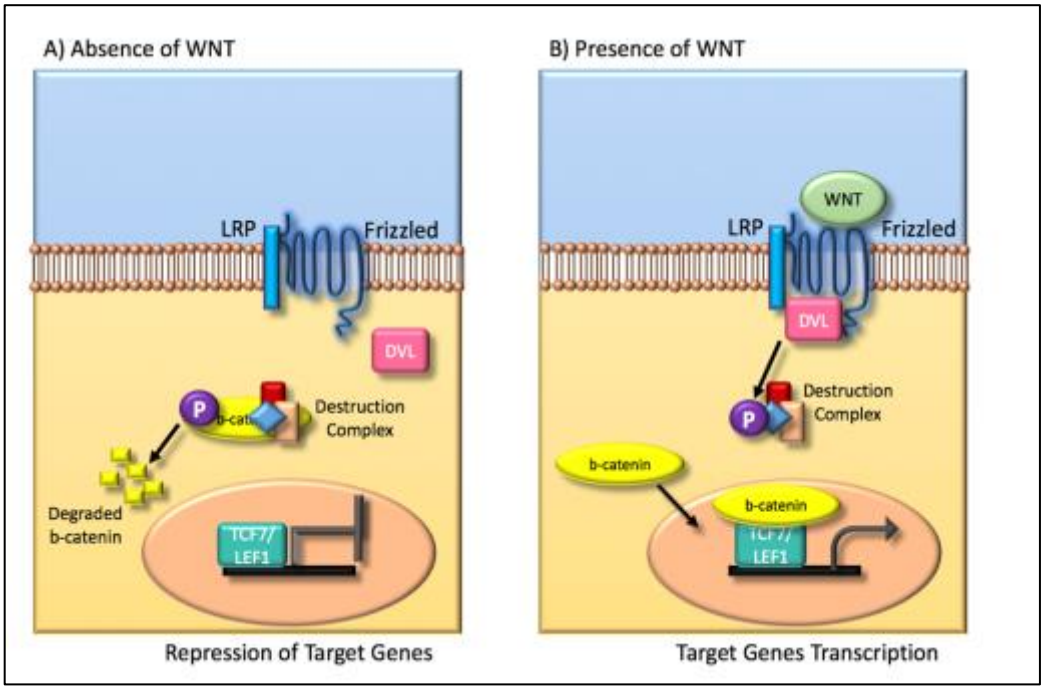


**Figure 1.5 The canonical WNT signaling pathway -** This pathway regulates levels of cytoplasmic b-catenin. A) In the absence of WNT, b-catenin signaling is turned off. GSK3B is a major component of the destruction complex and phosphorylates b-catenin marking it for degradation. In the absence of b-catenin, TCF/LEF repress target gene transcription. B) During active WNT signaling, WNT binds to FZD receptor and activates DVL. DVL phosphorylates GSK3B and inactivates the destruction complex resulting in stabilization of b-catenin. b-catenin translocates to the nucleus and binds to TCF/LEF resulting in upregulation of target genes.

*1.5.1 The role of WNT signaling in EMT and fibrosis*

Existing evidence from studies of tumorigenesis and development have confirmed the involvement of WNT/b-catenin signaling in inducing EMT, however the role of this pathway in the process of fibrogenesis requires further investigation. The first account of WNT signaling in fibrosis reported induced WNT4 expression in tubulointersititial fibrosis with high levels of expression observed in collagen 1 and a-SMA positive interstitial fibroblasts. WNT4 stabilized cytosolic b-catenin in myofibroblast cultures. WNT4 positive fibroblasts were transferred into the normal kidney resulting in accumulation of interstitial cells and disruption of the tubular epithelium [145]. Since then several studies have profiled WNT gene expression to better characterize the function of this pathway in the development fibrotic diseases. Studies of idiopathic pulmonary fibrosis [146,147], systemic sclerosis [148] and renal fibrosis [149,150] have demonstrated an upregulation of WNT related ligands and receptors. In particular, WNT1 and 4 have been consistently upregulated during fibrogenesis of the skin [151], lung [152], and kidney [145]. Furthermore, nuclear

accumulation of b-catenin and elevated expression of b-catenin target genes have been observed during pathogenesis of EMT and fibrosis suggesting an activation of this pathway [149,150,153].

Overexpression studies in vitro and in vivo have shed some light on the exact role of WNT/b-catenin signaling in fibrosis. Stabilization of b-catenin resulted in dermal fibrosis with a marked increase in number of myofibroblasts. Studies on inhibition of the WNT/b-catenin pathway have reinforced its role in EMT fibrosis and angiogenesis. Delivery of DKK-1 resulted in reduction in fibrosis and downregulated b-catenin in the kidney [149]. Treatment with an inhibitor of b-catenin (ICG-001) has been successful in reducing pulmonary [154], lens [155], and renal EMT and fibrosis [156]. ICG-001 specifically disrupts b-catenin mediated gene transcription. ICG-001 competes with b-catenin for binding to CBP and prevents b-catenin/CBP complex formation, thereby inhibiting b-catenin mediated gene expression [154]. There is limited evidence of the role of the WNT signaling pathway in the setting of peritoneal membrane injury. Levels of DKK-1 have been directly associated with fibroblast growth factor concentrations in the serum in patients undergoing PD [157]. A recent study explored the role of WNT signaling in EMT in the peritoneum. Administration of ICG-001 in a daily infusion model of peritoneal fibrosis resulted in reduced fibrosis and EMT both in vitro and in vivo. There was a marked reduction in submesothelial thickness and SNAIL1 with increased levels of E-cadherin [158].

*1.5.2 The role of WNT signaling in angiogenesis*

There is some evidence to support the role of WNT/b-catenin signaling in angiogenesis. WNT/b-catenin signaling regulates a number of different processes which are essential to angiogenesis including cell proliferation, branching morphogenesis, apoptosis and cell polarity [159]. Several studies have confirmed the expression of canonical WNT signaling in endothelial cells including a variety of WNT ligands and receptors [160] and this pathway was observed to be active in endothelial cells further suggesting its association with vascular development and remodeling [161]. Accumulating evidence has highlighted the importance of the WNT/b-catenin pathway in endothelial growth, differentiation and survival [159]. The canonical WNT/b-catenin pathway has been shown to enhance endothelial cell proliferation and WNT1 and WNT3a have been observed to induce proliferation and capillary stability in vitro. WNT5a on the other hand had opposing effects where it resulted in decreased cellular number and capillary length [162]. Studies of endothelial cell differentiation have demonstrated a role for WNT3A and WNT2 in promoting embryonic stem cells to differentiate into endothelial cells [163–166]. WNT5A was also observed to be required for endothelial cell differentiation by activating both b-catenin and protein kinase C [167]. More importantly, WNT/b-catenin signaling has also been demonstrated in regulating blood vessel assembly by controlling levels of VEGF. In studies of cancer, b-catenin stimulates tumor angiogenesis by upregulating VEGF [160,168,169] and therefore may be implicated in mediating blood vessel

sprouting. VEGF is also confirmed to be a downstream target of b-catenin signaling [160]. Intriguing evidence from studies of vascular disorders of the eye have also shown active canonical WNT signaling to be important in angiogenesis [168]. Norrin is an agonist for WNT/b-catenin signaling and has been implicated in governing angiogenesis in the retina by signaling through FZD4. Deficiencies in both Norrin and FZD4 have led to abnormal angiogenesis of the retina referred to as Norrie disease [159]. Collectively these studies have suggested the importance of WNT in different stages of vessel development, however further exploration of the exact role is still necessary [160].

### *1.5.3 The interaction between WNT signaling and TGFB during injury*

Expression of both TGFB and WNT/b-catenin has been confirmed in the development of EMT and fibrosis of different organs and the convergence of these pathways has been suggested [150]. Current studies have demonstrated some reciprocal transregulation between both pathways where TGFB controls components of the WNT/b-catenin pathway and vice versa [143]. In vitro studies of fibrosis suggest that b-catenin expression is dependent on TGFB [170] and TGFB can directly activate WNT/b-catenin signaling resulting in the accumulation of nuclear b-catenin [171]. Interaction of the TGFB and the WNT pathway at the nuclear level has been reported in recent studies of fibrosis highlighting the importance of SMAD3. Communication between the TGFB/SMAD3 pathway and b-catenin was observed to induce EMT in pulmonary fibrosis [171]. This interaction was confirmed in a study of dermal fibrosis where SMAD3 induced

b-catenin activity in the absence of TGFB [172]. In studies of EMT, TGFB has been shown to regulate transcription of LEF which complexes with SMAD2 and 4 to promote EMT by reducing E-cadherin [173]. EMT related genes SNAIL1 and SNAI2 have stimulated b-catenin /TCF complexes to induce TGFB resulting in EMT [174].

## 1.6 The Non-canonical WNT Signaling Pathway

Non-canonical WNT signaling involves two different signaling pathways independent of b-catenin including the planar cell polarity (PCP) pathway and the WNT/calcium pathway. The PCP pathway regulates rearrangement of the cytoskeleton important for cell polarization and migration during gastrulation. The PCP pathway involves signaling through non-canonical WNT proteins in concert with FZD independent of LRP5/6 resulting in the activation of small GTPases of the Rho family to modify the cytoskeleton. The WNT/$Ca^{2+}$ pathway uses FZD mediated signaling to release intracellular stores of calcium and activate genes regulating cell fate and migration [175]. This pathway is active during tumorigenesis, inflammation and has also been observed in the setting of skin and liver fibrosis [176]

### *1.6.1 Prototypical non-canonical WNT5A*

Classification of WNT ligands into canonical vs non-canonical is an area of continuous debate and much of the literature suggests that signaling is dependent on biological context. However, accumulating evidence has shown WNT5A to predominantly signal independently of b-catenin [177]. WNT5A has been

established to play a role in cell polarity via the planar cell polarity pathway in animal models of development (Figure 1.6A) [178]. Further investigation is required to determine the exact molecular mechanism by which WNT5A is signaling [178]. WNT5A also regulates intracellular levels of calcium by signaling through calmodulin-dependent protein kinase II (Figure 1.6B). This pathway is poorly understood, however WNT5A is observed to induce the activation of membrane associated calcium channels resulting in the influx of calcium. This influx results in signaling through CamkII and Protein Kinase C has been shown to inhibit b- catenin activity [177].

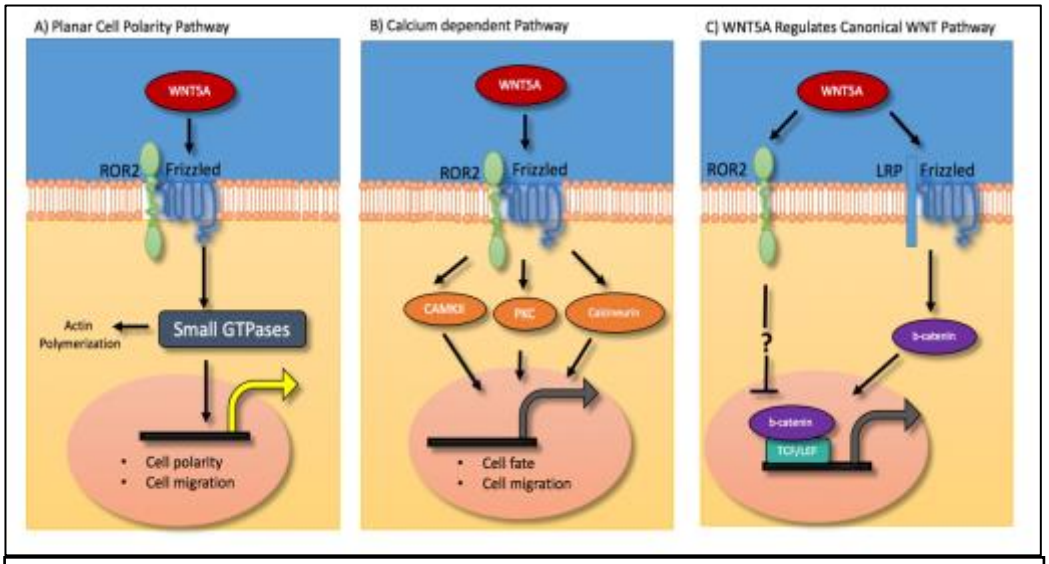



**Figure 1.6 The non-canonical WNT5A signaling pathway** - A) WNT5A binds to FZD/Ror2 to activate small GTPases leading to actin polymerization. These GTPases go on to regulate cell polarity and migration. B) WNT5A signals through FZD/Ror2 to induce PKC, CAMKII and Calcineurin. Calcineurin regulates genes controlling cell fate and migration. This pathway is not well understood. C) WNT5A signals through Ror2 to inhibit b-catenin activity. The mechanism behind this requires further investigation. WNT5A can also activate canonical WNT signaling through FZD/LRP resulting in b-catenin stabilization and stimulation of transcription.

In studies of tumorigenesis, WNT5A has been implicated to work as a tumor suppressor protecting against tumor progression but also as an oncogenic factor involved in metastasis [179]. Levels of WNT5A are significantly reduced in aggressive tumor cells in human breast cancer, melanoma and in cases of colorectal cancer and is implicated as a tumour suppressor [180,181]. WNT5A is suggested to inhibit colon cancer by suppressing cellular proliferation and attenuating epithelial to mesenchymal transition. On the other hand, WNT5A has been considered to act as a proto-oncogene in breast cancer, prostate cancer, pancreatic cancer and also in melanoma. Its expression is highly correlated with invasion and metastasis [182]. WNT5A has also been active in regulating vascular development in development as well [166].

There is also conflicting evidence describing the exact role of WNT5A in wound healing and the pathogenesis of fibrosis. In vitro studies of wound repair demonstrate that WNT5A contributes to the wound healing response by enhancing migration [183]. Contrary to this, there have been some accounts of WNT5A alone delaying migration and in the presence of FZD1 [184]. Accumulating evidence has characterized the expression of WNT5A in fibrosis. WNT5A was reported to be upregulated in sarcoidosis [185], liver fibrosis [176,186], lung fibrosis and acute lung injury [187–189]. Much of the evidence of the functional role of WNT5A come from in vitro studies of fibrosis or inflammatory diseases. Vuga et al showed that treatment of lung fibroblasts with WNT5A resulted in increased proliferation and prevented apoptosis. These effects

were mediated independently of b-catenin and WNT5A actually induced a decrease in b-catenin in these cells. WNT5A also resulted in increased levels of fibronectin in normal lung fibroblasts [189]. WNT5A was observed to interact with TGFB and other WNT ligands to induce ECM production by airway smooth muscle cells in a recent study of asthma [190]. Opposing results were observed in cardiac fibroblasts where WNT5A expression was associated with decreased my fibroblast markers in the presence of FZD1. In the presence of FZD2, WNT5A stimulated differentiation and an increase in expression of myofibroblast markers [184]. WNT5A did not increase activity of b-catenin in these cells [184]. The inconsistencies of these studies underline the complexity of WNT5A signaling and demonstrate its differential roles based on the receptor that is present.

*1.6.2 The interaction between WNT5A and Ror2*

Non-canonical WNT5A can signal via the FZD receptors 1- 5, however its co-receptors are not well defined and require further investigation. Receptor tyrosine kinase like orphan receptor 2 (Ror2) has been shown to be potential candidate receptor interacting with WNT5A during developmental processes [191]. Ror2 is part of the receptor tyrosine kinase superfamily (RTK) and is known to regulate cell migration, cellular proliferation and differentiation. WNT5A and Ror2 null mice exhibit similar phenotypes. Ror2 null mice have been observed to harbor deformities in skeletal development as well as abnormalities in the heart and lungs and do not survive beyond the perinatal period [183,192].

WNT5A and Ror2 have been demonstrated to interact physically and also functionally resulting in activation of the PCP and calcium pathway during developmental processes [193]. In the setting of wound healing and fibrosis, the protective effects of WNT5A have been attributed to its interaction with co-receptor Ror2. Previous reports show that WNT5A/Ror2 signaling is active during wound healing promoting rearrangement of microtubules and actin to promote cellular migration [183]. In vitro wound healing assays show that Ror2 mediates polarized cell migration during WNT5A induced wound closure and this is impaired when Ror2 is suppressed in cells [183]. Few studies have examined both WNT5A and Ror2 in the setting of fibrosis. In a study of renal fibrosis, WNT5A and Ror2 expression was induced following unilateral ureteral obstruction [194]. Expression of Ror2 was identified in tubular epithelial cells which co-expressed EMT related proteins such as SNAIL1 and MMP2. This study only characterizes the expression profile of WNT5A and Ror2, and therefore a follow up study is required to determine the exact effect of WNT5A/Ror2 signaling in renal fibrosis [194].

### *1.6.3 WNT5A/Ror2 Inhibits WNT/b-catenin Signaling*

WNT5A has been reported to have both antagonistic [195] and agonistic effects on the WNT/b-catenin pathway. The differential effects have been proposed to be dictated by the cell surface receptor. In studies of cancer, WNT5A has been observed to act as a tumor suppressor by antagonizing WNT/b-catenin signaling in the presence of Ror2 [180]. In vitro studies provide evidence of the

interaction between Ror2 and WNT5a in inhibiting WNT/b-catenin signaling. WNT5A potentiates WNT/b-catenin signaling by activating TCF in the presence of FZD4 [142,196,197]. These results suggest WNT5A regulates the WNT/b-catenin signaling pathway based on the cellular context and the receptor which is present. We hypothesize that WNT5A may antagonize WNT/b-catenin signaling through Ror2 and perhaps protect against injury. The mechanism by which WNT5A/Ror2 signaling attenuates WNT/b-catenin signaling requires further investigation. WNT5A has been proposed to downregulate WNT/b-catenin signaling by mediating the phosphorylation of TCF or by inhibiting co-activators such as p300 and CREB-binding protein. The latter hypothesis involves the interaction between WNT5A and Ror2 to downregulate b-catenin [197]. It has also been proposed that WNT5A may work independently of Ror2 by promoting the ubiquitination of b-catenin or it may antagonize the interaction between canonical WNT ligands and respective receptors [197]. Studies that have demonstrated the inhibitory effect of WNT5A on b-catenin signaling need to investigate the importance of Ror2 in mediated this effect. The exact role of WNT5A on canonical WNT/b-catenin signaling in peritoneal membrane injury has not yet been characterized.

# CHAPTER 2: HYPOTHESIS AND AIMS

## Purpose:

To investigate the mechanism by which peritoneal angiogenesis occurs.

## Aims

1. Determine the role of MMP9 in development of peritoneal angiogenesis.
2. Investigate the contribution of canonical WNT signaling to TGFB induced peritoneal angiogenesis.
3. Examine the role of non-canonical WNT5A and Ror2 in protecting against peritoneal angiogenesis.

## Hypothesis

We hypothesize that MMP9 is involved in peritoneal angiogenesis by cleavage of E-cadherin signaling through b-catenin to upregulate VEGF. We also hypothesize that canonical WNT signaling interacts with TGFB signaling to induce peritoneal angiogenesis and blocking this pathway may reduce injury. We hypothesize that non-canonical WNT5A may protect against peritoneal membrane injury and Ror2 mediates these effects.

# CHAPTER 3: EXPERIMENTAL SYSTEMS TO STUDY THE ORIGIN OF THE MYOFIBROBLAST IN PERTIONEAL FIBROSIS

Authors: Manreet Padwal, Peter Margetts

Corresponding Author: Peter Margetts, Division of Nephrology, St. Joseph's Hospital, Department of Medicine, McMaster University, Hamilton, ON, Canada L8P 4A6. Phone: (905) 525-9140 ext 32299, Fax: 905-540-6589, Email: margetts@mcmaster.ca

Citation: Padwal M, Margetts PJ. Experimental systems to study the origin of the myofibroblast in peritoneal fibrosis. *Kidney Research and Clinical Practice*. 2016;35(3):133-141. doi:10.1016/j.krcp.2016.07.003.

## PREFACE

The myofibroblast is the primary effector cell initiating peritoneal fibrosis and angiogenesis. In response to TGFB, this cell secretes extracellular matrix and proangiogenic factors during injury. The origin of the myofibroblast in peritoneal membrane injury is under current debate and is an area of active research. The myofibroblast has been proposed to come from resident fibroblasts, epithelial to mesenchymal transition, endothelial cells, perivascular cells and bone marrow. In previous research of peritoneal membrane injury, the myofibroblast has been suggested to derive from EMT. Experimental methods such as immunofluorescence have been commonly used to identify cells expressing both epithelial and mesenchymal markers. These studies have provided a foundation for our understanding of EMT, however this only provides a snapshot of this process. EMT has been recently challenged by lineage tracing studies. Because EMT is a dynamic process, this powerful technique can visualize the origin of the cell with molecular precision. These studies have suggested that the myofibroblast does not derive from epithelial cells, but instead comes from resident fibroblasts in the submesothelial zone. In this Chapter, we evaluate current experimental methods used to study the origin of the myofibroblast during peritoneal membrane injury and emphasize the importance of combining various methods to better understand the role of the mesothelium in this process.

The concept of this Review was developed by Dr. Peter Margetts and Manreet Padwal. Manreet Padwal completed a systematic review of current

literature. This involved an evaluation of experimental systems currently being used to study the origin of the myofibroblast in fibrosis. Manreet Padwal wrote the original draft of the manuscript and edited the manuscript based on reviewers' comments. Dr. Peter Margetts edited and revised the final manuscript.

**ABSTRACT**

Peritoneal fibrosis is one of the major complications occurring in long term peritoneal dialysis patients as a result of injury. Peritoneal fibrosis is characterized by submesothelial thickening and fibrosis which is associated with a decline in peritoneal membrane function. The myofibroblast has been identified as the key player involved in the development and progression of peritoneal fibrosis. Activation of the myofibroblast is correlated with expansion of the extracellular matrix and changes in peritoneal membrane integrity. Over the years, epithelial to mesenchymal transition (EMT) has been accepted as the predominant source of the myofibroblast. Peritoneal mesothelial cells have been described to undergo EMT in response to injury. Several animal and *in vitro* studies support the role of EMT in peritoneal fibrosis, however emerging evidence from genetic fate mapping studies has demonstrated myofibroblasts may be coming from resident fibroblasts and or pericytes/perivascular fibroblasts. In this review, we will discuss hypotheses currently surrounding the origin of the myofibroblast and highlight the experimental systems predominantly being used to investigate this.

## Introduction

Peritoneal dialysis (PD) is a widely used renal replacement therapy for patients with end stage renal disease [1;2]. The healthy peritoneum is a semipermeable membrane consisting of a superficial mesothelial layer, a basement membrane, and a thin submesothelial zone (Figure 3.1A) [3]. Long-term exposure to bioincompatible dialysis solutions, uremia [4], and intermittent peritonitis [5] leads to a low grade chronic inflammation triggering a reparative response. This response normally provides restoration of tissue structure with minimal loss of function. However, this reparative mechanism may become dysregulated. This results in tissue fibrosis with deposition of extracellular matrix (ECM), angiogenesis, and eventual membrane failure (Figure 3.1B). The appearance of myofibroblasts in the submesothelial tissue is a critical component of peritoneal membrane fibrosis and angiogenesis [6].

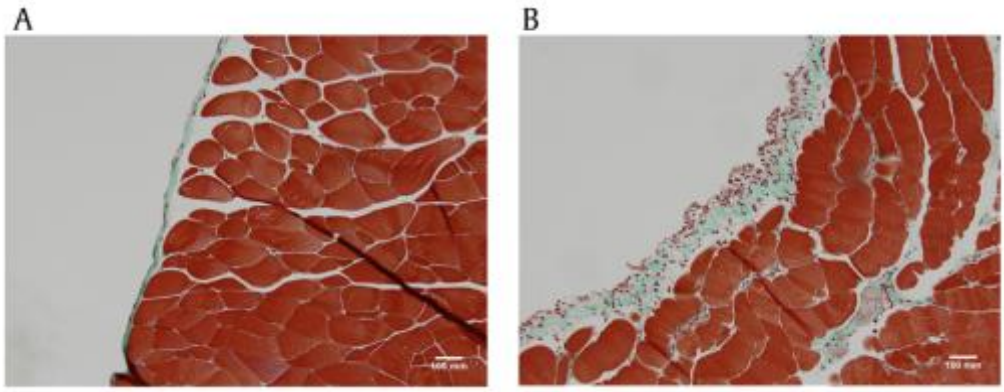



**Figure 3.1 Histology of the peritoneal membrane -** (A) Healthy mouse peritoneum taken from the anterior adbominal wall consists of superficial mesothelial layer and a thin submesothelial zone. (B) Exposure to an adenovirus expressing transforming growth factor beta results in submesothelial thickening, angiogenesis, and increased cell number in the peritoneum.

**The Myofibroblast in Fibrosis**

The myofibroblast is a specialized contractile cell that expresses both fibroblast and smooth muscle cell-like characteristics [7]. Myofibroblasts are motile cells and therefore possess stress fibers and ruffled cell membranes due to changes in the actin cytoskeleton [8]. Stress fibers are composed of bundles of actin microfilaments and contractile proteins. Actin microfilaments terminate at the cell surface in a specialized adhesion complex termed the fibronexus that becomes linked to extracellular fibronectin fibrils [8]. This creates a mechano-transduction system that allows for transmission of force from stress fibers to the extracellular matrix. Similarly, mechanical signals from the extracellular environment can activate intracellular signaling pathways [9]. Expression of alpha smooth muscle actin (a-SMA) is a key marker that distinguishes the myofibroblast

from the fibroblast [10]. Although the function of the myofibroblast has not fully been elucidated, the myofibroblast has mostly been studied for its role in wound healing and its presence during pathological conditions such as fibrosis. Originally, myofibroblasts were observed in granulation tissue of healing wounds. They were observed to produce the contractile force required during wound contraction. More recently, several studies have identified the myofibroblast as an important element in the production of extracellular matrix (ECM) and progression of fibrosis [8;11].

In a healthy environment, stromal cells including fibroblasts and perivascular cells remain quiescent, producing little ECM and few actin interactions between cells and cell to matrix [9]. In response to injury, myofibroblasts accumulate at the site of injury where they produce ECM in response to cytokines from local cells [7;9]. Transforming growth factor beta (TGFB) is a key cytokine that regulates expression of a-SMA expression and type 1 collagen in myofibroblast [10;12]. Moreover, the differentiation process of the myofibroblast is largely regulated by TGFB. In addition to TGFB, myofibroblasts are also stimulated by changes in the mechanical microenvironment and high extracellular stress [8;9]. During normal conditions, the ECM maintains its crosslinked structure, however tissue injury results in remodeling of the ECM and changes in the mechanical environment. In response to this change in ECM structure and TGFB signaling, precursor cells begin to acquire contractile stress fibers [8]. During this time, myofibroblasts expresses some features that are

similar to fibroblasts such as actin stress fibers. This transitional cell has been characterized as the protomyofibroblast [9;13]. The protomyofibroblast begins to acquire more contractile activity with the expression of a-SMA in the stress fibers and can now be characterized as a myofibroblast. ECM stiffness is one promoter of myofibroblast maturation [13;14]. During the final stages of wound healing, the ECM regains its normal structure and the myofibroblasts undergo apoptosis [9;13].

The last component of the wound healing response involves replacement of injured cells with new cells and resolution of scar tissue to preserve functional and structural integrity of the organ [15]. However, this is not always the case depending on type and duration of injury as well as the severity of damage. Often, chronic injury may result in the inability to replace scar tissue with functional tissue resulting in irreversible fibrosis [15]. Therefore fibrosis is largely characterized as accumulation of ECM including collagen, proteoglycans and fibronectin [11]. The myofibroblast is the primary effector cell that is activated during this process [11]. Myofibroblast populations have been noted in abnormal wound healing in several vital organs such as the kidney, lung, liver, and heart [12;16;17].

Myofibroblasts have also emerged as the main effector cell contributing to peritoneal fibrosis. The presence of the myofibroblast contributes to the loss of integrity of the peritoneal membrane in long term PD patients by inducing collagen deposition resulting in structural and functional changes in the peritoneal

membrane [18]. In PD patients, the myofibroblast is dominant in the peritoneal membrane even before fibrosis is present in contrast to normal healthy peritoneum [19].

It is challenging to study stromal cells; fibroblasts do not express any specific markers resulting in difficulties distinguishing them from other cells [11]. Several studies have used FSP1 (also known as S100A4) as fibroblast specific marker [20]. Recent evidence demonstrates FSP1 is not specific to fibroblasts and is also expressed in other cells such monocytes [41]. Myofibroblast expression of a-SMA is increasingly being used as method of identifying the myofibroblast. However, this marker still presents some challenges as a-SMA is also expressed by other cells of the mesenchymal lineage such as vascular smooth muscle cells [11].

**Origin of the myofibroblast: Current Hypotheses**

As the myofibroblast is the key effector cell in progression of fibrosis, it is of scientific and practical interest to elucidate the origin of this cell. This is a controversial area of research. In various research studies, myofibroblasts have been shown to derive from different cellular sources including resident fibroblasts [9;17], epithelial cells via epithelial to mesenchymal transition [21], endothelial cells (endothelial to mesenchymal transition) [22], perivascular cells [23], and from bone-marrow derived cells [20;24]. Interestingly, the original hypothesis was that myofibroblasts arose from locally residing mesenchymal cells such as resident fibroblasts and

pericytes/perivascular fibroblasts [9;17] based on studies of dermal wound healing [13;25] (Figure 3.2a). This was also supported in models of fibrosis such as scleroderma [26], liver and renal fibrosis [13;27]. However this view quickly changed as a result of accumulating evidence in support of conversion of local epithelial cells via EMT [28] (Figure 3.2B). The first piece of evidence comes from studies in vitro where epithelial cells can be transformed into myofibroblasts by exposing cells to wound healing cytokines such as TGFB [28]. Secondly, evidence from experimental fibrosis in animal models has provided us with snapshots of molecular markers of EMT in vivo [29]. Myofibroblasts appear in the submesothelial zone in experimental models of peritoneal fibrosis [18] and have been observed in biopsy samples in PD patients with peritoneal fibrosis [30]. The origin of the myofibroblasts in peritoneal membrane injury remains an area of active research. Early studies, including our own work, have pointed to EMT as a source of submesothelial myofibroblasts. More recently, these observations have been challenged by cell-fate tracing experiments [31]. There is also some experimental evidence that myofibroblasts can originate from the bone marrow and play a role in peritoneal fibrosis [20;24].

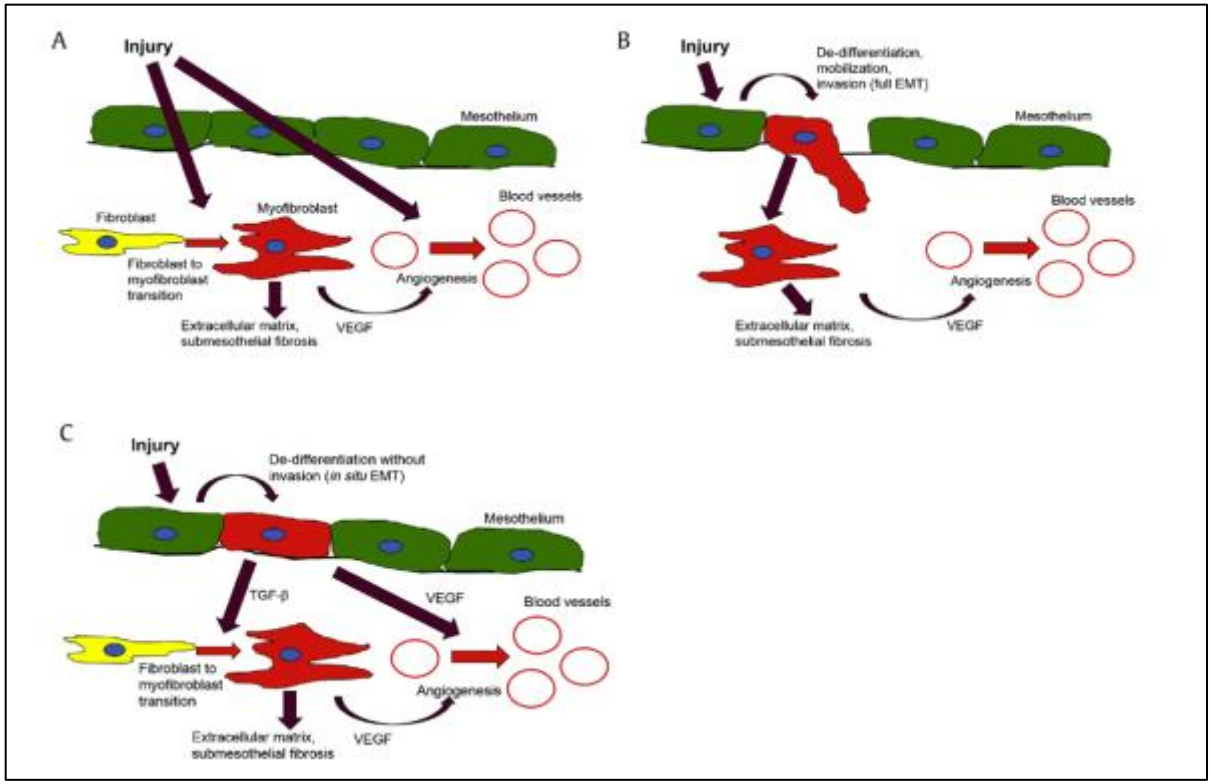



**Figure 3.2 Proposed theories on the origin of the myofibroblast, submesothelial fibrosis, and angiogenesis -** Peritoneal membrane injury occurs as a result of exposure to bioincompatible dialysate and intermittent inflammation or infection. (A) Although injury can damage the mesothelial cells, the mesothelium is not necessary for subsequent myofibroblast accumulation, submesothelial fibrosis, and angiogenesis, which is driven directly by the injury response. (B) In this model, full EMT occurs with injury inducing mesothelial transition and transitioned mesothelial cells migrate through the basement membrane into the submesothelial space and form a subset of the submesothelial myofibroblasts. These cells then drive submesothelial fibrosis and angiogenesis. (C) In this model, mesothelial transition occurs without migration (in situ EMT). These transitioned mesothelial cells are an essential source of growth factors (TGFB and VEGF) that then drive submesothelial fibrosis and angiogenesis.

**In vitro and ex vivo evidence for EMT**

The study of the origins of the myofibroblast began in vitro and moved into experimental animal models. Initial evidence supporting EMT comes from studies in vitro where epithelial cells have been induced to transform into myofibroblasts during conditions of injury [32;33]. This process has been extensively studied in renal fibrosis where primary tubular epithelial cells exposed to TGFB undergo EMT [34].

These studies have been fundamental in our understanding of the EMT process by defining the basic markers used to characterize EMT and the signaling pathways involved during this process [29]. EMT can be considered as a two-step process, the primary step being cellular transition followed by invasion into the interstitial tissue (Figure 3.2B) [28]. In vitro studies have outlined the process of cellular transition, but in standard 2 dimensional cell culture, cellular invasion has been more difficult to study; EMT is characterized by the loss of cell adhesion through downregulation of intercellular adhesion molecules such as E-cadherin, loss of cell polarity, rearrangement of the cytoskeleton with increased expression of a-SMA, and acquisition of migratory behavior (Figure 3.2B) [28;29]. During the last stage of EMT, the basement membrane can be degraded and the mesenchymal cell can migrate into the interstitial layers and deposit collagen resulting in fibrosis (Figure 3.2B). Metalloproteinases (MMPs) are usually

thought to be involved in degradation of the basement membrane and may facilitate invasion of transitioned cells [28;35].

The EMT cellular program is driven by initiating factors such as hypoxia, inflammation, or growth factors such as TGFB. These activate transcriptional regulators such as SNAIL1, ZEB, or TWIST [36]. These transcriptional regulators then initiate a genetic program that leads to the EMT phenotype [36].

Studies in vitro have been useful because they can demonstrate the transitional aspect of EMT by measuring cell movement and combining this with changes in cell phenotype [37]. Matrigel is a common substrate used to measure cellular invasion and studies have used this as a method of measuring cell movement [38]. However, cross-linked, mature basement membrane may not be susceptible to protease activity as is matrigel [39]. Similarly, collagen invasion assays have been used to study cell invasion against laminin, collagen 1 and collagen IV. However, in vivo, the triple helical structure of collagen is resistant to proteolytic attack [39]. Therefore analysis of invasive EMT is difficult to detect in vivo and investigators usually have to rely on recognition of certain markers that reflect transition [37].

EMT was first demonstrated in the human peritoneum after dialysis in a landmark paper in 2003 by Yanez-Mo and colleagues [40]. An ex vivo culture of mesothelial cells was developed from peritoneal effluent from patients on PD. They found that the cell morphology could be classified as epithelioid like or non-epithelioid like. The epithelioid cells demonstrated the usual markers such as

cytokeratin, E-cadherin and intracellular adhesion molecule 1. There was decreased E-cadherin and cytokeratin expression in cells with a fibroblast phenotype compared to those with a more epithelioid phenotype [40]. The epithelial markers also declined with time spent on PD. The gene expression of SNAIL1 was increased in transitioning and fibroblast like cells grown from peritoneal effluent. Confluent layers of mesothelial cells were also mechanically wounded and fibroblastic cells appeared in the layer of migrating cells near the wound. Cultured cells treated with TGFB displayed decreased amounts of E-cadherin and increased levels of SNAIL1. Biopsy specimen taken from 9 PD patients confirmed the results from the ex vivo mesothelial culture [40]. These results have been confirmed by a similar study of mesothelial cells also derived from peritoneal effluent [2]. These studies strongly suggest that myofibroblasts may originate from injured mesothelial cells undergoing epithelial to mesenchymal transition.

These ex-vivo cell based experimental results have been questioned by recent work in a rodent model of dialysate exposure [41]. Cho and colleagues found that the evaluation of EMT markers in the cells grown from the peritoneum of rats treated with PD solutions did not correlate well with the EMT observed in the peritoneal tissues [41]. Whether these results in a rat model can be applied to patients on PD is still an open question.

**Animal models to study peritoneal membrane injury**

Experimental animal models can recreate aspects of a pathophysiological condition and can thus help to identify mechanisms of disease and identify targets for therapeutic intervention. The extent to which an animal model of human disease actually simulates the disease state is always open to criticism [42].

Different animal models have been developed to study the underlying challenges in peritoneal dialysis including viability of the peritoneal membrane and biocompatibility of dialysis solutions [43]. Experimental animal models give us the ability to study mechanisms underlying peritoneal membrane injury and could lead to improvements in current techniques. Historically, investigators have used rat or rabbit to model PD generating acute and chronic models of PD. In a standard acute model of PD, fluid is infused by intraperitoneal injection or a temporary catheter into the peritoneal cavity in mice, rats, rabbit and sheep [44;45]. This model has been important in understanding properties of solute transport across the peritoneal membrane, changes in the interstitial matrix, and changes in attachment and morphology of mesothelial cells during injury [46-48]. The chronic infusion model of PD uses a peritoneal catheter implanted into rodents which then receive a daily or twice daily infusion of dialysis fluid for a span of 4-20 weeks [49]. Although this model closely mimics the progression of peritoneal membrane injury, the indwelling catheter has been shown to amplify the inflammatory response via a foreign body reaction [50].

The model used extensively in our lab and others involves gene transfer of TGFB into the rodent peritoneum [51]. TGFB is one of the major mediators of peritoneal fibrosis and angiogenesis and can be delivered to the peritoneum to model this condition. Adenovirus mediated gene transfer of TGFB has been used to model peritoneal fibrosis in mice [51] and rats [52]. This model has demonstrated changes in the functionality and structure of the peritoneum similar to what is seen in patients on long term PD. We have also a helper dependent adenovirus expressing TGFB to induce prolonged expression of the transgene and this resulted in changes in the peritoneal structure as observed in encapsulated peritoneal sclerosis (EPS) [53]. The helper dependent adenovirus allows for longer duration and lower expression of transgene with an attenuated immune response [53].

EPS is a rare disease that occurs in a small minority of PD patients [53]. Peritoneal fibrosis may be an underlying factor in development of EPS and therefore the study of EPS can also provide clues to understanding peritoneal fibrosis. One of the most commonly used agent to model EPS in animals is chlorohexidine gluconate [54]. Chlorohexidine was initially used as a topical antiseptic agent to prevent peritonitis but became associated with an increased risk of EPS and is now used as an experimental model of EPS. Daily intraperitoneal injections of chlorohexidine gluconate can induce peritoneal fibrosis and angiogenesis in mice and rats [55].

**Animal models and EMT**

Several animal models of peritoneal membrane injury have been generated which support the hypothesis that the epithelium is a source of myofibroblasts. Supported by extensive in vitro data for EMT, researchers have studied common peritoneal membrane injury models to confirm EMT in vivo [2;18]. The identification of in vivo EMT involves use of surrogate markers to identify changes in expression of epithelial markers, cell to cell adhesion molecules, cytoskeletal changes, and altered regulation of transcription factors associated with EMT [29;56]. Some of the major and most common markers used to identify EMT include loss of epithelial markers including E-cadherin and cytokeratin and gain of mesenchymal markers such as a-SMA and SNAIL1 [56]. The most common method that has been used to provide better visualization of the EMT process is immunofluorescence analysis of epithelial and mesenchymal markers [29]. Cells undergoing transition can then be identified by analyzing the co-localization of both epithelial and mesenchymal markers in cells in the peritoneal membrane [56]. However, these studies have received some criticism as static markers of EMT are commonly used. Changes in these markers may not be representative of EMT as EMT is a more transitory process [29]. Detecting EMT in vivo has presented several limitations. As mentioned previously, there are no specific markers for myofibroblasts, so immunohistochemistry is confusing due to non-specificity [56]. EMT is a reversible process so there may be stages where cells revert to their parental phenotype and lose their mesenchymal characteristics

[56]. There are also more advanced stages where epithelial cell characteristics are lost completely. Therefore, the detection of transitioning cells critically depends on the timing of detection after peritoneal membrane injury [56]. Detection of intermediate stages of EMT in injured tissue is a simple method that has been used in the past to identify transitioning cells in vivo [56]. Intermediate stages of EMT are characterized by cells expressing epithelial characteristics such as cytokeratin and E-cadherin but have also acquired mesenchymal markers such as a-SMA. Therefore these cells co-express epithelial and mesenchymal features at the same time [21] (Figure 3.3).

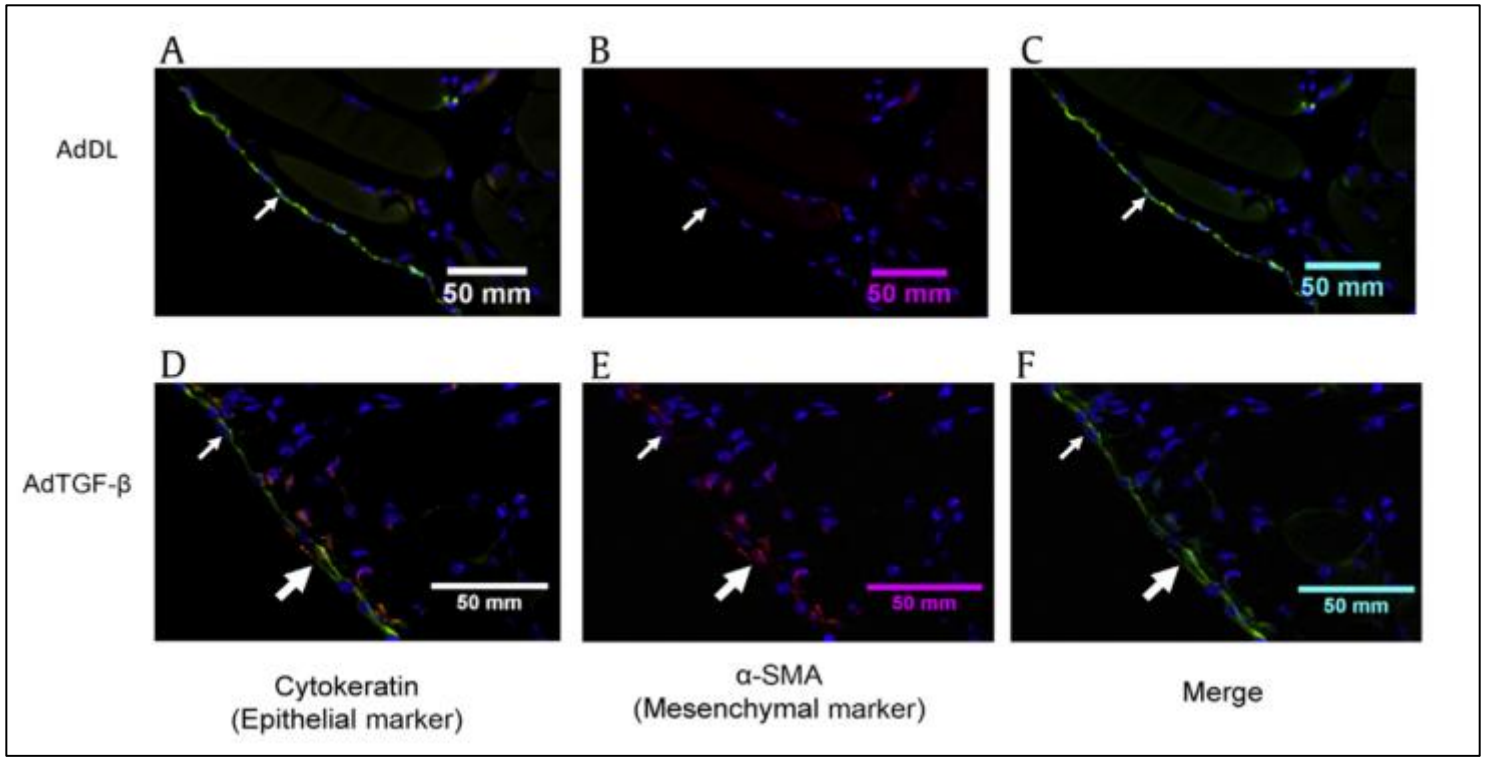



**Figure 3.3 EMT demonstrated by immunofluorescent labeling of the epithelial marker, cytokeratin, and the mesenchymal marker, a-SMA -** (A-C) Mouse peritoneum treated with control adenovirus (AdDL) does not display any dual-labeled cells and displays epithelial cells only (thin arrows). (D-F) Treatment of mouse peritoneum with AdTGF-b results in dual-labeled cells co-expressing epithelial (cytokeratin) and mesenchymal markers (a-SMA) (thick arrows). Some cells continue expressing cytokeratin and do not express a-SMA (thin arrows).

Aroeira et al. used these immunohistologic methods to identify EMT in vivo using an animal model of exposure to glucose based PD fluids to induce peritoneal fibrosis and angiogenesis over a period of 5 weeks [2]. This exposure was accompanied by a failure in ultrafiltration. After two weeks of PD fluid exposure, there was a loss of mesothelial cells from the peritoneum. This closely mimics the structural changes that occur in patients on PD [2]. Immunofluorescence staining for both an epithelial marker, cytokeratin, and the myofibroblast marker, a-SMA was used to identify mesothelial cells and myofibroblasts respectively. Dual-labelled cells expressing both cytokeratin and a-SMA were considered cells undergoing EMT in the intermediate stages. This analysis revealed that the loss of mesothelial cells from the peritoneal membrane surface coincided with the appearance of cells labelled with cytokeratin and a-SMA in the submesothelium [2]. We have also examined the process of EMT by combining analysis of EMT related genes and proteins along with immunofluorescence staining for EMT markers in our model of adenovirus mediated TGFB gene delivery (Figure 3.3). TGFB induces peritoneal fibrosis and angiogenesis in the rodent peritoneum creating a robust model of peritoneal membrane injury [18]. Rat peritoneum exposed to adenovirus expressing TGFB exhibited increased submesothelial thickness and angiogenesis. We observed an early increase in expression of SNAIL1, a-SMA, MMP2 and type 1 collagen. Laminin staining revealed a disruption of the basement membrane at an early time point. Dual staining revealed cells expressing both epithelial and myofibroblast

characteristics in the submesothelial layer [18]. In this study, along with other studies done in vivo, submesothelial a-SMA positive myofibroblasts without epithelial markings are of an unknown origin. They may be fully transformed epithelial cells, transformed fibroblasts or other cell types recruited locally or from the circulation. Therefore, these experiments suggest that at least some myofibroblasts in the submesothelial space may come from mesothelial cell conversion and supports the notion of EMT derived myofibroblasts.

The use of immunofluorescence labeling of epithelial and myofibroblast markers to identify cells undergoing EMT has been criticized. This provides only a static picture of the cellular response to injury and we have shown that this picture looks very different depending on the point of time in the injury response [34; 46]. Furthermore, there are criticisms of immunofluorescence studies including the non-specificity of antibodies used and image processing artifacts. Different models of peritoneal injury may yield different temporal patterns of damage and may thus make detection of EMT challenging. For example, the adenovirus mediated gene transfer model of TGFB yields a fairly rapid fibrogenic response, whereas daily exposure to PD fluids will lead to a more gradual onset of fibrosis and will likely be more difficult to detect EMT using dual immunofluorescence studies. Finally, different animal strains may have very different response to fibrogenic stimuli [36].

We have quantified the EMT response and find that very few cells express both mesenchymal and epithelial markers in the peritoneum after injury. In

general, we find only 1-2 cells per high power field to be dual labeled [51;57]. This could indicate that EMT is a rare event in peritoneal fibrosis, or that our 'snapshot' of peritoneal membrane injury captures only those cells in the process of transition, not those cells fully transitioned to a myofibroblast phenotype.

**Fate tracing experiments and evidence refuting EMT in peritoneal fibrosis**

An alternate method of visualizing the origin of cells in a complex injury response system has been developed using inducible genetic fate mapping. Emerging evidence from these studies sheds more light on the origin for the myofibroblast in tissue injury and throws into question the dominant role of EMT in the appearance of submesothelial myofibroblasts. Genetic fate mapping is a technique which allows researchers to detect the origin of cells with molecular precision [17;29]. Mammalian fate mapping studies have adopted the LoxP / Cre recombinase system. A mouse is constructed where a reporter molecule such as green or red fluorescent protein (GFP or RFP) is inserted in the genome. This reporter gene is silenced with an upstream STOP codon that is flanked by LoxP sites ('floxed')[17]. When this mouse is mated with a mouse that has a tissue specific promoter driving cre recombinase, the stop codon in that tissue is permanently removed, allowing for permanent expression of the reporter. The label is driven by a strong, constitutively active promoter. Therefore, all descendent cells will express this reporter molecule. Moreover, this system can be modified to generate inducible reporter expression or tissue specific reporter [17]. For example, in the kidney, podocyte specific gene expression can be achieved by

crossing reporter mice with a podocin promoter – Cre gene construct. Cre will be expressed in a tissue specific manner, resulting in permanent labeling of podoyctes. This system can be manipulated further by using an inducible promoter for the Cre gene. That way, Cre can be turned on in a cell specific manner by adding an inducing agent, such as tamoxifen for an estrogen receptor inducible promoter [17].

Peritoneal mesothelial cells are somewhat more difficult to label as a specific marker is not available. The closest available is a Wilms tumor -1 (WT1) promoter that labels mesothelial cells as well as developing podocytes. Recent studies by Chen and colleagues using this system will be outlined below [31].

Earlier work using lineage tracing came from models of kidney injury and fibrosis. Humphreys and colleagues used several different kidney specific promoter mice to demonstrate that myofibroblasts do not develop from injured tubular epithelial cells in vivo, however the in vitro response was similar to earlier reported studies [18]. They did arrive at the surprising result that the majority of interstitial myofibroblasts actually came from perivascular cells within the interstitium after kidney injury [23]. These striking results contradicted many previous studies suggesting EMT was a source of interstitial myofibroblasts after kidney injury.

Similar to Humphreys et al., Le Bleu et al also used genetically modified mice to track the origin and function of the myofibroblast [23;58]. Mice expressing yellow fluorescent protein driven by the a-SMA promoter mediated by

the LoxP / Cre system in a UUO model of renal fibrosis exhibited an accumulation of a-SMA+ myofibroblasts in the interstitium of fibrotic kidney [58]. Bone marrow transplant experiments using a-SMA-RFP mice and immunolabeling for a-SMA+ cells revealed that 35% of myofibroblasts in the interstitium of injured kidneys originated from the bone marrow and 65% originated from resident cells including pericytes. Interestingly, ablation of bone marrow derived myofibroblasts reduced kidney fibrosis, whereas the deletion of pericyte derived myofibroblasts did not [58]. Using mice expressing yellow fluorescent under the control of the γ-Glutamyl Transferase promoter (γGT) crossed with a-SMA RFP mouse, LeBleu and colleagues were able to demonstrate that a small number (about 5%) of interstitial myofibroblasts in the kidney arose from epithelial cells through EMT [58].

Similar studies have been recently carried out by Chen and colleagues in models of peritoneal fibrosis [31]. Transgenic mice were generated to conditionally express red fluorescent protein in WT1+ cells. The WT1 promoter was conditionally induced using tamoxifen to label mesothelial cells in adult mice. These authors used several models of peritoneal membrane injury including hypochlorite injection, exposure to dialysis fluid, and adenovirus mediated gene transfer of TGFB [31]. They observed that WT1 labeled the majority of mesothelial cells along with an occasional submesothelial cell of unknown type. Following injury, very few WT1+-RFP cells co-expressing a-SMA were found in the thickened laminin scar. WT1+-RFP cells remained in the mesothelial layer.

Type 1 collagen (COL1A) reporter mice were used to label collagen producing cells in the peritoneum. A-SMA / Col1A positive cells were found in the interstitium as myofibroblasts [31]. These were subsequently shown to originate from existing submesothelial fibroblasts. Peritoneal mesothelial cells expressed Col1A after injury, but not a-SMA, suggesting that the mesothelium participates in the injury response, but not through EMT [31]. Because this study is one of the first studies in the peritoneum to directly contradict EMT as an origin for myofibroblasts, confirmatory studies would be welcome. Furthermore, the expression of WT1 in the submesothelium is not as specific a cellular marker for mesothelium as kidney specific markers. The inducible WT1 promoter did not label all mesothelial cells but did label a proportion (15%) of submesothelial fibroblasts [31]. The fibroblast origin of myofibroblasts in this study was confirmed using a double transgenic Col1A1 reporter mouse with an inducible Col1A2 reporter construct. However, 65% of submesothelial fibroblasts were labelled with Col1A2 and these expanded to become roughly 65% of all myofibroblasts [31]. Therefore, the cell specific markers in the peritoneum are neither completely specific nor sensitive. This may have an impact on the results obtained. Finally, in all these lineage tracing experiments, it is theoretically possible for lineage promoter to become transcriptionally silenced [29].

**What is the role of epithelial cell transition in organ fibrosis?**

Studies of myofibroblasts in organ fibrosis, including peritoneal fibrosis, have focused on the origin of these cells in the interstitial or submesothelial tissue.

It is becoming increasingly clear, however, that the injurepithelium may play a critical role in directing organ repair without undergoing a full EMT process with transition and migration to the interstitium. Strong evidence for this comes from a recent paper by Lovisa and colleagues in evaluation of the role of the epithelium in directing renal fibrosis [59]. These researchers developed a mouse model where the key EMT transcription factors Twist or SNAIL1 were silenced specifically in the renal tubular epithelium. In a model of renal interstitial fibrosis, they demonstrated that silencing these transcription factors blocked the expression of a-SMA in the renal tubular epithelium. By preventing EMT, or at least cellular transition of epithelial cells, the extent of the renal interstitial fibrosis was abrogated [31].

In the work by Chen and colleagues using a Col1A reporter mouse, the injured mesothelial cells began to express collagen after peritoneal injury. They did not identify a-SMA staining of these cells [31]. In our own models, we have seen several instances where the peritoneal mesothelium undergoes a cellular transition and begins to express both cytokeratin and a-SMA, without migration of these cells to the interstitium (‘in situ’ EMT) [35] (Figure 3.2C). Using laser capture microdissection, injured peritoneal mesothelial cells expressed SNAIL1, a-SMA, and VEGF, suggesting these cells undergo EMT and elaborate growth factors that induce submesothelial resident cells to participate in the wound healing response [60]. The mesothelium is maintained in a healthy balance by a combination of pro- and anti-fibrotic factors. Among the anti-fibrotic factors is

bone morphogenic protein 7 which has been shown to be antagonized by the secreted factor Gremlin [61]. Recently, we overexpressed Gremlin in the peritoneum which led to in situ EMT. This cellular transition alone was able to drive the subsequent peritoneal fibrogenic response with matrix deposition and angiogenesis [57].

Therefore, recent evidence concerning the origin of myofibroblasts in organ fibrosis using cell fate tracing experiments may not be as contradictory with previous research as initially thought. Our previous work is consistent with the possibility that few of the myofibroblasts in the interstitial tissue are derived from the overlying epithelium. Despite this, there is increasing evidence that injured epithelial cells undergo a limited transition, or in situ EMT, and this may be sufficient to direct the subsequent interstitial or submesothelial fibrosis.

**Summary**

The origin of the myofibroblast requires further research as the recent evidence from fate mapping studies contradicts evidence from the past [23;31]. This could be attributed to technical difference between techniques and studies, or it could related to the development of the disease process and timing of pathogenesis. Research from in vitro studies of the origin of the myofibroblast have been useful in identifying surrogate markers of EMT including the loss of epithelial markers, E-cadherin and cytokeratin and gain of mesenchymal markers, a-SMA and SNAIL1. Using these surrogate markers, several animal models of peritoneal membrane injury have provided evidence in support of EMT as a

source of myofibroblasts [56]. Most commonly used, immunofluorescence methods have identified the transitioning cells in vivo by analyzing co-localization of epithelial and mesenchymal markers [29]. Moreover, most studies in vitro and in vivo support the injured mesothelial cell as the progenitor of the myofibroblast and there is one recent study with opposing results [31]. Studies of fibrosis in other organs also present evidence in support of EMT [28]. Recently some studies of renal fibrosis present results opposing epithelia as a major source of the myofibroblast, however still suggest a smaller portion of cells derive from EMT [18]. Therefore, confirmatory studies are necessary to further understand this process and to understand to what extent EMT is contributing to the myofibroblast population. This may be explained by the concept of in situ EMT. In other words, injured epithelial cells may not fully transition to yield a complete EMT and cells may only transition to an intermediate stage. A more specific method is required to define, and identify EMT as well as account for the disease model that is being used to truly understand its contribution to the origin of the myofibroblast. In the end, this may require using a combination of lineage tracing studies and EMT criteria. The role of 'in situ' EMT in driving peritoneal fibrosis could be further evaluated by deleting key EMT transcription factors, such as SNAIL1 or TWIST, in the mesothelium in a model of peritoneal fibrosis. Elucidating the origin of myofibroblast and better defining the role of the mesothelium will lead to development of new anti-fibrotic therapies and improvement of current treatment methods.

## Conflict of Interest

The authors have no conflict of interest with the material presented in this review.

## Acknowledgements

Dr. Margetts is funded by Kidney Foundation of Canada, St. Joseph's Healthcare, and Baxter Healthcare.

**References**


1. Aroeira,L.S., Aguilera,A., Selgas,R., Ramirez-Huesca,M., Perez-Lozano,M.L., Cirugeda,A., Bajo,M.A., del Peso,G., Sanchez-Tomero,J.A., Jimenez-Heffernan,J.A. et al 2005. Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis. 46:938-948.

2. Aroeira,L.S., Loureiro,J., Gonzalez-Mateo,G.T., Fernandez-Millara,V., del,P.G., Sanchez-Tomero,J.A., Ruiz-Ortega,M., Bajo,M.A., Lopez-Cabrera,M., and Selgas,R. 2008. Characterization of epithelial-to-mesenchymal transition of mesothelial cells in a mouse model of chronic peritoneal exposure to high glucose dialysate. Perit. Dial. Int. 28 Suppl 5:S29-S33.

3. Williams,J.D., Craig,K.J., Topley,N., Von Ruhland,C., Fallon,M., Newman,G.R., Mackenzie,R.K., Williams,G.T., and Peritoneal Biopsy Study Group. 2002. Morphologic changes in the peritoneal membrane of patients with renal disease. Journal of the American Society of Nephrology 13:470-479.

4. Honda,K., Hamada,C., Nakayama,M., Miyazaki,M., Sherif,A.M., Harada,T., and Hirano,H. 2008. Impact of uremia, diabetes, and peritoneal dialysis itself on the pathogenesis of peritoneal sclerosis: a quantitative study of peritoneal membrane morphology. Clin. J. Am. Soc. Nephrol. 3:720-728.

5. Kim,Y.L. 2009. Update on mechanisms of ultrafiltration failure. Perit. Dial. Int. 29 Suppl 2:S123-S127.

6. Kim,Y.L. 2009. Update on mechanisms of ultrafiltration failure. Perit. Dial. Int. 29 Suppl 2:S123-S127.

7. Duffield,J.S., Lupher,M., Thannickal,V.J., and Wynn,T.A. 2013. Host responses in tissue repair and fibrosis. Annu. Rev. Pathol. 8:241-276.

8. Tomasek,J.J., Gabbiani,G., Hinz,B., Chaponnier,C., and Brown,R.A. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat. Rev. Mol. Cell Biol. 3:349-363.

9. Hinz,B., Phan,S.H., Thannickal,V.J., Galli,A., Bochaton-Piallat,M.L., and Gabbiani,G. 2007. The myofibroblast: one function, multiple origins. Am. J. Pathol. 170:1807-1816.

10. Baum,J., and Duffy,H.S. 2011. Fibroblasts and myofibroblasts: what are we talking about? J. Cardiovasc. Pharmacol. 57:376-379.

11. Strutz,F., and Zeisberg,M. 2006. Renal fibroblasts and myofibroblasts in chronic kidney disease. J. Am. Soc. Nephrol. 17:2992-2998.

12. Ask,K., Bonniaud,P., Maass,K., Eickelberg,O., Margetts,P.J., Warburton,D., Groffen,J., Gauldie,J., and Kolb,M. 2008. Progressive pulmonary fibrosis is mediated by TGF-beta isoform 1 but not TGF-beta3. Int. J Biochem. Cell Biol. 40:484-495.

13. Hinz,B. 2007. Formation and function of the myofibroblast during tissue repair. J. Invest Dermatol. 127:526-537.

14. Hinz,B., and Gabbiani,G. 2003. Cell-matrix and cell-cell contacts of myofibroblasts: role in connective tissue remodeling. Thromb. Haemost. 90:993-1002.

15. Mutsaers,S.E., Bishop,J.E., McGrouther,G., and Laurent,G.J. 1997. Mechanisms of tissue repair: from wound healing to fibrosis. Int. J. Biochem. Cell Biol. 29:5-17.

16. Iwaisako,K., Brenner,D.A., and Kisseleva,T. 2012. What's new in liver fibrosis? The origin of myofibroblasts in liver fibrosis. J. Gastroenterol. Hepatol. 27 Suppl 2:65-68.

17. Grgic,I., Duffield,J.S., and Humphreys,B.D. 2012. The origin of interstitial myofibroblasts in chronic kidney disease. Pediatr. Nephrol. 27:183-193.

18. Margetts,P.J., Bonniaud,P., Liu,L., Hoff,C.M., Holmes,C.J., West-Mays,J.A., and Kelly,M.M. 2005. Transient overexpression of TGF-{beta}1 induces epithelial mesenchymal transition in the rodent peritoneum. J Am Soc Nephrol. 16:425-436.

19. Aroeira,L.S., Aguilera,A., Sanchez-Tomero,J.A., Bajo,M.A., del,P.G., Jimenez-Heffernan,J.A., Selgas,R., and Lopez-Cabrera,M. 2007. Epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions. J. Am. Soc. Nephrol. 18:2004-2013.

20. Iwano,M., Plieth,D., Danoff,T.M., Xue,C., Okada,H., and Neilson,E.G. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J. Clin. Invest 110:341-350.

21. Kalluri,R., and Weinberg,R.A. 2009. The basics of epithelial-mesenchymal transition. J. Clin. Invest. 119:1420-1428.

22. Piera-Velazquez,S., Li,Z., and Jimenez,S.A. 2011. Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am. J. Pathol. 179:1074-1080.

23. Humphreys,B.D., Lin,S.L., Kobayashi,A., Hudson,T.E., Nowlin,B.T., Bonventre,J.V., Valerius,M.T., McMahon,A.P., and Duffield,J.S. 2010. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am. J. Pathol. 176:85-97.

24. Loureiro,J., Aguilera,A., Selgas,R., Sandoval,P., Albar-Vizcaino,P., Perez-Lozano,M.L., Ruiz-Carpio,V., Majano,P.L., Lamas,S., Rodriguez-Pascual,F. et al 2011. Blocking TGF-beta1 protects the peritoneal membrane from dialysate-induced damage. J. Am. Soc. Nephrol. 22:1682-1695.

25. Desmouliere,A., Chaponnier,C., and Gabbiani,G. 2005. Tissue repair, contraction, and the myofibroblast. Wound. Repair Regen. 13:7-12.

26. Rajkumar,V.S., Howell,K., Csiszar,K., Denton,C.P., Black,C.M., and Abraham,D.J. 2005. Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. Arthritis Res. Ther. 7:R1113-R1123.

27. Desmouliere,A., Darby,I.A., and Gabbiani,G. 2003. Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. Lab Invest 83:1689-1707.

28. Kalluri,R., and Neilson,E.G. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. J. Clin. Invest 112:1776-1784.

29. Quaggin,S.E., and Kapus,A. 2011. Scar wars: mapping the fate of epithelial-mesenchymal-myofibroblast transition. Kidney Int. 80:41-50.

30. Braun,N., Fritz,P., Ulmer,C., Latus,J., Kimmel,M., Biegger,D., Ott,G., Reimold,F., Thon,K.P., Dippon,J. et al 2012. Histological criteria for encapsulating peritoneal sclerosis - a standardized approach. PLoS. ONE. 7:e48647.

31. Chen,Y.T., Chang,Y.T., Pan,S.Y., Chou,Y.H., Chang,F.C., Yeh,P.Y., Liu,Y.H., Chiang,W.C., Chen,Y.M., Wu,K.D. et al 2014. Lineage tracing reveals distinctive fates for mesothelial cells and submesothelial fibroblasts during peritoneal injury. J. Am. Soc. Nephrol. 25:2847-2858.

32. Greenburg,G., and Hay,E.D. 1982. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J. Cell Biol. 95:333-339.

33. Zuk,A., Matlin,K.S., and Hay,E.D. 1989. Type I collagen gel induces Madin-Darby canine kidney cells to become fusiform in shape and lose apical-basal polarity. J. Cell Biol. 108:903-919.

34. Fan,J.M., Ng,Y.Y., Hill,P.A., Nikolic-Paterson,D.J., Mu,W., Atkins,R.C., and Lan,H.Y. 1999. Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. Kidney Int. 56:1455-1467.

35. Patel,P., West-Mays,J., Kolb,M., Rodrigues,J.C., Hoff,C.M., and Margetts,P.J. 2010. Platelet derived growth factor B and epithelial mesenchymal transition of peritoneal mesothelial cells. Matrix Biol. 29:97-106.

36. Margetts,P.J., Hoff,C., Liu,L., Korstanje,R., Walkin,L., Summers,A., Herrick,S., and Brenchley,P. 2013. Transforming growth factor beta-induced peritoneal fibrosis is mouse strain dependent. Nephrol. Dial. Transplant. 28:2015-2027.

37. McLoughlin,R.M., and Topley,N. 2011. Switching on EMT in the peritoneal membrane: considering the evidence. Nephrol. Dial. Transplant. 26:12-15.

38. Joseph,M.J., Dangi-Garimella,S., Shields,M.A., Diamond,M.E., Sun,L., Koblinski,J.E., and Munshi,H.G. 2009. Slug is a downstream mediator of transforming growth factor-beta1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells. J. Cell Biochem. 108:726-736.

39. Sabeh,F., Li,X.Y., Saunders,T.L., Rowe,R.G., and Weiss,S.J. 2009. Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. J. Biol. Chem. 284:23001-23011.

40. Yanez-Mo,M., Lara-Pezzi,E., Selgas,R., Ramirez-Huesca,M., Dominguez-Jimenez,C., Jimenez-Heffernan,J.A., Aguilera,A., Sanchez-Tomero,J.A., Bajo,M.A., Alvarez,V. et al 2003. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N. Engl. J Med. 348:403-413.

41. Cho,J.H., Do,J.Y., Oh,E.J., Ryu,H.M., Park,S.Y., Kim,S.O., Hyun,S.H., Seo,H.J., Kim,G.H., Choi,J.Y. et al 2012. Are ex vivo mesothelial cells

representative of the in vivo transition from epithelial-to-mesenchymal cells in peritoneal membrane? Nephrol. Dial. Transplant. 27:1768-1779.

42. Seok,J., Warren,H.S., Cuenca,A.G., Mindrinos,M.N., Baker,H.V., Xu,W., Richards,D.R., McDonald-Smith,G.P., Gao,H., Hennessy,L. et al 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc. Natl. Acad. Sci. U. S. A 110:3507-3512.

43. Lameire,N., Van,B.W., Van,L.M., Wang,T., Heimburger,O., Bergstrom,J., Lindholm,B., Hekking,L.P., Havenith,C.E., and Beelen,R.H. 1998. Experimental models in peritoneal dialysis: a European experience. Kidney Int. 54:2194-2206.

44. Mactier,R.A., Khanna,R., Twardowski,Z., Moore,H., and Nolph,K.D. 1987. Contribution of lymphatic absorption to loss of ultrafiltration and solute clearances in continuous ambulatory peritoneal dialysis. J. Clin. Invest 80:1311-1316.

45. Mactier,R.A., and Khanna,R. 1989. Absorption of fluid and solutes from the peritoneal cavity. Theoretic and therapeutic implications and applications. ASAIO Trans. 35:122-131.

46. Zweers,M.M., Douma,C.E., de Waart,D.R., van der Wardt,A.B., Ho-dac-Pannekeet,M.M., Krediet,R.T., and Struijk,D.G. 1999. The standard peritoneal permeability analysis in the rabbit: a longitudinal model for peritoneal dialysis. Perit. Dial. Int. 19:56-64.

47. Krediet,R.T., Zemel,D., Imholz,A.L., and Struijk,D.G. 1994. Impact of surface area and permeability on solute clearances. Perit. Dial. Int. 14 Suppl 3:S70-S77.

48. Park,M.S., Heimburger,O., Bergstrom,J., Waniewski,J., Werynski,A., and Lindholm,B. 1994. Evaluation of an experimental rat model for peritoneal dialysis: fluid and solute transport characteristics. Nephrol. Dial. Transplant. 9:404-412.

49. Margetts,P.J., Kolb,M., Yu,L., Hoff,C.M., and Gauldie,J. 2001. A chronic inflammatory infusion model of peritoneal dialysis in rats. Perit. Dial. Int. 21 Suppl 3:S368-72.:S368-S372.

50. Flessner,M.F., Credit,K., Richardson,K., Potter,R., Li,X., He,Z., Hoskins,G., and Henegar,J. 2010. Peritoneal inflammation after twenty-week exposure to dialysis solution: effect of solution versus catheter-foreign body reaction. Perit. Dial. Int. 30:284-293.

51. Patel,P., Sekiguchi,Y., Oh,K.H., Patterson,S.E., Kolb,M.R., and Margetts,P.J. 2010. Smad3-dependent and -independent pathways are involved in peritoneal membrane injury. Kidney Int. 77:319-328.

52. Margetts,P.J., Kolb,M., Galt,T., Hoff,C.M., Shockley,T.R., and Gauldie,J. 2001. Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. J. Am. Soc. Nephrol. 12:2029-2039.

53. Liu,L., Shi,C.X., Ghayur,A., Zhang,C., Su,J.Y., Hoff,C.M., and Margetts,P.J. 2009. Prolonged peritoneal gene expression using a helper-dependent adenovirus. Perit. Dial. Int. 29:508-516.

54. Hoff,C.M. 2005. Experimental animal models of encapsulating peritoneal sclerosis. Perit. Dial. Int. 25 Suppl 4:S57-S66.

55. Ishii,Y., Sawada,T., Shimizu,A., Tojimbara,T., Nakajima,I., Fuchinoue,S., and Teraoka,S. 2001. An experimental sclerosing encapsulating peritonitis model in mice. Nephrology Dialysis Transplantation 16:1262-1266.

56. Zeisberg,M., and Duffield,J.S. 2010. Resolved: EMT produces fibroblasts in the kidney. J. Am. Soc. Nephrol. 21:1247-1253.

57. Siddique,I., Curran,S.P., Ghayur,A., Liu,L., Shi,W., Hoff,C.M., Gangji,A.S., Brimble,K.S., and Margetts,P.J. 2014. Gremlin promotes peritoneal membrane injury in an experimental mouse model and is associated with increased solute transport in peritoneal dialysis patients. Am. J. Pathol. 184:2976-2984.

58. LeBleu,V.S., Taduri,G., O'Connell,J., Teng,Y., Cooke,V.G., Woda,C., Sugimoto,H., and Kalluri,R. 2013. Origin and function of myofibroblasts in kidney fibrosis. Nat. Med. 19:1047-1053.

59. Lovisa,S., LeBleu,V.S., Tampe,B., Sugimoto,H., Vadnagara,K., Carstens,J.L., Wu,C.C., Hagos,Y., Burckhardt,B.C., Pentcheva-Hoang,T. et al 2015. Epithelial-to-mesenchymal transition induces cell cycle arrest and parenchymal damage in renal fibrosis. Nat. Med. 21:998-1009.

60. Zhang,J., Oh,K.H., Xu,H., and Margetts,P.J. 2008. Vascular endothelial growth factor expression in peritoneal mesothelial cells undergoing transdifferentiation. Perit. Dial. Int. 28:497-504.

61. Yanagita,M. 2012. Inhibitors/antagonists of TGF-beta system in kidney fibrosis. Nephrol. Dial. Transplant. 27:3686-3691.

# CHAPTER 4: MATRIX METALLOPROTEINASE 9 IS ASSOCIATED WITH PERITONEAL MEMBRANE SOLUTE TRANSPORT AND INDUCES ANGIOGENESIS THROUGH B –CATENIN SIGNALING

Authors: Manreet Padwal, Imad Siddique, Lili Wu, Katelynn Tang, Felix Boivin, Limin Liu, Jennifer Robertson, Darren Bridgewater, Judith West-Mays, Azim Gangji, Kenneth Scott Brimble, Peter J. Margetts

Corresponding Author: Peter Margetts, Division of Nephrology, St. Joseph's Hospital, Department of Medicine, McMaster University, Hamilton, ON, Canada L8P 4A6. Phone: (905) 525-9140 ext 32299, Fax: 905-540-6589, Email: margetts@mcmaster.ca

Citation: Manreet Padwal, Imad Siddique, Lili Wu, Katelynn Tang, Felix Boivin, Limin Liu, Jennifer Robertson, Darren Bridgewater, Judith West-Mays, Azim Gangji, Kenneth Scott Brimble, Peter J. Margetts; Matrix metalloproteinase 9 is associated with peritoneal membrane solute transport and induces angiogenesis through b-catenin signaling. *Nephrol Dial Transplant* 2017; 32 (1): 50-61. doi: 10.1093/ndt/gfw076

**PREFACE**

Peritoneal angiogenesis is one the major changes that occurs in long term peritoneal dialysis patients. Changes in peritoneal vasculature including an increase in surface area and vascular permeability results in changes in peritoneal solute transport. Increased solute transport is associated with a decline in ultrafiltration and increased risk of mortality. The goal of this work is to better understand the mechanism behind peritoneal membrane angiogenesis. Angiogenesis consists of remodeling of the extracellular matrix to allow for migration of endothelial cells and formation of new blood vessels. Matrix Metalloproteinases have been implicated in proteolysis of the extracellular matrix and MMP9 specifically degrades collagen IV which is a component of the basement membrane. We hypothesize that MMP9 is involved in development of peritoneal angiogenesis by cross-talk with WNT/b-catenin signaling. In this Chapter, we discuss the role of MMP9 in peritoneal angiogenesis. We proposed a novel mechanism by which MMP9 interacts with WNT/b-catenin signaling to induce angiogenesis in the peritoneum. We also discuss the role of MMP9 in peritoneal membrane function and its potential as a biomarker of peritoneal angiogenesis.

Manreet Padwal designed the study, performed all experiments and data analysis. The animal work was carried out with the assistance of Limin Liu. All adenoviruses were amplified by Limin Liu. The MMP2 and 9 knockout mice were provided to us by the West-Mays lab. Dr. Scott Brimble and Dr. Azim Gangji

developed the clinical study facilitating the collection of mesothelial cells and patient data. Manreet Padwal developed an ex vivo cell culture from patient peritoneal effluent. Histology support was carried out by Mary Jo Smith. Imad Siddique, Lili Wu and Dr. Felix Boivin assisted with immunohistochemistry. Katelynn Tang assisted with animal work and data analysis. Manreet Padwal wrote the original draft of the manuscript and edited the manuscript based on reviewers' comments. Dr. Peter Margetts contributed to the concept of this paper, interpretation of the data and reviewed the final manuscript.

**ABSTRACT**

*Background:* For patients using peritoneal dialysis, the peritoneal membrane can develop fibrosis and angiogenesis leading to ultrafiltration failure, chronic hypervolemia and increased risk of technique failure and mortality. Matrix metalloproteinases (MMP), and specifically the gelatinases (MMP2 and MMP9), may be involved in peritoneal membrane injury.

*Methods:* From stable PD patients, mesothelial cells were assayed for MMP gene expression. MMP9 was overexpressed in mouse peritoneum by adenovirus and $MMP9^{-/-}$ mice were subjected to transforming growth factor beta (TGFB) induced peritoneal fibrosis.

*Results:* MMP9 mRNA expression correlated with peritoneal membrane solute transport properties. Overexpression of MMP9 in the mouse peritoneum induced submesothelial thickening and angiogenesis. MMP9 induced mesothelial cell transition to a myofibroblast phenotype measured by increased alpha smooth muscle actin and decreased E-Cadherin expression. Angiogenesis was markedly reduced in $MMP9^{-/-}$ mice treated with an adenovirus expressing active transforming growth factor beta (TGFB) compared with wildtype mice. TGFB mediated E-cadherin cleavage was MMP9 dependent and E-cadherin cleavage led to b-catenin mediated signaling. A b-catenin inhibitor blocked the angiogenic response induced by AdMMP9.

*Conclusions:* Our data suggest that MMP9 is involved in peritoneal membrane injury possibly through cleavage of E-cadherin and induction of b-catenin

signaling. MMP9 is a potential biomarker for peritoneal membrane injury, and is a therapeutic target to protect the peritoneal membrane in peritoneal dialysis patients.

## INTRODUCTION

For peritoneal dialysis (PD) patients, the peritoneal membrane is injured by bioincompatible dialysis solutions, uremia, and peritonitis [1]. Loss of the mesothelial cell monolayer, submesothelial thickening and angiogenesis are associated with functional changes such as increased solute transport [2]. Increased solute transport has been associated with the failure of ultrafiltration [3] and increased risk of mortality [4]. The thickening of the submesothelial zone and the angiogenic response has been associated with accumulation of submesothelial myofibroblasts [5]. The origin of these myofibroblasts is controversial. We have shown that a portion of these cells derive from the mesothelium in a process of epithelial mesenchymal transition (EMT) [6]. Others have shown that the majority of submesothelial myofibroblasts derive from existing interstitial fibroblasts [7].

Metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in fibrotic and remodeling processes. MMP2 and 9 are gelatinases which degrade type IV collagen, an important structural component of the basement membrane [8]. Previous studies have demonstrated a role for MMP2 and MMP9 in cellular invasion as a component of EMT [9]. We have previously demonstrated that platelet derived growth factor B (PDGFB) induces a non-invasive EMT phenotype where the mesothelial cells transition but do not invade into the submesothelium [10]. This is in contrast to transforming growth factor beta (TGFB) induced peritoneal fibrosis which was associated with the appearance of transitioned epithelium in the submesothelial tissue [6]. TGFB

overexpression is associated with increased MMP2 and 9 expression [6] whereas PDGFB induced fibrosis did not increase gelatinase expression [10]. Therefore, gelatinases may be required to degrade the basement membrane to facilitate invasion of transitioned cells into the submesothelial zone.

MMP2 and 9 are angiogenic and are associated with increased activity of vascular endothelial growth factor (VEGF) [11]. They are also involved in cleavage of cell surface proteins and elaboration of growth factors from matrix stores [12]. E-cadherin is an epithelial cell adhesion protein found on peritoneal mesothelial cells and loss of E-cadherin is essential in EMT [13]. MMP9 has been shown to cleave the extracellular domain of E-cadherin resulting in an 80 kDa E-cadherin fragment [14]. The intracellular domain of E-cadherin is associated with b-catenin which connects to the cell's actin cytoskeleton. E-cadherin cleavage leads to increased concentration of cytosolic b-catenin which can then act as a transcription factor that regulates a variety of genes involved in cell growth and differentiation including MMPs, c-myc, and VEGF [15]. This study was aimed to identify the role of MMP9 in peritoneal membrane injury. Specifically, we analyzed mesothelial cells derived from peritoneal effluent from patients on PD to evaluate MMP9 expression and markers of peritoneal membrane function. Furthermore, we examined the effect of MMP9 on peritoneal angiogenesis, fibrosis and cellular invasion. We hypothesized that MMP9 induces peritoneal angiogenesis by cleavage of E-cadherin and signaling through b-catenin to upregulate VEGF.

## SUBJECTS and METHODS

### Human mesothelial cell culture

A 2L overnight dwell of peritoneal fluid effluent was taken from 33 stable PD patients with no peritonitis within 3 months of enrollment in the study. PD effluent was centrifuged and mesothelial cells were resuspended in F12 media and cultured. At the same clinic visit, a PET was carried out using a four hour dwell of 4.25% glucose solution as previously described [16]. Solute transport data including dialysate to plasma creatinine ratio (D/P creatinine) and a 4 hour to initial PD fluid glucose ratio ($D/D_0$ glucose) was obtained. Local ethics board approval was obtained and patients provide informed consent.

### Gene expression analysis

After 2 passages, human mesothelial cells were suspended in Trizol reagent and RNA was isolated. mRNA was evaluated using both nanoString nCounter (Seattle, WA) and quantitative real time PCR. NanoString technology has been previously described by Kulkarni and colleagues [17]. Briefly, customized probes for a panel of 31 genes associated with matrix metalloproteinase, cell differentiation, and angiogenesis, along with 4 housekeeping genes, were created (Supplemental table). We initially compared a subset of 7 MMP associated genes with D/P creatinine. We selected these genes based on a literature search of MMPs associated with fibrosis, and included representation of the major metalloproteinase families [18]. We then compared MMP9 gene expression with the entire 31 gene panel. Probes were hybridized to

100 ng of total RNA. Data were collected using the nCounter™ Digital Analyzer by counting individual barcodes. Positive and negative controls were included. mRNA counts were normalized using the geometric mean of the 4 housekeeping genes.

For quantitative real time PCR, 1µg of RNA obtained was reverse transcribed and gene expression for MMP9 was determined by ABI Prism 7500 Sequence Detector (Applied Biosystems, Life Technologies, Burlington, ON, Canada). Samples were run in duplicate and normalized to 18s.

**Recombinant Adenovirus**

We used three different adenoviruses in these experiments: AdTGFB, AdMMP9, and AdDL. AdDL is a null adenovirus used as viral control. AdTGFB expresses constitutively active TGFB1 [19]. AdMMP9 expresses human MMP9 [20]. Amplification and purification of adenoviruses was completed by CsCl gradient centrifugation and plaque-titred on 293 cells as previously described [21].

**Animal Experiments**

All animal studies were performed according to the Canadian Council on Animal Care guidelines with local ethics board approval. C57BL/6 mice received an injection of $3x10^8$ plaque forming units (pfu) of AdMMP9 (n=7 animals/group) and controls were treated with AdDL (n=8 animals/group). All adenoviruses were diluted in 100 µl of saline and administered by intraperitoneal injection (IP). One hour prior to euthanization, the animals received an intraperitoneal injection of 3

ml of 4.25% glucose dialysis solution (Dianeal, Baxter Healthcare, Mississauga, ON, Canada). Animals were euthanized 10 days after adenovirus injection.

An additional group of mice were treated with AdMMP9 (n=10 animals/group). At day 4, animals were administered with daily IP injections of ICG-001 (Millipore, Etobicoke, ON, Canada) (5mg/kg) for five days (n=5 animals/group). All animals received 3 ml IP injection of 4.25% Dianeal (Baxter Healthcare) an hour before euthanization Animals were euthanized 10 days after initial AdMMP9 injection.

Both $MMP2^{-/-}$ mice (Baylor College of Medicine, Houston, TX) [22] and $MMP9^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) [23] were on a C57BL/6 background. C57BL/6 (n=13 animals/group), $MMP2^{-/-}$ (n=13 animals/group), and $MMP9^{-/-}$ (n=13 animals/group) mice were administered with $0.8x10^8$ pfu of AdTGFB diluted to 100 ul of saline via the IP route. AdTGFB induced fibrosis and EMT in the peritoneum. Animals were also treated with control adenovirus (n=13 animals/group). As above, all animals received 3 ml IP injection of 4.25% Dianeal (Baxter Healthcare) an hour before euthanization which was carried out ten days after adenovirus infection.

For all experiments, the peritoneum was opened and the entire peritoneal fluid content was collected by pipette. The anterior abdominal wall was removed and stored in 4% neutral-buffered formalin. The omentum was taken and frozen in liquid nitrogen for protein analysis.

**Histology**

Peritoneal membrane samples from the anterior abdominal wall were initially fixed in 10% formalin for 48 hours and paraffin embedded. Fixed sections were stained with Masson's trichrome. Immunostaining for blood vessels was performed using vonWillebrand Factor antibody / Factor VIII (1:150) (Dako, Burlington, ON, Canada). Five images were taken per stained section from each animal. Northern Eclipse software was used to analyze the blood vessel density and submesothelial thickness. Photomicrographs (5-10 / section) were taken and vascular density was calculated in rectangles 100, 200 and 500 μm deep from the mesothelial cell layer. Submesothelial thickness was calculated by measuring the average distance from the superficial mesothelial cell layer to the muscle. Immunostaining of the laminin basement membrane was performed using a polyclonal antibody against laminin (1:50) (Millipore). For b-catenin, we used a monoclonal antibody that is specific for the non-phosphorylated or active b-catenin (Millipore) [24]. For quantification of b-catenin expression, photomicrographs (5-10 / section) were taken and positive stained cells were counted.

Immunofluorescence was completed using a monoclonal mouse anti-human a-SMA antibody (1:50) (Dako, Burlington, ON, Canada) with a Texas Red goat antimouse secondary antibody (1:100) (Molecular Probes, Life Technologies) followed by a FITC labeled monoclonal mouse antibody against pan-cytokeratin (1:50) (Sigma-Aldrich). To control for non-specific staining,

sections were exposed to the secondary antibody without the primary a-SMA antibody. To confirm dual-labeling, we also stained representative sections directly with a fluorescently labeled a-SMA antibody (Cy3, Sigma-Aldrich) (Supplemental figure 4.1). ImageJ (NIH) was used to analyze and quantify dual labeled cells in the mesothelial and submesothelial layer. Dual labelled cells co-expressing a-SMA and pancytokeratin were identified in the mesothelial cell layer and in the submesothelial cell layer from five high-power fields from each section and this was using a blinded approach. Dual labelled cells in the mesothelial layer were categorized as non-invading cells and dual labelled cells in the submesothelium were considered invading cells.

Mesothelial cells were grown on 22 x 22-mm glass cover slips in a 35 mm dish for immunofluorescence. The cells were fixed and permeabilized with 100% methanol and stained with a FITC labeled monoclonal mouse antibody against pan-cytokeratin (1:50) (Sigma-Aldrich, Oakville, ON, Canada).

**Protein Analysis**

Protein was extracted from frozen omental peritoneal tissue. Samples were electrophoresed on a 10% SDS-PAGE gel, and probed for E-cadherin (BD Biosciences, Mississauga, ON, Canada), E-cadherin fragment (Santa Cruz, Dallas, TX), MMP9 (Abcam, Toronto, ON, Canada), MMP2 (Santa Cruz) and VEGF (Milipore). Alpha tubulin (Abcam) and b-actin (Sigma Aldrich) were used as loading controls. For the peritoneal effluent, a non-specific band on coomassie blue stained blots was used as loading control.

Gelatin zymography was performed on an 8% SDS–PAGE with 0.1% gelatin (Sigma). Gels were soaked in 2.5% Triton X-100 for 30 min to remove SDS. They were then incubated in activation buffer (50 mM Tris–HCl;pH-8, 10 mMCaCl2, 5 μMZnSO4, and 150 mMNaCl) for 12 h at 37 °C. Following incubation, activity was observed using coomassie blue and quantified (ImageJ).

Peritoneal effluent concentration of VEGF was measured using a standard enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

**Statistical Analysis**

Data are presented as mean ± SD. Correlations were assessed by Spearman's co-efficient. A Bonferroni correction was used for multiple comparisons. For comparison between groups, Kolmogorov-Smirnov test was used to confirm normally distributed data. For normally distributed data, a t-test was used to compare between two groups and ANOVA with post-hoc Tukey's test was used for multiple groups. A Mann-Whitney test was used for non-normally distributed data.

## RESULTS

### *MMP9 is correlated with peritoneal membrane solute transport*

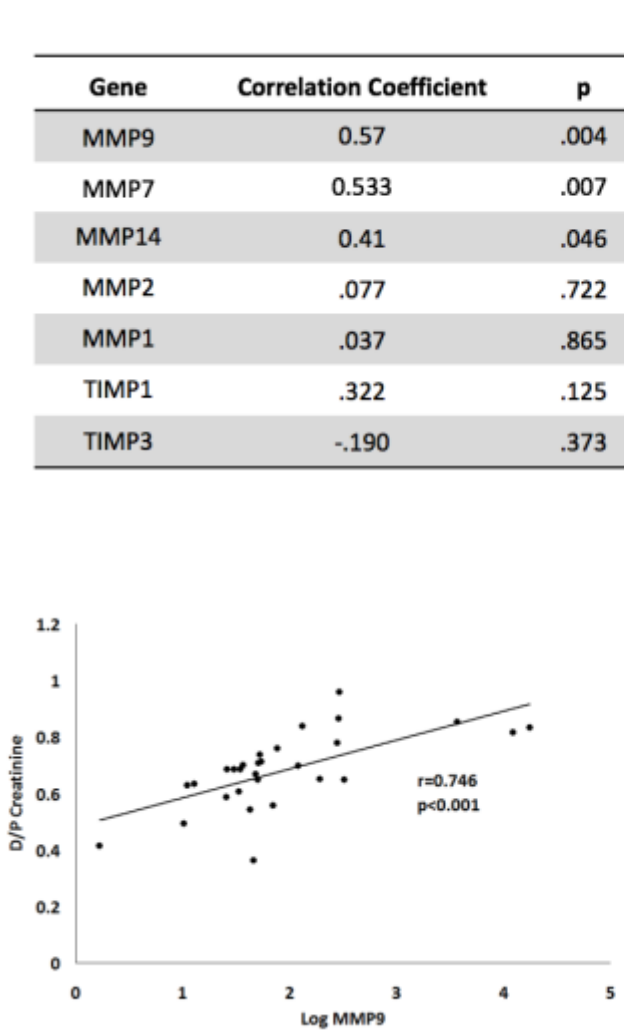
We studied a total of 33 stable PD patients. Average age of the patients was 62 (±13) years. Forty-five percent had diabetes. Average PD duration was 1.4 (±1.3) years. Using nanoString gene expression analysis, we measured 7 MMP

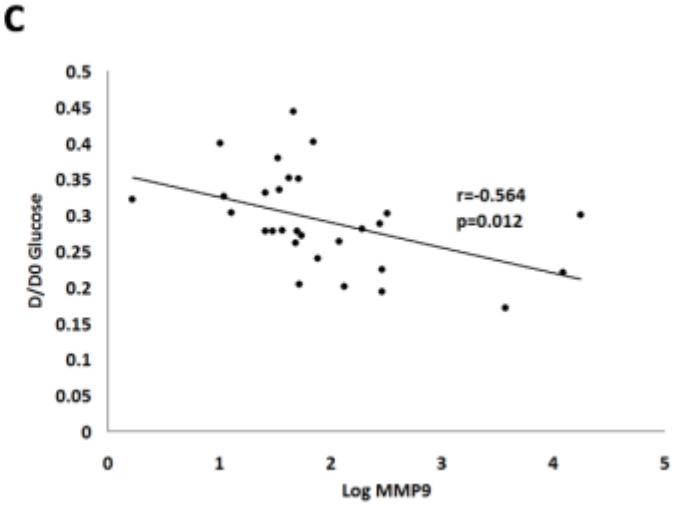
genes in ex vivo derived mesothelial cell culture and examined the association with the patients' peritoneal membrane solute transport properties (Figure 4.1A). Of our 7 gene MMP panel, MMP9 was found to be most strongly associated with D/P creatinine ($r=0.746$, $p<0.001$) but MMP7 was also significantly associated with solute transport (Figure 4.1A). Gene expression of MMP14 was correlated with solute transport in univariate analysis but is no longer significant after Bonferroni correction for multiple comparisons (Figure 4.1A). Notably, MMP2 gene expression did not correlate with solute transport (Figure 4.1A). We confirmed the association between ex vivo mesothelial cell gene expression of MMP9 and solute transport using quantitative PCR. MMP9 gene expression correlated with D/P creatinine (Figure 4.1B) and inversely with D/D0 glucose (Figure 3.1C). We compared MMP9 gene expression measured by nanoString with quantitative real time PCR and found a very close correlation ($r=0.804$, $p<0.001$) (Figure 4.1D). MMP9 gene expression in ex-vivo mesothelial cells was also higher in patients with diabetes than those without (1.0 vs 0.6, $p=0.01$, data not shown**).**

The peritoneal effluent initially contained about 5% mesothelial cells. After 2 passages in cell culture, the cells were virtually all cytokeratin positive mesothelial cells (Supplemental figure 4.2).

A

| Gene | Correlation Coefficient | p |
|---|---|---|
| MMP9 | 0.57 | .004 |
| MMP7 | 0.533 | .007 |
| MMP14 | 0.41 | .046 |
| MMP2 | .077 | .722 |
| MMP1 | .037 | .865 |
| TIMP1 | .322 | .125 |
| TIMP3 | -.190 | .373 |



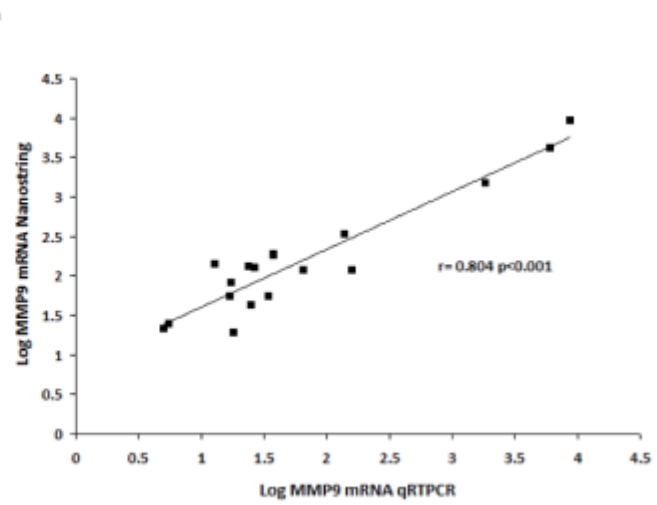



**Figure 4.1 MMP9 is associated with peritoneal membrane solute transport-** We measured the expression of 7 MMP related genes in ex vivo mesothelial cell culture from stable PD patients using nanoString. A) We found that MMP9 and MMP7 gene expression was highly associated with D/P creatinine. We confirmed this using quantitative real time PCR. MMP9 mRNA correlates with measures of solute transport including (B) D/P creatinine and (C) inversely with D/D0 glucose. D) MMP9 gene expression measured by nanoString correlated closely with MMP9 expression measured by real time PCR.

***MMP9 overexpression leads to submesothelial thickening and angiogenesis***

Because of this association between peritoneal membrane solute transport and mesothelial MMP9 gene expression, we used an adenovirus to overexpress MMP9 in the peritoneal membrane of mice (Figure 4.2A-B). The control adenovirus (AdDL) did not alter peritoneal membrane structure (Figure 4.2A). AdMMP9 led to submesothelial thickening, fibrosis, and angiogenesis. The mesothelial cells appear to be reactive with increased cell number and hypertrophy. These histologic changes were quantified from Factor VIII stained sections (Figure 4.2C, D, quantified in 4.2E, F). Submesothelial thickness increased by 2-fold ($p=0.004$) and angiogenesis increased by 3-fold ($p<0.001$) in response to AdMMP9. AdMMP9 induced a significant increase in VEGF protein concentration in the peritoneal effluent taken from these mice at the time of euthanasia (Figure 4.2G). We used zymography to confirm MMP9 expression after infection with AdMMP9 (Figure 4.2H, I) and noted that AdMMP9 also significantly upregulated MMP2 expression (Figure 4.2J).

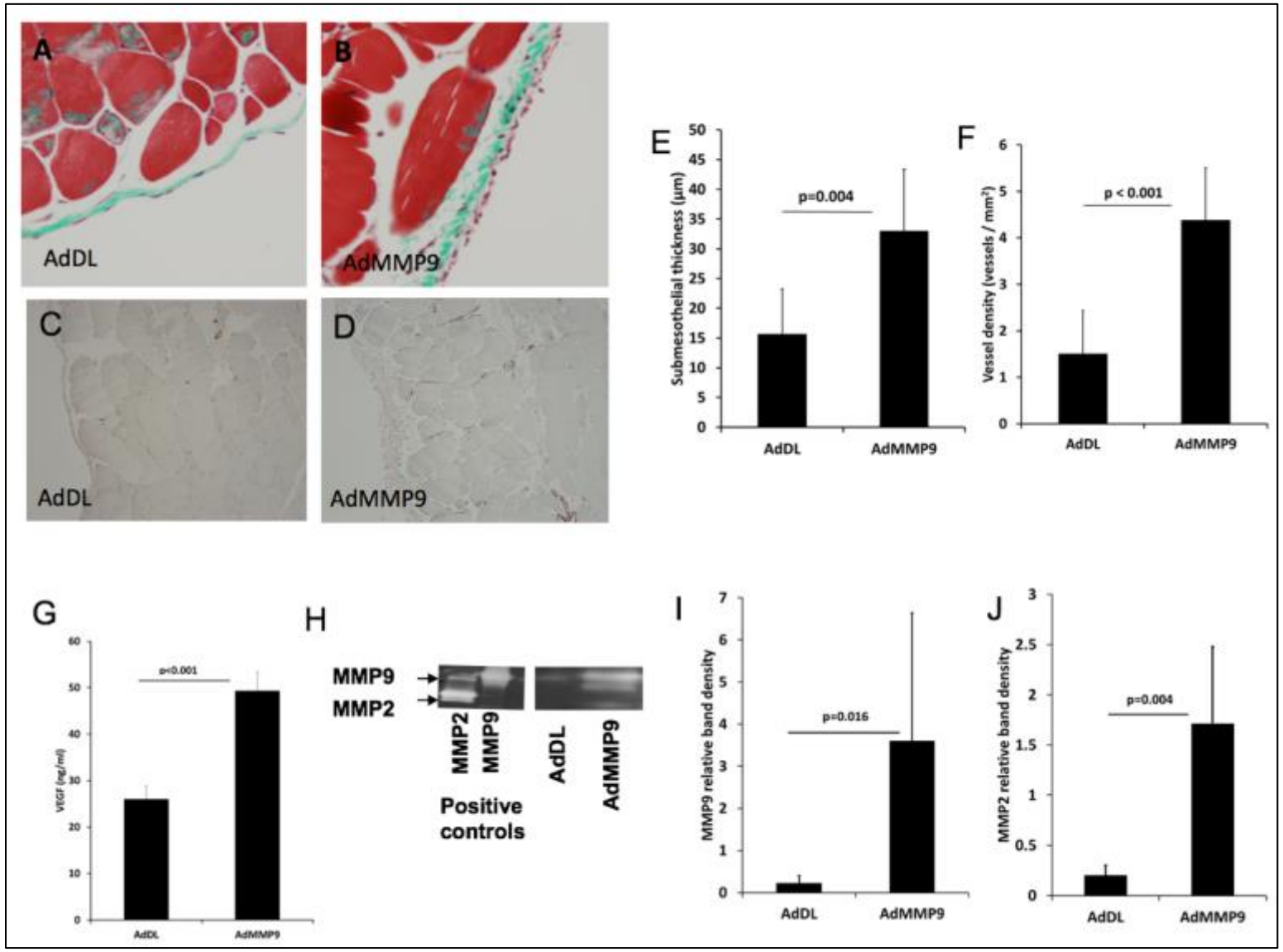

A
AdDL
B
AdMMP9
C
AdDL
D
AdMMP9
E
Submesothelial thickness (µm)
p=0.004
AdDL
AdMMP9
F
Vessel density (vessels / mm²)
p < 0.001
AdDL
AdMMP9
G
p<0.001
AdDL
AdMMP9
H
MMP9
MMP2
MMP2
MMP9
AdDL
AdMMP9
Positive controls
I
MMP9 relative band density
p=0.016
AdDL
AdMMP9
J
MMP2 relative band density
p=0.004
AdDL
AdMMP9

**Figure 4.2 Histology of the peritoneal membrane from C57Bl/6 mice 10 days after adenoviral gene transfer of control adenovirus (AdDL) or AdMMP9 -** A) AdDL does not significantly alter the peritoneal membrane histology. B) AdMMP9 led to submesothelial thickening and fibrosis (sections stained with Masson's trichrome, 200x magnification). C, D) Factor VIII stained sections of C57Bl/6 mouse peritoneum after adenoviral gene transfer of AdDL (C) or AdMMP9 (D) (100x magnification). E, F) Quantification of Factor VIII stained sections demonstrates an increase in submesothelial thickness after treatment with AdMMP9 (E) with an increase in blood vessel density (F). G) Increased angiogenesis was associated with a significant increase in VEGF measured in the peritoneal effluent. H) Representative zymogram from peritoneal effluent. The first two lanes are positive controls. I) Quantification shows increased MMP9 in mice receiving AdMMP9 along with an increase in MMP2 expression (J).

***MMP9 leads to mesothelial cell transition.***

We used immunofluorescence labelling of epithelial and mesenchymal markers to investigate the peritoneum for evidence of EMT and cellular invasion (Figure 4.3A-D). Control adenovirus treated animals showed cytokeratin positive mesothelium with no evidence of dual labeling with alpha-smooth muscle actin (a-SMA) (Figure 4.3A-C). Animals treated with AdMMP9 demonstrated an increase in dual labelled mesothelial cells suggestive of EMT (Figure 4.3D-F). There was no evidence of accumulation of submesothelial dual labeled cells suggesting a lack of mesothelial cell migration after transition (Figure 4.3H).

Decreased E-cadherin expression is a hallmark of mesothelial cell transition and we found that MMP9 overexpression led to a significant decrease of E-cadherin expression in peritoneal tissue (Figure 4.3I, J).

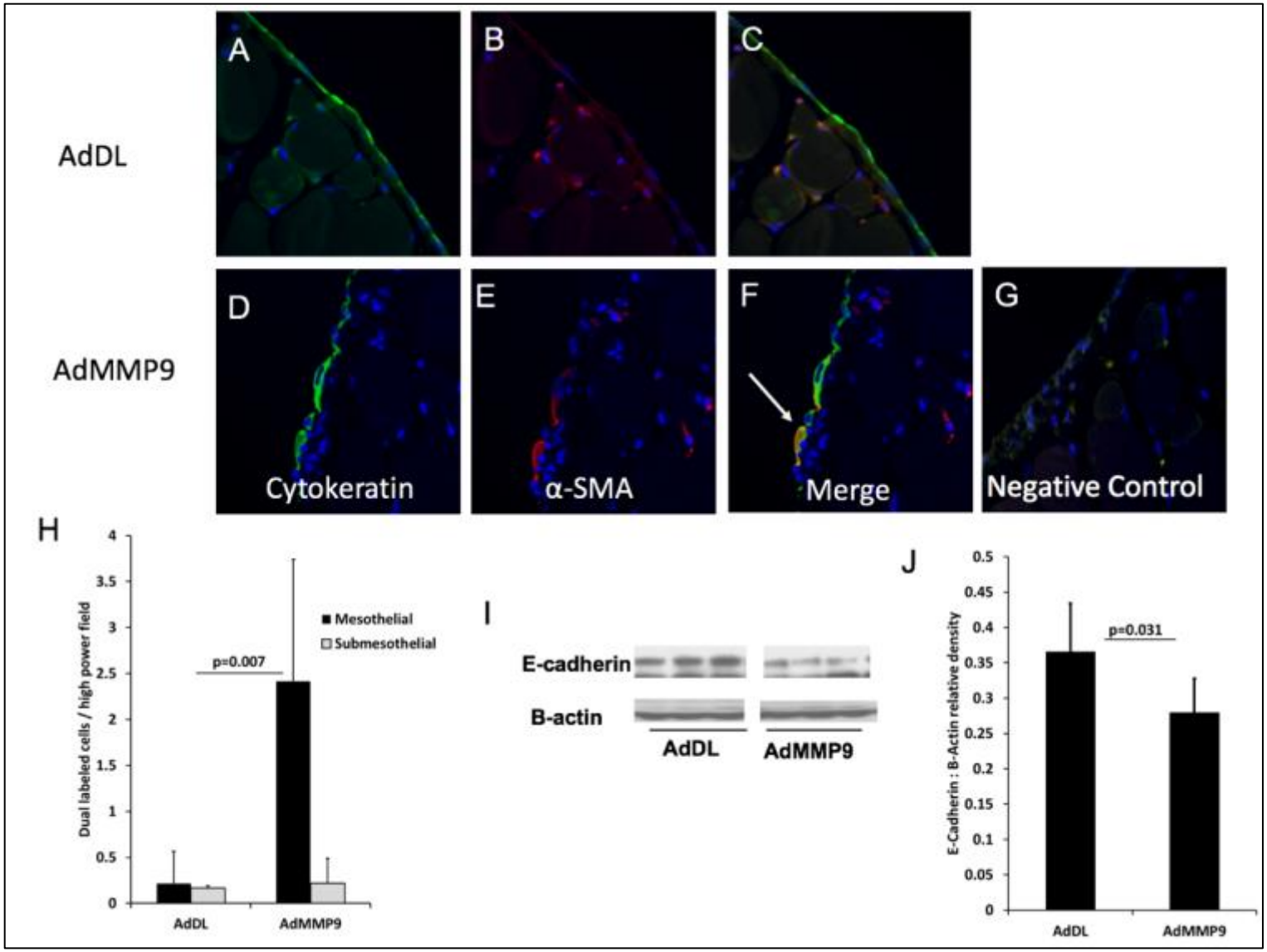

AdDL
AdMMP9
A
B
C
D
E
F
G
Cytokeratin
α-SMA
Merge
Negative Control
H
Dual labeled cells / high power field
Mesothelial
Submesothelial
p=0.007
AdDL
AdMMP9
I
E-cadherin
B-actin
AdDL
AdMMP9
J
E-Cadherin : B-Actin relative density
p=0.031
AdDL
AdMMP9

**Figure 4.3 Dual staining for cytokeratin and a-SMA in C57Bl/6 mice after adenoviral gene transfer -** A-C) Mice treated with AdDL display normal peritoneal membrane structure with cytokeratin positive mesothelial cells, minimal a-SMA expression seen in vascular structures, and no dual staining cells. (D-F) Infection with an adenovirus expressing MMP9 resulted in an increase in dual labelled cells in the mesothelial layer. (D-F, arrow in F). G) A negative control was used to confirm results were not due to non-specific staining. H) Quantification of dual labelled cells. MMP9 induces an increase of dual labelled mesothelial cells with no evidence of invasion into the submesothelial zone. I, J) E-cadherin expression was regulated by MMP9. (H) Representative blot. (I) Density analysis demonstrates that MMP9 alone induces a significant decrease in E-cadherin.

***Angiogenesis is inhibited in MMP9$^{-/-}$ mice***

Our results from human mesothelial cells suggested that MMP2 and MMP9 may have distinct actions with respect to peritoneal membrane injury (Figure 4.1). We induced peritoneal fibrosis in mice using an adenovirus expressing active TGFB1 in MMP2$^{-/-}$ and MMP9$^{-/-}$ mice (Figure 4.4A-F). We confirmed that the MMP2$^{-/-}$ and MMP9$^{-/-}$ mice were deficient in MMP2 and 9 respectively by western blot (Figure 4.4G). Control adenovirus treated MMP2$^{-/-}$ and MMP9$^{-/-}$ mice demonstrated normal histology (Figure 4.4A, C, E). There was a similar change in submesothelial thickness in response to AdTGFB in C57Bl/6, MMP2$^{-/-}$ and MMP9$^{-/-}$ mice (Figure 4.4B, D, F). All animals treated with AdTGFB exhibited a thickened submesothelial zone (quantified in figure 4.4H) and increased cell numbers. In addition, AdTGFB induced an increase in blood vessel density in C57Bl/6 (and MMP2$^{-/-}$ mouse peritoneum (Figure 4.4I). However, this angiogenic response to TGFB was not seen in the MMP9$^{-/-}$ mice.

ELISA analysis of peritoneal effluent revealed significantly increased levels of VEGF in C57Bl/6 and MMP2$^{-/-}$ mice treated with AdTGFB whereas MMP9$^{-/-}$ mice did not display a similar increase in VEGF (Figure 4.4J).

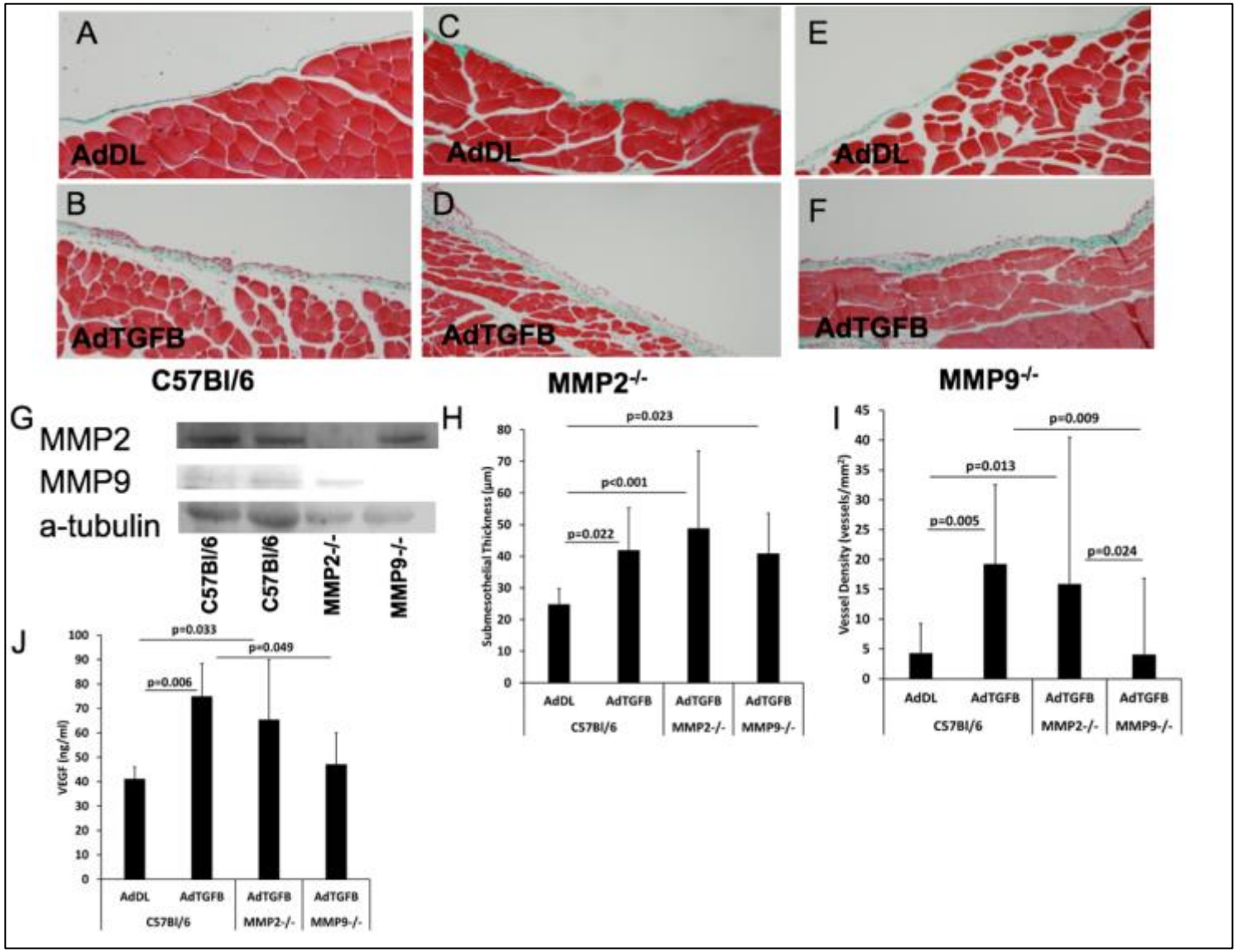

A
AdDL
C
AdDL
E
AdDL
B
AdTGFB
D
AdTGFB
F
AdTGFB
C57Bl/6
MMP2-/-
MMP9-/-
G
MMP2
MMP9
a-tubulin
C57Bl/6
C57Bl/6
MMP2-/-
MMP9-/-
H
Submesothelial Thickness (µm)
p=0.023
p<0.001
p=0.022
AdDL
AdTGFB
AdTGFB
AdTGFB
C57Bl/6
MMP2-/-
MMP9-/-
I
Vessel Density (vessels/mm²)
p=0.009
p=0.013
p=0.005
p=0.024
AdDL
AdTGFB
AdTGFB
AdTGFB
C57Bl/6
MMP2-/-
MMP9-/-
J
VEGF (ng/ml)
p=0.033
p=0.049
p=0.006
AdDL
AdTGFB
AdTGFB
AdTGFB
C57Bl/6
MMP2-/-
MMP9-/-

**Figure 4.4 Histology of the peritoneal membrane from C57Bl/6, MMP2-/- and MMP9-/- mice after adenoviral gene transfer of TGFB1-** (A-F) C57Bl/6, MMP2$^{-/-}$ and MMP9$^{-/-}$ mice appear to have similar responses to TGFB1. (G) Confirmation by western blot of MMP2 and MMP9 knock out status. (H) Quantification of submesothelial thickness displays no significant difference between wildtype, MMP2$^{-/-}$ and MMP9$^{-/-}$ mice treated with TGFB1. (I) Quantification of blood vessel density shows that TGFB1 resulted in an increase in blood vessel density in wildtype and MMP2$^{-/-}$ mice whereas MMP9$^{-/-}$ mice did not experience and increase in blood vessel density in response to AdTGFB1. (J) ELISA analysis of peritoneal effluent displays significantly increased levels of VEGF in C57Bl/6 mice and MMP2$^{-/-}$ mice treated with TGFB but not in MMP9$^{-/-}$ mice.

***TGFB induced mesothelial cell transition is reduced in MMP9$^{-/-}$ mice***

We evaluated the effect of MMP9 deficiency on mesothelial cell transition and invasion using dual immunofluorescence with cytokeratin and a-SMA to identify transitioned cells. TGFB1 induced mesothelial cell transition and invasion in C57Bl/6 mice (Figure 4.5A-C, quantified in figure 5G). MMP9$^{-/-}$ mice treated with TGFB1 exhibited significantly lower levels of transitioned cells with no evidence of cellular invasion (Figure 4.5D-F). Laminin staining suggested that MMP9$^{-/-}$ mice were protected against TGFB induced basement membrane degradation (Supplemental figure 4.3).

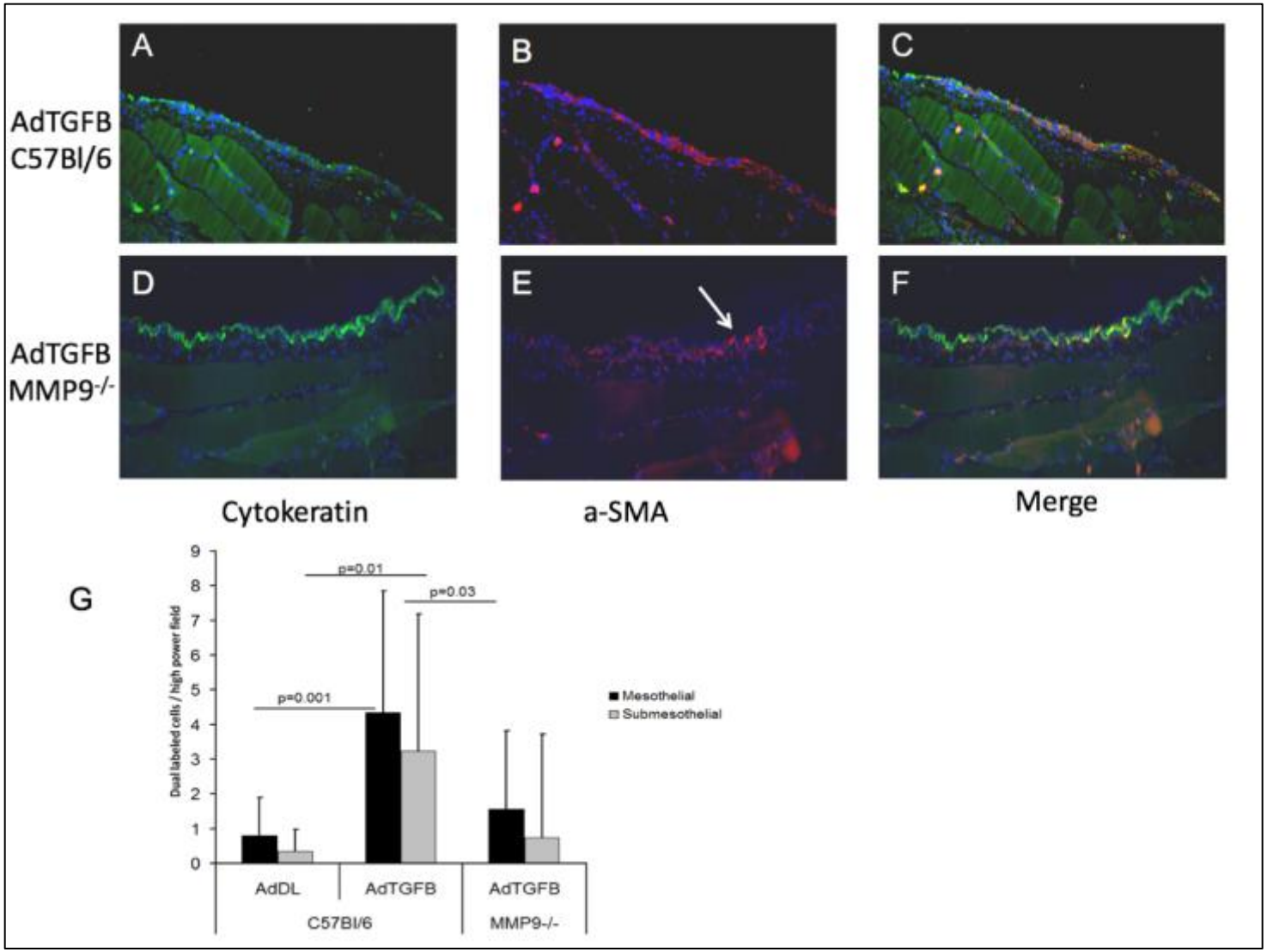
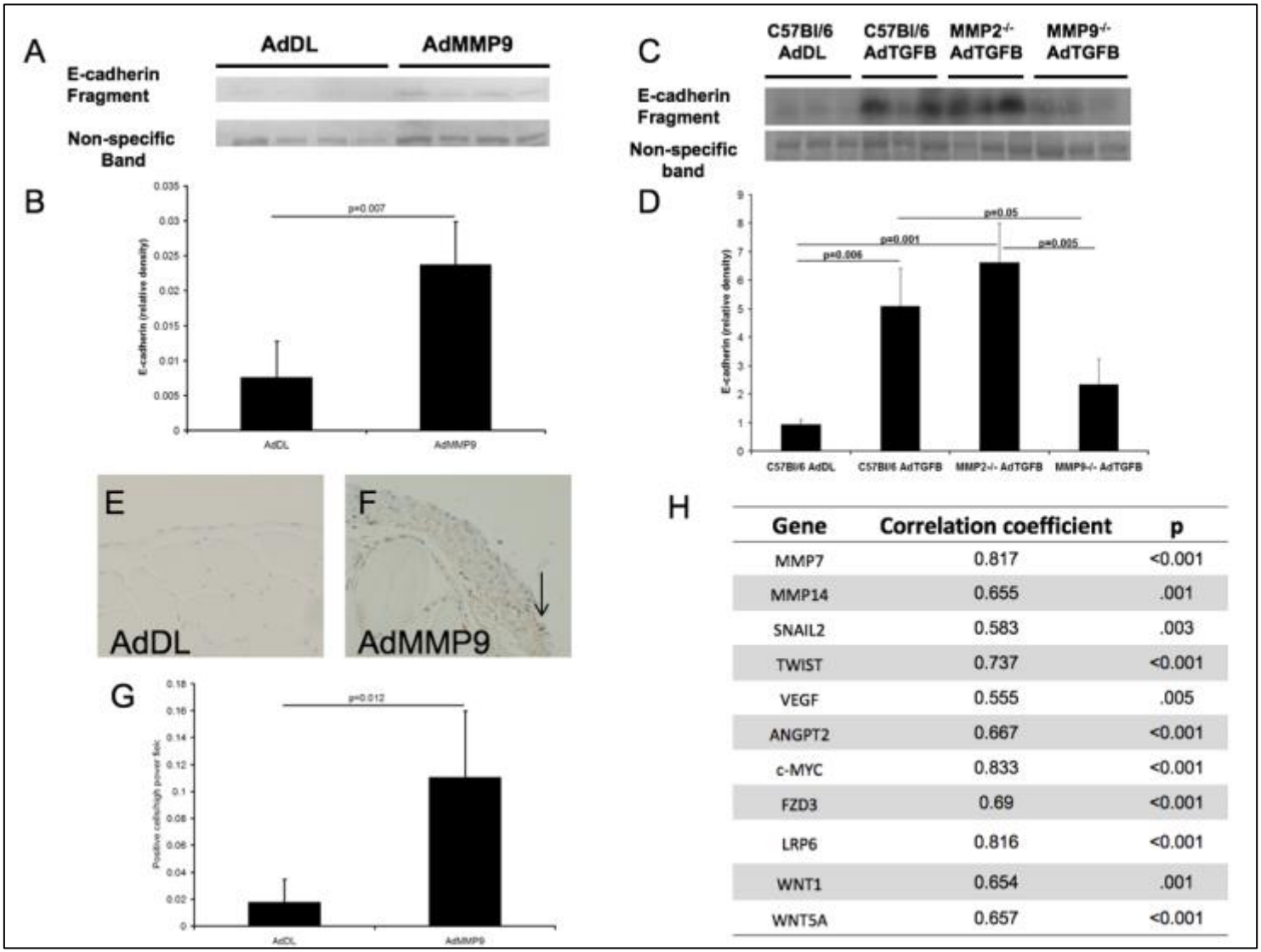

A
B
C
AdTGFB
C57Bl/6
D
E
F
AdTGFB
MMP9-/-
Cytokeratin
a-SMA
Merge
G
Dual labeled cells / high power field
p=0.01
p=0.03
p=0.001
Mesothelial
Submesothelial
AdDL
AdTGFB
AdTGFB
C57Bl/6
MMP9-/-

**Figure 4.5 Dual staining for cytokeratin and a-SMA in C57B1/6 and MMP9-/- mice -** (A-C) TGFB1 induces an increase in invasive dual labeled cells in C57Bl/6 mice. (D-F) A similar response was seen after TGFB1 treatment of $MMP9^{-/-}$ mice with occasional dual labeling of mesothelial cells (arrow in E). G) Quantification of dual staining. TGFB1 induces invasive (submesothelial) dual labelled cells. TGFB1 did not induce significant dual labeled cells in $MMP9^{-/-}$ mice.

***MMP9 cleaves E-cadherin***

As noted previously, AdMMP9 treated peritoneal tissue demonstrated a decrease in E-cadherin protein (Figure 4.3I, J) suggesting that MMP9 is involved in downregulation of E-cadherin. We determined whether MMP9 was involved in E-cadherin cleavage from the cell surface by measuring the amount of E-cadherin fragment (80kDa) in the peritoneal effluent from mice treated with AdMMP9. Western blot analysis revealed a 3-fold ($p=0.007$) increase in fragmented E-cadherin in response to MMP9 (Figure 4.6A, B). We also evaluated levels of E-cadherin fragment in the peritoneal effluent from TGFB-induced fibrosis in MMP2 or MMP9 deficient mice. Western blot analysis showed a significant increase in fragmented E-cadherin in the peritoneal effluent in C57Bl/6 and $MMP2^{-/-}$ animals treated with AdTGFB1 (Figure 4.6C, D). $MMP9^{-/-}$ mice treated with AdTGFB1 exhibited significantly lower levels (1.5 fold, $p=0.05$) of fragmented E-cadherin in the peritoneal effluent compared to C57Bl/6 mice treated with AdTGFB (Figure 4.6D).




H

| Gene | Correlation coefficient | p |
|---|---|---|
| MMP7 | 0.817 | <0.001 |
| MMP14 | 0.655 | .001 |
| SNAIL2 | 0.583 | .003 |
| TWIST | 0.737 | <0.001 |
| VEGF | 0.555 | .005 |
| ANGPT2 | 0.667 | <0.001 |
| c-MYC | 0.833 | <0.001 |
| FZD3 | 0.69 | <0.001 |
| LRP6 | 0.816 | <0.001 |
| WNT1 | 0.654 | .001 |
| WNT5A | 0.657 | <0.001 |

**Figure 4.6 MMP9 cleaves E-cadherin and activates b-catenin** - E-cadherin fragment (approx 80 kDa) was measured in the peritoneal effluent. (A) Representative blot form mice treated with AdDL or AdMMP9. (B) Analysis of band density normalized to a non-specific band on Coomasie blue stained sectiondemonstrates that MMP9 induces cleavage of E-cadherin. (C) Representative blot, normalized to a non-specific band on coomasie stained blot. (D) Analysis of band density demonstrates that TGFB1 induced E-Cadherin cleavage in C57Bl/6 mice and in $MMP2^{-/-}$ mice but there was significantly less E-Cadherin cleavage product in the peritoneal effluent of $MMP9^{-/-}$ mice treated with AdTGFB1. E, F) Staining of peritoneal tissue for active b-catenin. E) No active b-catenin was observed in the control adenovirus (AdDL) treated mouse peritoneum, whereas active b-catenin was observed after exposure to MMP9 in the mesothelial cells (arrow) and other submesothelial cells (F). (G) Quantification of b-catenin staining. MMP9 induces an increase in active b-catenin in mesothelial and submesothelial cells. (H) On a larger gene panel including WNT related genes, MMP9 gene expression was correlated with a number of genes including other metalloproteinases, along with genes associated with cellular transition, angiogenesis, and WNT signaling.

***MMP9 signals through b-catenin***

Our findings indicate that MMP9 is essential in cleaving E-cadherin and is associated with an upregulation of VEGF and angiogenesis in peritoneal membrane injury. We hypothesize that MMP9 induced E-cadherin cleavage may lead to dissociation of the E-cadherin / b-catenin complex and facilitate b-catenin translocation to the nucleus. To support this, we found an increase in active b-catenin seen in the peritoneal membrane in animals treated with AdMMP9 (Figure 4.6E, quantified in figure 4.6G). This was not observed in the control adenovirus treated mice (Figure 4.6F).

Consistent with our in vivo experiments, MMP9 expression in the patient-derived mesothelial cell culture was correlated with transcription factors

associated with cellular transition (SNAIL1, TWIST), and genes associated with angiogenesis (VEGF, angiopoietin 2) (Figure 4.6H). We also observed a correlation between MMP9 gene expression and other genes involved in the WNT signaling pathway including WNT1, WNT5A, and the WNT receptors FZD3 and LRP6. MMP7, VEGF and c-myc are known to be regulated by b-catenin signaling and were all significantly associated with MMP9 gene expression.

In order to directly assess the role of b-catenin in MMP9 induced angiogenesis, we blocked b-catenin with a specific t-cell factor/lymphoid enhancer factor (TCF/LEF) inhibitor, ICG-001[25] in mice after infection with AdMMP9. Analysis of blood vessel density in the mouse peritoneum after adenoviral gene transfer confirmed that MMP9 induces an increase in blood vessel density in the peritoneum (Figure 4.7A, B). ICG-001 inhibited this angiogenic response (Figure 4.7C, quantified in figure 4.7E). ICG-001 did not induce significant changes in submesothelial thickness (Figure 4.7D). Animals treated with ICG-001 demonstrated a 4-fold ($p=0.023$) reduction in VEGF protein measured in the peritoneal tissue by western blot (Figure 4.7F, G).

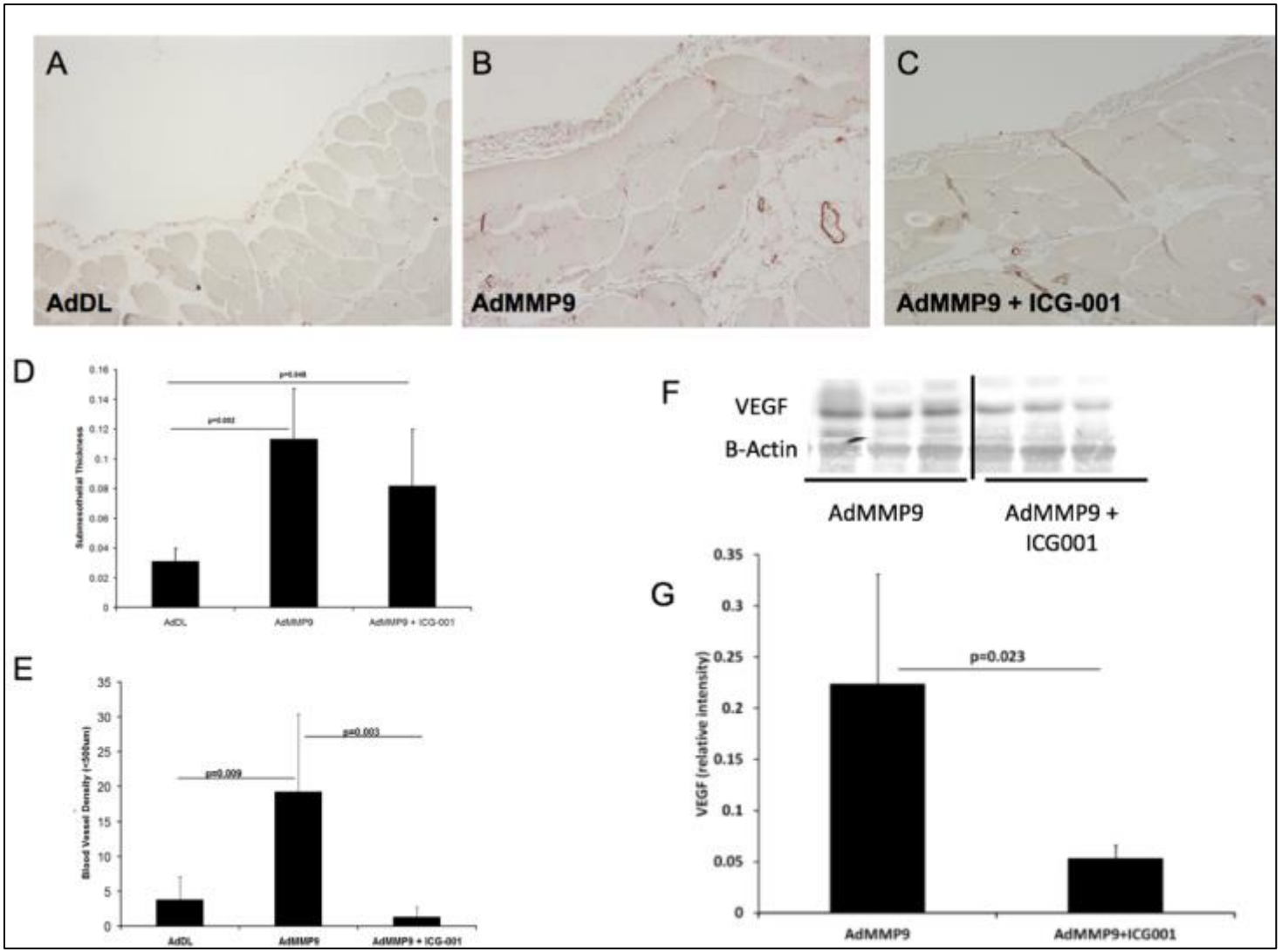

A
AdDL
B
AdMMP9
C
AdMMP9 + ICG-001
D
Submesothelial Thickness
AdDL
AdMMP9
AdMMP9 + ICG-001
E
Blood Vessel Density (<50um)
p=0.009
p=0.003
AdDL
AdMMP9
AdMMP9 + ICG-001
F
VEGF
B-Actin
AdMMP9
AdMMP9 + ICG001
G
VEGF (relative intensity)
p=0.023
AdMMP9
AdMMP9+ICG001

**Figure 4.7 Factor VIII stained sections of C57Bl/6 mouse peritoneum after adenoviral gene transfer -** (A) AdDL is a control virus and demonstrates the normal mouse peritoneum. (B) MMP9 induces an increase in blood vessel density and submesothelial thickness in the peritoneum (C) ICG-001 inhibits the angiogenic response to MMP9. ICG-001 does not induce a significant reduction in submesothelial thickness. (D) Quantification of submesothelial thickness. E) Quantification of angiogenesis. F, G) VEGF expression in peritoneal tissue is reduced by ICG-001 treatment. F) Representative blot quantified in (G).

## DISCUSSION

The peritoneal membrane is a lifeline for patients with end stage kidney disease on PD therapy. Injury to the peritoneum results in both structural and functional changes. Blood vessel formation is of critical importance because it is associated with high transport status [26] and a decline in peritoneal membrane function. In our study, we measured gene expression of MMPs in mesothelial cells derived from overnight effluent from stable PD patients. Ex-vivo mesothelial cell MMP9 mRNA expression was significantly correlated with peritoneal membrane solute transport in stable PD patients. Mesothelial cell gene expression of the associated gelatinase MMP2 did not correlate with solute transport properties.

Several recent studies have evaluated biomarkers of early peritoneal membrane injury in order to optimize renal replacement therapy [27, 28]. Our observations suggest that ex-vivo mesothelial cell MMP9 mRNA expression may be a useful biomarker for peritoneal membrane injury for PD patients but this

result needs to be confirmed in a larger cohort of stable PD patients with longitudinal follow up.

Recent studies by Barreto [28], Cho [29], and Hirahara [30] have demonstrated that MMP2 measured in the peritoneal effluent correlated with peritoneal membrane solute in large population studies. In the study by Barreto et al, MMP2 was measured in 86 stable PD patients and the intraperitoneal production of MMP2 was estimated [28]. In larger studies by Cho and colleagues and Hirahara and colleagues, total MMP2 in the peritoneal effluent was measured by ELISA [29], [30]. In our study, we evaluated both MMP2 and MMP9 gene expression in mesothelial cells derived from the peritoneal effluent. This ensured that the MMP expression reflects local intraperitoneal production. The close correlation between mesothelial cell MMP9 expression and peritoneal solute transport suggests that mesothelial derived MMP9 may be a useful biomarker, and may be directly involved in the pathology of peritoneal membrane injury.

Our results are discordant to the other 3 studies that have identified MMP2 as a potential biomarker for peritoneal membrane injury [27, 28]. The MMP9 enzyme is found at low concentration in the peritoneal effluent whereas MMP2 is found at a much higher concentration and is thus more easily measured [27, 28]. Furthermore, our results suggest that MMP2 is induced by MMP9 in the peritoneal tissues (Figure 4.2J) suggesting that locally active MMP9 may induce MMP2 which can then be measured in the PD effluent. This suggests that mesothelial derived MMP9 may be an earlier indicator of peritoneal membrane

injury. Hirahara and colleagues have also shown that in an animal model of peritoneal fibrosis, the majority of MMP2 detected in the peritoneal effluent is not active, but in situ film zymography shows extensive gelatinase activation within the injured peritoneal membrane [31], which suggests local activation of gelatinases within the peritoneal tissue.

We have identified a novel mechanism for MMP9 mediated peritoneal angiogenesis. Specifically, our results suggest that MMP9 leads to E-cadherin cleavage and activation of b-catenin signaling. Cleavage of a 50-84 kDa sized N-terminal fragment of E-cadherin is an essential step in epithelial cell transition and has been studied extensively in the setting of epithelial malignancies [32]. MMP9 has been reported to cleave an 80 kDa E-cadherin fragment in ovarian carcinoma cells [33]. Our results agree with these studies. We found that AdMMP9 treated mice had increased amounts of E-cadherin fragment in the peritoneal effluent (Figure 4.6A,B). Similarly, we found that wildtype and MMP2$^{-/-}$ mice treated with AdTGFB1 had high levels of E-cadherin fragment in the peritoneal effluent; this was greatly reduced in MMP9$^{-/-}$ mice exposed to TGFB1 (Figure 4.6C, D).

Several studies have identified that cleavage of E-cadherin can lead to b-catenin signaling [32, 34-36]. b-catenin is sequestered by E-cadherin at the cell membrane as part of the adherens junction. With E-cadherin cleavage, b-catenin is released into the cytosol. Cytosolic b-catenin is rapidly degraded and activation of the canonical WNT pathway is required to inhibit b-catenin ubiquitination [37]. Therefore, we hypothesize that in MMP9-induced peritoneal injury, there is likely

activation of WNT signaling that facilitates b-catenin mediated gene expression. In support of this, we found a correlation between MMP9 gene expression in patient-derived mesothelial cells and gene expression of other WNT signaling molecules such as WNT1 and the receptors FZD3 and LRP5. There was simultaneous activation of several b-catenin regulated genes including MMP7, MMP14, VEGF and c-myc (Figure 4.6H).

Previous studies have identified the role of MMP9 in angiogenesis in the setting of tumor cell metastasis [38], fibrosis [39], and ischemia [40]. MMPs have been hypothesized to be involved in degrading the basement membrane of blood vessels to allow endothelial cells the ability to migrate and form new blood vessels [41]. MMPs may also contribute to angiogenesis through the release of ECM bound pro-angiogenic factors [40] including VEGF. Our results support an angiogenic function of MMP9 as we observed increases in both VEGF and angiogenesis when MMP9 was overexpressed in the mouse peritoneum. The angiogenic response to peritoneal overexpression of TGFB was attenuated in $MMP9^{-/-}$ but not $MMP2^{-/-}$ mice. We did not assess the association between MMPs and uremia due to the lack of uremia in this model.

Furthermore, our results suggest a novel mechanism by which MMP9 induces VEGF to promote blood vessel growth. We propose that MMP9 induces b-catenin signaling which increases expression of VEGF. We observed nuclear localization of active b-catenin in peritoneal tissues after overexpression of AdMMP9 (Figure 4.6F, quantified in 4.6G). Further, we used the b-catenin

signaling inhibitor ICG-001 in the peritoneum of mice treated with AdMMP9. ICG-001 prevented MMP9 induce angiogenesis and blocked MMP9 associated increase in tissue concentration of VEGF (Figure 4.7).

Previous studies have demonstrated a role for SMAD signaling in TGFB induced VEGF expression [42]. Our data suggests both SMAD and MMP9 may be involved in regulating VEGF expression. In fact, these pathways have been linked by observations that SMAD and b-catenin cooperate in nuclear signaling [43].

MMPS have been involved in the remodelling of the ECM, but their role in fibrosis has not been fully elucidated. Several studies describe MMPs to contribute to progression of fibrosis [44, 45], however recent evidence demonstrates MMPs may have a more protective effect [46]. This discordance may be attributed to differences in function of diverse types of MMPs as collagenases degrade collagen and may be more protective and gelatinases degrade basement membrane and may be more injurious.

The role of EMT in fibrosis has become increasingly controversial. Studies in both the kidney [47] and the peritoneum [7] suggest that transition of mesothelial cells does not contribute significantly to the accumulation of submesothelial myofibroblasts characteristic of fibrosis. Despite these observations, the epithelium likely has a key role in driving peritoneal fibrosis indirectly and this process may require epithelial cellular transition without migration into the submesothelium [48]. Aroeira and colleagues previously found

that the cells grown from the peritoneum of PD patients take on different morphologies, from epithelial-like to fibroblast-like [5]. They demonstrated an association between cell morphology and peritoneal membrane transport that we were unable to replicate in our patients (Supplemental figure 4.4).

Abnormal expression of MMP9 has been implicated in induction of EMT [44] and fibrosis [49]. Tan and colleagues found that MMP9 causes EMT of renal tubular epithelial cells in vitro [50]. Our observations support these findings. We found infection with AdMMP9 induced co-localization of epithelial and mesenchymal markers in cells suggesting an EMT process (Figure 4.3). We also found the TGFB induced EMT response was attenuated in MMP9$^{-/-}$ mice (Figure 4.5).

There is extensive data from cancer research to suggest that MMP2 [51] and MMP9 [52] are directly involved in cellular invasion after EMT. It is clear that MMP2 and 9 can effectively degrade basement membrane proteins in an in vitro assay using material such as matrigel [32, 53]. However, cross-linked, mature basement membrane may not be susceptible to gelatinase activity [54]. Our study demonstrated potential degradation of basement membrane in response to AdMMP9 and AdTGFB. Laminin stained sections from these mice demonstrated areas of decreased or absent basement membrane, whereas the control adenovirus treated mice demonstrated a continuous basement membrane structure. (Supplemental Figure 4.2). Transitioned mesothelium do not constitute a large component of submesothelial myofibroblasts after peritoneal membrane

injury [7] and our data do not support a role for MMP9 in cellular invasion (Figure 4.3H).

Therefore, in the setting of peritoneal membrane injury, MMP9 appears to have a role in the induction of EMT but does not facilitate cellular invasion. MMP9 also has a role in peritoneal membrane angiogenesis and we have identified a novel b-catenin mediated pathway for this response. This expands potential therapeutic options for preservation of the peritoneal membrane in PD patients by targeting the metalloproteinase or WNT pathways. Furthermore, mesothelial cell MMP9 mRNA may be a potential biomarker for peritoneal membrane injury and could eventually be used to guide PD therapy.

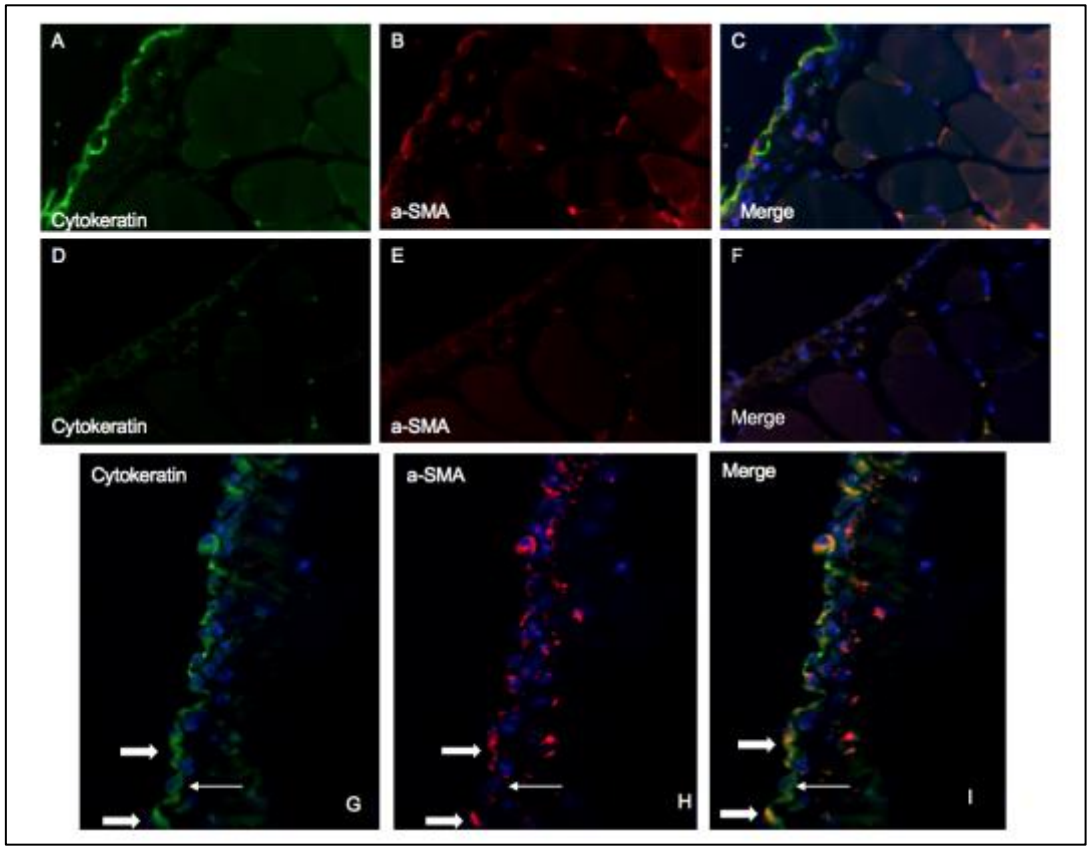
**ACKNOWLEDGEMENT**

This work was supported by the Kidney Foundation of Canada. Additional support was available through St. Joseph's Healthcare, Hamilton.

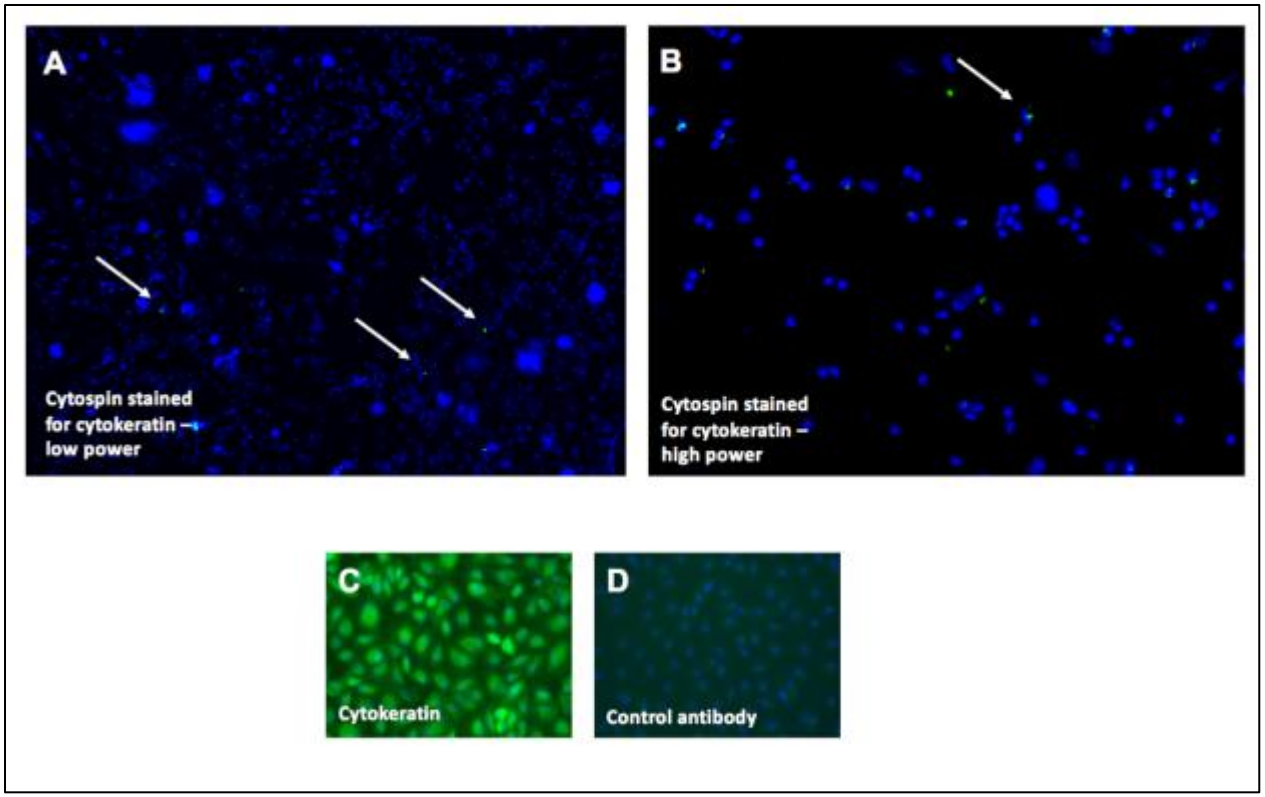
**CONFLICT OF INTEREST**

The results in this paper have not been previously published except in abstract form. The authors have no conflict of interest with respect to the findings included in this paper.

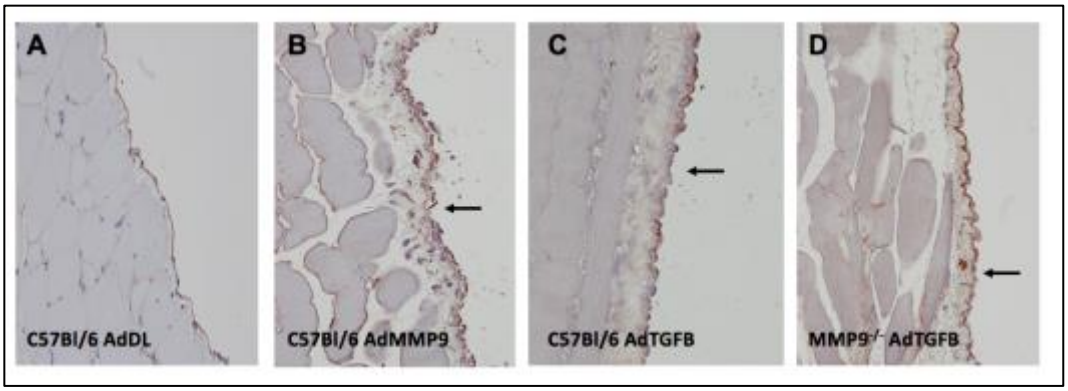
**SUPPLEMENTARY MATERIAL**



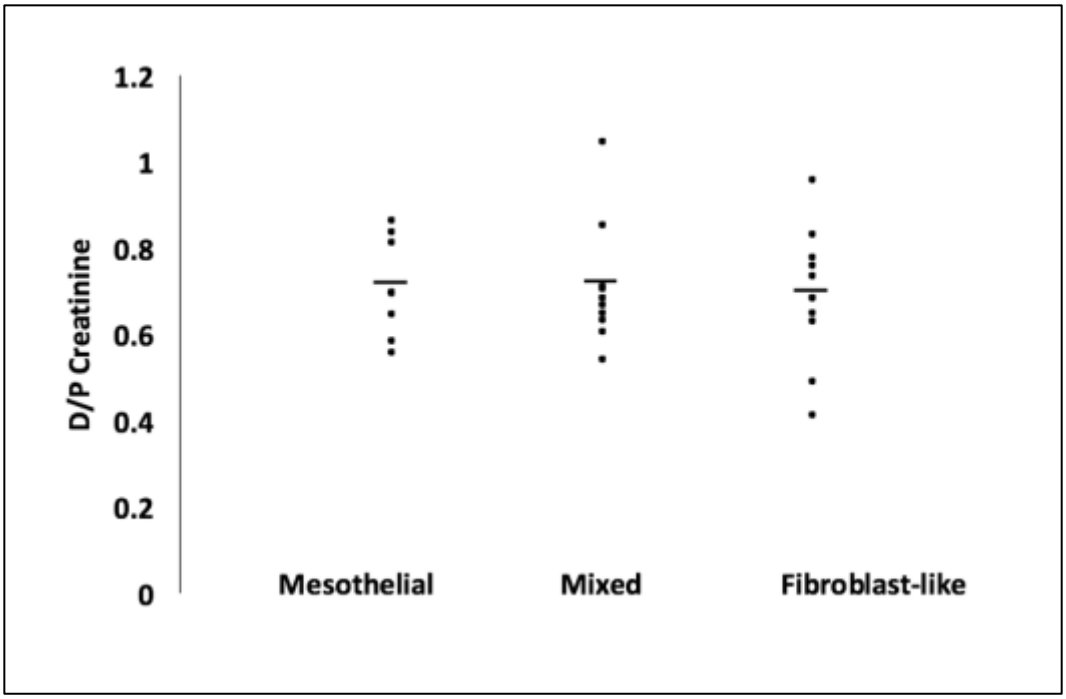

**Supplemental Figure 4.1 – Immunofluorescence with fluorescently labeled a-SMA antibody** (A-C) Positive staining for cytokeratin / alpha-SMA (D-F) Control staining without primary a-SMA antibody and with isotype control for cytokeratin. G-I) Dual staining using a second primary antibody for a-SMA (a-SMA-cy3 (Sigma) demonstrates similar staining pattern. Thick arrow: dual labeled cells. Thin arrow: single-labeled cytokeratin positive mesothelial cell.




**Supplemental Figure 4.2 – Immunofluorescence for cytokeratin in ex vivo mesothelial cells** A, B) An aliquot of cells from PD fluid was plated by cytospin and stained with cytokeratin antibody. A) Low power and B) high power images show rare (approximately 5%) of cells are cytokeratin positive mesothelial cells. C) After culture and 2 passages, virtually 100% of the cells are cytokeratin positive mesothelial cells. D) Similar cell culture with non-specific fluorescent antibody to control for non-specific staining.




**Supplemental Figure 4.3 - Laminin stained sections of mouse peritoneum after adenoviral gene transfer** (A) C57Bl/6 mice treated with control virus demonstrate normal submesothelial basement membrane architecture. (B) C57Bl/6 mice treated with AdMMP9 demonstrated an expansion of submesothelial tissue with some disruptions in the basement membrane. (C) C57Bl/6 mice treated with AdTGFB also demonstrated a disrupted basement membrane with submesothelial thickening. (D) MMP9$^{-/-}$ mice treated with AdTGFB also showed some areas of possible degradation in the basement membrane.




**Supplemental Figure 4.4 – Cell Type and Peritoneal Membrane function** There was no significant correlation correlation between cell morphology and d/p creatinine.

**References**

1. Margetts PJ, Brimble KS. Peritoneal dialysis, membranes and beyond. Curr Opin. Nephrol. Hypertens 2006;15(6):571-576

2. Williams JD, Craig KJ, Topley N, *et al.* Morphologic changes in the peritoneal membrane of patients with renal disease. J. Am. Soc. Nephrol 2002;13(2):470-479

3. Davies SJ. Longitudinal relationship between solute transport and ultrafiltration capacity in peritoneal dialysis patients. Kidney Int 2004;66(6):2437-2445

4. Brimble KS, Walker M, Margetts PJ, *et al.* Meta-analysis: peritoneal membrane transport, mortality, and technique failure in peritoneal dialysis. J Am Soc Nephrol 2006;17(9):2591-2598

5. Aroeira LS, Aguilera A, Selgas R, *et al.* Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis 2005;46(5):938-948

6. Margetts PJ, Bonniaud P, Liu L, *et al.* Transient overexpression of TGF-{beta}1 induces epithelial mesenchymal transition in the rodent peritoneum. J Am Soc Nephrol 2005;16(2):425-436

7. Chen YT, Chang YT, Pan SY, *et al.* Lineage tracing reveals distinctive fates for mesothelial cells and submesothelial fibroblasts during peritoneal injury. J Am Soc Nephrol 2014;25(12):2847-2858

8. Catania JM, Chen G, Parrish AR. Role of matrix metalloproteinases in renal pathophysiologies. Am J Physiol Renal Physiol. 2007;292(3):F905-911. Epub 2006 Dec 2026.

9. Zhao XL, Sun T, Che N, *et al.* Promotion of hepatocellular carcinoma metastasis through matrix metalloproteinase activation by epithelial-mesenchymal transition regulator Twist1. J Cell Mol Med. 2011;15(3):691-700. doi: 610.1111/j.1582-4934.2010.01052.x.

10. Patel P, West-Mays J, Kolb M, *et al.* Platelet derived growth factor B and epithelial mesenchymal transition of peritoneal mesothelial cells. Matrix Biol 2010;29(2):97-106

11. Belotti D, Paganoni P, Manenti L, *et al.* Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth

factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. Cancer Res. 2003;63(17):5224-5229.

12. Bauvois B. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. Biochim Biophys Acta. 2012;1825(1):29-36. doi: 10.1016/j.bbcan.2011.1010.1001. Epub 2011 Oct 1012.

13. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008;65(23):3756-3788. doi: 3710.1007/s00018-00008-08281-00011.

14. Korol A, Pino G, Dwivedi D, *et al.* Matrix metalloproteinase-9-null mice are resistant to TGF-beta-induced anterior subcapsular cataract formation. Am J Pathol. 2014;184(7):2001-2012. doi: 2010.1016/j.ajpath.2014.2003.2013. Epub 2014 May 2006.

15. Qu B, Liu BR, Du YJ, *et al.* Wnt/beta-catenin signaling pathway may regulate the expression of angiogenic growth factors in hepatocellular carcinoma. Oncol Lett. 2014;7(4):1175-1178. Epub 2014 Jan 1127.

16. Twardowski ZJ. Clinical value of standardized equilibration tests in CAPD patients. Blood Purif 1989;7(2-3):95-108

17. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. Curr. Protoc. Mol. Biol 2011;Chapter 25:Unit25B.10. doi: 10.1002/0471142727.mb25b10s94.:Unit25B

18. Ravanti L, Kahari VM. Matrix metalloproteinases in wound repair ( review). Int. J Mol. Med 2000;6(4):391-407

19. Sime PJ, Xing Z, Graham FL, *et al.* Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin. Invest 1997;100(4):768-776

20. Leifler KS, Svensson S, Abrahamsson A, *et al.* Inflammation induced by MMP-9 enhances tumor regression of experimental breast cancer. J Immunol. 2013;190(8):4420-4430. doi: 4410.4049/jimmunol.1202610. Epub 1202013 Mar 1202615.

21. Xing Z, Ohkawara Y, Jordana M, *et al.* Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. J. Clin. Invest 1996;97(4):1102-1110

22. Kheradmand F, Rishi K, Werb Z. Signaling through the EGF receptor controls lung morphogenesis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP2. J Cell Sci. 2002;115(Pt 4):839-848.

23. Coussens LM, Tinkle CL, Hanahan D, *et al.* MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell. 2000;103(3):481-490.

24. Sarin S, Boivin F, Li A, *et al.* beta-Catenin overexpression in the metanephric mesenchyme leads to renal dysplasia genesis via cell-autonomous and non-cell-autonomous mechanisms. Am. J. Pathol 2014;184(5):1395-1410

25. Emami KH, Nguyen C, Ma H, *et al.* A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A. 2004;101(34):12682-12687. Epub 12004 Aug 12616.

26. Numata M, Nakayama M, Nimura S, *et al.* Association between an increased surface area of peritoneal microvessels and a high peritoneal solute transport rate. Perit. Dial. Int 2003;23(2):116-122

27. Barreto DL, Coester AM, Struijk DG, *et al.* Can effluent matrix metalloproteinase 2 and plasminogen activator inhibitor 1 be used as biomarkers of peritoneal membrane alterations in peritoneal dialysis patients? Perit Dial Int. 2013;33(5):529-537. doi: 510.3747/pdi.2012.01063. Epub 02013 Sep 01061.

28. Lopes Barreto D, Struijk DG, Krediet RT. Peritoneal Effluent MMP-2 and PAI-1 in Encapsulating Peritoneal Sclerosis. Am J Kidney Dis. 2015;65(5):748-753. doi: 710.1053/j.ajkd.2014.1010.1022. Epub 2014 Dec 1017.

29. Cho Y, Johnson DW, Vesey DA, *et al.* Dialysate interleukin-6 predicts increasing peritoneal solute transport rate in incident peritoneal dialysis patients. BMC. Nephrol 2014;15(1):8-15

30. Hirahara I, Inoue M, Okuda K, *et al.* The potential of matrix metalloproteinase-2 as a marker of peritoneal injury, increased solute transport, or progression to encapsulating peritoneal sclerosis during peritoneal dialysis--a multicentre study in Japan. Nephrol. Dial. Transplant 2007;22(2):560-567

31. Hirahara I, Ogawa Y Fau - Kusano E, Kusano E Fau - Asano Y, *et al.* Activation of matrix metalloproteinase-2 causes peritoneal injury during peritoneal dialysis in rats. (0931-0509 (Print))

32. Zheng G, Lyons JG, Tan TK, *et al.* Disruption of E-cadherin by matrix metalloproteinase directly mediates epithelial-mesenchymal transition downstream of transforming growth factor-beta1 in renal tubular epithelial cells. Am J Pathol. 2009;175(2):580-591. doi: 510.2353/ajpath.2009.080983. Epub 082009 Jul 080989.

33. Symowicz J, Adley BP, Gleason KJ, *et al.* Engagement of collagen-binding integrins promotes matrix metalloproteinase-9-dependent E-cadherin ectodomain shedding in ovarian carcinoma cells. Cancer Res. 2007;67(5):2030-2039.

34. Mei JM, Borchert GL, Donald SP, *et al.* Matrix metalloproteinase(s) mediate(s) NO-induced dissociation of beta-catenin from membrane bound E-cadherin and formation of nuclear beta-catenin/LEF-1 complex. Carcinogenesis. 2002;23(12):2119-2122.

35. Dwivedi A, Slater SC, George SJ. MMP-9 and -12 cause N-cadherin shedding and thereby beta-catenin signaling and vascular smooth muscle cell proliferation. Cardiovasc Res. 2009;81(1):178-186. doi: 110.1093/cvr/cvn1278. Epub 2008 Oct 1013.

36. Yamashita CM, Dolgonos L, Zemans RL, *et al.* Matrix metalloproteinase 3 is a mediator of pulmonary fibrosis. Am J Pathol. 2011;179(4):1733-1745. doi: 1710.1016/j.ajpath.2011.1706.1041. Epub 2011 Aug 1724.

37. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17(1):9-26. doi: 10.1016/j.devcel.2009.1006.1016.

38. Mira E, Lacalle RA, Buesa JM, *et al.* Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface. J Cell Sci. 2004;117(Pt 9):1847-1857.

39. Heissig B, Rafii S, Akiyama H, *et al.* Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. J Exp Med. 2005;202(6):739-750. Epub 2005 Sep 2012.

40. Bendeck MP. Macrophage matrix metalloproteinase-9 regulates angiogenesis in ischemic muscle. Circ Res. 2004;94(2):138-139.

41. van Hinsbergh VW, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc Res. 2008;78(2):203-212. Epub 2007 Dec 2012.

42. Patel P, Sekiguchi Y, Oh KH, *et al.* Smad3-dependent and -independent pathways are involved in peritoneal membrane injury. Kidney Int. 2010;77(4):319-328. doi: 310.1038/ki.2009.1436. Epub 2009 Dec 1032.

43. Tian X, Zhang J, Tan TK, *et al.* Association of beta-catenin with P-Smad3 but not LEF-1 dissociates in vitro profibrotic from anti-inflammatory effects of TGF-beta1. J Cell Sci. 2013;126(Pt 1):67-76. doi: 10.1242/jcs.103036. Epub 102012 Nov 103030.

44. Tan TK, Zheng G, Hsu TT, *et al.* Matrix metalloproteinase-9 of tubular and macrophage origin contributes to the pathogenesis of renal fibrosis via macrophage recruitment through osteopontin cleavage. Lab Invest. 2013;93(4):434-449. doi: 410.1038/labinvest.2013.1033. Epub 2013 Jan 1028.

45. Zhao H, Dong Y, Tian X, *et al.* Matrix metalloproteinases contribute to kidney fibrosis in chronic kidney diseases. World Journal of Nephrology;2(3):84-89

46. Fielding CA, Jones GW, McLoughlin RM, *et al.* Interleukin-6 signaling drives fibrosis in unresolved inflammation. Immunity. 2014;40(1):40-50. doi: 10.1016/j.immuni.2013.1010.1022. Epub 2014 Jan 1019.

47. Humphreys BD, Lin SL, Kobayashi A, *et al.* Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am. J. Pathol 2010;176(1):85-97

48. Wu CF, Chiang WC, Lai CF, *et al.* Transforming growth factor beta-1 stimulates profibrotic epithelial signaling to activate pericyte-myofibroblast transition in obstructive kidney fibrosis. Am. J. Pathol 2013;182(1):118-131

49. Pardo A, Selman M. Role of matrix metaloproteases in idiopathic pulmonary fibrosis. Fibrogenesis Tissue Repair. 2012;5(Suppl 1 Proceedings of Fibroproliferative disorders: from biochemical analysis to targeted therapiesPetro E Petrides and David Brenner):S9. eCollection 2012.

50. Tan TK, Zheng G, Hsu TT, *et al.* Macrophage matrix metalloproteinase-9 mediates epithelial-mesenchymal transition in vitro in murine renal tubular

cells. Am J Pathol. 2010;176(3):1256-1270. doi: 1210.2353/ajpath.2010.090188. Epub 092010 Jan 090114.

51. Riches K, Morley ME, Turner NA, *et al.* Chronic hypoxia inhibits MMP-2 activation and cellular invasion in human cardiac myofibroblasts. J Mol Cell Cardiol. 2009;47(3):391-399. doi: 310.1016/j.yjmcc.2009.1006.1002. Epub 2009 Jun 1011.

52. Zhao S, Ma W, Zhang M, *et al.* High expression of CD147 and MMP-9 is correlated with poor prognosis of triple-negative breast cancer (TNBC) patients. Med Oncol. 2013;30(1):335. doi: 310.1007/s12032-12012-10335-12034. Epub 12012 Dec 12022.

53. Joseph MJ, Dangi-Garimella S, Shields MA, *et al.* Slug is a downstream mediator of transforming growth factor-beta1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells. J. Cell Biochem 2009;108(3):726-736

54. Sabeh F, Li XY, Saunders TL, *et al.* Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. J Biol Chem. 2009;284(34):23001-23011. doi: 23010.21074/jbc.M23109.002808. Epub 002009 Jun 002819.

# CHAPTER 5: THE WNT SIGNALING PATHWAY IS ACTIVATED DURING PERITONEAL MEMBRANE INJURY

Authors: Manreet Padwal, Genyang Cheng, Limin Liu, Felix Boivin, A.S. Gangji, K.S. Brimble, Darren Bridgewater, Peter J. Margetts

Corresponding Author: Peter Margetts, Division of Nephrology, St. Joseph's Hospital, Department of Medicine, McMaster University, Hamilton, ON, Canada L8P 4A6. Phone: (905) 525-9140 ext 32299, Fax: 905-540-6589, Email: margetts@mcmaster.ca

Citation: Submitted to American Journal of Physiology Renal Physiology

**PREFACE**

In the previous Chapter, we established a role for MMP9 in mediating peritoneal membrane angiogenesis. Moreover, we observed MMP9 to be directly associated with increased peritoneal membrane solute transport. We suggested a novel mechanism by which MMP9 cleaves E-cadherin and releases membrane bound b-catenin. We propose that active WNT signaling stabilizes free b-catenin and allows for upregulation of angiogenesis related genes particularly VEGF. Therefore, we further investigate WNT/b-catenin signaling in peritoneal membrane injury in this chapter. We hypothesize that TGFB interacts with canonical WNT signaling to induce peritoneal membrane injury and blocking this pathway may reduce injury. We examine WNT related ligands and receptors in patient derived mesothelial cells and observe a strong correlation between WNT1 and solute transport. In a TGFB model of peritoneal membrane injury, we also observe activation of WNT/b-catenin signaling. We confirm the role of WNT/b-catenin in TFGB induced peritoneal angiogenesis by using two different inhibitors. Our study supports current literature of the adverse effects of WNT/b-catenin signaling in other fibrotic diseases.

Manreet Padwal designed the study, performed all experiments and data analysis. The animal work was carried out by Manreet Padwal and Genyang Cheng. AdTGFB was amplified by Limin Liu. Adenovirus expressing DKK-1 was provided by Dr. Calvin Kuo, Stanford University and was amplified by Manreet Padwal with the assistance of Limin Liu. Dr. Scott Brimble and Dr.

Azim Gangji developed the clinical study facilitating the collection of mesothelial cells and patient data. Manreet Padwal developed an ex vivo cell culture from patient peritoneal effluent. Manreet Padwal performed all other experiments and Dr. Genyang Cheng assisted with these. Histology support was carried out by Mary Jo Smith and Dr. Felix Boivin assisted with completion of immunohistochemistry for b-catenin. Manreet Padwal completed all data analysis and wrote the original draft of the manuscript. Dr. Peter Margetts contributed to the concept of this paper, interpretation of the data, Nanostring analysis and reviewed the final manuscript.

**ABSTRACT**

The WNT signaling pathway is involved in wound healing and fibrosis. We evaluated the WNT signaling pathway in peritoneal membrane injury. We assessed WNT1 protein expression in the peritoneal effluents of 54 stable peritoneal dialysis (PD) patients and WNT-related gene expression in ex vivo mesothelial cell cultures from 21 PD patients. In a transforming growth factor beta (TGFB) mediated animal model of peritoneal fibrosis, we evaluated regulation of the WNT pathway and the effect of WNT inhibition on peritoneal fibrosis and angiogenesis.

WNT1 protein and gene expression was positively correlated with peritoneal membrane solute transport in PD patients. WNT1 gene expression was associated with genes involved in cellular transition such as SNAIL1 and TWIST. In the mouse peritoneum, TGFB-induced peritoneal fibrosis was associated with increased expression of WNT2, WNT4 and WNT11 and decreased expression of WNT7B and WNT8A. Peritoneal b-catenin protein was significantly upregulated after infection with AdTGFB. Elements of the WNT signaling pathway were increased after exposure to AdTGFB. Treatment with a b-catenin inhibitor (ICG-001) in mice with AdTGFB-induced peritoneal fibrosis resulted in attenuation of peritoneal angiogenesis and reduced vascular endothelial growth factor. Similar results were also observed with the WNT antagonist Dickkopf related protein (DKK) 1. In addition to this, DKK-1 blocked epithelial to mesenchymal transition and increased levels of the cell adhesion protein E-cadherin.

We provide evidence that WNT signaling is active in the setting of experimental peritoneal fibrosis and WNT1 correlates with patient peritoneal membrane solute transport. Intervention in this pathway is a possible therapy for peritoneal membrane injury.

## INTRODUCTION

Peritoneal dialysis (PD) is a renal replacement therapy that uses the semipermeable peritoneal membrane to provide dialysis and ultrafiltration. Peritoneal membrane injury is a consequence of peritonitis or long-term exposure to bioincompatible PD fluid. With injury, the peritoneal membrane undergoes structural changes including the denudation of the mesothelial cells, thickening of the submesothelial zone, and angiogenesis. These structural changes correlate with increased solute transport and a loss of ultrafiltration [1]. Ultrafiltration dysfunction has been identified as a contributing factor of modality failure [2,3] and reduced peritoneal ultrafiltration is associated with increased mortality [4,5]. Increased solute transport and reduced peritoneal ultrafiltration leads to hypervolemia, which has been associated with increased comorbidity, hypertension, and arterial stiffness [6].

The role of transforming growth factor beta (TGFB) is a key contributor to peritoneal membrane injury [7]. TGFB induces collagen deposition [8], activates fibroblasts [9] and promotes angiogenesis in the submesothelial zone of the peritoneal membrane [7]. Epithelial mesenchymal transition (EMT) describes the transition of mesothelial cells to a mesenchymal phenotype with loss of intercellular adhesion, loss of epithelial markers, and increased expression of mesenchymal proteins [10]. Although the role of EMT in peritoneal fibrosis is controversial [11], TGFB has been shown to induce EMT of peritoneal

mesothelial cells [12] and these transitioned cells are a source of angiogenic and fibrogenic growth factors [13].

The WNT signaling pathway plays a complementary role to TGFB in promoting fibrosis in many different organ systems [14], however its role is yet to be elucidated in the development of peritoneal membrane injury. This pathway is comprised of 19 WNT proteins, 9 Frizzled (FZD) receptors, several co-receptors including lipoprotein receptor proteins (LRP) 5 and 6 and the orphan tyrosine kinase receptor ROR2 [15]. WNT signaling is categorized as either canonical or non-canonical, depending on whether b-catenin is activated [16]. Cytoplasmic levels of b-catenin are tightly regulated by a protein complex which includes glycogen synthase kinase (GSK) 3B. When active, this complex facilitates b-catenin ubiquitination and proteolysis. During canonical WNT signaling, WNTs bind to the FZD receptor which phosphorylates disheveled (DVL) which in turn phosphorylates and inactivates GSK3b. Stabilized b-catenin then translocates to the nucleus and acts as a signal transduction factor inducing genes involved in cellular proliferation and differentiation. b -catenin activated genes include c-myc, matrix metalloproteinase (MMP)7, and vascular endothelial growth factor (VEGF)[26]. Secreted frizzled-related proteins (SFRP) and Dickkopf (DKK) proteins act as inhibitors to WNT signaling [16].

There is limited evidence of the role of the WNT signaling pathway in the setting of peritoneal membrane injury [17,18]. In our study, we found that WNT1 protein and gene expression in primary mesothelial cell culture directly correlated

with peritoneal membrane function in stable PD patients. We also confirmed activation of WNT pathways in an experimental model of peritoneal membrane injury. Our results indicate that there is an interaction between TGFB and b-catenin in mediating peritoneal membrane injury and we have identified a novel mechanism contributing to EMT and angiogenesis in the peritoneum.

## METHODS

### Recombinant Adenovirus

AdTGFB was created with TGFB1 cDNA mutated at residues 223 and 225, so that the transgene product does not bind to the latency-associated protein and is therefore biologically active [45]. AdDKK-1 was kindly provided by Dr. Calvin Kuo, Stanford University. A null adenovirus (AdDL) was used as a control. Amplification and purification of adenoviruses was performed using CsCl gradient centrifugation as previously described [46].

### Human Mesothelial Cell Culture

A 2L overnight dwell of PD effluent was obtained from 54 stable PD patients free from peritonitis for at least 3 months before enrollment. A peritoneal equilibration test (PET) was performed at the same clinic visit. The PET was carried out using a four hour dwell of 4.25% glucose solution as previously described [19]. Solute transport data measured by dialysate to plasma creatinine ratio (d/p creatinine). Demographic information including age, time on PD therapy, and presence of diabetes was also recorded. For 21 of these patients, we developed an ex vivo mesothelial cell culture as previously described [20].

**Animal Experiments**

All animal studies were performed according to the Canadian Council on Animal Care guidelines. Mice (C57BL/6, 5-6 weeks; Harlan, Indianapolis, IN, USA) received an intraperitoneal (IP) injection of $1 \times 10^8$ plaque forming units (pfu) of first generation AdTGFB or AdDL. AdTGFB was diluted to 100 mL in phosphate-buffered saline. Animals (n=6 animals/group) were injected with a peritoneal dwell of 3 ml of 4.25% glucose dialysis solution (Dianeal, Baxter, Mississauga ON Canada) 30 minutes prior to euthanasia 4 and 10 days after infection with adenovirus.

In the next experiment, $1 \times 10^8$ pfu of AdTGFB was administered to C57Bl/6 mice via IP injection. Starting at day 4, animals were given daily IP injections of ICG-001 (Millipore, Etobicoke, ON, Canada) (5mg/kg) for the following five days (n=5 animals/group). In the final experiment, C57Bl/6 mice received an IP injection of $1 \times 10^8$ pfu of AdTGFB + AdDL, AdDKK-1 + AdDL, or AdTGFB + AdDKK-1 (n=5 animals/group). Animals were euthanized 10 days after infection with AdTGFB, with a 4.25% PD solution peritoneal dwell 30 minutes prior.

The entire anterior abdominal wall was then removed from euthanized mice and divided with the lower section placed in 10% formalin for histological analysis and the upper section was placed in 1 mL Easy-blue$^{TM}$ reagent (Fraggabio, North York, ON, Canada) for RNA isolation. Omental tissue was taken and frozen in liquid nitrogen for protein analysis.

**Gene Expression Analysis**

Peritoneal mRNA was extracted from sacrificed animals by immersion of the parietal peritoneal surface in the Easy-blue™ reagent (Froggabio) for 20 minutes. mRNA was also extracted from the ex vivo mesothelial cell culture. Concentration of extracted mRNA was determined by Agilent 2100 Bioanalyzer using RNA Nano Chips (Agilent Technologies, Mississauga, ON, Canada). Samples were diluted to 20ng/ul for the Nanostring assay. NanoString technology has been previously described by Kulkarni and colleagues [47]. Briefly, customized probes were created for a panel 31 human and 49 mouse WNT signaling related genes, genes related to cellular transition, fibrosis, angiogenesis, and 4 housekeeping genes (Supplemental tables). mRNA counts were normalized using the geometric mean of the 4 housekeeping genes. A codeset consists of gene-specific capture probes and reporter probes tagged with a specific fluorescent barcode. Probes were hybridized to 100 ng of total RNA. nCounter™ Digital Analyzer was used to count the individual barcodes. Positive and negative controls were included. We compared a subset of 6 WNT genes to D/P creatinine.

**Histology**

Tissue samples were fixed in a sufficient amount of 10% neutral-buffered formalin for 48 hours, followed by 70% ethanol. The tissue samples were then processed, paraffin embedded, and cut in 5-um sections. Sections were stained with Masson's trichrome. Sections were also stained using vonWillebrand Factor antibody / Factor VIII (Dako, Burlington, ON, Canada) to measure blood vessel density. Five images were taken per stained section from each animal. Northern

Eclipse software (Empix Imaging, Mississauga, ON, Canada) was used to quantify blood vessel density and thickness of the submesothelial zone. Immunhistochemistry was completed for the non-phosphorylated form or active form of b-catenin using (Millipore) [48]. B-catenin positive cells were counted and quantified in photomicrographs using Image J (5 images per section).

We used immunofluorescence for a-SMA and pancytokeratin to identify EMT in the mesothelial and submesothelial layers. We used a monoclonal mouse anti-human a-SMA antibody (1:50) (Dako, Burlington, ON, Canada) as a mesenchymal marker with a Texas Red goat anti-mouse secondary antibody (1:100) (Molecular Probes, Life Technologies). Following this, a FITC labeled monoclonal mouse antibody was used against the epithelial marker pan-cytokeratin (1:50) (Sigma-Aldrich, Oakville, ON, Canada). Cells expressing both a-SMA and pancytokeratin were categorized as dual labelled cells undergoing EMT and were quantified in the mesothelial cell layer and in the submesothelial cell layer from five high-power fields from each section using a blinded approach.

**Protein Analysis**

Protein was extracted from frozen omental tissue by homogenization in a standard lysis buffer with proteinase inhibitors. Equal amounts of the protein were run on a 8% or 10% SDS-PAGE gel, transferred to a membrane, and probed with Dvl2 (1:1000, cell signaling ), b-catenin (1:1000, cell signaling ), p-GSK3B (1:1000, cell signaling ), GSK3B (1:1000, cell signaling ) and alpha-tubulin (1:1000, Sigma), VEGF (1:1000, Millipore), E-cadherin (1:1000, BD

Biosciences). Equal volumes of PD effluent were run on 10% SDS-PAGE gel and probed with WNT1 (1:200, Santa Cruz). Coomasie blue staining of the western blot was used to obtain a non-specific band for the loading control.

**Statistics**

Significance of correlations were evaluated using Spearman`s coefficient. Bonferroni correction was applied for multiple testing. Differences between AdTGFB and AdDL treated mice were evaluated using t-test. Differences between multiple groups were assessed using ANOVA with Tukey`s post hoc analysis. Analysis was carried out using SPSS v 22.0 (IBM, Armonk NY).

## RESULTS

### *WNT1 is correlated with peritoneal solute transport properties*

We evaluated peritoneal effluents from 54 stable peritoneal dialysis patients. These patients were 63 ± 12 (± standard deviation) years old, 44% had diabetes, and they had been on peritoneal dialysis therapy for 1.6 ± 1.2 years. From an overnight dwell the night before a standard peritoneal equilibrium test [19], we established a mesothelial cell culture as previously described [20] and took a PD effluent sample. Using NanoString RNA analysis, we characterized the expression of WNT genes in the ex-vivo mesothelial cell culture from 21 patients in this cohort. We chose to analyze the expression of these genes based on existing literature [14,21,22]. WNT1 mRNA extracted from ex vivo cell culture correlated with peritoneal membrane solute transport measured by dialysate to plasma (D/P)

creatinine (r=0.639, p=0.002). Therefore, higher levels of WNT1 mRNA were associated with higher levels of solute transport (Figure 5.1A, B). WNT2 and WNT3a were also correlated with D/P creatinine but did not meet significance after Bonferroni correction for multiple testing (corrected $p<0.008$) (Figure 5.1A).

In order to confirm this association between peritoneal WNT1 and peritoneal membrane solute transport, we evaluated WNT1 protein concentration in the peritoneal effluent from the entire cohort. WNT1 protein concentration in PD effluent and WNT1 gene expression in ex vivo mesothelial cells were significantly correlated (r=0.606, p=0.005) (Figure 5.1C). Furthermore, WNT1 protein concentration in the peritoneal effluent correlated with solute transport measured by D/P creatinine (r=0.486, $p<0.001$) (Figure 5.1D).

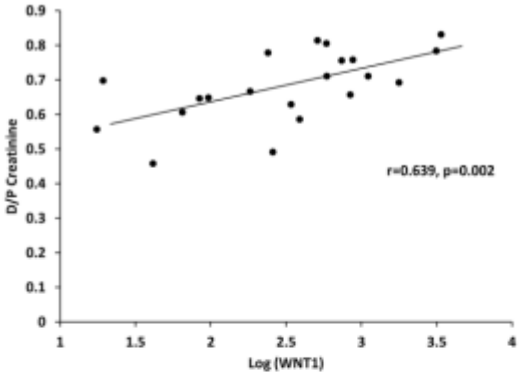
Because WNT1 gene and protein expression demonstrated the strongest association with peritoneal membrane function, we evaluated the correlation between WNT1 peritoneal mesothelial cell gene expression and the expression of 31 fibrosis-related genes. Thirteen genes significantly correlated with WNT1 gene expression (Figure 5.1E). WNT1 gene expression correlated with the expression of other WNTs including WNT2, WNT3A, and WNT11, along with WNT inhibitor SFRP2 and the receptor FZD3. WNT1 gene expression also correlated with regulatory genes involved in EMT - TWIST and SNAIL1 – and genes associated with fibrosis and angiogenesis - MMP7, MMP9, VEGF, angiopoietin (ANGPT) 1, and ANGPT2. Several known targets of WNT signaling including c-

MYC [24], TWIST [24], SNAIL1 [24], MMP7 [25], MMP9 [23], and VEGF [23] were correlated with WNT1 expression.

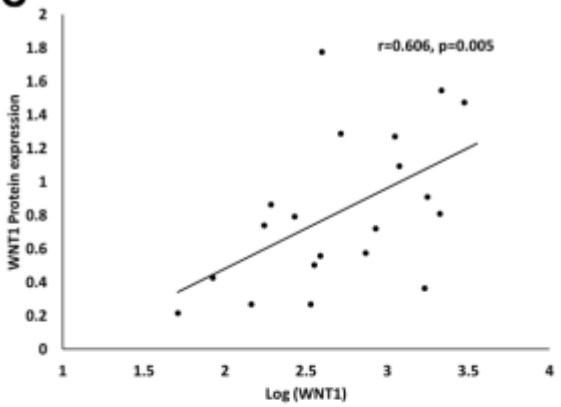
**A**

| Gene | r | p |
|---|---|---|
| WNT1 | 0.64 | .002 |
| WNT2 | 0.49 | .026 |
| WNT3a | 0.47 | .030 |
| WNT11 | 0.36 | .110 |
| WNT5a | 0.31 | .170 |
| WNT4 | 0.10 | .670 |

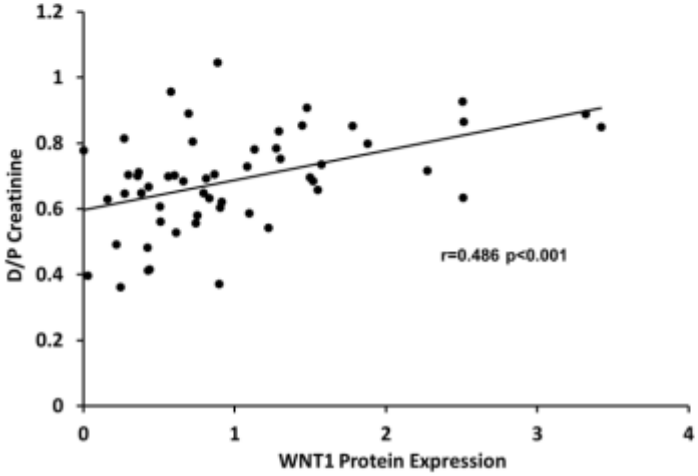
**B**




**C**




**D**




**E**

| Gene | r | p | Role | Known WNT target |
|---|---|---|---|---|
| WNT2 | 0.80 | <0.001 | WNT | |
| WNT3A | 0.71 | <0.001 | WNT | |
| WNT11 | 0.67 | <0.001 | WNT | |
| SFRP2 | 0.86 | <0.001 | WNT regulator | |
| FZD3 | 0.66 | <0.001 | WNT regulator | |
| TWIST | 0.84 | <0.001 | EMT | Yes |
| SNAIL1 | 0.66 | <0.001 | EMT | Yes |
| c-MYC | 0.56 | .005 | EMT | Yes |
| MMP9 | 0.68 | <0.001 | Fibrosis, angiogenesis | Yes |
| MMP7 | 0.71 | <0.001 | Fibrosis, angiogenesis | Yes |
| VEGFA | 0.64 | .001 | Angiogenesis | Yes |
| ANGPT1 | 0.62 | .001 | Angiogenesis | |
| ANGPT2 | 0.61 | .002 | Angiogenesis | |

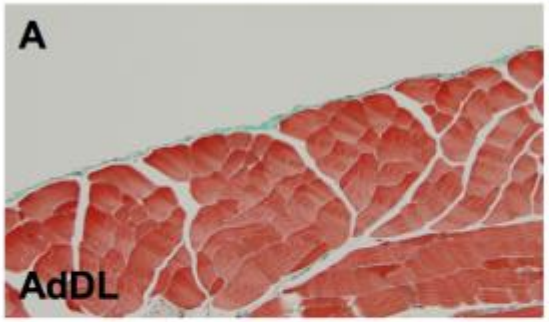
**Figure 5.1 WNT1 is correlated with peritoneal solute transport properties-** A) Correlation between expression of selected WNT genes in ex-vivo mesothelial cell culture and D/P creatinine. WNT1 expression was significantly associated with D/P creatinine. WNT2 and WNT3a were also associated, but did not meet significance with correction for multiple testing. B) Graph of log(WNT1) and d/p creatinine. C) WNT1 gene expression from ex-vivo mesothelial cells correlated with WNT1 protein expression in the PD effluent. D) WNT1 protein expression in the peritoneal effluent correlated with D/P creatinine. E) From a panel of 31 fibrosis related genes, associations with WNT1 gene expression in ex-vivo mesothelial cells are shown.

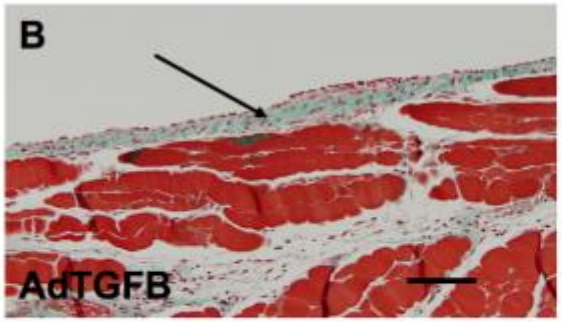
***WNT Signaling is upregulated in an experimental model of peritoneal injury***

We used an adenovirus to overexpress TGFB in the mouse peritoneal membrane and examined the changes in genes associated with fibrosis based on a NanoString 49 gene panel. Control adenovirus (AdDL) had no effect on peritoneal membrane histology (Figure 5.2A) whereas overexpression of TGFB resulted in increased submesothelial thickening and extracellular matrix deposition (Figure 5.2B). Ten genes were at least 2-fold significantly upregulated 4 days after administration of AdTGFB (Figure 5.2C). Of these, 4 genes remained significantly elevated 10 days after AdTGFB infection. WNT4 was significantly upregulated 4 and 10 days after AdTGFB infection and WNT2 was significantly upregulated 4 days after AdTGFB infection. WNT1 was not significantly upregulated in response to AdTGFB. The WNT modulating protein SFRP1 was also significantly elevated 4 days after induction of fibrosis.

Six genes were found to be significantly downregulated by at least 2 fold after AdTGFB. WNT7B and WNT8A were downregulated at 4 and 10 days after

infection (Figure 5.2 E, F). SFRP4 expression was significantly suppressed 4 days after AdTGFB administration.




C

| Gene | Fold Change AdTGFB vs AdDL, Day 4 | p |
|---|---|---|
| TIMP1 | 10.26 | <0.001 |
| IL-6 | 6.65 | <0.001 |
| A-SMA | 4.40 | 0.019 |
| WNT4 | 3.12 | <0.001 |
| SNAIL1 | 2.58 | 0.009 |
| MMP14 | 2.57 | 0.001 |
| TIMP3 | 2.26 | 0.001 |
| SFRP1 | 2.19 | 0.001 |
| P4H | 2.12 | <0.001 |
| WNT2 | 2.01 | 0.015 |

D

| Gene | Fold Change AdTGFB vs AdDL, Day 10 | p |
|---|---|---|
| TIMP1 | 13.70 | 0.041 |
| MMP3 | 4.39 | <0.001 |
| SNAIL1 | 3.36 | 0.030 |
| MMP14 | 2.49 | 0.005 |
| MMP2 | 2.05 | 0.014 |
| WNT4 | 2.04 | 0.014 |

E

| Gene | Fold Change AdTGFB vs AdDL, Day 4 | p |
|---|---|---|
| SFRP4 | 0.49 | 0.023 |
| WNT5A | 0.47 | <0.001 |
| ANGPT1 | 0.38 | 0.001 |
| WNT8A | 0.35 | 0.002 |
| WNT7B | 0.34 | 0.025 |
| BMP4 | 0.32 | 0.002 |

F

| Gene | Fold Change AdTGFB vs AdDL, Day 10 | p |
|---|---|---|
| WNT8A | 0.35 | 0.001 |
| WNT7b | 0.09 | 0.027 |

**Figure 5.2 - Histology of the peritoneal membrane 10 days after infection with adenovirus** A) AdDL (control adenovirus) or B) AdTGFB. TGFB overexpression is associated with submesothelial thickening and fibrosis (arrow). C) Genes significantly upregulated 4 or 10 days after TGFB overexpression (D) (fold change compared with control adenovirus treated animals). E) Genes significantly downregulated 4 or 10 days after TGFB overexpression (F). Genes shown include those with significant difference in expression ($p < 0.05$) and more than 2 fold up or downregulation. Photomicrographs are stained with Masson's trichrome, bar = 100 um. TIMP – tissue inhibitor of metalloproteinase, IL – interleukin, a-SMA – alpha smooth muscle actin, P4H – prolyl hydroxylase 4, ANGPT – angiopoietin, BMP – bone morphogenic protein.

***WNT/b-catenin signaling is activated during peritoneal membrane injury***

In mice administered AdTGFB, we assessed for changes in b-catenin protein expression. B-catenin stained sections of mouse peritoneum revealed that expression of active, nuclear b-catenin was increased in mice treated with AdTGFB (Figure 5.3A and B). By western blot, there was a 2.5 fold increase in peritoneal tissue protein concentration of b-catenin in the peritoneum at both time points (Figure 5.3C). DVL2, part of the WNT signaling pathway, was increased 5 fold after exposure to AdTGFB at day 4 and this was less pronounced at day 10 (2.5 fold increase) (Figure 5.3D). GSK3B is phosphorylated and inactivated by canonical WNT signaling. There was a significant 2.5 fold increase of phosphorylated GSK3B at day 4. These effects were less prominent at day 10 (Figure 5.3E).

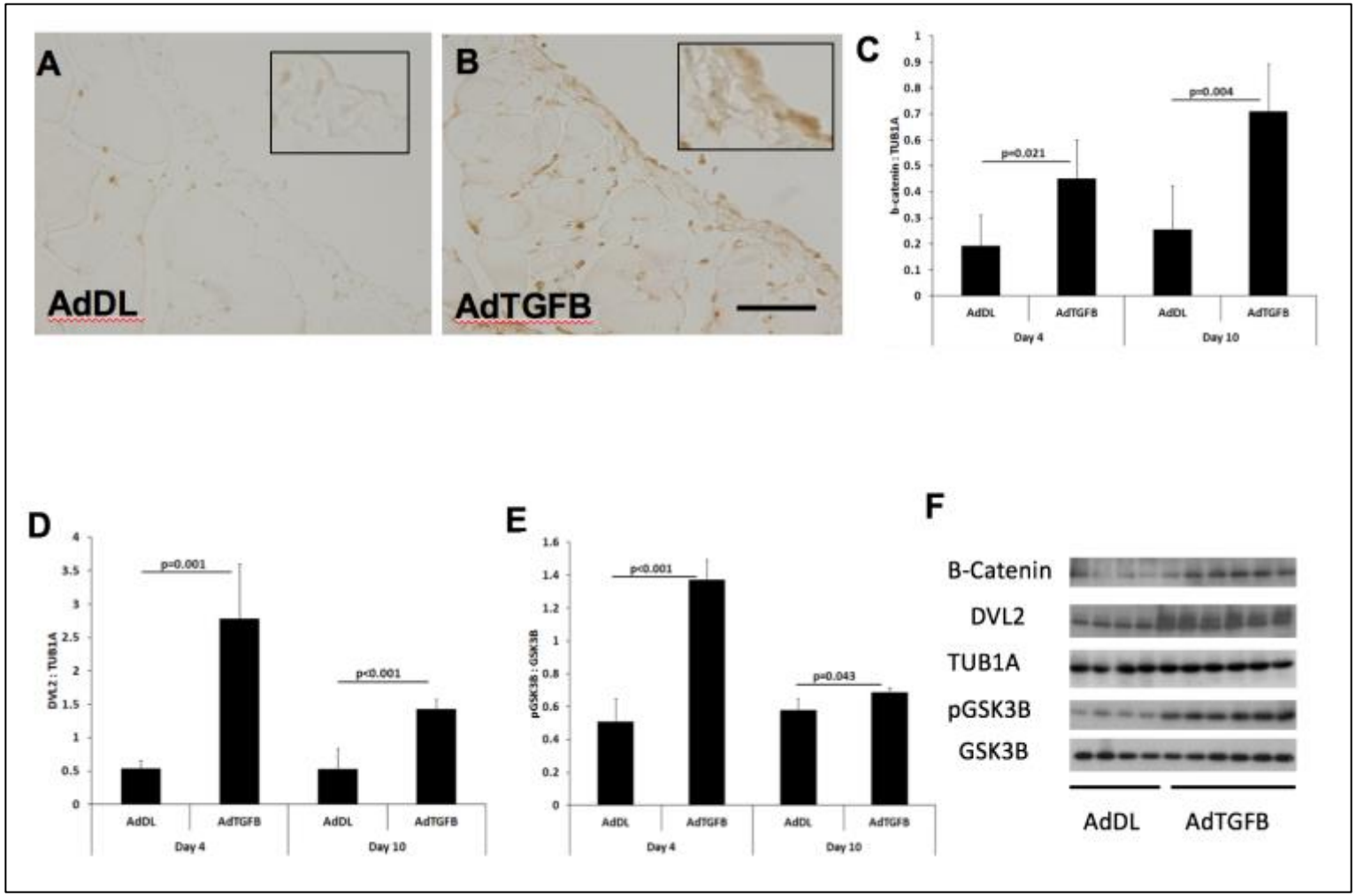

A
AdDL
B
AdTGFB
C
b-catenin : TUB1A
p=0.021
p=0.004
AdDL
AdTGFB
AdDL
AdTGFB
Day 4
Day 10
D
DVL2 : TUB1A
p=0.001
p<0.001
AdDL
AdTGFB
AdDL
AdTGFB
Day 4
Day 10
E
pGSK3B : GSK3B
p<0.001
p=0.043
AdDL
AdTGFB
AdDL
AdTGFB
Day 4
Day 10
F
B-Catenin
DVL2
TUB1A
pGSK3B
GSK3B
AdDL
AdTGFB

**Figure 5.3 WNT/b-catenin signaling is activated during peritoneal membrane injury -** A, B) Sections of mouse peritoneum infected with AdDL (A) or AdTGFB (B) and stained for active b-catenin. b-catenin expression was increased in the peritoneal tissues of mice treated with AdTGFB. Inset is a magnified area of mesothelium showing staining. Bar = 100 um. C) Western blot of peritoneal tissues demonstrates increase b-catenin concentration 4 and 10 days after infection with AdTGFB. Similar results were seen with other WNT signaling elements DVL2 (D) and pGSK3B (E). F) Representative western blot. TUB1A – alpha tubulin.

***TGFB signals through b-catenin to stimulate angiogenesis***

We have previously demonstrated that TGFB induces angiogenesis during peritoneal membrane injury [7] and have shown that MMP9 induced peritoneal angiogenesis is related to b-catenin signaling [20]. The link between WNT/b-catenin signaling and VEGF induced angiogenesis has also been observed in other model systems [26]. We hypothesize that a similar connection exists between WNT/b-catenin signaling and changes in peritoneal membrane vasculature.

Mice with AdTGFB induced peritoneal fibrosis were co-treated with an adenovirus expressing DKK-1 (AdDKK-1), or the b-catenin inhibitor ICG-001 [27]. AdDL and AdDKK-1 had little effect on the peritoneal membrane seen 10 days after infection (Figure 5.4A, B). As seen previously, TGFB induced a thickening of the submesothelial zone (Figure 5.4C). The addition of ICG-001 or AdDKK-1 had little effect on the gross appearance of TGFB-induced peritoneal membrane fibrosis (Figure 5.4D, E). Immunostaining for non-phosphorylated or active b-catenin was used to confirm the role of DKK-1 in antagonizing the WNT/b-catenin signaling pathway (Fig 5.4F, G). DKK-1 induced a marked

decrease in b-catenin expression in both mesothelial and submesothelial cells in the peritoneum. Positive staining was quantified in Figure 4H.

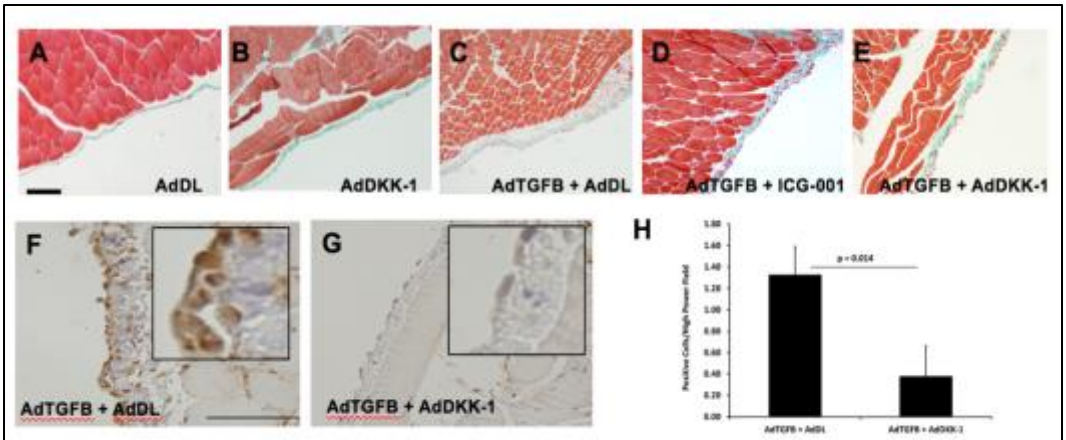



**Figure 5.4 TGFB activates b-catenin -** (A-E) Trichrome stained sections of C57Bl/6 mouse peritoneum 10 days after adenoviral gene transfer of control adenovirus (AdDL), AdDKK-1, AdTGFB+AdDL, AdTGFB + ICG-001, TGFB+AdDKK-1. F, G) Staining for active b-catenin demonstrates significant downregulation in animals treated with both AdTGFB and AdDKK-1. Inset shows magnified details of mesothelium. H) Staining quantified. Bar = 100 um.

Submesothelial thickening and peritoneal membrane angiogenesis were quantified (Figure 5.5A, B). There was no observed difference in submesothelial thickness between mice treated with AdTGFB, or co-treated with AdTGFB and ICG-001, or AdTGFB and AdDKK-1 (Figure 5.5A). TGFB alone induced angiogenesis in the peritoneum, and both ICG-001 and DKK-1 inhibited this angiogenic response (Figure 5.5B). We measured protein concentrations of VEGF in mice peritoneal effluent and found reduced levels of VEGF protein in ICG-001and AdDKK-1 treated mice compared to AdTGFB (Figure 5.5C, D).

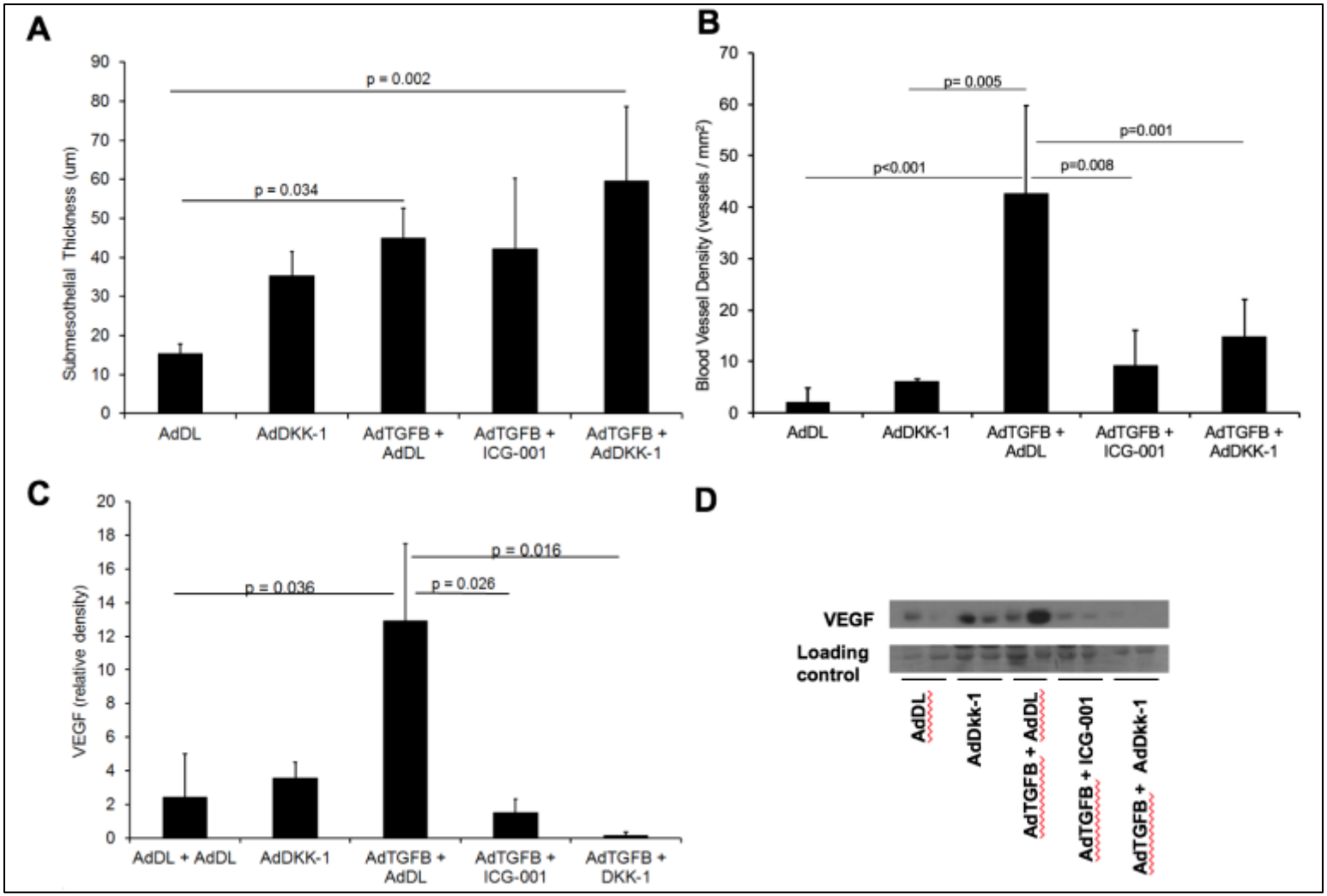

A
Submesothelial Thickness (um)
p = 0.002
p = 0.034
AdDL
AdDKK-1
AdTGFB + AdDL
AdTGFB + ICG-001
AdTGFB + AdDKK-1
B
Blood Vessel Density (vessels / mm²)
p= 0.005
p=0.001
p<0.001
p=0.008
AdDL
AdDKK-1
AdTGFB + AdDL
AdTGFB + ICG-001
AdTGFB + AdDKK-1
C
VEGF (relative density)
p = 0.016
p = 0.036
p = 0.026
AdDL + AdDL
AdDKK-1
AdTGFB + AdDL
AdTGFB + ICG-001
AdTGFB + DKK-1
D
VEGF
Loading control
AdDL
AdDkk-1
AdTGFB + AdDL
AdTGFB + ICG-001
AdTGFB + AdDkk-1

**Figure 5.5 TGFB signals through b-catenin to stimulate angiogenesis -** A) Quantification of submesothelial thickness demonstrates that neither DKK-1 nor ICG-001 affects submesothelial thickening in response to TGFB. B) TGFB induces a significant increase in blood vessel density in the peritoneum. Both ICG-001 and DKK-1 inhibit this angiogenic response. Peritoneal effluent VEGF is significantly increased in response to AdTGFB. Treatment with ICG-001 and DKK-1 inhibits this response. D) Representative blot of VEGF expression in peritoneal fluid.

***TGFB promotes EMT via WNT/b-catenin signaling***

TGFB has been observed to stimulate EMT resulting in loss of the epithelial phenotype and acquisition of mesenchymal characteristics [10]. During peritoneal membrane injury, EMT in the mesothelial cell involves loss of cell adhesion proteins such as E-cadherin, reorganization of the cytoskeleton and increase expression of alpha smooth muscle actin (a-SMA) resulting in a more myofibroblast phenotype [12]. Although it is controversial whether the transitioned mesothelial cells invade into the submesothelium [11], these transitioned cells do express pro-fibrotic and pro-angiogenic factors such as VEGF [28]. There is evidence that WNT/b-catenin signaling may augment the TGFB pathway in promoting EMT and we hypothesized that TGFB may be interacting with the WNT/b-catenin pathway to promote EMT [10].

We used immunofluorescence labelling of epithelial and mesenchymal markers to identify cells undergoing EMT in the mesothelium and submesothelium (Figure 5.6A-C). AdTGFB induced dual labeled cells expressing both cytokeratin and a-SMA in the mesothelium and submesothelium (Figure 5.6A, C). This was inhibited by DKK-1 (Figure 5.6B, C).

Decreased E-cadherin expression is a hallmark of mesothelial cell transition [29] and TGFB has been observed to downregulate the expression of E-cadherin in peritoneal tissue. We observed an increase in E-cadherin in response to DKK-1 (Figure 5.6D, E).

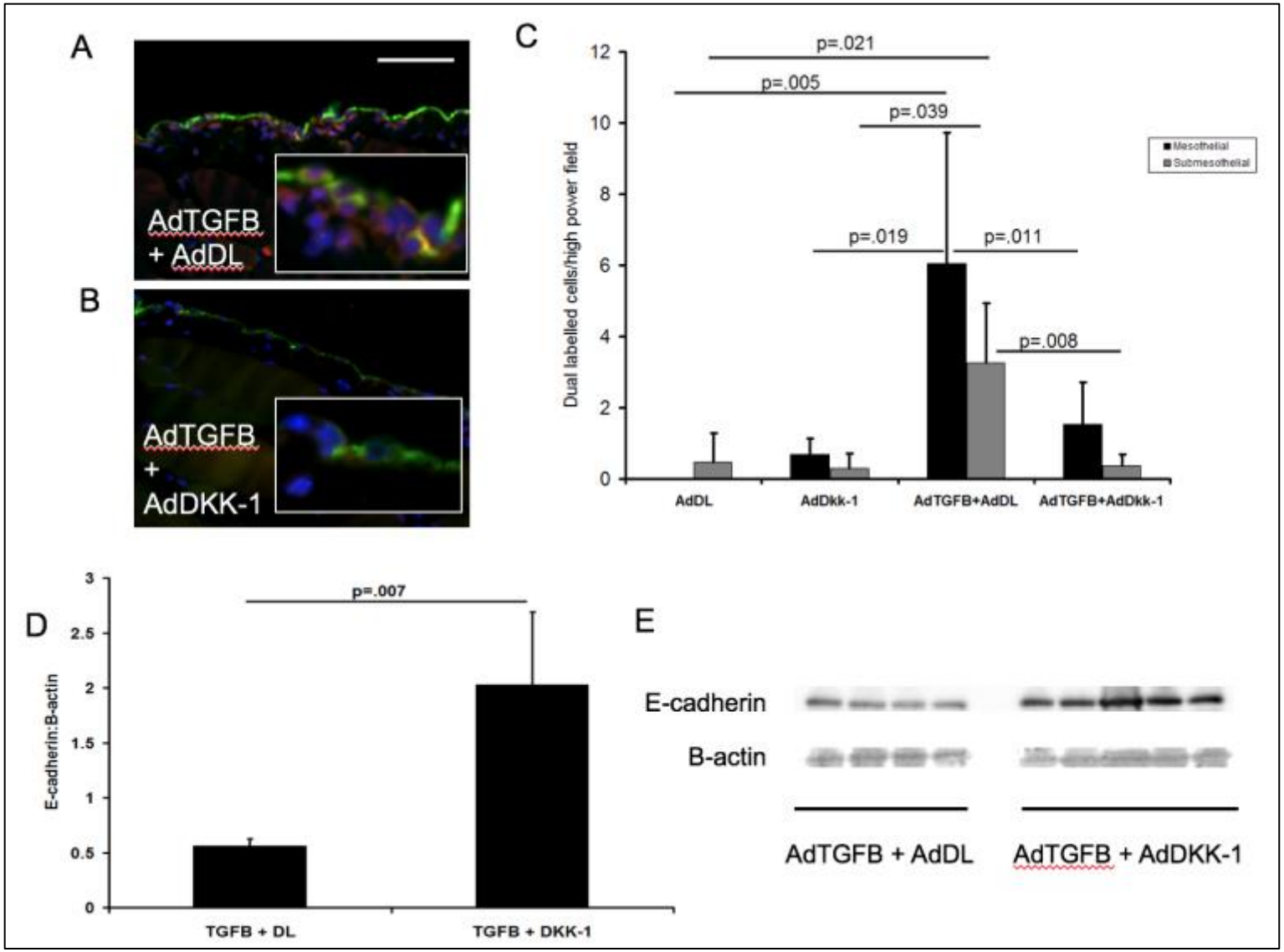

A
AdTGFB
+ AdDL
B
AdTGFB
+
AdDKK-1
C
p=.021
p=.005
p=.039
p=.019
p=.011
p=.008
Dual labelled cells/high power field
Mesothelial
Submesothelial
AdDL
AdDkk-1
AdTGFB+AdDL
AdTGFB+AdDkk-1
D
p=.007
E-cadherin:B-actin
TGFB + DL
TGFB + DKK-1
E
E-cadherin
B-actin
AdTGFB + AdDL
AdTGFB + AdDKK-1

**Figure 5.6 TGFB promotes EMT via WNT/b-catenin signaling -** Dual staining for cytokeratin and a-SMA in C57Bl/6 mice after adenoviral gene transfer. A) Four days after AdTGFB infection, there is an increase in dual-labeled (cytokeratin, a-SMA) cells which was reduced by co-infection with AdDKK-1 (B). Inset shows magnified view of mesothelium with dual labeling (A). C) Quantification of dual stained cells shows DKK-1 treatment decreases the number of these cells in the in mesothelium (p=0.011) and submesothelium (p=.008) compared with AdTGFB alone. D) Protein extracted from omental tissue shows that co-infection with AdDKK-1 preserves E-Cadherin expression which is downregulated by TGFB. E) Representative western blot.

## DISCUSSION

Our results suggest that, in PD patients, WNT1 is associated with peritoneal membrane solute transport measured by D/P creatinine. Peritoneal membrane solute transport has been directly associated with peritoneal vascularization [30] and we demonstrate in an animal model of peritoneal membrane injury that WNT signaling blockade reduces peritoneal angiogenesis.

We have previously shown that increased D/P creatinine is associated with increased risk of mortality in PD patients [31]. Therefore, this observed association between WNT1 and D/P creatinine suggests that the WNT signaling pathway may be an important target for intervention to prevent chronic damage to the peritoneal membrane and improve outcomes for PD patients. Furthermore, WNT1 may be a biomarker for clinical outcomes in PD patients.

The association between an injury marker and peritoneal membrane solute transport may be spurious as the marker may be passively transported from the blood to the peritoneal effluent [32]. We found that WNT1 gene is expressed in ex vivo mesothelial cells culture, and the expression in these cells correlated with

WNT1 protein concentration in the peritoneal effluent (Figure 5.1). These data suggest that WNT1 is locally produced in the peritoneal tissues which supports a causative role in peritoneal membrane injury.

Ex vivo mesothelial cells cultured from overnight PD effluent have been described previously [33,34]. We have shown that the initial cell collection from the peritoneal effluent includes about 5% mesothelial cells, but after 2 passages, the cells are virtually 100% cytokeratin positive mesothelial cells [20]. It has previously been shown that the phenotype of cultured peritoneal mesothelial cells (epithelial-like, non-epitheloid) correlates with peritoneal solute transport properties [34], and ex-vivo mesothelial cell expression of VEGF correlates with peritoneal membrane solute transport [35]. Using the same methodology, we demonstrated that MMP9 correlates with peritoneal membrane solute transport [20]. The research outlined above uses this same procedure to identify the role of WNT signaling in peritoneal membrane injury in PD patients and confirmed this in an animal model.

The WNT signaling pathway as a component of peritoneal membrane dysfunction has not been explored in detail. In our own research, we have shown that MMP9 cleaves E-cadherin, and this initiates a signaling cascade that leads to peritoneal membrane angiogenesis [20]. This response was blocked by the b-catenin inhibitor ICG-001 [20]. Zhang and colleagues previously outlined the role of WNT/b-catenin signaling in EMT of peritoneal mesothelial cells [17]. Ji and colleagues recently demonstrated that b-catenin contributes to EMT in a model of

dialysate induced peritoneal fibrosis. Their results confirm our findings with a reduction in peritoneal membrane injury using ICG-001 [36]. Our research extends these observations from a mouse model of peritoneal membrane injury to PD patients samples.

Our results differ from those of Ji and colleagues as they demonstrated a decrease in fibrosis in response to b-catenin inhibitor, ICG-001 [36]. In our work, ICG-001 did not have any significant effect on fibrosis. The difference may be explained by the models used. Ji and colleagues used a chronic infusion model involving daily exposure to peritoneal dialysis fluid for a period of 30 days [36]. In this model, peritoneal dialysis fluid directly injures the mesothelial cells resulting in TGFB production and epithelial to mesenchymal transition. TGFB production from these injured mesothelial cells is likely an important component of peritoneal membrane fibrosis inducing myofibroblasts to secrete ECM. In this model, ICG-001 was administered at the earliest stage of injury as it was combined with delivery of PD fluid and is likely to act on initial injury to the mesothelium. Therefore, in this model ICG-001 prevented both peritoneal EMT and fibrosis [36]. Our model directly induces TGFB by adenovirus gene transfer to the peritoneum and circumvents the step of initial injury to the mesothelium. TGFB works in a positive feedback loop to induce injury to the mesothelium resulting in EMT and greater production of TGFB. Therefore, blocking b-catenin signaling does not have significant effects on peritoneal fibrosis. This model is more suitable for studying the development of peritoneal angiogenesis. We were

able to demonstrate a causative role of WNT signaling on TGFB-induced angiogenesis by directly blocking this pathway using DKK1 or ICG-001.

In peritoneal membrane injury, EMT refers to transition of mesothelial cells to a myofibroblast phenotype with subsequent migration of these cells into the interstitium where they play a role in fibrosis and angiogenesis [12]. There have been mixed findings with regard to the role of EMT in peritoneal membrane injury using lineage tracing experiments. Chen and colleagues found that submesothelial myofibroblasts were derived from existing fibroblasts [11]. Lua and colleagues, using the similar lineage tracing techniques, found that 17% of submesothelial myofibroblasts were derived from transformed mesothelial cells [37].

Our research suggests that mesothelial cells may undergo transition with decreased E-cadherin expression and increased a-SMA associated with increased EMT related transcription factors such as SNAIL1 [38]. Migration of these transformed mesothelial cells into the interstitium is variable depending on the extent of injury to the peritoneal tissues. Further, we have demonstrated that transitioned mesothelial cells are a source for VEGF, and thus promote peritoneal angiogenesis [13,35]. Therefore, we believe that EMT, whether invasive or ‘in situ’ is an important first step in promoting peritoneal membrane changes including fibrosis and angiogenesis. Our data suggest that blockade of WNT signaling through AdDKK1 significantly reduced evidence of EMT through a reduction in dual labeled a-SMA / cytokeratin cells and an increase in E-cadherin

expression. Our results are supported by a recent study by Ji and colleagues where chlorhexidine induced peritoneal membrane injury and EMT was reversed with ICG-001 [36].

The WNT profile in ex vivo mesothelial cell culture and in the mouse model of peritoneal fibrosis were different. We saw an association between WNT1, 2, and 3A with D/P creatinine as a marker of peritoneal membrane injury. In the mouse model, TGFB1 induced an increased expression of WNT4 and WNT2, but WNT1 was unchanged. In previous animal models of renal fibrosis, multiple WNT ligands are upregulated simultaneously [39]. There are redundancies in action of WNTs, and some WNT ligands may be upregulated in response to the injury and not have a direct causal role [40]. Therefore, it is not unexpected that a different WNT profile would emerge in an animal model vs human pathology.

Ours is the first study to identify a role for WNT signaling in peritoneal membrane angiogenesis. There is extensive evidence of WNT involvement in angiogenesis in other biologic processes. b-catenin has been shown to stimulate tumor angiogenesis by upregulating VEGF [41] and also regulates vascular development during embryogenesis [42]. We previously observed MMP9 to induce angiogenesis in the peritoneal membrane by cleaving E-cadherin and signaling through b-catenin [20]. In this study, we investigated the direct role of the WNT/b-catenin pathway in TGFB induced peritoneal membrane injury. We used two different inhibitors to silence WNT/b-catenin signaling in the mouse peritoneum and examine the effect on peritoneal angiogenesis. ICG-001 acts by

suppressing b-catenin associated t-cell factor/lymphoid enhancer factor signaling by obstructing the interaction with cyclic AMP response element-binding protein [43]. DKK-1 sequesters the LRP5/6 co-receptor of the frizzled receptor complex resulting in suppression of the WNT/b-catenin signaling pathway [44]. We demonstrate that TGFB mediates angiogenesis by signaling through b-catenin, however we did not observe any direct effect on peritoneal fibrosis. This is likely due to the fact that, in our model, fibrosis is driven directly by TGFB1 driven by the adenovirus gene transfer. This process is unlikely to be affected by WNT signaling, whereas downstream TGFB-mediated processes such as angiogenesis, are WNT dependent.

Peritoneal fibrosis is an irreversible process that still does not have effective treatment available. Peritoneal fibrosis and angiogenesis lead to increased solute transport, decreased ultrafiltration failure, and eventual peritoneal membrane failure. A better understanding of these phenomena is required to improve the current therapeutic strategies and understanding the role of WNT signaling may provide some insight on the mechanisms behind peritoneal membrane injury to improve PD in the future.

**ACKNOWLEDGEMENT**

This research was supported by the Kidney Foundation of Canada, Baxter Healthcare USA, and St. Joseph's Healthcare, Hamilton, ON, Canada.

**References**


1 Devuyst O, Margetts PJ, Topley N: The pathophysiology of the peritoneal membrane. J Am Soc Nephrol 2010;21:1077–1085.

2 Teitelbaum I: Ultrafiltration failure in peritoneal dialysis: a pathophysiologic approach. Blood Purif 2015;39:70–73.

3 Quinn RR, Ravani P, Hochman J: Technique failure in peritoneal dialysis patients: insights and challenges. Perit Dial Int 2010;30:161–162.

4 Brown EA, Davies SJ, Rutherford P, Meeus F, Borras M, Riegel W, et al.: Survival of functionally anuric patients on automated peritoneal dialysis: the European APD Outcome Study. J Am Soc Nephrol 2003;14:2948–2957.

5 Jansen MAM, Termorshuizen F, Korevaar JC, Dekker FW, Boeschoten E, Krediet RT: Predictors of survival in anuric peritoneal dialysis patients. Kidney Int 2005;68:1199–1205.

6 Kwan BC-H, Szeto C-C, Chow K-M, Law M-C, Cheng MS, Leung C-B, et al.: Bioimpedance spectroscopy for the detection of fluid overload in Chinese peritoneal dialysis patients. Perit Dial Int 2014;34:409–416.

7 Margetts PJ, Kolb M, Galt T, Hoff CM, Shockley TR, Gauldie J: Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. J Am Soc Nephrol 2001;12:2029–2039.

8 Iglesias-de la Cruz MC, Ziyadeh FN, Isono M, Kouahou M, Han DC, Kalluri R, et al.: Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes. Kidney Int 2002;62:901–913.

9 Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994;331:1286–1292.

10 Lamouille S, Xu J, Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014;15:178–196.

11 Chen Y-T, Chang Y-T, Pan S-Y, Chou Y-H, Chang F-C, Yeh P-Y, et al.: Lineage tracing reveals distinctive fates for mesothelial cells and submesothelial fibroblasts during peritoneal injury. J Am Soc Nephrol 2014;25:2847–2858.

12 Margetts PJ, Bonniaud P, Liu L, Hoff CM, Holmes CJ, West-Mays JA, et al.:Transient overexpression of TGFB-1 induces epithelial mesenchymal transition in the rodent peritoneum. J Am Soc Nephrol 2005: 16: 425-436.

13 Zhang J, Oh K-H, Xu H, Margetts PJ: Vascular endothelial growth factor expression in peritoneal mesothelial cells undergoing transdifferentiation. Perit Dial Int 2008;28:497–504.

14 Guo Y, Xiao L, Sun L, Liu F: Wnt/beta-catenin signaling: a promising new target for fibrosis diseases. Physiol Res 2012;61:337–346.

15 Cheng C, Yeh J, Fan T-P, Smith SK, Charnock-Jones DS: Wnt5a-mediated non-canonical Wnt signaling regulates human endothelial cell proliferation and migration. Biochem Biophys Res Commun 2008;365:285–290.

16 Widelitz R: Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors 2005;23:111–116.

17 Zhang F, Liu H, Liu F, Peng Y, Chen M, Liu Y, et al.: New insights into the pathogenesis and treatment of peritoneal fibrosis: a potential role of Wnt/beta-catenin induced epithelial to mesenchymal transition and stem cells for therapy. Med Hypotheses 2013;81:97–100.

18 Le SJ, Gongora M, Zhang B, Grimmond S, Campbell GR, Campbell JH, et al.: Gene expression profile of the fibrotic response in the peritoneal cavity. Differentiation 2010;79:232–243.

19 Twardowski ZJ: PET--a simpler approach for determining prescriptions for adequate dialysis therapy. Adv Perit Dial 1990;6:186–191.

20 Padwal M, Siddique I, Wu L, Tang K, Boivin F, Liu L, et al.: Matrix metalloproteinase 9 is associated with peritoneal membrane solute transport and induces angiogenesis through beta-catenin signaling. Nephrol Dial Transplant 2017;32:50–61.

21 Kaminski N, Rosas IO: Gene expression profiling as a window into idiopathic pulmonary fibrosis pathogenesis: can we identify the right target genes? Proc Am Thorac Soc 2006;3:339–344.

22 Selman M, Pardo A, Kaminski N: Idiopathic pulmonary fibrosis: aberrant recapitulation of developmental programs? PLoS Med 2008;5:e62.

23 Yang H-L, Tsai Y-C, Korivi M, Chang C-T, Hseu Y-C: Lucidone Promotes the Cutaneous Wound Healing Process via Activation of the PI3K/AKT, WNT/b-catenin, NF-Kappab Signaling pathways. Biochem Biophys Acta 2017; 1864: 151-168.

24 Nambotin SB, Tomimaru Y, Merle P, Wands JR, Kim M: Functional consequences of WNT3/Frizzled7-mediated signaling in non-transformed hepatic cells. Oncogenesis 2012;1:e31.

25 Dey N, Young B, Abramovitz M, Bouzyk M, Barwick B, De P, et al.: Differential activation of Wnt-beta-catenin pathway in triple negative breast cancer increases MMP7 in a PTEN dependent manner. PLoS One 2013;8:e77425.

26 Qu B, Liu B-R, DU Y-J, Chen J, Cheng Y-Q, Xu W, et al.: Wnt/beta-catenin signaling pathway may regulate the expression of angiogenic growth factors in hepatocellular carcinoma. Oncol Lett 2014;7:1175–1178.

27 Hao S, He W, Li Y, Ding H, Hou Y, Nie J, et al.: Targeted inhibition of beta-catenin/CBP signaling ameliorates renal interstitial fibrosis. J Am Soc Nephrol 2011;22:1642–1653.

28 Siddique I, Curran SP, Ghayur A, Liu L, Shi W, Hoff CM, et al.: Gremlin promotes peritoneal membrane injury in an experimental mouse model and is associated with increased solute transport in peritoneal dialysis patients. Am J Pathol 2014;184:2976–2984.

29 Zeisberg M, Neilson EG: Mechanisms of tubulointerstitial fibrosis. J Am Soc Nephrol 2010;21:1819–1834.

30 Numata M, Nakayama M, Nimura S, Kawakami M, Lindholm B, Kawaguchi Y: Association between an increased surface area of peritoneal microvessels and a high peritoneal solute transport rate. Perit Dial Int 2003;23:116–122.

31 Brimble KS, Walker M, Margetts PJ, Kundhal KK, Rabbat CG: Meta-analysis: peritoneal membrane transport, mortality, and technique failure in peritoneal dialysis. J Am Soc Nephrol 2006;17:2591–2598.

32 Zweers MM, de Waart DR, Smit W, Struijk DG, Krediet RT: Growth factors VEGF and TGF-beta1 in peritoneal dialysis. J Lab Clin Med 1999;134:124–132.

33 Ruiz-Carpio V, Sandoval P, Aguilera A, Albar-Vizcaino P, Perez-Lozano ML, Gonzalez-Mateo GT, et al.: Genomic reprograming analysis of the Mesothelial to Mesenchymal Transition identifies biomarkers in peritoneal dialysis patients. Sci Rep 2017; 7: 44941.

34 Yanez-Mo M, Lara-Pezzi E, Selgas R, Ramirez-Huesca M, Dominguez-Jimenez C, Jimenez-Heffernan JA, et al.: Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 2003;348:403–413.

35 Aroeira LS, Aguilera A, Selgas R, Ramirez-Huesca M, Perez-Lozano ML, Cirugeda A, et al.: Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis 2005;46:938–948.

36 Ji S, Deng H, Jin W, Yan P, Wang R, Pang L, et al.: Beta-catenin participates in dialysate-induced peritoneal fibrosis via enhanced peritoneal cell epithelial-to-mesenchymal transition. FEBS Open Bio 2017;7:265–273.

37 Lua I, Li Y, Pappoe LS, Asahina K: Myofibroblastic Conversion and Regeneration of Mesothelial Cells in Peritoneal and Liver Fibrosis. Am J Pathol 2015;185:3258–3273.

38 Patel P, West-Mays J, Kolb M, Rodrigues J-C, Hoff CM, Margetts PJ: Platelet derived growth factor B and epithelial mesenchymal transition of peritoneal mesothelial cells. Matrix Biol 2010;29:97–106.

39 He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y: Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 2009;20:765–776.

40 DiRocco DP, Kobayashi A, Taketo MM, McMahon AP, Humphreys BD: Wnt4/beta-catenin signaling in medullary kidney myofibroblasts. J Am Soc Nephrol 2013;24:1399–1412.

41 Easwaran V, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, et al.: beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res 2003;63:3145–3153.

42 Zerlin M, Julius MA, Kitajewski J: Wnt/Frizzled signaling in angiogenesis. Angiogenesis 2008;11:63–69.

43 Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, et al.: A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci USA 2004;101:12682–12687.

# CHAPTER 6: THE ROLE OF WNT5A AND ROR2 IN PERITONEAL MEMBRANE INJURY

Authors: Manreet Padwal, Limin Liu, Peter J. Margetts

Corresponding Author: Peter Margetts, Division of Nephrology, St. Joseph's Hospital, Department of Medicine, McMaster University, Hamilton, ON, Canada L8P 4A6. Phone: (905) 525-9140 ext 32299, Fax: 905-540-6589, Email: margetts@mcmaster.ca

Citation: In preparation for submission

**PREFACE**

WNT signaling can be separated into the canonical b-catenin dependent pathway and the non-canonical b-catenin independent pathway. In the previous Chapter, we elucidated the role of canonical WNT/b-catenin signaling in peritoneal membrane injury. The WNT ligands have been sorted as canonical vs non-canonical based on their ability to activate b-catenin. Non-canonical WNT signaling has been observed to regulated cell polarity and intracellular levels of calcium. Interestingly, some non-canonical WNT signaling members such as WNT5A have the ability to inhibit canonical WNT/b-catenin signaling. This is of particular interest because we previously showed that aberrant WNT/b-catenin signaling induces peritoneal membrane injury. Accumulating evidence have shown WNT5A to inhibit b-catenin dependent signaling in the presence of receptor Ror2. WNT5A has been observed to have deleterious effects in the presence of FZD receptors. We hypothesize that non-canonical WNT5A and Ror2 may protect against peritoneal membrane injury. In this Chapter, we confirm the role of WNT5A and Ror2 in protecting against peritoneal membrane injury. We also demonstrate that WNT5A can block canonical WNT/b-catenin signaling in TGFB induced peritoneal membrane injury.

Manreet Padwal designed the study, performed all experiments and data analysis. AdTGFB was amplified by Limin Liu. Adenovirus expressing WNT5A was provided by Dr.Tong Chuan He from The University of Chicago Medical Center and was amplified by Manreet Padwal with the assistance of Limin Liu.

Histology support was carried out by Mary Jo Smith. Manreet Padwal wrote the original draft of the manuscript. Dr. Peter Margetts contributed to the concept of this paper, interpretation of the data, and reviewed the final manuscript.

**ABSTRACT**

Patients on peritoneal dialysis are at risk of developing peritoneal fibrosis and angiogenesis which can lead to dysfunction of the peritoneal membrane. Recent evidence has identified cross-talk between transforming growth factor beta (TGFB) and the WNT/b-catenin pathway to induce fibrosis and angiogenesis. Limited evidence exists describing the role of non-canonical WNT signaling in peritoneal membrane injury. Non-canonical WNT5A is suggested to have different effects depending on the cell surface receptor that is present. WNT5A has been implicated in antagonizing canonical WNT/b-catenin signaling in the presence of receptor tyrosine kinase like orphan receptor (Ror2). We co-expressed TGFB and WNT5A using adenovirus and examined its role in the development of peritoneal fibrosis and angiogenesis. The treatment of mouse peritoneum with AdTGFB and AdWNT5A resulted in a decrease in submesothelial thickness. We also observe a decrease in angiogenesis and reduced levels of vascular endothelial growth factor (VEGF). Our observations demonstrated that WNT5A blocks WNT/b-catenin signaling by decreasing levels of phosphorylated glycogen synthase kinase 3 beta (GSK3B). To examine the function of Ror2, we overexpressed TGFB and used an siRNA against Ror2 in MET5A cells. In MET5A cells, levels of VEGF and fibronectin were significantly increased in response to Ror2 silencing. Our results suggest that WNT5A protects against peritoneal injury by antagonizing the WNT/b-catenin pathway and this may depend on receptor context.

## INTRODUCTION

Peritoneal dialysis (PD) is a common treatment for patients with chronic kidney disease or end stage renal disease [1]. The healthy peritoneum is a semipermeable membrane consisting of a superficial mesothelial layer, a basement membrane and a thin submesothelial zone [2,3]. During PD, hyperosmotic dialysis fluid is used to generate a concentration gradient, which drives diffusion of fluids and dissolved substance across the peritoneal membrane. This results in the removal of wastes and toxins from the blood [4]. Continuous time spent on PD results in two major structural changes in the peritoneum including thickening of the submesothelium and expansion of the membrane vasculature [1,2]. Studies have also documented a change in peritoneal membrane function recorded as an increase in solute transport [5]. The increase in angiogenesis is associated with an increase in solute transport rate and a decline in the ultrafiltration capacity [3,4,6]. Decreased ultrafiltration capacity, combined with loss of residual renal function, may predict for subclinical volume expansion which is associated with inflammation and cardiac hypertrophy [7].

Transforming growth factor beta (TGFB) is a predominant cytokine involved in the initiation and progression of peritoneal fibrosis [3,4]. Mesothelial cells secrete TGFB in response to injury from dialysis solutions and possible infection [4]. Active TGFB has been observed to induce changes in peritoneal structure including thickening of the submesothelial zone and angiogenesis. In conjunction with TGFB, the WNT signaling family has also been reported to play

an important role in the initiation and progression of fibrosis in different organ systems [8,9]. WNT signaling can be separated into canonical signaling and non-canonical signaling; canonical WNT signaling utilizes a b-catenin dependent pathway. Canonical WNT signaling has been observed to interact with the TGFB pathway to potentiate fibrosis in the kidney [8,9], liver [10], lungs [11] and the skin [12]. The WNT family of proteins can be loosely separated into canonical vs non-canonical WNT ligands and WNT5A is considered a prototypical non-canonical WNT protein [13].

The role of WNT signaling in peritoneal membrane injury has been recently clarified. We examined the interaction between TGFB and WNT signaling in the development of peritoneal fibrosis and angiogenesis. We observed WNT/b-catenin signaling to be active during TGFB induced peritoneal membrane injury and our results suggest cross-talk with the TGFB pathway during the development of injury. We used DKK-1 to inhibit WNT/b-catenin signaling and observed a decrease in both angiogenesis and also epithelial to mesenchymal transition (EMT) [14]. In a similar study of dialysis infused peritoneal fibrosis, b-catenin was shown to induce EMT [15]. Our results concur with reports of WNT signaling in other fibrotic diseases [9-11, 16-18].

The majority of studies in fibrotic diseases have focused on the role of b-catenin dependent signaling. There are few studies on the pathological role of non-canonical WNT5A in fibrosis. Physiologically, WNT5A has been observed to regulate cell polarity and intracellular calcium levels through the planar cell

polarity pathway (PCP) and calmodulin-dependent protein kinase II pathway, respectively [19]. During acute wound repair, the fibroblast deposits extracellular matrix and myofibroblasts migrate to mediate contraction of the wound. In vitro studies of wound healing have demonstrated that WNT5A induces cell migration and assists in wound closure suggesting a potential role in preventing fibrosis [13, 20, 21]. A few studies have revealed elevated levels of WNT5A in fibrosis of the liver [22] and in patients with idiopathic pulmonary fibrosis and acute lung injury [23-25]. Although these studies shed some light on the gene expression profile of WNT5A, further investigation is required to understand why WNT5A is upregulated during wound healing and fibrosis and if it is exerting a positive or negative effect. Emerging studies in fibrosis suggest that WNT5A contributes to progression of fibrosis by inducing fibroblast proliferation [26-28] and by inducing matrix production [29,30]. In the studies that have analyzed receptor environment, the Frizzled (FZD) family of receptors have been involved in mediating the injurious effects of WNT5A [31].

In studies of tumorigenesis, WNT5A has been reported to have opposing effects. WNT5A has been shown to work as a tumour suppressor in certain types of cancer [32-34]. In contrast to this, WNT5A has been associated with the progression of many cancer types and its expression is highly related to invasion and metastasis [21-35]. Moreover, in the process of epithelial to mesenchymal transition (EMT) WNT5A been reported to suppress EMT [33], but also induce EMT during tumor development [36]. WNT5A has also been described to

regulate vascular development during normal embryogenesis playing a role in endothelial cell differentiation as well as during tumor development [37]. Overall, the adverse effects of WNT5A seem to occur in the presence of FZD [36] and we hypothesize that the role of WNT5A may be depend on receptor context.

WNT5A has been observed to mediate signaling through receptor tyrosine kinase- like orphan receptor 2 (Ror2) and through FZD 3, 4, 5 and LRP5/6. WNT5A and Ror2 have been demonstrated to interact physically and also functionally by activating the PCP pathway to regulate the cytoskeleton during embryogenesis [38]. Ror2 can also interact with several canonical WNT proteins. In addition to this, accumulating evidence suggests that WNT5A can have both antagonistic and agonistic effects on the WNT/b-catenin pathway based on the receptor it is signaling through [13,39]. Mikels et al investigated the signaling of WNT5A in the presence of two different receptors, Ror2 or FZD4 [13]. Ror2 was observed to interact with WNT5A to inhibit the WNT/b-catenin pathway. However, in the presence of FZD 4, WNT5A potentiated WNT/b-catenin signaling [13,40].

There is very little evidence of the role of WNT5A and Ror2 in peritoneal membrane injury. Aberrant WNT/b-catenin signaling has been established to contribute to progressive fibrosis in several different organs [8,10,41]. Therefore, we hypothesize WNT5A may block canonical WNT signaling and protect against development of injury. In our study we examined the function of WNT5A in TGFB induced peritoneal membrane injury. We found WNT5A protected against

fibrosis and angiogenesis in the mouse peritoneum by inhibiting the WNT/b-catenin pathway. We evaluated whether WNT5A signaling differs based on receptor context. We blocked Ror2 using an siRNA and overexpressed TGFB in both MET5A cells and mesothelial cells from patients. We found that WNT5A signals through Ror2 to regulate levels of VEGF and fibronectin.

## METHODS

### Recombinant Adenovirus

The construction of the adenovirus vector AdTGFB has been previously described. AdTGFB expresses constitutively active TGFB1 [42]. AdTGFB was created with TGFB1 cDNA mutated at residues 223 and 225, so that the transgene product does not bind to the latency-associated protein and is therefore biologically active. A null adenovirus (AdDL) was used as a control. AdWNT5A expressing murine WNT5a was a kind gift from Dr. Tong Chuan He. Amplification and purification of adenoviruses was performed using CsCl gradient centrifugation as previously described. Titration of adenovirus was completed using 293 cells as previously described [42].

### Animal Experiments

All animal studies were performed according to the Canadian Council on Animal Care guidelines. Mice (C57BL/6, 5-6 weeks; Harlan, Indianapolis, IN, USA) were injected intrapertioneally with $1 \times 10^8$plaque forming units (pfu) of first generation AdTGFB + AdDL (n=4 animals/group) or AdWNT5A + AdDL

(n=4 animals/group). AdTGFB or AdWNT5A was diluted to 100 mL in phosphate-buffered saline. Animals were infused with a peritoneal dwell of 4.25% glucose dialysis solution an hour prior to sacrifice. Animals were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, ON, Canada) and sacrificed at day 10.

At sacrifice, the entire anterior abdominal wall was then removed and divided into two sections. The lower section was placed in 10% formalin for histological analysis and the upper section was placed in 1 mL Easy-blue$^{TM}$ reagent (Froggabio) for RNA isolation. Omental tissue was taken and frozen in liquid nitrogen for protein analysis.

**Cell Culture Experiments**

Human mesothelial cells were cultured from peritoneal effluent obtained from one patient undergoing peritoneal dialysis. PD effluent was centrifuged and mesothelial cells were resuspended in F12 media and cultured. Human MET5A cells were cultured in M199 media (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada).

Human MET5A cells (ATCC, Manassas, VA, USA) and mesothelial cells from patients were transfected with small interfering RNA (siRNA) against Ror2 (Stealth, Invitrogen, Burlington, ON, Canada) or nontargetting siRNA (Fisher Scientific, Ottawa, ON, Canada). Twenty four hours after transfection, serum starved cells were treated with either rTGFB (R&D Systems, Burlington, ON,

Canada) at 2.5ng/mL for 8 hours. Cells were harvested to collect supernatant, protein and RNA.

**Histology**

Tissue samples were fixed in a sufficient amount of 10% neutral-buffered formalin for 48 hours, followed by 70% ethanol. The tissue samples were then processed, paraffin embedded, and cut in 5-mm sections. Sections were stained with Masson's trichrome. Sections were also stained using vonWillebrand Factor antibody / Factor VIII (Dako, Burlington, ON, Canada) to measure blood vessel density. Five images were taken per stained section from each animal. Image J was used to quantify blood vessel density and thickness of the submesothelial zone.

**Immunofluorescence**

Immunofluorescence was performed by using a monoclonal mouse anti-human α smooth muscle action (α -SMA) antibody (1:50) (Dako, Burlington, ON, Canada) with a Texas Red goat anti-mouse secondary antibody (1:100) (Molecular Probes, Life Technologies) followed by a FITC-labeled monoclonal mouse antibody against pan-cytokeratin (1:50) (Sigma-Aldrich, Oakville, ON, Canada). Quantification of dual labeled cells in the mesothelial and submesothelial zones was completed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Cells co-expressing a-SMA and pan-cytokeratin were classified as dual labeled. Dual labeled cells were identified in the mesothelial cell layer and in the submesothelial cell layer from five high-power fields from each section using a blinded approach.

**Protein Analysis**

Protein was extracted from frozen omental tissue by homogenization in a standard lysis buffer with proteinase inhibitors. Equal amounts of the protein were run on a 8% or 10% SDS-PAGE gel, transferred to a membrane, and probed with VEGF (1:1000, Millipore), E-cadherin (1:1000, BD Biosciences), PGSK3B (1:1000, Cell Signaling), GSK3B (1:1000, Cell Signaling) Fibronectin (1:1000, Sigma), a-SMA (1:1000, Dako), B-actin (1:1000, Sigma ) and alpha-Tubulin (1:1000, Sigma).

Concentration of human VEGF was measured in cell culture supernatant using a standard ELISA kit (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) according to the manufacturer's instructions.

**Gene expression analysis**

RNA was extracted from MET5A cells according to manufacturer's instructions (Invitrogen, Burlington, ON, Canada). The concentration of RNA obtained was measured by nanodrop. 1μg of RNA obtained was reverse transcribed by RT-PCR. Gene expression of VEGF was determined by 7500 Real Time PCR (Applied Biosystems, Foster City, CA). Samples were run in duplicate and normalized to 18s housekeeping gene. A standard curve generated from pooled mRNA samples was used for comparative quantification. Results are reported relative to 18s RNA.

**Statistical Analysis**

Differences between two groups were evaluated using t-test. Differences between multiple groups were assessed using ANOVA with Tukey`s post hoc analysis. Analysis was carried out using SPSS v 22.0 (IBM, Armonk NY).

## RESULTS

### *WNT5A inhibits TGFB induced fibrosis and angiogenesis*

We co-expressed TGFB and WNT5A to understand its effect on peritoneal fibrosis. Adenoviral gene transfer of TGFB induced a significant thickening of the submesothelial region, 10 days after infection. AdDL displayed normal peritoneal membrane structure ($p<0.01$). Animals that were co-treated with TGFB and WNT5A exhibited a marked reduction in submesothelial thickness ($p <0.001$) (Figure 6.1E). We also assessed angiogenesis by analyzing changes in blood vessel density and VEGF protein. Analysis of blood vessels stained for factor VIII confirmed that AdTGFB induced an increase in blood vessel density in the peritoneum ($p=0.001$) (Figure 6.1H). AdWNT5A alone did not induce an increase in angiogenesis and appeared similar to control adenovirus. Co-expression of AdTGFB and AdWNT5A inhibited this response as we observed a reduction in blood vessel density ($p <0.01$) (Figure 6.1G, quantified in Figure 6.1H). We also examined levels of VEGF protein in the peritoneal tissue by western blot analysis

and we observed a decrease in VEGF in response to AdTGFB and AdWNT5A ($p = 0.042$) (Figure 6.1 I, J).

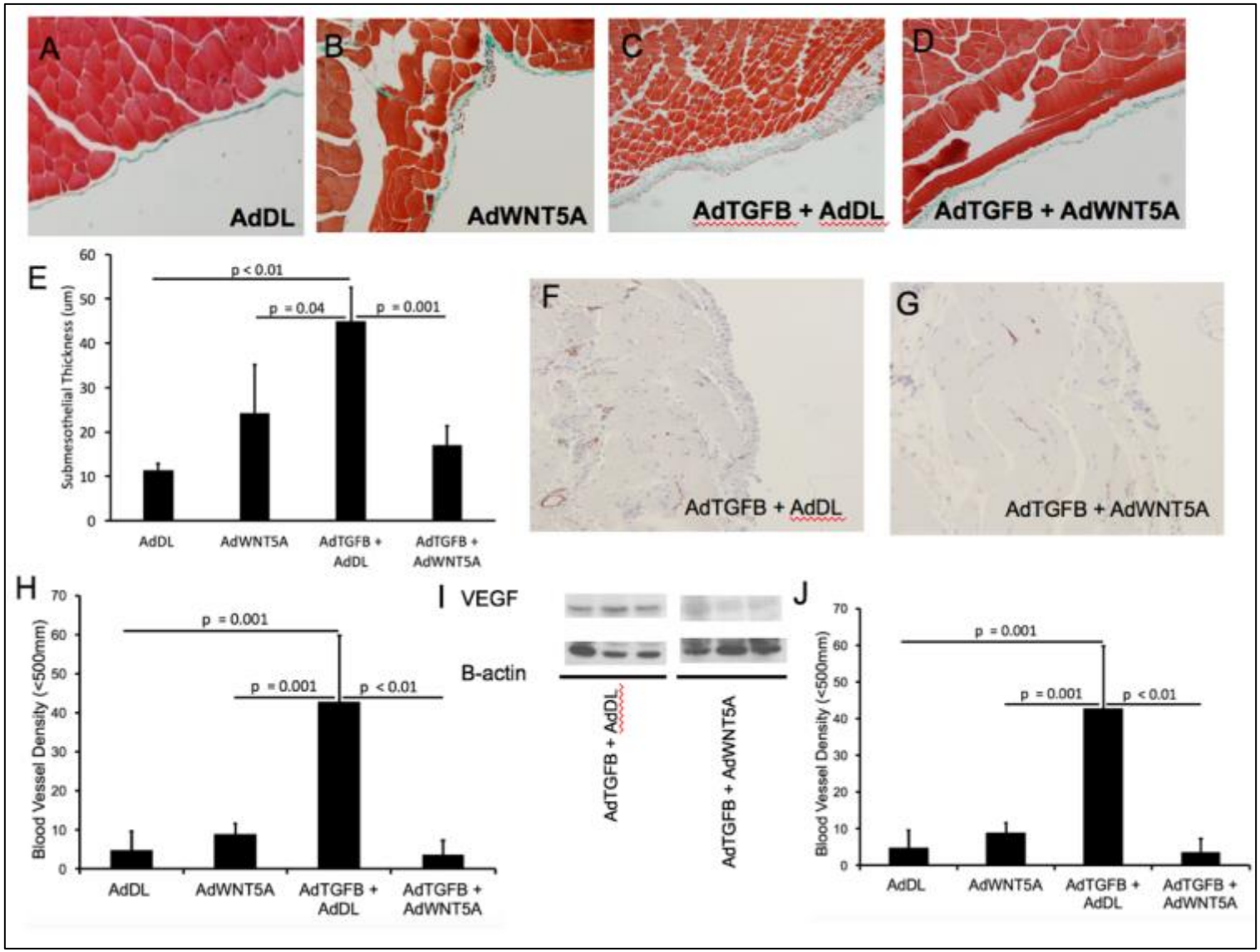

A
AdDL
B
AdWNT5A
C
AdTGFB + AdDL
D
AdTGFB + AdWNT5A
E
Submesothelial Thickness (um)
p < 0.01
p = 0.04
p = 0.001
AdDL
AdWNT5A
AdTGFB + AdDL
AdTGFB + AdWNT5A
F
AdTGFB + AdDL
G
AdTGFB + AdWNT5A
H
Blood Vessel Density (<500mm)
p = 0.001
p = 0.001
p < 0.01
AdDL
AdWNT5A
AdTGFB + AdDL
AdTGFB + AdWNT5A
I
VEGF
B-actin
AdTGFB + AdDL
AdTGFB + AdWNT5A
J
Blood Vessel Density (<500mm)
p = 0.001
p = 0.001
p < 0.01
AdDL
AdWNT5A
AdTGFB + AdDL
AdTGFB + AdWNT5A

**Figure 6.1 WNT5A Protects against TGFB induce peritoneal angiogenesis -** (A-D) Trichrome stained sections of C57Bl/6 mouse peritoneum after adenoviral gene transfer of AdDL, AdWNT5A, AdTGFB + AdDL and AdTGFB + AdWnt5a. (E) Co-expression of WNT5A and TGFB results in a decreased submesothelial thickness (p=0.013). (F-G) Factor VIII stained sections of mouse pertioneum treated with AdTGFB + DL and AdTGFB + AdWNT5A H) TGFB induces an increase in blood vessel density in the pertioneum. Ad TGFB + AdWnt5a inhibits this angiogenic response. I) Representative blot (J) Wnt5a inhibits levels of VEGF in the omentum.

***WNT5A inhibits EMT in mesothelial cells***

We completed immunofluorescent dual staining for cytokeratin and a-SMA as a measure of EMT in the peritoneal membrane. Addition of TGFB resulted in an increase of mesothelial cells co-expressing cytokeratin and a-SMA ($p = 0.014$). Treatment with TGFB and WNT5A suppressed dual labelled cells in the mesothelial layer ($p = 0.027$). Dual labelled cells in the submesothelial zone were not significantly affected.

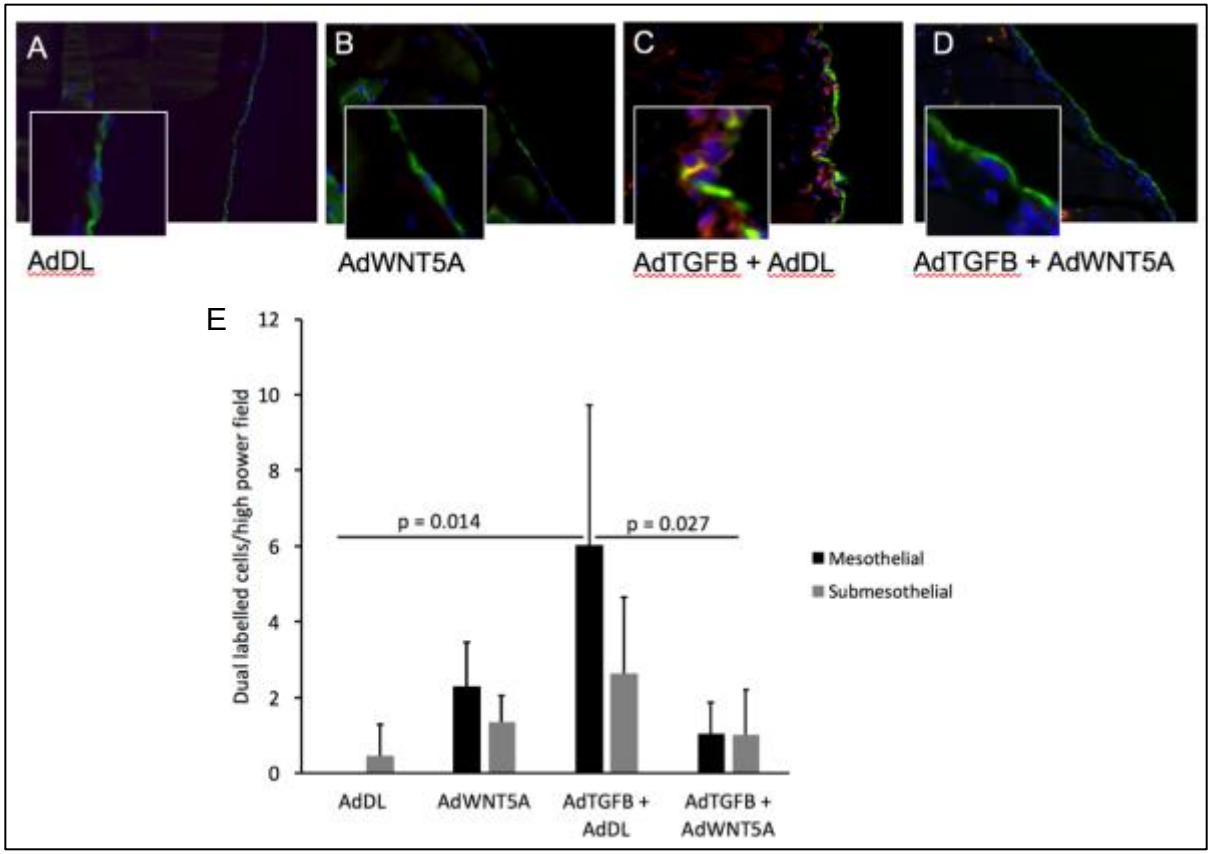



**Figure 6.2 WNT5A inhibits dual labeled cells in the mesothelial layer -** Immunofluorescence for cytokeratin and a-SMA in C57Bl/6 mice after adenoviral gene delivery. A) AdDL treated mice demonstrate normal peritoneal membrane structure with cells expressing cytokeratin and little a-SMA. B) Mice treated with AdWNT5A also exhibit normal structure similar to controls. C) Infection with AdTGFB increase dual labeled cells in the mesothelial layer. D) Co-infection with AdTGFB and AdWNT5A resulted in a significant decrease in dual labeled cells in the mesothelial layer. E) Quantification of dual labelled cells demonstrates that WNT5A inhibits mesothelial cell transition.

***WNT5A antagonizes WNT/b-catenin signaling***

We observed that co-expression of WNT5A and TGFB attenuated fibrosis and angiogenesis and therefore exhibits protection against peritoneal membrane injury. Studies have inconsistently reported WNT5A as either inducing or protecting against injury in the setting of wound healing. Furthermore, some studies have reported the antagonism between b-catenin dependent and independent signaling. These studies demonstrate that WNT5A activity is dependent on the receptor context. In the presence of FZD, WNT5A activates WNT/b-catenin and in the presence of Ror2, WNT5A inhibits WNT/b-catenin [13]. We hypothesized that WNT5A is antagonizing WNT/b-catenin signaling to reduce fibrosis and angiogenesis. To assess this, we analyzed levels of phosphorylated glycogen synthase kinase 3 beta (PGSK3B) in animals co-treated with AdWNT5A and AdTGFB and compared this to animals treated with AdTGFB alone. Activation of WNT signaling results in phosphorylation of GSK3B and stabilization of b-catenin [10]. Western blot analysis revealed a marked decrease in relative PGSK3B in response to AdTGFB and AdWNT5A compared to AdTGFB alone ($p=0.008$) (Figure 6.2A,B).

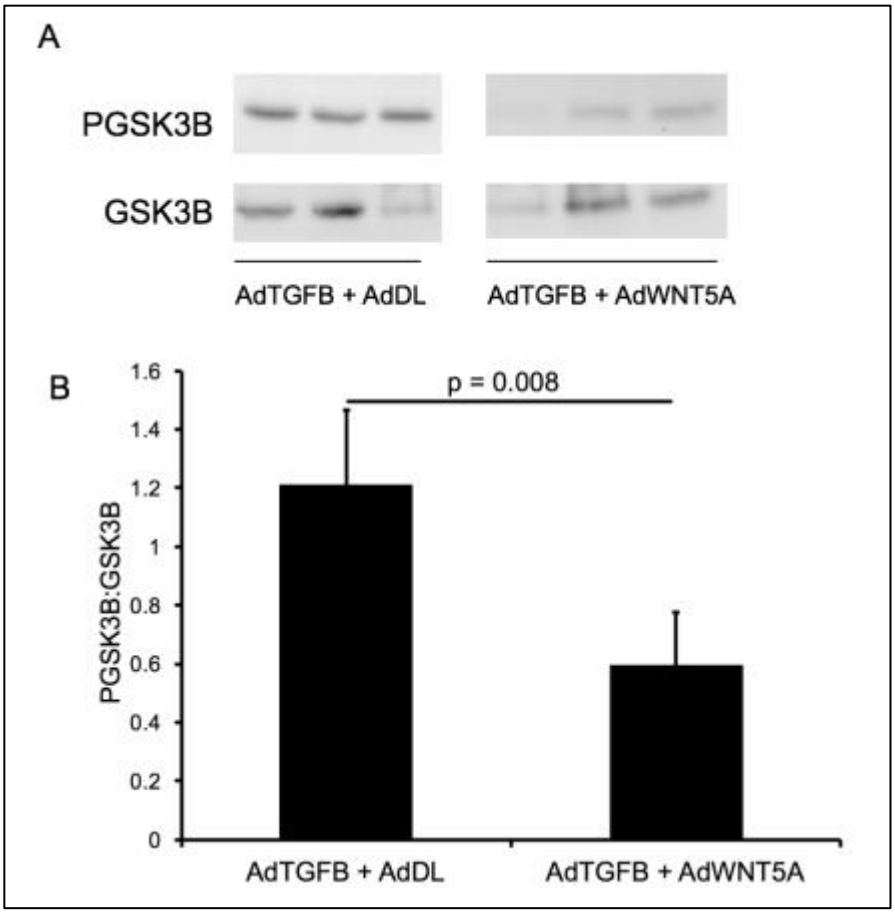



**Figure 6.3 WNT5A inhibits canonical WNT/b-catenin signaling -** (A) Representative blot (B) Western blot analysis revealed a marked decrease in relative PGSK3B in response to WNT5A compared to TGFB (p=0.008).

***WNT5A signals through Ror2 to regulate fibrosis and angiogenesis***

WNT5A appeared to inhibit fibrosis and angiogenesis in our peritoneal fibrosis model. We therefore investigated the mechanism by which WNT5A downregulates fibrosis and angiogenesis related mediators. WNT5A has been documented to signal through two different families of receptors including Ror2 and FZD. Existing evidence suggests that WNT5A has differential roles based on

the receptor context and inhibits WNT/b-catenin signaling in the presence of Ror2 [13]. We hypothesized that WNT5A may be signaling through Ror2 to protect against angiogenesis. To examine this, we exposed MET5A cells to recombinant TGFB and used an siRNA to block Ror2. Ror2 was successfully silenced. Scrambled RNA was included in the control group. TGFB induced an increase in fibronectin ($p=0.002$) and this effect was amplified with an siRNA against Ror2 ($p<0.01$) (Figure 6.3A, B). We observed increased levels of VEGF mRNA in response to TGFB. Furthermore, blocking Ror2 resulted in an even greater augmentation of VEGF mRNA ($p = 0.009$) (Figure 6.3C). Similarly, we analyzed levels of VEGF protein in supernatant from cultured mesothelial cells and observed the same effect ($p<0.001$). TGFB induced an increase in VEGF protein and this effect was exacerbated in response to an siRNA against Ror2 ($p<0.001$) (Figure 6.3D).

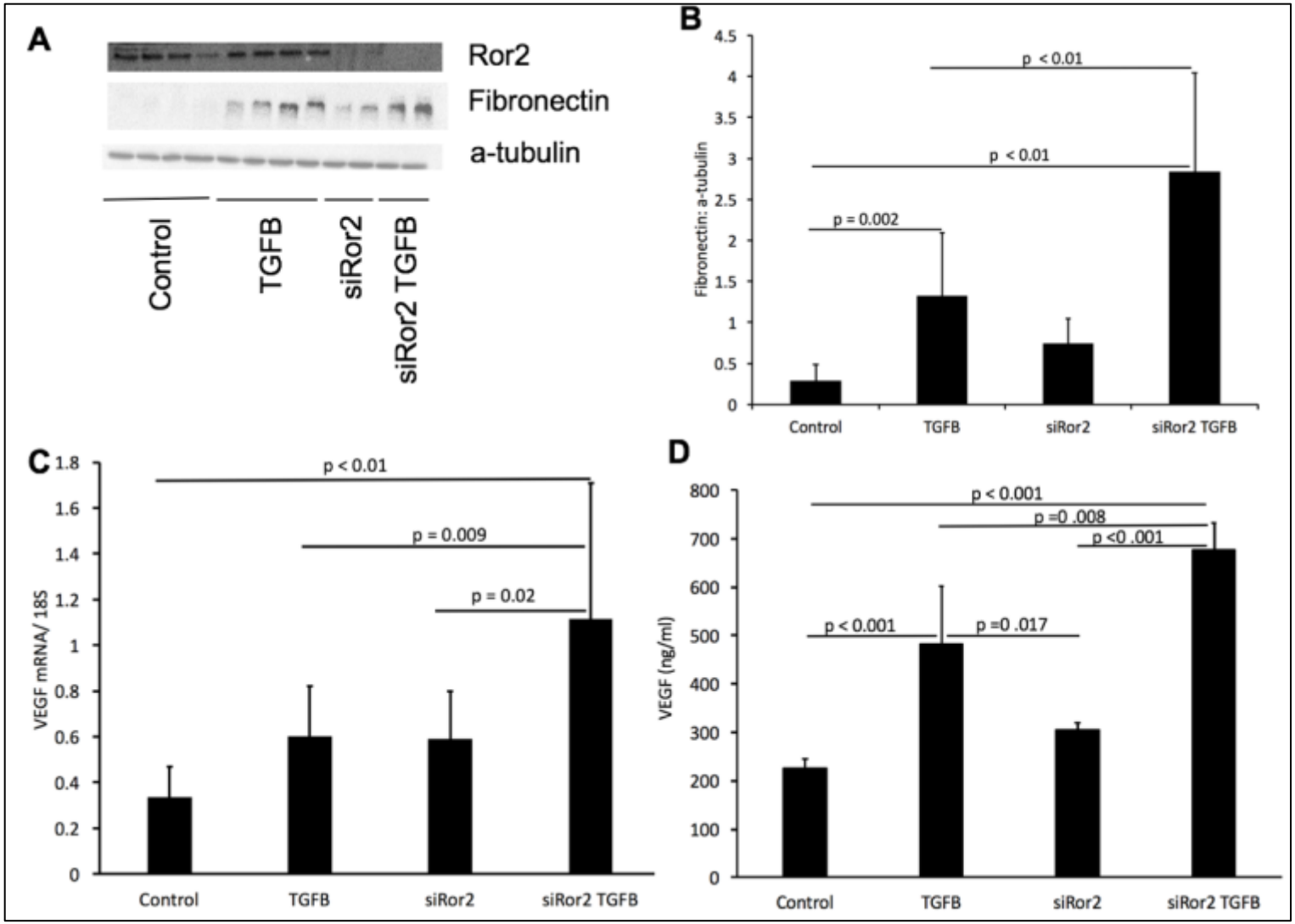

A
Ror2
Fibronectin
a-tubulin
Control
TGFB
siRor2
siRor2 TGFB
B
Fibronectin: a-tubulin
p < 0.01
p < 0.01
p = 0.002
Control
TGFB
siRor2
siRor2 TGFB
C
VEGF mRNA/ 18S
p < 0.01
p = 0.009
p = 0.02
Control
TGFB
siRor2
siRor2 TGFB
D
VEGF (ng/ml)
p < 0.001
p =0 .008
p <0 .001
p < 0.001
p =0 .017
Control
TGFB
siRor2
siRor2 TGFB

**Figure 6.4 WNT5A signals through Ror2 to regulate fibrosis and angiogenesis -** (A) Representative Blot (B) Western blot analysis of fibronectin demonstrated an increase in response to recombinant TGFB ($p=0.002$). Blocking Ror2 exacerbated this effect ($p<0.01$) (C) Quantitative analysis of VEGF mRNA: TGFB induced an increase in VEGF mRNA. When using an siRNA to block Ror2, there was a further increase in VEGF mRNA ($p=0.009$) (D) ELISA analysis of VEGF protein in cell culture supernatant. TGFB induced an increase in VEGF protein levels ($p<0.001$). When using an siRNA to block Ror2, there was an even greater effect on the increase in VEGF protein ($p= 0.008$).

We repeated this experiment in mesothelial cells cultured from peritoneal effluent from a patient sample. Analysis of VEGF protein in the supernatant of these mesothelial cells demonstrated a similar trend in response to TGFB as observed in the MET5A cells ($p<0.01$) (Figure 6.5). With the addition of siRor2, levels of VEGF were found to be increased compared to TGFB alone, however this was not significant (Figure 6.5). Collectively, these results suggest Ror2 is involved in protecting against angiogenesis by regulating levels of VEGF gene and protein expression in both MET5A cells and mesothelial cells cultured from patient peritoneal effluent.

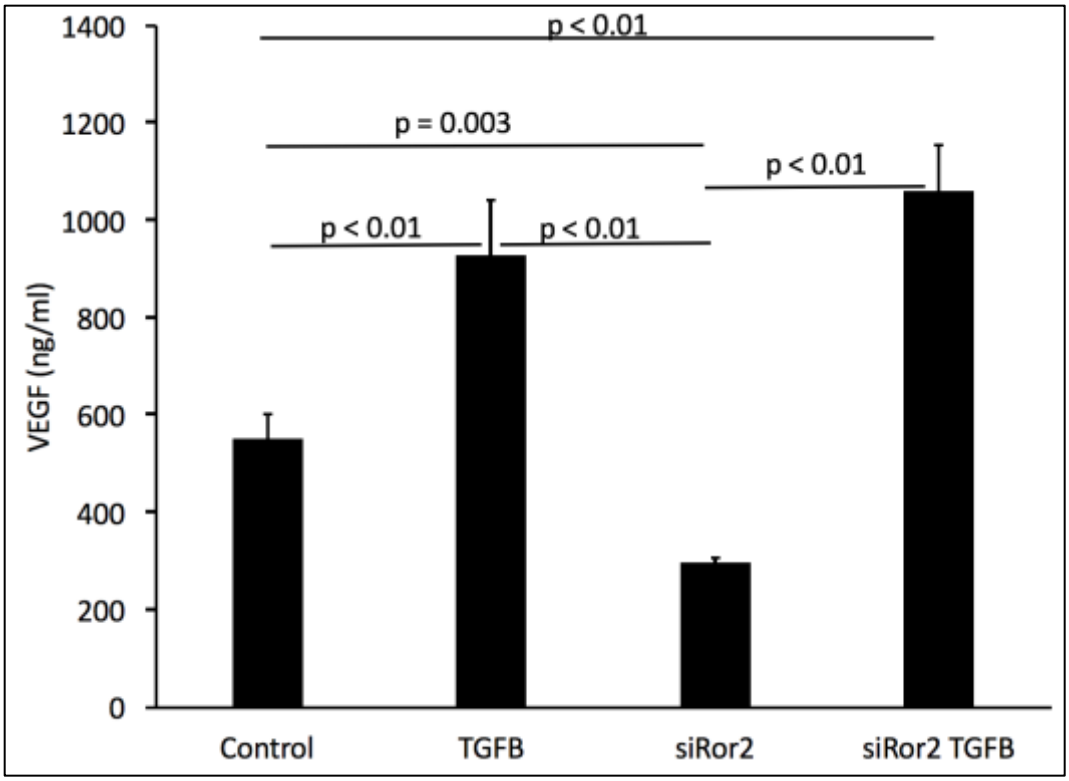



**Figure 6.5 ELISA analysis for VEGF from human mesothelial cells cultured from patient peritoneal effluent -** Recombinant TGFB induced an increase in VEGF protein ((p=0.003). Similar to what was observed with the MET5A cells, there is an in VEGF when cells were treated with TGFB and an siRNA against Ror2 although not significant.

## DISCUSSION

Patients undergoing peritoneal dialysis begin to develop a change in peritoneal membrane structure including fibrosis and angiogenesis. Peritoneal fibrosis has been associated with a decline in ultrafiltration capacity by altering hydraulic conductance of the membrane [42]. Expansion of peritoneal vasculature is also suggested to be associated with increased solute transport or leakiness of the membrane [4]. High solute transport results in increased reabsorption of

glucose and loss of the solute gradient that drives ultrafiltration [3]. Although fibrosis and angiogenesis are interrelated events, angiogenesis is suggested to have a greater impact on solute transport and may be a more important therapeutic target [4].

In this study, we assessed the role of WNT5A, in the development of both fibrosis and angiogenesis and observed it protects against both elements of peritoneal membrane injury. Addition of WNT5A to the mouse peritoneum resulted in reduced submesothelial thickness and blood vessel density. Accumulating evidence has described WNT5A to exhibit distinct effects depending on the cell surface receptor that is present. Studies of angiogenesis report that WNT5A can either stimulate [44] or inhibit endothelial cells [45] and its effect may be attributed to receptor context. Emerging functional studies in fibrosis have suggested a more deleterious role for WNT5A in fibrosis [26] and most have shown WNT5A to exacerbate injury in the presence of FZD receptors. For instance, in a study of asthma development WNT5A and TGFB resulted in ECM production in airway smooth muscle cells and these cells demonstrated high levels of FZD8. This WNT receptor was observed to regulate TGFB induced expression of collagen and fibronectin. Minimal levels of Ror2 expression were observed in these cells and Ror2 was not further investigated [29]. In other studies, WNT5A has been observed to mediate cell migration in the presence of FZD1, 2 and 7 [46,47]. In our study, we examined the receptor environment in injured mesothelial cells by blocking Ror2. Ror2 mediated protective effects in

injured mesothelial cells. Overexpression of TGFB induced an increase in fibronectin and VEGF. When Ror2 was silenced, levels of fibronectin and VEGF protein were exacerbated. In addition to this, we observed a similar response in mesothelial cells cultured from patients on PD.

In contrast to our observations, WNT5A and Ror2 were shown to contribute to injury in a recent study of renal fibrosis [30]. WNT5A and Ror2 were both elevated after unilateral ureteral obstruction (UUO) and Ror2 expression was associated with expression of EMT related proteins. Ror2 heterozygotes were generated to partially reduce Ror2 and exposed to UUO, but did not yield altered levels of EMT markers. However, lower levels of Ror2 did result in reduced damage to the basement membrane [30]. Although this study suggests that Ror2 is contributing to injury, it is unclear how this receptor is functioning in the context of WNT5A. It may be acting as a co-receptor alongside FZD and stimulating a different pathway. Moreover, Ror2 heterozygotes still express Ror2 to some degree. Interestingly, Ror2 heterozygotes showed an increase in Dvl2 which is a component of both canonical and non-canonical WNT signaling. In our study, we directly co-expressed WNT5A with TGFB and observed protection against both angiogenesis and fibrosis. In vitro, we used siRNA against Ror2 successfully silenced Ror2 to a greater extent. Overall, further investigation is required to understand the mechanism by which Ror2 is acting and which downstream pathways it may be activating or inhibiting during the development of injury. Moreover, several transcription factors regulate the expression of WNT5A which

is different in each cell type; thus surrounding signaling pathways require closer analysis [31].

It has become apparent that WNT5A can also regulate b-catenin dependent signaling. Mikels et al., observed the dual nature of WNT5A where it could both activate and inhibit the canonical WNT/ b-catenin signaling pathway depending on the cell surface receptor that was present. In the presence of FZD4, WNT5A increased b-catenin levels. Paradoxically, in the presence of Ror2, WNT5A suppressed b-catenin activity in vitro [13]. Therefore, WNT5A can lead to activation of two discrete pathways based on receptor context and these observations were validated in other studies [48]. Levels of b-catenin are controlled by a complex of proteins including GSK3B which marks b-catenin for ubiquitination and degradation. When WNT signaling is turned on, GSK3B is phosphorylated and inactivated resulting in stabilization of b-catenin and upregulation of its target genes. We found WNT5A induced a decrease in PGSK3B and may be implicated in antagonizing WNT/b-catenin signaling. Our results agree with other studies where WNT5A has inhibited canonical WNT signaling in the development of cancer [49]. Other groups have reported that WNT5A blocks transcriptional activity of b-catenin and does not directly impact levels of PGSK3B or b-catenin protein levels [13,38,50,51]. Our results demonstrate a more upstream effect as we observed WNT5A to reduce levels of PGSK3B. The molecular mechanism by which WNT5A regulates WNT/b-catenin signaling requires further investigation.

The peritoneal membrane is a lifeline for patients with end stage kidney disease on peritoneal dialysis therapy. We know that injury to the peritoneum increases the risk of technique failure and mortality. This current research expands potential therapeutic options for preservation of the peritoneal membrane in PD patients. Based on our findings, we suggest WNT5A blocks the canonical WNT pathway to reduce both fibrosis and angiogenesis in the peritoneum and Ror2 is a potential candidate for mediating these effects. Although further investigation is necessary, the dual nature of WNT5A may be used as a method of targeting and blocking aberrant WNT/b-catenin signaling to prevent progression of injury.

**References**

1 Aroeira LS, Aguilera A, Selgas R, Ramirez-Huesca M, Perez-Lozano ML, Cirugeda A, et al.: Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis 2005;46:938–948.

2 Williams JD, Craig KJ, Topley N, Von Ruhland C, Fallon M, Newman GR, et al.: Morphologic changes in the peritoneal membrane of patients with renal disease. J Am Soc Nephrol 2002;13:470–479.

3 Margetts PJ, Brimble KS: Peritoneal dialysis, membranes and beyond. Curr Opin Nephrol Hypertens 2006;15:571–576.

4 Margetts PJ, Bonniaud P: Basic mechanisms and clinical implications of peritoneal fibrosis. Perit Dial Int 2003;23:530–541.

5 Lambie M, Chess J, Donovan KL, Kim YL, Do JY, Lee HB, et al.: Independent effects of systemic and peritoneal inflammation on peritoneal dialysis survival. J Am Soc Nephrol 2013;24:2071–2080.

6 Davies SJ: Longitudinal relationship between solute transport and ultrafiltration capacity in peritoneal dialysis patients. Kidney Int 2004;66:2437–2445.

7 Hassan K, Hassan D, Shturman A, Rubinchik I, Fadi H, Shadi H, et al.: The impact of sub-clinical over-hydration on left ventricular mass in peritoneal dialysis patients. Int J Clin Exp Med 2015;8:5890–5896.

8 He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y: Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 2009;20:765–776.

9 Wang D, Dai C, Li Y, Liu Y: Canonical Wnt/beta-catenin signaling mediates transforming growth factor-beta1-driven podocyte injury and proteinuria. Kidney Int 2011;80:1159–1169.

10 Guo Y, Xiao L, Sun L, Liu F: Wnt/beta-catenin signaling: a promising new target for fibrosis diseases. Physiol Res 2012;61:337–346.

11 Zhou B, Liu Y, Kahn M, Ann DK, Han A, Wang H, et al.: Interactions between beta-catenin and transforming growth factor-beta signaling pathways mediate epithelial-mesenchymal transition and are dependent on

the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). J Biol Chem 2012;287:7026–7038.

12 Akmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, et al.: Activation of canonical WNT signaling is required for TGFB-mediates fibrosis. Nat Commun 2012;3:735

13 Mikels A, Minami Y, Nusse R: Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. J Biol Chem 2009;284:30167–30176.

14 Padwal M, Cheng G, Liu L, Boivin F, Gangji A, Brimble S, Bridgewater D, Margetts P: The WNT Signaling Pathway is Activated During Peritoneal Membrane Injury. Submitted to Am J Physiol Renal Physiol 2017;

15 Ji S, Deng H, Jin W, Yan P, Wang R, Pang L, et al.: Beta-catenin participates in dialysate-induced peritoneal fibrosis via enhanced peritoneal cell epithelial-to-mesenchymal transition. FEBS Open Bio 2017;7:265–273.

16 Enzo MV, Rastrelli M, Rossi CR, Hladnik U, Segat D: The Wnt/beta-catenin pathway in human fibrotic-like diseases and its eligibility as a therapeutic target. Mol Cell Ther 2015;3:1.

17 Selman M, Pardo A, Kaminski N: Idiopathic pulmonary fibrosis: aberrant recapitulation of developmental programs? PLoS Med 2008;5:e62.

18 Kaminski N, Rosas IO: Gene expression profiling as a window into idiopathic pulmonary fibrosis pathogenesis: can we identify the right target genes? Proc Am Thorac Soc 2006;3:339–344.

19 Veeman MT, Axelrod JD, Moon RT: A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 2003;5:367–377.

20 Nomachi A, Nishita M, Inaba D, Enomoto M, Hamasaki M, Minami Y: Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A. J Biol Chem 2008;283:27973–27981.

21 Kikuchi A, Yamamoto H, Sato A, Matsumoto S: Wnt5a: its signaling, functions and implication in diseases. Acta Physiol (Oxf) 2012;204:17–33.

22 Cheng JH, She H, Han Y-P, Wang J, Xiong S, Asahina K, et al.: Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2008;294:G39-49.

23 Villar J, Cabrera-Benitez NE, Ramos-Nuez A, Flores C, Garcia-Hernandez S, Valladares F, et al.: Early activation of pro-fibrotic WNT5A in sepsis induced acute lung injury. Crit Care 2014;18:568.

24 Baarsma HA, Skronska-Wasek W, Mutze K, Ciolek F, Wagner DE, John-Schuster G, et al.: Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD. J Exp Med 2017;214:143–163.

25 Newman DR, Sills WS, Hanrahan K, Ziegler A, Tidd KM, Cook E, et al.: Expression of WNT5A in Idiopathic Pulmonary Fibrosis and Its Control by TGF-beta and WNT7B in Human Lung Fibroblasts. J Histochem Cytochem 2016;64:99–111.

26 Vuga LJ, Ben-Yehudah A, Kovkarova-Naumovski E, Oriss T, Gibson KF, Feghali-Bostwick C, et al.: WNT5A is a regulator of fibroblast proliferation and resistance to apoptosis. Am J Respir Cell Mol Biol 2009;41:583–589.

27 Dong S, Wu C, Hu J, Wang Q, Chen S, Zhirong W, et al.: Wnt5a Promotes Cytokines Production and Cell Proliferation in Human Hepatic Stellate Cells Independent of Canonical Wnt Pathway. Clin Lab 2015;61:537–547.

28 Chatani N, Kamada Y, Kizu T, Ogura S, Furuta K, Egawa M, et al.: Secreted frizzled-related protein 5 (Sfrp5) decreases hepatic stellate cell activation and liver fibrosis. Liver Int 2015;35:2017–2026.

29 Kumawat K, Menzen MH, Bos IST, Baarsma HA, Borger P, Roth M, et al.: Noncanonical WNT-5A signaling regulates TGF-beta-induced extracellular matrix production by airway smooth muscle cells. FASEB J Off Publ Fed Am Soc Exp Biol 2013;27:1631–1643.

30 Li X, Yamagata K, Nishita M, Endo M, Arfian N, Rikitake Y, et al.: Activation of Wnt5a-Ror2 signaling associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis. Genes Cells 2013;18:608–619.

31 Beljaars L, Daliri S, Dijkhuizen C, Poelstra K, Gosens R: WNT-5A regulates TGF-beta-related activities in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2017;312:G219–G227.

32 Leris ACA, Roberts TR, Jiang WG, Newbold RF, Mokbel K: WNT5A expression in human breast cancer. Anticancer Res 2005;25:731–734.

33 Cheng R, Sun B, Liu Z, Zhao X, Qi L, Li Y, et al.: Wnt5a suppresses colon cancer by inhibiting cell proliferation and epithelial-mesenchymal transition. J Cell Physiol 2014: 229:1908-1917

34 Ying J, Li H, Yu J, Ng KM, Poon FF, Wong SCC, et al.,: WNT5A exhibits tumor-supressive activity through antagonizing the Wnt/beta-catenin signaling pathway, and is frequently methylated in correctal cancer. Clin Cancer Res 2008; 14:55-61

35 Kikuchi A, Yamamoto H: Tumor formation due to abnormalities in the beta-catenin-independent pathway of Wnt signaling. Cancer Sci 2008;99:202–208.

36 Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath G: A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. Cell 2014;159:844–856.

37 Franco CA, Liebner S, Gerhardt H: Vascular morphogenesis: a Wnt for every vessel? Curr Opin Genet Dev 2009;19:476–483.

38 Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, et al.: The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signaling pathway. Genes Cells 2003;8:645–654.

39 Torres MA, Yang-Snyder JA, Purcell SM, DeMarais AA, McGrew LL, Moon RT: Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. J Cell Biol 1996;133:1123–1137.

40 Schambony A, Wedlich D: Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. Dev Cell 2007;12:779–792.

41 Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, et al.: Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. Am J Pathol 2003;162:1495–1502.

42 Sime PJ, Xing Z, Graham FL et al. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997; 100: 768–776

43 Cina D, Patel P, Bethune JC, Thoma J, Rodriguez-Lecompte JC, Hoff CM, et al.: Peritoneal morphological and functional changes associated with platelet-derived growth factor B. Nephrol Dial Transplant 2009;24:448–457.

44 Goodwin AM, Kitajewski J, D'Amore PA: Wnt1 and Wnt5a affect endothelial proliferation and capillary length; Wnt2 does not. Growth Factors 2007;25:25–32.

45 Masckauchan TNH, Agalliu D, Vorontochikhina M, Ahn A, Parmalee NL, Li CM, et al.: Wnt5a signaling induces proliferation and survival of endothelial cells in vitro and expression of MMP-1 and Tie-2. Mol Biol Cell 2006;17:5163-5172

46 Nishita M, Itsukushima S, Nomachi A, Endo M, Wang Z, Inaba D, et al.: Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating Dishevelled polymerization. Mol Cell Biol 2010;30:3610–3619.

47 Laeremans H, Rensen SS, Ottenheijm HCJ, Smits JFM, Blankesteijn WM: Wnt/frizzled signaling modulates the migration and differentiation of immortalized cardiac fibroblasts. Cardiovasc Res 2010;87:514–523.

48 Yuan Y, Niu CC, Deng G, Li ZQ, Pan J, Zhao C, et al.: The Wnt5a/Ror2 noncanonical signaling pathway inhibits canonical Wnt signaling in K562 cells. Int J Mol Med 2011;27:63–69.

49 Yuzugullu H, Benhaj K, Ozturk N, Senturk S, Celik E, Toylu A, et al.: Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. Mol Cancer 2009;8:90.

50 Ishitani T, Kishida S, Hyodo-Miura J, Ueno N, Yasuda J, Waterman M, et al.: The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol 2003;23:131–139.

51 Ishitani T, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, et al.: The TAK1-NLK-MAPK-related pathway antagonizes signaling between beta-catenin and transcription factor TCF. Nature 1999;399:798–802.

# CHAPTER 7: DISCUSSION

Peritoneal membrane injury is a common complication observed in patients undergoing long-term peritoneal dialysis. This condition consists of thickening of the submesothelial layer and expansion of the peritoneal vasculature as a result of bioincompatibility of dialysis fluids and recurring episodes of peritonitis [6]. The observed changes in peritoneal membrane structure are associated with changes in peritoneal membrane function. The increase in blood vessel density is thought to be the major contributor of changes in peritoneal membrane filtration [28]. The presence of a higher number of blood vessels, changes in surface area and permeability of these vessels results in an increase in solute transport across the peritoneal membrane [10,26]. High solute transport is associated with reabsorption of glucose and volume expansion increasing the risk of hypertension and related cardiovascular events [198]. Furthermore high solute transport is also associated with reduced osmotic conductance and poorer clinical outcomes [26,28]. As a result of these complications, many of the patients on PD must be switched to hemodialysis or receive a transplant which may not be readily available [199]. Therefore, it is important to understand how we can better protect and preserve the peritoneal membrane for patients on PD.

TGFB and the appearance of the myofibroblast have been recognized as the main initiators of both peritoneal fibrosis and angiogenesis [55]. The mechanism by which TGFB induces injury in the peritoneal membrane has not fully been elucidated. In previous work, we have generated an adenoviral vector expressing TGFB and have used this to create a model of peritoneal membrane

injury. Addition of AdTGFB to the mouse peritoneum has recapitulated the changes in peritoneal membrane structure observed in patients on PD including submesothelial thickening and increased blood vessel density [55]. The adenovirus can effectively transfer the candidate gene to the peritoneum and yield high levels of transient and stable gene expression. In addition to this, adenoviral vectors can hold large segments of DNA which are easy to manipulate and transfer into non proliferating cells [200]. In this model, active TGFB is maximum at day 4 and day 7 up until day 14. Significant changes in peritoneal membrane structure begin to appear at day 4 and this includes increased peritoneal thickness, angiogenesis and cellular proliferation. These changes are more pronounced at day 7 [55]. This included an increase in levels of VEGF which peaked at day 7 after exposure to AdTGFB. This model also mimics changes in peritoneal membrane function with significant reductions in ultrafiltration from day 4 to 21 until day 28. Other studies have confirmed that the adenovirus itself does not induce any acute changes and we have observed similar effects with replication-deficient adenoviral vectors. Using a control adenovirus, we confirmed the changes in structure and function in the peritoneum were not virus-induced effects [55]. Another commonly used model is the chronic infusion model of PD which involves daily infusion of dialysis fluid into the rat peritoneum over a period of 5 weeks. Although, this model successfully replicates components of peritoneal membrane injury, the indwelling catheter has resulted in amplification of the inflammatory response [7].

In our model of TGFB induced injury, angiogenesis was associated with changes in peritoneal membrane function. This suggests a link between vascularization and solute transport. Elevated levels of VEGF and increased albumin transport at day 4 suggests that VEGF may induce increased permeability of existing blood vessels. Moreover, the sustained increase in blood vessel density and decrease in ultrafiltration also indicates that solute transport is associated with vascular surface area [55]. Angiogenesis is considered the leading contributor to increases in solute transport and volume expansion leading to poorer clinical outcomes [28]. Therefore, this animal model allows us to measure angiogenesis and angiogenic factors. We have used immunohistochemistry for von Willebrand factor as a measure for angiogenesis and we have also assessed gene and protein expression of angiogenic factors such as VEGF directly in the peritoneal membrane, omental tissue and peritoneal dialysis effluent.

**The Role of MMP9 and WNT Signaling in Peritoneal Angiogenesis**

As angiogenesis of the peritoneal membrane is considered a primary contributor of ultrafiltration failure, our research is focused on understanding the underlying mechanism behind this process [13]. These findings can be applied to similar areas of biomedical research such as the pathogenesis of vascular diseases and vascularization of tumours [244, 245]. One of the important steps during angiogenesis involves clearance of the extracellular matrix for the formation of new vessels. Proteolysis of the basement membrane and remodeling of the matrix facilitates the migration and invasion of new endothelial cells [203]. Matrix

metalloproteinases play an important role in remodeling of the extracellular matrix and MMP2 and 9 have been consistently recognized for their ability to degrade collagen IV of the basement membrane [73]. Much of the evidence on the function of MMPs in vascular formation comes from studies of malignancy [202] and other vascular diseases [204]. Several studies involving knockout of MMP2 and 9 in tumors demonstrate defects in vessel formation and development. MMP2 and 9 have also been shown to play a role in regulating angiogenesis during normal processes such as embryogenesis [204] and wound repair [205]. In support of these studies, our work has shown evidence of the proangiogenic role of MMP9 in TGFB induced peritoneal membrane injury. We did not, however, observe MMP2 to have a significant effect on angiogenesis suggesting that MMP9 is a more critical component to focus on. The discrete effects of MMP2 and 9 also indicate an independent role for each metalloproteinase.

We have discovered a novel mechanism by which MMP9 upregulates VEGF to induce angiogenesis (Figure 7.1). Substantial evidence exists for the role of MMPs in vascular remodeling via upregulation of VEGF [204]. MMPs have also been proposed to induce angiogenesis via other mechanisms such as proteolysis of the basement membrane and type1 collagen for endothelial cell migration and modification of PDGF [204]. Of all the proposed mechanisms implicated in angiogenesis, VEGF is a critical factor that has been observed to induce endothelial cell proliferation, sprouting and assembly and also affects vessel permeability [110]. MMP9 mediates the release of matrix sequestered

VEGF in studies of ovarian [110], breast [105], and gastric cancer [202] resulting in tumor angiogenesis.

Although these studies have demonstrated the proteolytic release of VEGF by MMPs, our study is the first to demonstrate an interaction between MMP9 and the WNT/b-catenin signaling pathway in inducing VEGF (Figure 7.1) [110]. In our proposed mechanism, we suggest that MMP9 cleaves E-cadherin and activates b-catenin signaling to induce VEGF. Our work provides evidence of E-cadherin cleavage by MMP9 and our observations are supported by studies of cancer [118]. E-cadherin is a cell adhesion protein bound to the cytoskeleton via b-catenin [126]. We suggest that MMP9 cleaves E-cadherin and releases membrane bound b-catenin. In the absence of WNT signaling, cytoplasmic b-catenin is phosphorylated and degraded. We propose that activation of canonical WNT signaling stabilizes free b-catenin allowing it translocate to the nucleus to upregulate VEGF (Figure 7.1). We examined the interaction between MMP9 and WNT signaling by overexpressing MMP9 and used ICG-001 to inhibit activation of the WNT pathway. ICG-001 blocks the WNT pathway by disrupting b-catenin mediated gene transcription [206]. ICG-001 effectively blocked MMP9 induced angiogenesis and resulted in a reduction of VEGF levels in the peritoneal membrane. MMP9 gene expression in patient mesothelial cells was also associated with gene expression of several WNT related ligands and receptors. We propose VEGF is coming from the injured mesothelium and is acting in a paracrine manner. We have demonstrated that both MMP9 and WNT signaling

cooperate with TGFB to induce angiogenesis. Other studies expand on our findings by further examining the interactions between MMPs and WNT signaling. Evidence from these studies has shown that MMPs are both upstream activators of WNT/b-catenin signaling and downstream targets of b-catenin [207–209] suggesting a possible positive feedback loop by which b-catenin could amplify MMP signaling. We have shown MMP9 induces nuclear translocation of b-catenin, but have not examined upstream of this pathway. Thus, combining our evidence with these studies prompts further exploration of the interaction between MMP9 and WNT/b-catenin signaling to delineate this potential feedback mechanism. Combining this work with studies of peritoneal membrane function will allow for a better understanding of the contribution of angiogenesis to ultrafiltration failure.

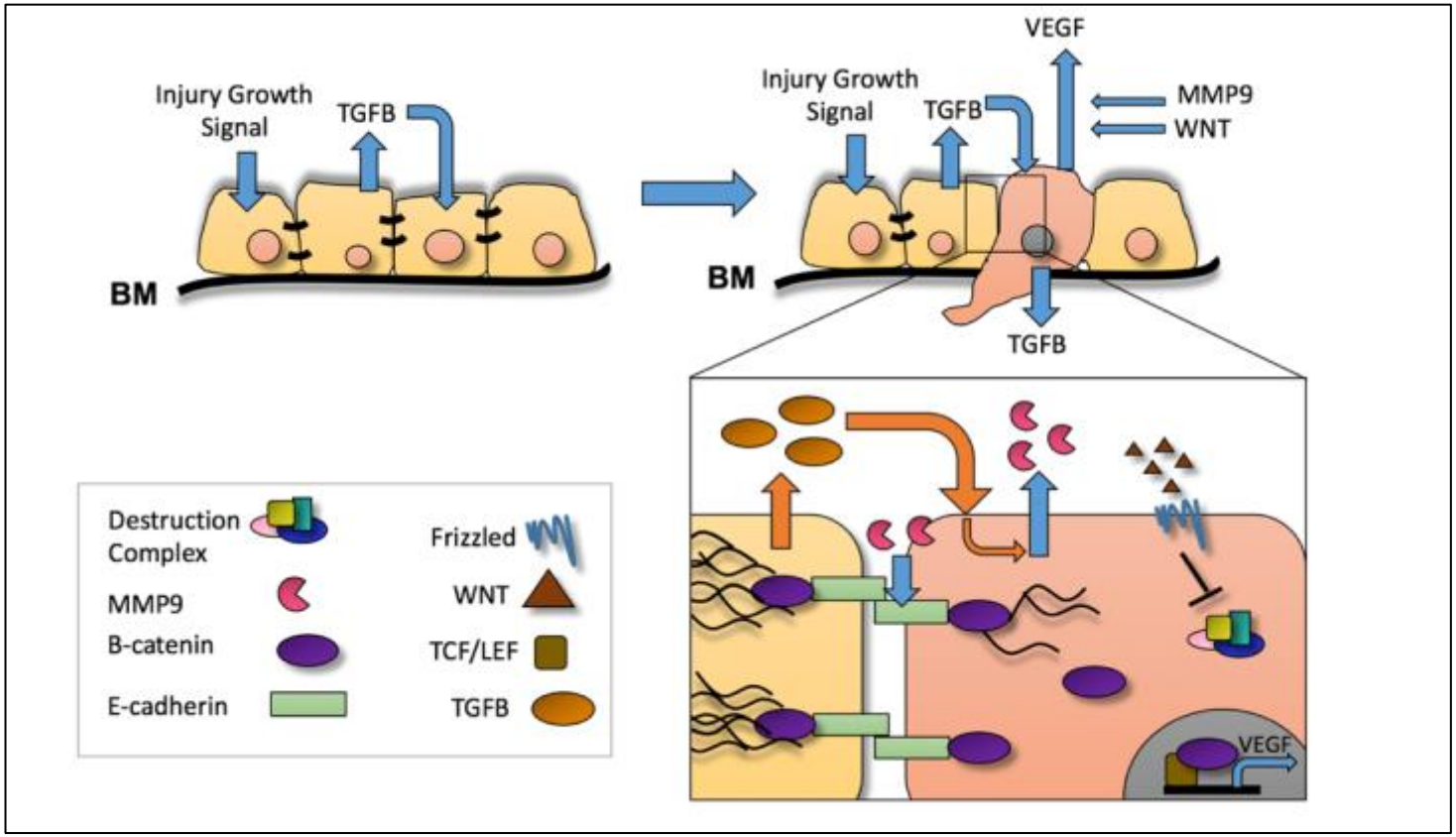



**Figure 7.1 Mechanism of MMP9 and b-catenin Signaling to induce angiogenesis -** Injury to the mesothelial cells results in production of TGFB which induces EMT. TGFB induces secretion of MMP9 in the transitioning cell. E-cadherin is anchored to the cytoskeleton via b-catenin. MMP9 facilitates cleavage of E-cadherin which releases b-catenin into the cytosol. At the same time TGFB induces an increase in WNT/ b-catenin signaling. Cross-talk with WNT signaling stabilizes b-catenin which translocates to the nucleus and upregulates proangiogenic genes such as VEGF.

Our findings suggest that the TGFB pathway induces activation of the canonical WNT signaling pathway. Several reports have investigated the mechanism by which these pathways may interact. Reciprocal transregulation has been observed in studies of embryogenesis and malignancy where WNT factors can regulate members of the TGFB family and conversely TGFB can regulate WNT ligand expression [174]. For instance, TGFB has been observed to downregulate levels of DKK-1 [143]. In addition, TGFB and WNT signaling have also been shown to co-regulate gene expression [210]. In recent studies of fibrosis, the convergence of the TGFB and WNT pathway has been observed in the nucleus. In pulmonary fibrosis, ICG-001 was shown to block EMT and fibrosis. In this study, the TGFB/SMAD3 pathway was observed to interact with b-catenin to induce effects of EMT [171]. In dermal fibrosis, overexpression of SMAD3 induces b-catenin activity in the absence of TGFB [172]. ICG-001 also blocked renal fibrosis independently of SMAD2 signaling [156]. Collectively, these data indicate that TGFB is interacting with the WNT pathway and in the future, we would like to understand how these factors cooperatively induce angiogenesis.

The proangiogenic role of MMP9 is important in peritoneal membrane injury because expansion of peritoneal membrane vasculature is the main factor that leads to increased solute transport and failure of ultrafiltration [28]. Therefore, it is necessary to understand the exact function of MMP9 in peritoneal angiogenesis. We developed an ex vivo cell culture of human mesothelial cells

from patient peritoneal dialysis effluent. We used Nanostring technology to characterize the gene expression of MMPs and WNT related genes in this ex vivo cell culture. We also looked to see if these genes were associated with properties of peritoneal membrane function measured by D/P creatinine and D/D0 glucose. We found that MMP9 was most strongly correlated with D/P creatinine or peritoneal membrane solute transport compared to other MMPs including MMP2. In other studies of peritoneal membrane solute transport, MMP2 in peritoneal effluent was observed to be associated with solute transport, however MMP9 was found at lower concentrations [97–99]. In these studies, peritoneal effluent may include systemic amounts of MMP2, therefore confounding the final assessment. To account for this, we have measured the local production of MMP9 directly from patient mesothelial cells. We found that MMP9 gene expression in human mesothelial cells is correlated with expression of WNT related ligands and receptors as well as VEGF. From our panel of WNT signaling genes, we observed the strongest correlation between WNT1 and peritoneal membrane solute transport among other WNT ligands. We provide the first look at peritoneal membrane function and WNT related proteins and receptors.

The WNT signaling pathway can be separated into the canonical and non-canonical pathway. The canonical pathway is well characterized and involves signaling via b-catenin whereas the non-canonical pathway exerts its effects independently of b-catenin. Some studies have separated the different WNT ligands and receptors into canonical or non-canonical based on their activity in

each respective pathway. Prototypically, WNT1, WNT2 and WNT3A are considered canonical WNTs and have been observed to signal through the b-catenin dependent pathway [211]. WNT4, WNT5A and WNT11 have been documented to mostly participate in b-catenin independent signaling and therefore are considered non-canonical WNT ligands [211]. Physiologically, activation of WNT/b-catenin signaling is mainly observed in vasculature of developing organisms but is silenced in adults [212–214].

We characterized WNT signaling in peritoneal membrane injury and saw that it induces angiogenesis, but it did not significantly impact fibrosis. Active canonical and non-canonical WNT signaling have been implicated in studies of development and human vascular diseases. b-catenin has been shown to directly regulate the expression of VEGF and when inhibited, VEGF is decreased [215]. WNT/b-catenin promotes proliferation and survival of endothelial cells [163]. The WNT system has significant importance in vascular morphogenesis during development and during endothelial cell differentiation [136]. Cultured endothelial cells have been shown to express many WNT family members including WNT ligands but also FZD receptors and co-receptors confirming that these cells can respond to WNT signaling [216]. Abnormal levels of b-catenin have been associated with angiogenesis in vascular disorders of the eye including Norrie disease [159] and diabetic retinopathy [217]. In Norrie disease, deficiencies in FZD4/Norrin signaling resulted in defective vascular development of the retina [159]. WNT signaling was also found to be active in the retinas of

diabetic human and animal models. Blocking WNT signaling using a novel inhibitor resulted in decreased levels of VEGF and decreased endothelial cell migration [217]. We found that the TGFB pathway interacts with the WNT/b-catenin pathway to induce injury. WNT signaling is proposed to act through several different mechanisms which include both regulating angiogenic factors such as VEGF and directly acting on endothelial cell migration. Our work has shown that WNT/b-catenin signaling induces an increase in VEGF and blood vessel density. Future investigation is necessary to investigate the mechanism of interaction between the TGFB and WNT pathway during peritoneal membrane injury.

**The role of MMP9 and WNT Signaling in EMT and Fibrosis**

The role for MMPs in extracellular matrix remodeling is well established [103]. Traditionally, MMPs have been implicated in reducing fibrosis due to their ability to degrade ECM. However accumulating evidence has shown MMPs to have both stimulatory and inhibitory functions. MMP2 has been shown to be anti-fibrotic in the kidney [218] and liver [219]. On the other hand, MMP9 has mostly been observed as having pro-fibrotic properties in the kidney [94], lung [220] and lens tissue [221]. Studies of lung fibrosis have reported conflicting results of the function of MMP9 and therefore further investigation is critical [222,223]. Our work has shown MMP9 to be a mediator of angiogenesis with less effect on fibrosis. The inconsistencies on the role of MMP9 in fibrosis may be attributed to the difference in function of each MMP. Moreover, future studies need to focus

on the regulation of these proteins in addition to their functionality. During fibrosis, MMPs are regulated at the level of transcription but also post-translationally by other enzymes [224]. Moreover, diverse MMPs play distinct roles and can contribute to fibrosis in a number of different ways including regulation of ECM, activating or deactivating the myofibroblast, controlling level of inflammation and degree of injury or repair [224].

In chronic fibrotic conditions, the resolution phase of wound healing is defective and the fibrotic matrix is not effectively cleared. At this point, a-SMA positive myofibroblasts continue to deposit collagen and is the key effector cell contributing to injury. It is unclear where these myofibroblasts originate from, but EMT is a potential contributing process. EMT in fibrosis has recently become a contentious area of study due to new evidence from lineage tracing studies. This powerful technique allows researchers to determine the origin of the myofibroblast. An acceptable marker to label peritoneal cells has not been found, however Wilms' Tumor 1 (WT1) has been used to identify mesothelial cells thus far [68]. A recent study using this system suggested that the myofibroblast found in the submesothelial zone does not come from transitioning mesothelial cells but instead from local submesothelial fibroblasts [68]. These results challenge the current notion of EMT as a major source of the myofibroblast. However, the specificity of this marker prompts further investigation as it did not label all mesothelial cells.

EMT can be broken into two processes, one which includes epithelial cell transition and the second involves invasion of the transitioned cell. We hypothesize epithelial cell transition is occurring without invasion (In situ EMT). The mesothelial cells may be undergoing an in situ EMT involving cellular transition without invasion into the submesothelium. These transitioning cells are producing growth factors such as TGFB and VEGF contributing to both fibrosis and angiogenesis. Our work has shown evidence for MMP9 induced EMT in the peritoneal membrane. Aberrant expression of MMP9 has also been observed to induce EMT of renal tubular epithelial cells and in studies of cancer [87,91]. In these reports, MMP2 and 9 were also shown to degrade the basement membrane to facilitate cellular invasion [113,124].

Using adenoviral delivery of TGFB, we have also developed an in vivo model of EMT and observed that TGFB induces a full EMT phenotype. In a follow-up study, we constructed an adenovirus expressing PDGFB. PDGF plays a multitude of roles in a number of different biological contexts. PDGF can induce cellular proliferation and fibrosis but is also involved in modulating blood vessel growth. We have found PDGF to induce a non-invasive phenotype of EMT which is comprised of cellular transition without invasion. This non-invasive EMT phenotype was associated with a lack of MMP2 and 9 activities and therefore we suggested that they are involved in facilitating invasion of transitioned cells.

WNT1 has been observed to be upregulated in progressive fibrosis of the lung, heart and skin. In adriamycin nephropathy, WNT1 gene expression

exacerbated podocyte injury and proteinuria [150]. During TGFB induced peritoneal membrane injury, we observed activation of WNT/b-catenin signaling denoted by increased expression of WNT related genes and proteins. Furthermore, our results suggest that WNT/b-catenin signaling pathway is interacting with the TGFB pathway to induce injury. Only one other study has looked at WNT/b-catenin and its function in peritoneal fibrosis [158]. This study uses a dialysate induced model of peritoneal fibrosis and detected increased levels of b-catenin, GSK3B and SNAIL1 expression in response to injury. Similar to our study, they used ICG-001 to inhibit WNT/b-catenin activation and this reversed the levels of pGSK3B, b-catenin and SNAIL1. In addition, levels of E-cadherin were rescued suggesting WNT/b-catenin induces EMT during injury [158]. ICG-001 has been used in other studies of fibrosis and has inhibited renal interstitial fibrosis after UUO [156], bleomycin induced lung fibrosis [154] and dermal fibrosis [225]. We also used DKK-1 to inhibit WNT/b-catenin signaling which acts by binding to co-receptor LRP6. EMT was also significantly reduced in response to DKK-1 verifying that TGFB is interacting with WNT/b-catenin signaling to induce injury. ICG-001 and DKK-1 only provide a knock down approach to blocking WNT/b-catenin signaling. A tissue specific knockout mouse would be a more suitable model that could be used to validate our results.

**WNT5A Protects Against Peritoneal Membrane Injury**

WNT5A has the ability to activate both the b-catenin dependent pathway as well as two b-catenin independent pathways including the PCP and the

WNT/calcium pathway. WNT5A has been documented to exhibit differential effects depending on receptor context. WNT5A has the ability to signal through FZD 3, 4 and 5 and also through Ror2. Studies of WNT5A in angiogenesis and fibrosis have shown conflicting results and this may be attributed to receptor context. WNT5A can stimulate or inhibit angiogenesis during embryogenesis [226] and cancer development [227]. Recent studies have come to profile the gene expression of WNT5A in fibrosis and have found it to be upregulated in the liver and the lung [188]. Furthermore, functional studies have reported deleterious effects for WNT5A in fibrosis and inflammatory diseases, almost always in the presence of FZD receptors [189]. Inconsistent reports from studies of malignancy have also shown a dual role for WNT5A capable of inducing and attenuating EMT [228,229]. Our work shows that WNT5A inhibits peritoneal fibrosis and angiogenesis and therefore suggests a more protective effect. Moreover, we saw that WNT5A attenuated EMT in mesothelial cells in TGFB induced injury. The conflicting data from existing studies led us to hypothesize that WNT5A signaling is dependent on the cell surface receptor that is present and it may exhibit differential effects as a result of this. In the studies that examined receptor environment, WNT5A usually had adverse effects in the presence of FZD receptors [228,230,231]. Future studies should focus on examining receptor environment to validate this hypothesis.

Ror2 may be a potential candidate by which WNT5A mediates protective effects. WNT5A directly interacts with Ror2 to activate the PCP and calcium

pathway inducing polarized cell migration in developmental processes as well as during wound healing. Ror2 has been shown to induce formation of lamelliopodia and reorganize the MTOC for cellular migration and is potentially protective during the wound healing response [183]. WNT5A-Ror2 signaling may become dysregulated during this process resulting in abnormal wound healing and fibrosis. Interestingly, WNT5A-Ror2 signaling has been observed to attenuate the b-catenin dependent WNT signaling pathway. In the presence of FZD/LRP5/6, WNT5A activates b-catenin signaling. In the presence of Ror2, WNT5A has been observed inhibit b-catenin signaling [142]. In our work, we observed a reduction in b-catenin activity in response to WNT5A during peritoneal membrane injury. However, the mechanism of signaling of WNT5A in the presence of FZD or Ror2 must be clarified. Our work sheds some light on the effects of Ror2 in TGFB induced injury. We overexpressed TGFB and blocked Ror2 using an siRNA in mesothelial cells. We observed an exaggerated increase in levels of VEGF and fibronectin when Ror2 was silenced. Therefore, this suggests that Ror2 protects against angiogenesis and fibrosis. Other canonical WNT ligands also have the ability to physically and functionally interact with Ror2 including WNT3A and therefore it is important to examine the surrounding biological environment [193,232]. These studies suggest that WNT5A may act in separate pathways to provide different outcomes on angiogenesis and fibrosis.

Our work has demonstrated that non-canonical and canonical signaling potentially have discrete effects. The non-canonical pathway inhibited both

fibrosis and angiogenesis but the canonical pathway did not affect fibrosis. This finding is difficult to reconcile but may be attributed to the interaction of WNT5A with other surrounding signaling pathways. We observed WNT5A to inhibit PGSK3B supporting its ability to block WNT/b-catenin signaling. However, we propose WNT5A may go beyond this effect by interacting with other key players such as TGFB. Furthermore, the effect of WNT5A becomes more complex based on receptor environment and we have demonstrated the importance of Ror2 in regulating both fibronectin and VEGF. The linkage between WNT5A and TGFB has been studied in carcinogenesis and recently fibrosis. In studies of malignancy, TGFB has been shown to directly induce WNT5A and act as a tumor suppressor [233,234]. In human aortic smooth muscle cells from asthmatic patients, TGFB induced upregulation of WNT5A [190]. In lung fibroblasts, co-regulation of WNT5A by WNT7B and TGFB was also observed. These studies suggest a more pro-fibrotic role for WNT5A, lacking crucial evidence on the surrounding receptor environment. [187]. Therefore, greater exploration is required to examine the interaction between WNT5A and Ror2 in combination with TGFB to shed light the mechanism behind blocking both fibrosis and angiogenesis in the peritoneum. This would be imperative to understanding the different effects seen by canonical and non-canonical WNT signaling.

**Limitations and Future Directions**

In patients on peritoneal dialysis, vascular remodeling occurs in response to bioincompatible dialysis fluids and infection. This research will improve our understanding of peritoneal injury in patients on peritoneal dialysis and potentially predict outcome of a patient on PD. Our work has shed some light on the relative contribution of MMP9 and WNT signaling in angiogenesis of the peritoneal membrane. This is important because angiogenesis is associated with an increase in solute transport which can lead to failure of ultrafiltration and poorer clinical outcomes. Therefore, identifying the underlying mechanisms contributing to angiogenesis will improve the long term utility of this therapy and also make it safer for patients.

Our work will also help in identifying useful biomarkers to predict the transport status and filtration capacity of the peritoneal membrane for each patient. The direct correlation of MMP9 and WNT1 with peritoneal membrane and solute transport is an important finding in peritoneal dialysis research because these proteins can potentially be used as early markers of peritoneal membrane injury. This could shape treatment options and dialysis regimens for patients with chronic kidney disease creating a more personalized level of care. Another option is directly targeting MMP9 and potential MMP inhibitors do exist [235], however this avenue presents the concern of specificity and issues with disrupting normal physiological processes [224]. With targeting MMPs, we also have to consider substrate specificity, regulation by other MMPs and inter-MMP compensation

[205,224]. WNT signaling based therapeutics have already been moving into clinical trials in cancer and osteoporosis, however there are no current treatments on the market that reverse fibrosis. This is because the WNT signaling pathway has not been clearly defined in fibrosis. This includes clarifying information on the interaction of individual protein interfaces as well as ligand-receptor complexes. The efficacy of ICG-001 has been demonstrated in studies of malignacy and the use the clinical equivalent, PRI-724 is under current clinical trials of colon and pancreatic cancer as well as myeloid malignancies [236]. We demonstrated that ICG-001 successfully blocked TGFB induced angiogenesis and therefore may offer a new therapeutic approach in the future.

It is conceivable that other vascular pathologies may stem from abnormal MMP9 and WNT/b-catenin signaling. On a broader scale, this research will not only impact the basic understanding of peritoneal biology, but also biomedical research and medicine. This will lead to the discovery of several novel chemical inhibitors of both MMPs and WNT/b-catenin signaling to protect peritoneal membrane injury and diseases of similar pathogenesis.

**References**

1 Perl J, Bargman JM: Peritoneal dialysis: from bench to bedside and bedside to bench. Am J Physiol Renal Physiol 2016;311:F999–F1004.

2 Juergensen E, Wuerth D, Finkelstein SH, Juergensen PH, Bekui A, Finkelstein FO: Hemodialysis and peritoneal dialysis: patients' assessment of their satisfaction with therapy and the impact of the therapy on their lives. Clin J Am Soc Nephrol 2006;1:1191–1196.

3 Rabindranath KS, Adams J, Ali TZ, Daly C, Vale L, Macleod AM: Automated vs continuous ambulatory peritoneal dialysis: a systematic review of randomized controlled trials. Nephrol Dial Transplant 2007;22:2991–2998.

4 Nagy JA, Jackman RW: Anatomy and Physiology of the Peritoneal Membrane. Semin Dial 1998;11:49–56.

5 Mutsaers SE: The mesothelial cell. Int J Biochem Cell Biol 2004;36:9–16.

6 Williams JD, Craig KJ, Topley N, Von Ruhland C, Fallon M, Newman GR, et al.: Morphologic changes in the peritoneal membrane of patients with renal disease. J Am Soc Nephrol 2002;13:470–479.

7 Flessner MF, Credit K, Richardson K, Potter R, Li X, He Z, et al.: Peritoneal inflammation after twenty-week exposure to dialysis solution: effect of solution versus catheter-foreign body reaction. Perit Dial Int 2010;30:284–293.

8 Aroeira LS, Aguilera A, Sanchez-Tomero JA, Bajo MA, del Peso G, Jimenez-Heffernan JA, et al.: Epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions. J Am Soc Nephrol 2007;18:2004–2013.

9 Mateijsen MA, van der Wal AC, Hendriks PM, Zweers MM, Mulder J, Struijk DG, et al.: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. Perit Dial Int 1999;19:517–525.

10 Davies SJ, Phillips L, Naish PF, Russell GI: Peritoneal glucose exposure and changes in membrane solute transport with time on peritoneal dialysis. J Am Soc Nephrol 2001;12:1046–1051.

11 Cho Y, Johnson DW, Craig JC, Strippoli GFM, Badve S V, Wiggins KJ: Biocompatible dialysis fluids for peritoneal dialysis. Cochrane database Syst Rev 2014;CD007554.

12 Margetts PJ, Brimble KS: Peritoneal dialysis, membranes and beyond. Curr Opin Nephrol Hypertens 2006;15:571–576.

13 Margetts PJ, Bonniaud P: Basic mechanisms and clinical implications of peritoneal fibrosis. Perit Dial Int 2003;23:530–541.

14 Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994;331:1286–1292.

15 Honda K, Nitta K, Horita S, Yumura W, Nihei H, Nagai R, et al.: Accumulation of advanced glycation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultra-filtration. Nephrol Dial Transplant 1999;14:1541–1549.

16 Nakamoto M: Pathogenesis of peritoneal fibrosis and peritoneal small vessel changes. Perit Dial Int 1996;16 Suppl 1:S39-41.

17 Combet S, Miyata T, Moulin P, Pouthier D, Goffin E, Devuyst O: Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long-term peritoneal dialysis. J Am Soc Nephrol 2000;11:717–728.

18 Krediet RT: Evaluation of peritoneal membrane integrity. J Nephrol 1997;10:238–244.

19 Struijk DG, Krediet RT, Koomen GC, Boeschoten EW, Hoek FJ, Arisz L: A prospective study of peritoneal transport in CAPD patients. Kidney Int 1994;45:1739–1744.

20 van Hinsbergh VWM, Koolwijk P: Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc Res 2008;78:203–212.

21 Shibuya M: Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes Cancer 2011;2:1097–1105.

22 Hippenstiel S, Krull M, Ikemann A, Risau W, Clauss M, Suttorp N: VEGF induces hyperpermeability by a direct action on endothelial cells. Am J Physiol 1998;274:L678-84.

23 Zweers MM, Douma CE, de Waart DR, van der Wardt AB, Ho-dac-Pannekeet MM, Krediet RT, et al.: The standard peritoneal permeability analysis in the rabbit: a longitudinal model for peritoneal dialysis. Perit Dial Int 1999;19:56–64.

24 Twardowski ZJ: Clinical value of standardized equilibration tests in CAPD

patients. Blood Purif 1989;7:95–108.

25 Pannekeet MM, Imholz AL, Struijk DG, Koomen GC, Langedijk MJ, Schouten N, et al.: The standard peritoneal permeability analysis: a tool for the assessment of peritoneal permeability characteristics in CAPD patients. Kidney Int 1995;48:866–875.

26 Davies SJ: Longitudinal relationship between solute transport and ultrafiltration capacity in peritoneal dialysis patients. Kidney Int 2004;66:2437–2445.

27 Brimble KS, Walker M, Margetts PJ, Kundhal KK, Rabbat CG: Meta-analysis: peritoneal membrane transport, mortality, and technique failure in peritoneal dialysis. J Am Soc Nephrol 2006;17:2591–2598.

28 Davies SJ: Peritoneal solute transport--we know it is important, but what is it? Nephrol Dial Transplant 2000;15:1120–1123.

29 Cueto-Manzano AM: Rapid solute transport in the peritoneum: physiologic and clinical consequences. Perit Dial Int 2009;29 Suppl 2:S90-5.

30 Demidova-Rice TN, Hamblin MR, Herman IM: Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: normal and chronic wounds: biology, causes, and approaches to care. Adv Skin Wound Care 2012;25:304–314.

31 Broughton G 2nd, Janis JE, Attinger CE: The basic science of wound healing. Plast Reconstr Surg 2006;117:12S–34S.

32 Sinno H, Prakash S: Complements and the wound healing cascade: an updated review. Plast Surg Int 2013;2013:146764.

33 Diegelmann RF, Evans MC: Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 2004;9:283–289.

34 Xue M, Jackson CJ: Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring. Adv wound care 2015;4:119–136.

35 Wynn TA, Ramalingam TR: Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med 2012;18:1028–1040.

36 Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3:349–363.

37 Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat M-L, Gabbiani G: The myofibroblast: one function, multiple origins. Am J Pathol 2007;170:1807–1816.

38 Hinz B: Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 2007;127:526–537.

39 Gabbiani G: The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 2003;200:500–503.

40 Plum J, Hermann S, Fussholler A, Schoenicke G, Donner A, Rohrborn A, et al.: Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. Kidney Int Suppl 2001;78:S42-7.

41 Margetts PJ, Bonniaud P, Liu L, Hoff CM, Holmes CJ, West-Mays JA, et al.: Transient overexpression of TGF-{beta}1 induces epithelial mesenchymal transition in the rodent peritoneum. J Am Soc Nephrol 2005;16:425–436.

42 Humphreys BD, Lin S-L, Kobayashi A, Hudson TE, Nowlin BT, Bonventre J V, et al.: Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol 2010;176:85–97.

43 Loureiro J, Aguilera A, Selgas R, Sandoval P, Albar-Vizcaino P, Perez-Lozano ML, et al.: Blocking TGF-beta1 protects the peritoneal membrane from dialysate-induced damage. J Am Soc Nephrol 2011;22:1682–1695.

44 Piera-Velazquez S, Li Z, Jimenez SA: Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am J Pathol 2011;179:1074–1080.

45 Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003;112:1776–1784.

46 Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, et al.: Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A 1986;83:4167–4171.

47 Meng X-M, Nikolic-Paterson DJ, Lan HY: TGF-beta: the master regulator of fibrosis. Nat Rev Nephrol 2016;12:325–338.

48 Wynn TA: Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest 2007;117:524–529.

49 Wang T, Ghen YG, Ye RG, Mai WY, Zhen ZH, Li HQ: Enhanced expression of TGF-beta 1 by peritoneal macrophages in CAPD patients. Adv Perit Dial 1995;11:11–14.

50 Mlambo NC, Hylander B, Brauner A: Increased levels of transforming growth factor beta 1 and basic fibroblast growth factor in patients on CAPD: a study during non-infected steady state and peritonitis. Inflammation 1999;23:131–139.

51 Wang T, Chen YG, Ye RG, Li SL, Li HQ, Chen YX: Effect of glucose on TGF-beta 1 expression in peritoneal mesothelial cells. Adv Perit Dial 1995;11:7–10.

52 Medcalf JF, Walls J, Pawluczyk IZ, Harris KP: Effects of glucose dialysate on extracellular matrix production by human peritoneal mesothelial cells (HPMC): the role of TGF-beta. Nephrol Dial Transplant 2001;16:1885–1892.

53 Lai KN, Lai KB, Szeto CC, Lam CW, Leung JC: Growth factors in continuous ambulatory peritoneal dialysis effluent. Their relation with peritoneal transport of small solutes. Am J Nephrol 1999;19:416–422.

54 Gangji AS, Brimble KS, Margetts PJ: Association between markers of inflammation, fibrosis and hypervolemia in peritoneal dialysis patients. Blood Purif 2009;28:354–358.

55 Margetts PJ, Kolb M, Galt T, Hoff CM, Shockley TR, Gauldie J: Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. J Am Soc Nephrol 2001;12:2029–2039.

56 Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, et al.: Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 1994;269:6271–6274.

57 Thiery JP: Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15:740–746.

58 Yanez-Mo M, Lara-Pezzi E, Selgas R, Ramirez-Huesca M, Dominguez-Jimenez C, Jimenez-Heffernan JA, et al.: Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 2003;348:403–413.

59 Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al.: The transcription factor snail is a repressor of E-cadherin gene expression

in epithelial tumour cells. Nat Cell Biol 2000;2:84–89.

60 Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al.: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000;2:76–83.

61 Carver EA, Jiang R, Lan Y, Oram KF, Gridley T: The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol Cell Biol 2001;21:8184–8188.

62 Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420–1428.

63 Dobbie JW: Pathogenesis of peritoneal fibrosing syndromes (sclerosing peritonitis) in peritoneal dialysis. Perit Dial Int 1992;12:14–27.

64 Jimenez-Heffernan JA, Aguilera A, Aroeira LS, Lara-Pezzi E, Bajo MA, del Peso G, et al.: Immunohistochemical characterization of fibroblast subpopulations in normal peritoneal tissue and in peritoneal dialysis-induced fibrosis. Virchows Arch 2004;444:247–256.

65 Aroeira LS, Loureiro J, Gonzalez-Mateo GT, Fernandez-Millara V, del Peso G, Sanchez-Tomero JA, et al.: Characterization of epithelial-to-mesenchymal transition of mesothelial cells in a mouse model of chronic peritoneal exposure to high glucose dialysate. Perit Dial Int 2008;28 Suppl 5:S29-33.

66 Aroeira LS, Aguilera A, Selgas R, Ramirez-Huesca M, Perez-Lozano ML, Cirugeda A, et al.: Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. Am J Kidney Dis 2005;46:938–948.

67 Del Peso G, Jimenez-Heffernan JA, Bajo MA, Aroeira LS, Aguilera A, Fernandez-Perpen A, et al.: Epithelial-to-mesenchymal transition of mesothelial cells is an early event during peritoneal dialysis and is associated with high peritoneal transport. Kidney Int Suppl 2008;S26-33.

68 Chen Y-T, Chang Y-T, Pan S-Y, Chou Y-H, Chang F-C, Yeh P-Y, et al.: Lineage tracing reveals distinctive fates for mesothelial cells and submesothelial fibroblasts during peritoneal injury. J Am Soc Nephrol 2014;25:2847–2858.

69 Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence

that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002;110:341–350.

70 Patel P, West-Mays J, Kolb M, Rodrigues J-C, Hoff CM, Margetts PJ: Platelet derived growth factor B and epithelial mesenchymal transition of peritoneal mesothelial cells. Matrix Biol 2010;29:97–106.

71 Devuyst O, Margetts PJ, Topley N: The pathophysiology of the peritoneal membrane. J Am Soc Nephrol 2010;21:1077–1085.

72 McCawley LJ, Matrisian LM: Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today 2000;6:149–156.

73 Klein T, Bischoff R: Physiology and pathophysiology of matrix metalloproteases. Amino Acids 2011;41:271–290.

74 Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al.: MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998;93:411–422.

75 Mohan R, Chintala SK, Jung JC, Villar WVL, McCabe F, Russo LA, et al.: Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. J Biol Chem 2002;277:2065–2072.

76 Kobayashi T, Kim H, Liu X, Sugiura H, Kohyama T, Fang Q, et al.: Matrix metalloproteinase-9 activates TGF-beta and stimulates fibroblast contraction of collagen gels. Am J Physiol Lung Cell Mol Physiol 2014;306:L1006-15.

77 Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, et al.: Gelatinase B functions as regulator and effector in leukocyte biology. J Leukoc Biol 2001;69:851–859.

78 Zhao X, Sun T, Che N, Sun D, Zhao N, Dong X, et al.: Promotion of hepatocellular carcinoma metastasis through matrix metalloproteinase activation by epithelial-mesenchymal transition regulator Twist1. J Cell Mol Med 2011;15:691–700.

79 Schmalfeldt B, Prechtel D, Harting K, Spathe K, Rutke S, Konik E, et al.: Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. Clin Cancer Res 2001;7:2396–2404.

80 Hanemaaijer R, Verheijen JH, Maguire TM, Visser H, Toet K, McDermott

E, et al.: Increased gelatinase-A and gelatinase-B activities in malignant vs. benign breast tumors. Int J cancer 2000;86:204–207.

81 Sheen-Chen SM, Chen HS, Eng HL, Sheen CC, Chen WJ: Serum levels of matrix metalloproteinase 2 in patients with breast cancer. Cancer Lett 2001;173:79–82.

82 Mehner C, Hockla A, Miller E, Ran S, Radisky DC, Radisky ES: Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 2014;5:2736–2749.

83 Lakka SS, Rajan M, Gondi C, Yanamandra N, Chandrasekar N, Jasti SL, et al.: Adenovirus-mediated expression of antisense MMP-9 in glioma cells inhibits tumor growth and invasion. Oncogene 2002;21:8011–8019.

84 Joseph MJ, Dangi-Garimella S, Shields MA, Diamond ME, Sun L, Koblinski JE, et al.: Slug is a downstream mediator of transforming growth factor-beta1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells. J Cell Biochem 2009;108:726–736.

85 Cheng S, Pollock AS, Mahimkar R, Olson JL, Lovett DH: Matrix metalloproteinase 2 and basement membrane integrity: a unifying mechanism for progressive renal injury. FASEB J Off Publ Fed Am Soc Exp Biol 2006;20:1898–1900.

86 Cheng S, Lovett DH: Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation. Am J Pathol 2003;162:1937–1949.

87 Orlichenko LS, Radisky DC: Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development. Clin Exp Metastasis 2008;25:593–600.

88 Ho JE, Liu C, Lyass A, Courchesne P, Pencina MJ, Vasan RS, et al.: Galectin-3, a marker of cardiac fibrosis, predicts incident heart failure in the community. J Am Coll Cardiol 2012;60:1249–1256.

89 Nathu Z, Dwivedi DJ, Reddan JR, Sheardown H, Margetts PJ, West-Mays JA: Temporal changes in MMP mRNA expression in the lens epithelium during anterior subcapsular cataract formation. Exp Eye Res 2009;88:323–330.

90 Dancer RCA, Wood AM, Thickett DR: Metalloproteinases in idiopathic pulmonary fibrosis. Eur Respir J 2011;38:1461–1467.

91 Tan TK, Zheng G, Hsu T-T, Lee SR, Zhang J, Zhao Y, et al.: Matrix metalloproteinase-9 of tubular and macrophage origin contributes to the pathogenesis of renal fibrosis via macrophage recruitment through osteopontin cleavage. Lab Invest 2013;93:434–449.

92 Lenz O, Elliot SJ, Stetler-Stevenson WG: Matrix metalloproteinases in renal development and disease. J Am Soc Nephrol 2000;11:574–581.

93 Yang J, Shultz RW, Mars WM, Wegner RE, Li Y, Dai C, et al.: Disruption of tissue-type plasminogen activator gene in mice reduces renal interstitial fibrosis in obstructive nephropathy. J Clin Invest 2002;110:1525–1538.

94 Wang X, Zhou Y, Tan R, Xiong M, He W, Fang L, et al.: Mice lacking the matrix metalloproteinase-9 gene reduce renal interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol 2010;299:F973-82.

95 Tan TK, Zheng G, Hsu T-T, Wang Y, Lee VWS, Tian X, et al.: Macrophage matrix metalloproteinase-9 mediates epithelial-mesenchymal transition in vitro in murine renal tubular cells. Am J Pathol 2010;176:1256–1270.

96 Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, et al.: Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. Kidney Int 2002;62:137–146.

97 Hirahara I, Inoue M, Okuda K, Ando Y, Muto S, Kusano E: The potential of matrix metalloproteinase-2 as a marker of peritoneal injury, increased solute transport, or progression to encapsulating peritoneal sclerosis during peritoneal dialysis--a multicentre study in Japan. Nephrol Dial Transplant 2007;22:560–567.

98 Lopes Barreto D, Struijk DG, Krediet RT: Peritoneal Effluent MMP-2 and PAI-1 in Encapsulating Peritoneal Sclerosis. Am J Kidney Dis 2014;65:748–753.

99 Cho Y, Johnson DW, Vesey DA, Hawley CM, Pascoe EM, Clarke M, et al.: Higher Dialysate Matrix Metalloproteinase-2 Levels Are Associated With Peritoneal Membrane Dysfunction. Perit Dial Int 2014;36:16–25.

100 Hirahara I, Ogawa Y, Kusano E, Asano Y: Activation of matrix metalloproteinase-2 causes peritoneal injury during peritoneal dialysis in rats. Nephrol Dial Transplant 2004;19:1732–1741.

101 Ingber DE, Folkman J: How does extracellular matrix control capillary morphogenesis? Cell 1989;58:803–805.

102 Bendeck MP: Macrophage matrix metalloproteinase-9 regulates angiogenesis in ischemic muscle. Circ Res 2004;94:138–139.

103 Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al.: Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993;4:197–250.

104 Heissig B, Rafii S, Akiyama H, Ohki Y, Sato Y, Rafael T, et al.: Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. J Exp Med 2005;202:739–750.

105 Mira E, Lacalle RA, Buesa JM, de Buitrago GG, Jimenez-Baranda S, Gomez-Mouton C, et al.: Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface. J Cell Sci 2004;117:1847–1857.

106 Johnson C, Sung H-J, Lessner SM, Fini ME, Galis ZS: Matrix metalloproteinase-9 is required for adequate angiogenic revascularization of ischemic tissues: potential role in capillary branching. Circ Res 2004;94:262–268.

107 Nakamura T, Kuwai T, Kim J-S, Fan D, Kim S-J, Fidler IJ: Stromal metalloproteinase-9 is essential to angiogenesis and progressive growth of orthotopic human pancreatic cancer in parabiont nude mice. Neoplasia 2007;9:979–986.

108 Bauvois B: New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. Biochim Biophys Acta 2012;1825:29–36.

109 Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al.: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737–744.

110 Belotti D, Paganoni P, Manenti L, Garofalo A, Marchini S, Taraboletti G, et al.: Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. Cancer Res 2003;63:5224–5229.

111 Lee S, Jilani SM, Nikolova G V, Carpizo D, Iruela-Arispe ML: Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 2005;169:681–691.

112 Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, et al.:

Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002;109:625–637.

113 Symowicz J, Adley BP, Gleason KJ, Johnson JJ, Ghosh S, Fishman DA, et al.: Engagement of collagen-binding integrins promotes matrix metalloproteinase-9-dependent E-cadherin ectodomain shedding in ovarian carcinoma cells. Cancer Res 2007;67:2030–2039.

114 Kemler R: From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet 1993;9:317–321.

115 Gumbiner BM: Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 1996;84:345–357.

116 Takeichi M: Cadherin cell adhesion receptors as a morphogenetic regulator. Science 1991;251:1451–1455.

117 Charrasse S, Meriane M, Comunale F, Blangy A, Gauthier-Rouviere C: N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. J Cell Biol 2002;158:953–965.

118 David JM, Rajasekaran AK: Dishonorable discharge: the oncogenic roles of cleaved E-cadherin fragments. Cancer Res 2012;72:2917–2923.

119 Slaton JW, Karashima T, Perrotte P, Inoue K, Kim SJ, Izawa J, et al.: Treatment with low-dose interferon-alpha restores the balance between matrix metalloproteinase-9 and E-cadherin expression in human transitional cell carcinoma of the bladder. Clin Cancer Res 2001;7:2840–2853.

120 Gao H, Lan X, Li S, Xue Y: Relationships of MMP-9, E-cadherin, and VEGF expression with clinicopathological features and response to chemosensitivity in gastric cancer. Tumour Biol 2017;39:1010428317698368.

121 Akdeniz O, Akduman D, Haksever M, Ozkarakas H, Muezzinoglu B: Relationships between clinical behavior of laryngeal squamous cell carcinomas and expression of VEGF, MMP-9 and E-cadherin. Asian Pac J Cancer Prev 2013;14:5301–5310.

122 Cowden Dahl KD, Symowicz J, Ning Y, Gutierrez E, Fishman DA, Adley BP, et al.: Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. Cancer Res 2008;68:4606–4613.

123 Zuo J-H, Zhu W, Li M-Y, Li X-H, Yi H, Zeng G-Q, et al.: Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. J Cell Biochem 2011;112:2508–2517.

124 Zheng G, Lyons JG, Tan TK, Wang Y, Hsu T-T, Min D, et al.: Disruption of E-cadherin by matrix metalloproteinase directly mediates epithelial-mesenchymal transition downstream of transforming growth factor-beta1 in renal tubular epithelial cells. Am J Pathol 2009;175:580–591.

125 Dwivedi DJ, Pino G, Banh A, Nathu Z, Howchin D, Margetts P, et al.: Matrix metalloproteinase inhibitors suppress transforming growth factor-beta-induced subcapsular cataract formation. Am J Pathol 2006;168:69–79.

126 Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, et al.: E-cadherin/beta-catenin complex and the epithelial barrier. J Biomed Biotechnol 2011;2011:567305.

127 Tian Y-C, Fraser D, Attisano L, Phillips AO: TGF-beta1-mediated alterations of renal proximal tubular epithelial cell phenotype. Am J Physiol Renal Physiol 2003;285:F130-42.

128 Masszi A, Di Ciano C, Sirokmany G, Arthur WT, Rotstein OD, Wang J, et al.: Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol 2003;284:F911-24.

129 Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA: Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. J Am Soc Nephrol 2004;15:390–397.

130 Bedi S, Vidyasagar A, Djamali A: Epithelial-to-mesenchymal transition and chronic allograft tubulointerstitial fibrosis. Transplant Rev (Orlando) 2008;22:1–5.

131 Thannickal VJ, Toews GB, White ES, Lynch JP 3rd, Martinez FJ: Mechanisms of pulmonary fibrosis. Annu Rev Med 2004;55:395–417.

132 Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, et al.: Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 2005;166:1321–1332.

133 Rygiel KA, Robertson H, Marshall HL, Pekalski M, Zhao L, Booth TA, et al.: Epithelial-mesenchymal transition contributes to portal tract

fibrogenesis during human chronic liver disease. Lab Invest 2008;88:112–123.

134 Orsulic S, Huber O, Aberle H, Arnold S, Kemler R: E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. J Cell Sci 1999;112 ( Pt 8:1237–1245.

135 Masszi A, Fan L, Rosivall L, McCulloch CA, Rotstein OD, Mucsi I, et al.: Integrity of cell-cell contacts is a critical regulator of TGF-beta 1-induced epithelial-to-myofibroblast transition: role for beta-catenin. Am J Pathol 2004;165:1955–1967.

136 MacDonald BT, Tamai K, He X: Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009;17:9–26.

137 Widelitz R: Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors 2005;23:111–116.

138 van Amerongen R, Fuerer C, Mizutani M, Nusse R: Wnt5a can both activate and repress Wnt/beta-catenin signaling during mouse embryonic development. Dev Biol 2012;369:101–114.

139 van Amerongen R, Mikels A, Nusse R: Alternative wnt signaling is initiated by distinct receptors. Sci Signal 2008;1:re9.

140 He X, Semenov M, Tamai K, Zeng X: LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development 2004;131:1663–1677.

141 Bryja V, Andersson ER, Schambony A, Esner M, Bryjova L, Biris KK, et al.: The extracellular domain of Lrp5/6 inhibits noncanonical Wnt signaling in vivo. Mol Biol Cell 2009;20:924–936.

142 Mikels A, Minami Y, Nusse R: Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. J Biol Chem 2009;284:30167–30176.

143 Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, et al.: Activation of canonical Wnt signalling is required for TGF-beta-mediated fibrosis. Nat Commun 2012;3:735.

144 Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J: Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. J Cell Sci 2008;121:737–746.

145 Surendran K, McCaul SP, Simon TC: A role for Wnt-4 in renal fibrosis. Am J Physiol Renal Physiol 2002;282:F431-41.

146 Kaminski N, Rosas IO: Gene expression profiling as a window into idiopathic pulmonary fibrosis pathogenesis: can we identify the right target genes? Proc Am Thorac Soc 2006;3:339–344.

147 Selman M, Pardo A, Kaminski N: Idiopathic pulmonary fibrosis: aberrant recapitulation of developmental programs? PLoS Med 2008;5:e62.

148 Gardner H, Shearstone JR, Bandaru R, Crowell T, Lynes M, Trojanowska M, et al.: Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. Arthritis Rheum 2006;54:1961–1973.

149 He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y: Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 2009;20:765–776.

150 Wang D, Dai C, Li Y, Liu Y: Canonical Wnt/beta-catenin signaling mediates transforming growth factor-beta1-driven podocyte injury and proteinuria. Kidney Int 2011;80:1159–1169.

151 Beyer C, Schramm A, Akhmetshina A, Dees C, Kireva T, Gelse K, et al.: beta-catenin is a central mediator of pro-fibrotic Wnt signaling in systemic sclerosis. Ann Rheum Dis 2012;71:761–767.

152 Konigshoff M, Kramer M, Balsara N, Wilhelm J, Amarie OV, Jahn A, et al.: WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. J Clin Invest 2009;119:772–787.

153 Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, et al.: Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. Am J Pathol 2003;162:1495–1502.

154 Henderson WRJ, Chi EY, Ye X, Nguyen C, Tien Y, Zhou B, et al.: Inhibition of Wnt/beta-catenin/CREB binding protein (CBP) signaling reverses pulmonary fibrosis. Proc Natl Acad Sci U S A 2010;107:14309–14314.

155 Taiyab A, Korol A, Deschamps PA, West-Mays JA: beta-Catenin/CBP-Dependent Signaling Regulates TGF-beta-Induced Epithelial to Mesenchymal Transition of Lens Epithelial Cells. Invest Ophthalmol Vis Sci 2016;57:5736–5747.

156 Hao S, He W, Li Y, Ding H, Hou Y, Nie J, et al.: Targeted inhibition of beta-catenin/CBP signaling ameliorates renal interstitial fibrosis. J Am Soc Nephrol 2011;22:1642–1653.

157 Yamada S, Tsuruya K, Tokumoto M, Yoshida H, Ooboshi H, Kitazono T: Factors associated with serum soluble inhibitors of Wnt-beta-catenin signaling (sclerostin and dickkopf-1) in patients undergoing peritoneal dialysis. Nephrology (Carlton) 2015;20:639–645.

158 Ji S, Deng H, Jin W, Yan P, Wang R, Pang L, et al.: Beta-catenin participates in dialysate-induced peritoneal fibrosis via enhanced peritoneal cell epithelial-to-mesenchymal transition. FEBS Open Bio 2017;7:265–273.

159 Parmalee NL, Kitajewski J: Wnt signaling in angiogenesis. Curr Drug Targets 2008;9:558–564.

160 Easwaran V, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, et al.: beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res 2003;63:3145–3153.

161 Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, Hassan AB, et al.: Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A 2003;100:3299–3304.

162 Goodwin AM, Kitajewski J, D'Amore PA: Wnt1 and Wnt5a affect endothelial proliferation and capillary length; Wnt2 does not. Growth Factors 2007;25:25–32.

163 Wright M, Aikawa M, Szeto W, Papkoff J: Identification of a Wnt-responsive signal transduction pathway in primary endothelial cells. Biochem Biophys Res Commun 1999;263:384–388.

164 Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM: Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. Am J Physiol Heart Circ Physiol 2003;285:H1917-38.

165 Masckauchan TNH, Agalliu D, Vorontchikhina M, Ahn A, Parmalee NL, Li C-M, et al.: Wnt5a signaling induces proliferation and survival of endothelial cells in vitro and expression of MMP-1 and Tie-2. Mol Biol Cell 2006;17:5163–5172.

166 Goodwin AM, D'Amore PA: Wnt signaling in the vasculature. Angiogenesis 2002;5:1–9.

167 Yang D-H, Yoon J-Y, Lee S-H, Bryja V, Andersson ER, Arenas E, et al.: Wnt5a is required for endothelial differentiation of embryonic stem cells and vascularization via pathways involving both Wnt/beta-catenin and

protein kinase Calpha. Circ Res 2009;104:372–379.

168 Zerlin M, Julius MA, Kitajewski J: Wnt/Frizzled signaling in angiogenesis. Angiogenesis 2008;11:63–69.

169 Zhao S, Ma W, Zhang M, Tang D, Shi Q, Xu S, et al.: High expression of CD147 and MMP-9 is correlated with poor prognosis of triple-negative breast cancer (TNBC) patients. Med Oncol 2013;30:335.

170 Sun Q, Guo S, Wang C-C, Sun X, Wang D, Xu N, et al.: Cross-talk between TGF-beta/Smad pathway and Wnt/beta-catenin pathway in pathological scar formation. Int J Clin Exp Pathol 2015;8:7631–7639.

171 Zhou B, Liu Y, Kahn M, Ann DK, Han A, Wang H, et al.: Interactions between beta-catenin and transforming growth factor-beta signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). J Biol Chem 2012;287:7026–7038.

172 Sato M: Upregulation of the Wnt/beta-catenin pathway induced by transforming growth factor-beta in hypertrophic scars and keloids. Acta Derm Venereol 2006;86:300–307.

173 Nawshad A, Medici D, Liu C-C, Hay ED: TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex. J Cell Sci 2007;120:1646–1653.

174 Medici D, Hay ED, Olsen BR: Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. Mol Biol Cell 2008;19:4875–4887.

175 Gomez-Orte E, Saenz-Narciso B, Moreno S, Cabello J: Multiple functions of the noncanonical Wnt pathway. Trends Genet 2013;29:545–553.

176 Cheng JH, She H, Han Y-P, Wang J, Xiong S, Asahina K, et al.: Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2008;294:G39-49.

177 De A: Wnt/Ca2+ signaling pathway: a brief overview. Acta Biochim Biophys Sin (Shanghai) 2011;43:745–756.

178 Qian D, Jones C, Rzadzinska A, Mark S, Zhang X, Steel KP, et al.: Wnt5a functions in planar cell polarity regulation in mice. Dev Biol 2007;306:121–133.

179 Leris ACA, Roberts TR, Jiang WG, Newbold RF, Mokbel K: WNT5A expression in human breast cancer. Anticancer Res 2005;25:731–734.

180 Cheng R, Sun B, Liu Z, Zhao X, Qi L, Li Y, et al.: Wnt5a suppresses colon cancer by inhibiting cell proliferation and epithelial-mesenchymal transition. J Cell Physiol 2014;229:1908–1917.

181 Ying J, Li H, Yu J, Ng KM, Poon FF, Wong SCC, et al.: WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer. Clin Cancer Res 2008;14:55–61.

182 Kikuchi A, Yamamoto H: Tumor formation due to abnormalities in the beta-catenin-independent pathway of Wnt signaling. Cancer Sci 2008;99:202–208.

183 Nomachi A, Nishita M, Inaba D, Enomoto M, Hamasaki M, Minami Y: Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A. J Biol Chem 2008;283:27973–27981.

184 Laeremans H, Rensen SS, Ottenheijm HCJ, Smits JFM, Blankesteijn WM: Wnt/frizzled signalling modulates the migration and differentiation of immortalized cardiac fibroblasts. Cardiovasc Res 2010;87:514–523.

185 Levanen B, Wheelock AM, Eklund A, Grunewald J, Nord M: Increased pulmonary Wnt (wingless/integrated)-signaling in patients with sarcoidosis. Respir Med 2011;105:282–291.

186 Rashid ST, Humphries JD, Byron A, Dhar A, Askari JA, Selley JN, et al.: Proteomic analysis of extracellular matrix from the hepatic stellate cell line LX-2 identifies CYR61 and Wnt-5a as novel constituents of fibrotic liver. J Proteome Res 2012;11:4052–4064.

187 Newman DR, Sills WS, Hanrahan K, Ziegler A, Tidd KM, Cook E, et al.: Expression of WNT5A in Idiopathic Pulmonary Fibrosis and Its Control by TGF-beta and WNT7B in Human Lung Fibroblasts. J Histochem Cytochem 2016;64:99–111.

188 Villar J, Cabrera-Benitez NE, Ramos-Nuez A, Flores C, Garcia-Hernandez S, Valladares F, et al.: Early activation of pro-fibrotic WNT5A in sepsis-induced acute lung injury. Crit Care 2014;18:568.

189 Vuga LJ, Ben-Yehudah A, Kovkarova-Naumovski E, Oriss T, Gibson KF, Feghali-Bostwick C, et al.: WNT5A is a regulator of fibroblast

proliferation and resistance to apoptosis. Am J Respir Cell Mol Biol 2009;41:583–589.

190 Kumawat K, Menzen MH, Bos IST, Baarsma HA, Borger P, Roth M, et al.: Noncanonical WNT-5A signaling regulates TGF-beta-induced extracellular matrix production by airway smooth muscle cells. FASEB J Off Publ Fed Am Soc Exp Biol 2013;27:1631–1643.

191 Forrester WC: The Ror receptor tyrosine kinase family. Cell Mol Life Sci 2002;59:83–96.

192 Oldridge M, Fortuna AM, Maringa M, Propping P, Mansour S, Pollitt C, et al.: Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. Nat Genet 2000;24:275–278.

193 Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, et al.: The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells 2003;8:645–654.

194 Li X, Yamagata K, Nishita M, Endo M, Arfian N, Rikitake Y, et al.: Activation of Wnt5a-Ror2 signaling associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis. Genes Cells 2013;18:608–619.

195 Torres MA, Yang-Snyder JA, Purcell SM, DeMarais AA, McGrew LL, Moon RT: Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. J Cell Biol 1996;133:1123–1137.

196 Schambony A, Wedlich D: Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. Dev Cell 2007;12:779–792.

197 Mikels AJ, Nusse R: Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol 2006;4:e115.

198 Hassan K, Hassan D, Shturman A, Rubinchik I, Fadi H, Shadi H, et al.: The impact of sub-clinical over-hydration on left ventricular mass in peritoneal dialysis patients. Int J Clin Exp Med 2015;8:5890–5896.

199 Twardowski ZJ: PET--a simpler approach for determining prescriptions for adequate dialysis therapy. Adv Perit Dial 1990;6:186–191.

200 Vorburger SA, Hunt KK: Adenoviral gene therapy. Oncologist 2002;7:46–

59.

201 Tsaousi A, Mill C, George SJ: The Wnt pathways in vascular disease: lessons from vascular development. Curr Opin Lipidol 2011;22:350–357.

202 Deryugina EI, Quigley JP: Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature. Matrix Biol 2015;44–46:94–112.

203 Rundhaug JE: Matrix metalloproteinases and angiogenesis. J Cell Mol Med 2005;9:267–285.

204 Page-McCaw A, Ewald AJ, Werb Z: Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 2007;8:221–233.

205 Caley MP, Martins VLC, O'Toole EA: Metalloproteinases and Wound Healing. Adv wound care 2015;4:225–234.

206 Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, et al.: A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A 2004;101:12682–12687.

207 Tan RJ, Zhou D, Zhou L, Liu Y: Wnt/beta-catenin signaling and kidney fibrosis. Kidney Int Suppl 2014;4:84–90.

208 Wu B, Crampton SP, Hughes CCW: Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity 2007;26:227–239.

209 Qu B, Liu B-R, DU Y-J, Chen J, Cheng Y-Q, Xu W, et al.: Wnt/beta-catenin signaling pathway may regulate the expression of angiogenic growth factors in hepatocellular carcinoma. Oncol Lett 2014;7:1175–1178.

210 Guo X, Wang X-F: Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res 2009;19:71–88.

211 Nusse R, Clevers H: Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell 2017;169:985–999.

212 Corada M, Nyqvist D, Orsenigo F, Caprini A, Giampietro C, Taketo MM, et al.: The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. Dev Cell 2010;18:938–949.

213 Clevers H: Wnt/beta-catenin signaling in development and disease. Cell

2006;127:469–480.

214 Huang H, He X: Wnt/beta-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol 2008;20:119–125.

215 Delgado ER, Bahal R, Ly D, Monga SPS: The role of wnt/β-catenin signaling in regulating angiogenesis in hepatocellular carcinoma. FASEB J 2013;27:471.1-471.1.

216 Goodwin AM, Sullivan KM, D'Amore PA: Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. Dev Dyn 2006;235:3110–3120.

217 Zhang B, Abreu JG, Zhou K, Chen Y, Hu Y, Zhou T, et al.: Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in the serine proteinase inhibitor family. Proc Natl Acad Sci U S A 2010;107:6900–6905.

218 Takamiya Y, Fukami K, Yamagishi S, Kaida Y, Nakayama Y, Obara N, et al.: Experimental diabetic nephropathy is accelerated in matrix metalloproteinase-2 knockout mice. Nephrol Dial Transplant 2013;28:55–62.

219 Onozuka I, Kakinuma S, Kamiya A, Miyoshi M, Sakamoto N, Kiyohashi K, et al.: Cholestatic liver fibrosis and toxin-induced fibrosis are exacerbated in matrix metalloproteinase-2 deficient mice. Biochem Biophys Res Commun 2011;406:134–140.

220 Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, et al.: Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). J Exp Med 2001;194:809–821.

221 Korol A, Pino G, Dwivedi D, Robertson J V, Deschamps PA, West-Mays JA: Matrix metalloproteinase-9-null mice are resistant to TGF-beta-induced anterior subcapsular cataract formation. Am J Pathol 2014;184:2001–2012.

222 Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000;14:163–176.

223 Cabrera S, Gaxiola M, Arreola JL, Ramirez R, Jara P, D'Armiento J, et al.: Overexpression of MMP9 in macrophages attenuates pulmonary fibrosis induced by bleomycin. Int J Biochem Cell Biol 2007;39:2324–2338.

224 Giannandrea M, Parks WC: Diverse functions of matrix metalloproteinases during fibrosis. Dis Model Mech 2014;7:193–203.

225 Beyer C, Reichert H, Akan H, Mallano T, Schramm A, Dees C, et al.: Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. Ann Rheum Dis 2013;72:1255–1258.

226 Franco CA, Liebner S, Gerhardt H: Vascular morphogenesis: a Wnt for every vessel? Curr Opin Genet Dev 2009;19:476–483.

227 Ekstrom EJ, Bergenfelz C, von Bulow V, Serifler F, Carlemalm E, Jonsson G, et al.: WNT5A induces release of exosomes containing pro-angiogenic and immunosuppressive factors from malignant melanoma cells. Mol Cancer 2014;13:88.

228 Gujral TS, Chan M, Peshkin L, Sorger PK, Kirschner MW, MacBeath G: A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. Cell 2014;159:844–856.

229 Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, et al.: Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 2002;1:279–288.

230 Arderiu G, Espinosa S, Pena E, Aledo R, Badimon L: Monocyte-secreted Wnt5a interacts with FZD5 in microvascular endothelial cells and induces angiogenesis through tissue factor signaling. J Mol Cell Biol 2014;6:380–393.

231 Nishita M, Itsukushima S, Nomachi A, Endo M, Wang Z, Inaba D, et al.: Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating Dishevelled polymerization. Mol Cell Biol 2010;30:3610–3619.

232 Hikasa H, Shibata M, Hiratani I, Taira M: The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development 2002;129:5227–5239.

233 Serra R, Easter SL, Jiang W, Baxley SE: Wnt5a as an effector of TGFbeta in mammary development and cancer. J Mammary Gland Biol Neoplasia 2011;16:157–167.

234 Roarty K, Baxley SE, Crowley MR, Frost AR, Serra R: Loss of TGF-beta or Wnt5a results in an increase in Wnt/beta-catenin activity and redirects mammary tumour phenotype. Breast Cancer Res 2009;11:R19.

235 Cathcart J, Pulkoski-Gross A, Cao J: Targeting Matrix Metalloproteinases

in Cancer: Bringing New Life to Old Ideas. Genes Dis 2015;2:26–34.

236 Wiese M, Walther N, Diederichs C, Schill F, Monecke S, Salinas G, et al.: The beta-catenin/CBP-antagonist ICG-001 inhibits pediatric glioma tumorigenicity in a Wnt-independent manner. Oncotarget 2017;8:27300–27313.