Université de Montréal

Understanding the Role of the Matricellular Protein SMOC-2 in Renal Cell Carcinoma

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Thèse présenté en vue de l'obtention
du grade de M. Sc en Pharmacologie

Août, 2019

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée

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Résumé

Les proteins matricellulaires (MPs) sont des macromolécules non structurales de la matrice extracellulaire (ECM) qui sont induites de façon transitoire lors du développement, de la réparation et du remodelage tissulaire et lors de l'inflammation. L'expression des MPs peut être déclenchée par des dommages tissulaires aigus, et leur expression à long terme peut contribuer à certaines maladies chroniques. Les MPs agissent principalement pour médier les événements du remodelage tissulaire en facilitant les interactions et les signaux à partir de l'ECM vers l'environnement cellulaire avoisinant.

En utilisant des données de RNA-seq provenant de deux modèles distincts de dommages rénaux, soit l'Acide Folique (FA) ou l'Obstruction Urétérale Unilatérale (UUO), nous avons analysé les profils d'expressions de plusieurs familles bien connues de MPs lors des blessures aigues et chroniques. Nous révélons de nouvelles MPs impliquées dans les dommages rénaux et présentons de nouveaux réseaux entre les membres de chaque famille de MPs, en utilisant des outils bioinformatiques. L'expression de l'ARNm de certaines MPs a été confirmée par immuno-buvardage de type Western (WB).

Afin d'approfondir notre connaissance des mécanismes de réparation tissulaire et de remodelage de la matrice, nous avons choisi SMOC-2 comme MP modèle dans l'étude des carcinomes cellulaires rénaux (RCC), cancers qui présentent de fortes tendances métastatiques. Nous avons démontré que la surexpression de SMOC-2 ainsi que le traitement avec la protéine recombinante de lignées cellulaires RCC (786-O, et ACHN) induisent un profil métastatique de transition épithélio-mésenchymateuse (EMT) par WB et des tests fonctionnels. Nous avons également démontré que l'inhibition de SMOC-2 par siRNA donne les résultats opposés.

L'ensemble de nos travaux utilise la compréhension des patrons d'expressions temporels des MPs pour améliorer notre compréhension des mécanismes et conditions qui supportent une activation persistante dans des états pathologiques chroniques. Globalement, notre étude sur SMOC-2 offre une perspective ainsi qu'un modèle intéressant pour l'étude et la caractérisation de nouvelles MPs dans des maladies impliquant le remodelage et la réparation de la matrice.

Mot Clès: protéine matricellulaire, transition épithéliale-mésenchymateuse, acide folique, obstruction unilatérale de l'uretère, matrice extracellulaire, carcinome à cellules rénales, métastase du cancer.

Abstract

Matricellular proteins (MPs) are non-structural ECM macromolecules induced transiently during development, tissue repair and remodeling, and inflammation. Expression of MPs can be triggered by acute tissue injury and their sustained expression can contribute to chronic disease. MPs primarily act to mediate tissue remodeling events by facilitating interactions and signals from the ECM to the surrounding cellular niche.

Using published RNA-seq data from two distinct models of kidney injury, Folic Acid (FA) and Unilateral Ureteral Obstruction (UUO), we analyzed the expression profile of various members of well-known MP families during the acute and fibrotic injury phases. We reveal novel MPs implicated in renal injury and present informative networks between members of each MP family using bioinformatic tools. mRNA expression of select candidate MPs were confirmed by Western blot.

To extend our understanding of translatable mechanisms in repair and matrix remodeling, we chose SMOC-2 as our MP model to study in Renal Cell Carcinoma (RCC) which has strong metastatic tendencies. SMOC-2 overexpression and recombinant protein treatment of RCC cell lines (786-O, ACHN) were shown to induce a metastatic EMT profile by Western blot analysis, supported by functional assays (proliferation, migration). Silencing SMOC-2 by siRNA showed the contrary results.

Taken together, our work utilizes the understanding of temporal expression patterns of MPs to gain insight into mechanisms and conditions that support persistent activation in chronic injury states. Overall, our work with SMOC-2 provides a valuable perspective and template to approach studying and characterizing novel MPs in diseases involving pathological matrix remodeling and repair.

Key Words: matricellular protein, epithelial-to-mesenchymal transition, folic acid, unilateral ureteral obstruction, extracellular matrix, renal cell carcinoma, cancer metastasis.

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List of Abbreviations

AKI – acute kidney injury
α-SMA – α-alpha smooth muscle actin
BUN – blood urea nitrogen
CKD – chronic kidney diease
DPC – days post coitum
DE – differentially expressed
ECM – extracellular matrix
EGF – epidermal growth factor
EMT – epithelial-to-mesenchymal transition
FA – folic acid
FBS – fetal bovine serum
GEO – gene expression omnibus
GAG – glycosaminoglycan
GO – gene ontology
i.p – intraperitoneal injection
KUPKB – kidney and urinary pathway knowledge base
LOX – lysyl oxidase
MP – matricellular protein
MMP – matrix metalloprotease
PDGF – platelet derived growth factor
RCC – renal cell carcinoma
SPARC – secreted protein acidic and rich in cysteine
SIBLING – small integrin-binding ligand, N-linked glycoprotein
SLRP - Small leucine-rich proteoglycan
SMOC-2 – secreted modular calcium binding protein-2
STRING – search tool for the retrieval of interacting genes/proteins
THBS - thrombospondin
TN - tenascin
UUO – unilateral ureteral obstruction
VEGF – vascular endothelial growth factor

Acknowledgements

I would like to first extend my greatest gratitude to both my professors, Dr. Vincent Pichette and Dr. Casimiro Gerarduzzi. Thank you Dr. Pichette for taking me on as a student and giving me the opportunity to pursue scientific research and opening your lab space to us.

Thank you to Dr. Gerarduzzi for taking me on as his first student and being my friend, mentor and fellow Raptors fan throughout this journey. Thank you for all the opportunities and challenges you gave me and for always pushing me to see my potential.

Thank you to Nathalie Henley, the super research assistant. Thank you especially for helping me navigate the complexities and intricacies of HMR. I am most grateful for all the guidance and support you provided and always having answers to my constant "Hey Nathalie….". Thank you for teaching me to appreciate the little details in experiments.

Thank to you to all my lab mates over the course of time. Each of you brought your own perspectives and personalities, and it was great to have more company in the lab.

And lastly, thank you to the research community and staff of HMR. I am sorry I cannot list everyone but it has been a pleasure meeting and working with everyone. I hope our paths cross again someday.

1.0 Introduction

The idea of matricellular proteins is credited to the group of Paul Bornstein and their 1991 manuscript titled "Extracellular Proteins That Modulate Cell-Matrix Interactions"[1]. The proteins described by Bornstein have come to be known today as matricellular proteins (MPs) and have been appreciated and recognized as important contributors to the plethora of ways cells communicate and regulate the surrounding extracellular environment. The term 'matricellular' is used to describe a particular group of extracellular matrix (ECM) proteins that do not contribute directly to the formation of structural elements, but instead serve to modulate cell–matrix interactions and cell function[2]. MPs work in an orchestrated manner with major structural proteins (ie. collagens, fibronectin, proteoglycans) to provide the heterogenous ECM scaffold for cellular functions. The primary cell functions governed by MPs include migration and chemotaxis, matrix (dis)-assembly, signal transduction of diffusible small proteins (ie. cytokines and growth factors) and supporting a state of intermediate cell adhesion to allow rapid response to external stimuli requiring increased adhesion or de-adhesion[2].

1.1 Extracellular Matrix Composition & Function

The extracellular matrix (ECM) is a non-cellular component present in all tissues and organs that provides a physical framework for cellular constituents and acts in a reciprocal manner towards the surroundings of cell populations by mediating biochemical and biomechanical cues. The ECM is not simply a static structure but is better understood as a highly dynamic environment that reflects the needs and demands of the local cellular environment or in some cases the whole tissue. Broadly, the ECM is made of water, proteins and polysaccharides, but each tissue has a unique heterogenous composition and topology generated during development[3]. Owing to the various demands of each unique organ, mechanical parameters such as compressive strength, elasticity and density are affected by the nearby cells as the delicate balance of ECM components adapts to the changing demands of the tissue.

The two major classes of macromolecules in the ECM are proteoglycans and fibrous proteins[3]. Proteoglycans are proteins that have been post-translationally modified by glycosylation by covalent linkage of glycosaminoglycan (GAG) chains to a peptide core and fill the majority of the interstitial space by forming linked matrices to provide resistance towards compressive forces[3]. Three main families of proteoglycan proteins in the ECM are small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell surface proteoglycans[3]. In addition to being structural proteins, proteoglycans also serve as mediators of ECM signal transduction as they are upstream of intracellular signaling cascades owing to their localization[3, 4]. Fibrous proteins of the ECM are predominantly collagen, fibronectin, elastin and laminin which compose the main structural proteins that provide tensile strength to tissues to enable a physical response to mechanical forces[3, 5]. Collagens are a ubiquitous protein in vertebrae secreted primarily by local fibroblasts and are the most abundant protein in the ECM. Fibrous type collagens type I, II, III are the major forms in the ECM and form a rod like triple helix structure made of three α-chains in a hetero-or homotrimeric fashion with context dependent variable length[3, 6]. Covalent cross-linking of collagen fibers mediated by lysyl oxidases and association with the other fibrous proteins in the ECM allow tissues like the skin, heart and bones to resist sheer, tensile and pressure forces to maintain function. The delicate balance of collagen production and degradation in the ECM is often perturbed in pathologies like organ fibrosis with excess collagen deposition causing tissue stiffening or Ehlers-Danlos syndrome in which genetic collagen mutations lead to frequent joint dislocations and hypermobility.

Fibronectin is an abundant modular glycoprotein of the ECM that assembles into dimers to create a branched mesh network that extends and connects adjacent cells and can facilitate binding to collagen, tethering to cell surface integrins and other fibronectin proteins through its various modular domains[7]. Its own dimeric assembly is a cell mediated process and begins as thin fibrils which eventually cluster together to form more robust and thicker bundle structures from the multimerization of individual dimers[7]. These mature fibronectin fibrils have remarkable tensile strength as they can be stretched several times beyond its resting length by cell traction forces, which has also been shown to expose hidden integrin binding domains for adaptive cell signaling[3, 7].

The capacity of the ECM to induce intracellular pathways and signaling cascades is mediated primarily by binding to different heterodimeric integrins on the cell surface. Integrin binding domains on matrix proteins can recognize unique integrin combinations associated with the desired response or induce ligand occupied integrin translocation. For example, $\alpha_v\beta_3$ integrins were found to predominate at the cell periphery for formation of focal adhesions and stress fibers, whereas $\alpha_5\beta_1$ integrins were observed to associate with intracellular actin bundles to transmit cell generated tension forces[7, 8]. The fibronectin network itself can also act as a mechano-regulator and indicator of tissue stiffness as conformational changes can expose cryptic integrin binding sites. Processes like cell spreading and adhesion can adapt to different levels of stiffness as seen with experiments showing predominant $\alpha_5\beta_1$ interactions in maintaining cell adhesion and resisting detachment but preferential $\alpha_v\beta_3$ binding on stiffer substrates[3, 9].

Elastin's are another type of fibrous protein found in the ECM and have important functions in allowing tissues to recoil from stretching forces, as their name suggests. Formation of elastic fibers occur by lysyl oxidase mediated crosslinking of the precursor, tropoelastin and are modified with fibrillin's to maintain structural integrity[3, 10].

Laminins are large heterotrimeric multimodal proteins, perhaps best known for their role in basement membrane formation but also act to reinforce the ECM through association with other matrix macromolecules[11]. Laminins, like the other major fibril proteins of the ECM, connect to the surrounding cells via integrins to fulfill their mechanical anchoring properties but to also mediate cell migration and adhesion. Of the three subunits, α, β, and γ, the α-chain plays a significant role in mediating biological processes, especially organogenesis during embryo development[12].

Lastly, within the ECM are also non-structural proteins, known as MPs that are secreted specifically into the pericellular matrix, a sub-compartment of the ECM directly adjacent to the cell membrane[2]. MPs are present in a context dependent manner typically during development, tissue injury and repair processes. The presence and expression of MPs in the ECM are widespread throughout many organs and produced by cell types ranging from fibroblasts to

macrophages due to their multimodal functions and have been shown to have contradicting properties depending on the context.

1.2 Matricellular Protein Function and Integrin Signaling

In contrast to the common structural ECM proteins, MPs are not constitutively expressed. Instead, MPs are both spatially and temporally expressed at specific sites during times of active remodeling such as in development, tissue repair or in response to an injury or chronic disease states[13]. From a therapeutic aspect, the context specific expression can be exploited to target MPs during aberrant repair or chronic disease states in which MPs are most active, with hopes that resolution of the complication will also signal their innate downregulation and thus leading to fewer side effects.

The main functions of MPs are primarily to mediate ECM remodeling processes such as synthesis, contraction or proteolytic degradation as well as trigger intracellular events to stimulate proliferation, cell migration and invasion[14]. Like classical ECM proteins, MPs can also interact with matrix components by their distinct functional domains that allow them to anchor and contribute to the intricate network of the ECM[15].

Integrin signaling is the primary mechanism by which MPs exert their function on the surrounding cells to communicate extracellular signals to initiate intracellular responses, allowing cells to quickly adapt to the changing external environment[16, 17]. Integrins are categorized as transmembrane cell surface receptors but also provide mechanical anchoring to both the ECM and cytoskeleton[16]. Moreover, they influence a broad range of cellular functions from proliferation, migration, differentiation and importantly confers mechano-sensing capability to cells[18]. As such, deregulation of integrin signaling is often observed in the development of pathological processes where it is adapted to instead sustain pathologies like chronic inflammation and cancer[18].

Integrin receptors are composed of two subunits, α and β, that non-covalently interact to form a functional heterodimeric receptor to bridge the ECM and intracellular cytoskeleton

through a helical transmembrane domain, extracellular domain and a short cytoplasmic tail[14, 19]. In vertebrates, there exists 18 α and 8 β subunits that assemble into 24 unique αβ heterodimer structures[18, 19]. Integrins are ubiquitously expressed by all cells but typically only express a subset of the many αβ heterodimer combinations. The integrin profile is relevant to the cell type but can also be influenced by the current surrounding ECM composition[19, 20]. The most abundantly produced integrin subunits have been found to be α_v and β_1, due to their ability to adapt and pair with several other α- and β- subunits[18]. For example, the β_1 subunit has been shown to be essential in fibroblasts to regulate wound repair events such as wound closure and de-novo ECM secretion and organization[21]. Major processes such as cell migration rely on large ECM proteins such as fibronectin that can also serve as ligands to integrin receptors (ie. $\alpha_v\beta_3$, $\alpha_5\beta_1$, , $\alpha_4\beta_1$). Moreover, MPs also function primarily through integrin mediated signaling by direct physical interactions at specific binding sites, which has been exploited for therapeutic benefits[22].

Integrins also exhibit bidirectional signaling, a unique feature among transmembrane receptors that allow them to bind both extracellular and intracellular proteins and signaling molecules to exert their function[16, 18]. The intracellular to extracellular signaling cascade, or so-called inside-out activation of integrin by cytoskeletal proteins induces conformational changes that promotes high affinity ligand binding and integrin clustering by oligomerization of various α and β subunits[17]. Similarly, outside-in signaling begins with ligand binding in the extracellular domains to cluster with other bound integrins on the plasma membrane to form structures known as focal adhesions. Focal adhesions are connected to the cytoskeleton and high local concentrations is one way integrins can activate downstream intracellular responses and influence the cell cytoskeleton[17]. Important pathways initiated by integrin mediated signaling include the Focal adhesion kinases (FAK), ERK, JNK, Akt and PI3K[23]. These extensive and broad acting pathways are the drivers of many major cell processes like proliferation, survival, motility, and matrix remodeling (Figure 1).

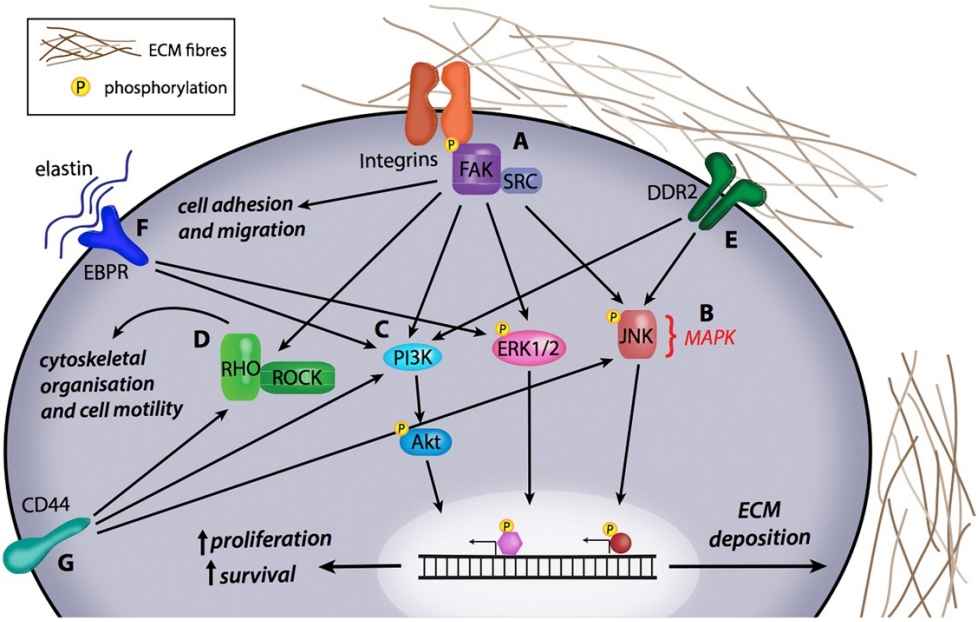

Figure 1: Integrin pathways activated by ECM protein interactions. Gene expression changes that influence proliferation, survival, cell motility and matrix remodeling triggered by ECM protein-integrin interaction. Figure sourced from Hastings et al[23].

As stated, both ECM structural proteins like fibronectin and MPs can interact with integrins to trigger intracellular downstream signaling. For example, CCN1 is a well characterized MP expressed in diverse cell types such as fibroblasts, smooth muscle cells, and monocytes[24]. Recognition and binding of CCN1 occurs by different integrin receptors dependent not only on the cell type but the intended cellular response, as MPs are multimodal. For instance, in fibroblasts, CCN1 engages the integrins $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ for cell adhesion, migration and DNA synthesis/proliferation respectively[24]. Therefore, expression of cell-specific integrin receptors is one mechanism by which cells can discriminate between the multifunctional role of MPs.

1.3 Matricellular Protein Families

Within the last two decades, many MPs have been discovered and placed within distinct families based on shared structural similarities and/or functional domains[25]. The major MP families are **S**ecreted **P**rotein **A**cidic and **R**ich in **C**ysteine (**SPARC**), **CCN**, Thrombospondin (**THBS**), **S**mall **I**ntegrin-**B**inding **Li**gand, **N**-linked **G**lycoprotein (**SIBLING**) and Tenascin (**TN**). MPs have been well-studied for their important function in regulating tissue repair mechanisms but emerging roles for MPs in pathologies involving dysregulated remodeling are becoming increasingly common[2, 26]. MPs have been implicated in a variety of pathologies ranging from skeletal development, fibrosis, cancer and metastasis with disparate and context dependent roles of MPs within the same family for added complexity[25, 27, 28].

Most notably and best described is the SPARC family with eight members (SPARC, SPARCL1, SMOC1-2, SPOCK1-3, FSTL1) that all share a follistatin-like domain and an extracellular calcium binding E-F hand domain[29]. Among one of original MPs first described, SPARC or otherwise referred to as Osteonectin or BM-40 served as the prototype for understanding the counter-intuitive functions of MPs in the ECM as it showed de-adhesive activity, but later re-defined as a state of "intermediate adhesion"[30, 31].

The CCN family encompasses six proteins designated CCN1-6, though many alternative names exist for each member, and are unified by a cysteine-knot module (apart from CCN5) composed of a series of 38 cysteine residues with strict conservation in position and number[32]. Moreover, CCN proteins have a tetra-modular structure, allowing for up to four independent and unique interactions working in a synergistic or antagonistic manner, hence the mosaic structure of CCN proeins[32].

Thrombospondins (THBS), specifically THBS-1, was also amongst the first described MPs, which has now expanded to five total members, namely THBS1-4 and COMP. The hallmark of all THBS proteins lies in the highly conserved globular C-terminal domain of each peptide that consists of a calcium binding domain and a variable number of repeating epidermal growth factor (EGF)-like domains that serve as the THBS signature[33]. Variability between THBS

proteins come from the N-terminal domain which confer functional differences between family members, such as the anti-angiogenic properties of THBS1-2 attributed to their properdin domain[33].

The SIBLING family consist of 5 defining proteins (OPN/SPP1, BSP, DMP1, DSPP and MEPE) with OPN being the most characterized of the set. All SIBLING proteins contain an integrin binding Arg-Gly-Asp (RGD) motif important for cell adhesion and its overall hydrophilic structure[34]. Most SIBLING proteins also activate specific matrix metalloproteases (MMPs) that play important roles in ECM degradation[35]. However, SIBLING proteins remain understudied in the context of cancer, with the exception of OPN and BSP which have been associated with tumor aggressiveness in processes of invasion, metastasis and angiogenesis[34], and in development of mineralized tissue such as bone and dentin[34].

Tenascin-C (TN-C) is the founding member of the TN family and was the third original MP first described by the Bornstein group. The TN family has now expanded to five proteins, TN-C, TNX-A, TNX-B, TN-R and TN-N, that all share a N-terminal heptad repeats, EGF-like repeats and fibronectin type-III repeats[36]. Tenascins can assemble into large oligomeric proteins, primarily forming trimers via the highly conserved heptad repeats but formation of large symmetrical hexamers from two trimers is seen in the case of TN-C[36]. Less is known about the other members, but functional capabilities of TN-C includes its ability to bind directly to fibronectin to modulate cell adhesion properties and as a integrin-binding ligand to trigger intracellular pathways affecting cell spreading[36, 37]. Moreover, *de-novo* expression of TN-C in wound healing, chronic inflammation and cancer exemplifies its wide ranging capabilities from its initial discovery[36, 38].

Active research in the field of MPs has shed light on their diverse functions and continues to show novel contexts in which MPs have not yet been considered (Table 1). Though our knowledge of familiar MPs is constantly expanding into new research domains, there still remains much to be explored for lesser known proteins. For example, considering splice variants of MPs is something that may be important for understanding contradictory roles of MPs in different contexts and differences between family members[39]. With increasing appreciation for

MPs, emphasis on finding links to disease by adapting screening studies to focus on MPs has also helped to expand the growing body of knowledge.

Table 1: Functional Roles and Characteristics of Matricellular Protein Families

MP Family	MP Family Members	Biological and Cellular Functions	ECM Interactions	Clinical Implications
SPARC[40-42]	Sparc Sparcl1 Smoc1,2 Spock1,2,3 Fstl1	- Follistatin-like domain - Extracellular Ca^{2+} binding domain - Contradictory cell-type dependent regulation of ECM synthesis/proteolysis, invasion, motility, adhesion, cell growth - Modulation of growth factors (VEGF, TGFβ, bFGF)	- Regulator of collagen fibril architecture (Sparcl1, Sparc) - Context dependent induction of MMP activity influencing tumorigenesis - Regulator of structural ECM protein expression (Fibronectin, collagen)	- Warrdenburg Anopthalmia Syndrome (SMOC-1) - Rheumatoid arthritis (Fstl1) - Osteogenesis imperfecta (Sparc)
Thrombospondins (THBS)[33, 43, 44]	Thbs1,2,3,4 Comp	- Conserved calcium binding domain - Distinguishing C-terminal EGF-like domains - Activation of latent TGFβ - Endogenous angiogenesis inhibitor (Thbs1,2)	- Ca^{2+} dependent cell attachment to ECM glycoproteins & proteoglycans - Collagen matrix assembly - Formation of fibrin clots	- Osteoarthritis, (Comp) - Pseudoachondroplasia (Comp) - Myocardial infarction (Thbs4)
Tenascins (TN)[36, 38]	TN-C TNX-A,B TN-R TN-N	- Large glycoproteins (200-400 kDa) - Epidermal growth factor (EGF) -like repeats - Trimeric and hexameric oligomerization structures	- Variable effect on cell adhesion and motility via fibronectin - Mechanosensitive expression via actin, collagen and fibronectin dynamics	- Ehlers-Danlos Syndrome (Tnx-B) - Tumorigenesis (TN-C)
CCN[45-48]	CCN1 (CYR61) CCN2 (Ctgf) CCN3 (Nov) CCN 4,5,6 (Wisp1,2,3)	- Tetra-modular structure with cysteine-knot module - Mediators and targets of Wnt, BMP, TGF-pathways - Extensive context dependent function in adhesion, angiogenesis, proliferation, development and tumorigenesis	- Direct interaction and inducible synthesis of ECM components (fibronectin, heparin, collagen) - Stimulation of fibroblast spreading/adherence to fibronectin	- Atrioventricular septal defects and nephroblastoma (CCN3) - Scleroderma (CCN2) - Breast carcinoma (CCN5)
SIBLING[34, 35]	Spp1 Bsp Dmp1 Dspp Mepe	- Share a Arg-Gly-Asp (RGD) cell binding domain - Share a conserved acidic serine- and aspirate-rich motif (ASARM) for functional activity - All coded on single chromosome (4q) - Functions in bone and matrix mineralization - Principally expressed in bone and dentin	- Mediate ECM mineralization by regulating $[Ca^{2+}]$ and $[PP_i/P_i]$ - Activation of matrix metalloproteases (MMP)	- Hypophosphataemic Rickets (Dmp1) - Dentinogenesis imperfectra II/III (Dspp) - Cancer proliferation and aggressiveness (Spp1)

1.3.1 SMOC-2 as a Model MP

Secreted Modular Calcium binding protein-2 (SMOC-2) was first described by the Hartmann lab in 2003 and is part of the SPARC family of matricellular proteins[49]. It is encoded on human chromosome 6q27 and has highly conserved intron and exon structure to the murine equivalent on chromosome 17[49]. SMOC-2 contains a homologous extracellular calcium binding and follistatin-like domain shared among all SPARC family members, two thyroglobulin-like domains and a SMOC-protein unique domain[49]. Moreover, an N-terminal signal consensus sequence and peptidase cleavage site along with the absence of a transmembrane hydrophobic domain allows it to be secreted into the ECM like other members of the SPARC family[49] (Figure 2).

Figure 2: SMOC-protein domains. Image sourced from Hartmann et al[49]

By Northern blotting and RT-PCR, SMOC-2 was found to be expressed widely in ovary, testis, heart, spleen, thymus, lung, liver, kidney, skin, colon, pancreas and skeletal muscle[49, 50]. Expression of SMOC-2 has been shown to be detected in mice as early as 8.5 dpc (days post-coitum)[51] and mRNA detectable at 12.5 dpc in the development of facial and cranial features and limbs[51, 52]. Moreover, SMOC-2 mutations have been shown to be linked to human dental developmental defects despite no other observed complication at birth[50]. Beyond developmental processes, SMOC-2 expression has also been found to be highly upregulated in murine models of renal fibrosis[53] and metastatic colorectal cancer[54]. However, conflicting observations have been made showing its downregulation in tumor development in ovarian[55], pancreatic[56], breast[57] and thyroid[58] cancer by microarray studies. The contradicting roles of SMOC-2 simply speak to its highly context dependent function and our only partial understanding of its role in various diseases.

The molecular functions of SMOC-2 range from mediating cell cycle progression[59] and growth factor signals to regulating mitogenic and angiogenic processes[60]. It is also shown to influence cell growth, migration and attachment[51, 61]. The broad functions and involvement in various biological processes highlight not only the breadth of SMOC-2, but importance and capability of matricellular proteins on a broader scale as both mediators and main drivers to trigger intracellular changes from the ECM.

Supporting cellular attachment and anchoring to the ECM is an important function of matricellular proteins. SMOC-2 was shown to promote adhesion of keratinocytes exclusively by its EC domain[51]. The EC domain is important for extracellular Ca^{+2} binding to enhance affinity for binding ECM ligands/proteins and as such, removal of soluble Ca^{+2} by EDTA chelation completely abolishes cell attachment[51]. SMOC-2 overexpression was also shown to stimulate keratinocyte migration in the presence of fibronectin but not BSA in a modified Boyden chamber assay[51]. SMOC-2 overexpressing endothelial cells also showed increased migration with bFGF[60] used as a chemoattractant, showing an enhanced pro-angiogenic and migratory phenotype when coupled with potent growth factors.

To regulate cell processes like cell attachment and migration, SMOC-2 signals from the ECM are translated by integrin receptors at the cell surface. Specifically, the $\alpha_v\beta_1$ integrin-subunit complex is important in promoting cell attachment, while inhibition of only the β_1 subunit is sufficient to drastically reduce adhesive capability of keratinocytes[51]. Moreover, $\alpha_v\beta_1$ has been shown to be highly expressed on the surface of activated fibroblasts to bind TGFβ-1[62] to trigger intracellular cascades like the Smad pathway[62, 63], Snail[64, 65] and Rho-like GTPases[65] which all can promote cell migration and attachment by either directly influencing gene expression.

SMOC-2 has also been shown to have pro-angiogenic effects in endothelial cells (HUVEC) overexpressing a Myc-SMOC-2 fusion protein using a adenoviral vector (Ad-Myc-SMOC-2), as indicated by increased projections in Matrigel coated wells[60]. Complementary to the pro-angiogenic effects, these HUVEC cells also showed increased DNA synthesis measured

by [^{3}H]thymidine incorporation in a dose dependent manner with VEGF and bFGF (basic Fibroblast Growth Factor) in Ad-SMOC-2 transduced cells compared to the Ad-GFP control[60].

Overall, SMOC-2 remains a understudied MP and our understanding of its functions pales in comparison to other members in the SPARC family. Emerging research in the field of MPs will hopefully spark interest in studying the lesser known proteins.

1.4 Tissue Repair Process Overview

In all mammalian organ systems, the wound repair process occurs by an overlapping three step process, beginning with inflammation, followed by a proliferative phase and concludes with matrix remodeling events[66]. However, the timing of these events are also tightly regulated to ensure prompt repair but also distinct completion of each phase to avoid prolonged states of inflammation or matrix remodeling which is the underlying cause of many other pathologies.

The first responders to the wound site include recruitment of, but are not limited to, platelets, fibrin and fibronectin that act as a hemostatic plug and provide the first basis of a provisional matrix for incoming inflammatory cells to further build upon[67]. Local release of growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), TGFβ-1, pro-inflammatory cytokines like IL-1β, IL6[68, 69] act as stimulants to transform resident monocytes to macrophages, recruit neutrophils to the wound site and initiate the development of granulation tissue using the provisional matrix as a foundation. As the primary purpose of the inflammation phase is to clear damaged cellular debris and prevent infection, pro-inflammatory signals must be properly sequestered once they served their purpose to transition into the next phase and avoid a state of chronic inflammation.

Following inflammation, the next major theme in the repair process is the proliferative phase which is important to regenerate the wound site by replacing the provisional matrix with granulation tissue that forms a new foundation. Local concentration of aforementioned wound

repair signals trigger migration and increased proliferation of cells such as, keratinocytes, fibroblasts, granulocytes and surviving epithelial cells to help fill the wound gap and build upon the granulation tissue[69]. Moreover, anti-inflammatory M2 macrophages can secrete factors such as EGF to promote re-epithelization and nitric oxide for wound contraction[69]. Migration is aided by the loss of physical tension and cell-cell contact during injury and stops once this contact is re-established and new adhesion structures restored. This wave of migratory cells work in essence to replace the provisional matrix laid down during hemostasis to make way for deposition of structural ECM proteins like collagen and fibronectin that is secreted by fibroblasts, which also releases degradative proteases such as MMPs[69, 70]. The presence of these two major ECM structures distinguish the temporary provisional matrix from the lasting granulation tissue[71, 72] and provide a basic scaffold for further cell adhesion, migration, growth and differentiation to occur through the subsequent phases of wound repair. Another important aspect during the proliferative phase is angiogenesis, which is the process of forming new blood vessels to support the growing tissue and is initiated by growth factors like PDGF and vascular endothelial growth factor (VEGF). The local concentration of these factors work to attract endothelial cells of nearby vessels to migrate and proliferate towards this source of angiogenic stimuli[69].

The last phase to conclude the repair process is a compilation of matrix remodeling events with the goal of restoring functionality to the injured tissue[73]. The injured parenchymal tissue orchestrates the repair process by promoting both epithelial and fibroblast transformation to their effector forms to mediate remodeling. A major event during this phase include restoring the tensile forces that hold the tissue together. This primarily occurs by TGFβ-1 driven fibroblast differentiation into myofibroblasts that express α-smooth muscle actin (α-SMA) which is incorporated into de-novo contractile bundles of actin of the myofibroblast, coined stress fibers[69, 74]. These stress fibers provide an intracellular link to the ECM via transmembrane integrin receptors to form large structures known as focal adhesion complexes[74, 75]. Moreover, the rapidly deposited collagen backbone, composed of predominately collagen IV is converted to collagen I which provides greater tensile strength after cross-linking[66, 69, 74, 76]. Re-organization and alignment of the present ECM materials to restore normal tissue architecture can still be actively occurring from 6 months – 1 year post injury, depending on the severity of the insult[66, 73] and can

only regain up to 80% of the tensile strength in the case of dermal wounds[77]. The conclusion of the remodeling phase must also signal the shutdown of inflammatory, proliferative, regenerative and matrix protein secretory pathways as their prolonged activation can cause scar formation, overly stiffened/inflexible tissue and long term pathologies associated with dysregulated or imperfect repair such as organ fibrosis[69, 73].

MPs are involved during all three stages of the repair process and targeted disruption of MP expression or function in murine models report altered inflammation responses, reduced angiogenesis at the wound site and impaired matrix deposition[78]. For example, THBS-2 null mice were able to recover from a full thickness excisional wound but with abnormal neovascularization within the wound bed and a disorganized ECM in the dermal layer compared to wildtype. Though interestingly, although the repair process occurred faster in THBS-2 null mice, the end result appeared highly irregular[78]. Another example is the absence of OPN, which significantly impaired the synthesis and organization of collagen I protein in cardiac remodeling after myocardial infarction that resulted in reduced strength of the repaired ECM[79]. Collectively, these studies highlight the implications of MP absence during wound repair at varying stages that result in sub-optimal restoration of the injured tissue.

1.4.1 Fibrosis – Deregulated Repair

Fibrosis is defined as the stiffening or scarring of tissue that leads to the destruction of the organ architecture and impairment of function associated with excessive deposition of ECM components[80, 81]. Fibrosis affects nearly every tissue in the body including but not limited to, bone marrow, kidney, heart, intestine, lung and skin[80]. Existing therapies are limited to only helping manage or slow the progression of fibrosis as there are currently no treatments to stop or completely reverse fibrotic tissue in any organ[82]. One note of positivity is the histological similarities between different organs after fibrotic onset, providing hope for potentially elucidating and targeting common mechanism(s) to be effective across many organs. However, organ specific triggers and pathological impact on overall health still vary depending on the severity and progression in afflicted tissues.

As the ECM is a dynamic structure, normal up-and downregulated production of ECM materials is crucial for cells to adapt quickly to subtle and transient changes in its surroundings[83]. There is consensus in the literature that the overproduction of these ECM materials come from activated fibroblasts (myofibroblasts) that secrete primarily collagen type I and IV and fibronectin in an unregulated manner[3, 82]. Without proper management or degradation, this deposition and accumulation over time causes elevated mechanical stress, tension and reduced elasticity to the entire ECM network. The differentiation of originally reparative fibroblasts, resident epithelial cells and even circulating bone marrow derived mesenchymal stem cells[3, 84] into myofibroblasts from this dysregulated system creates a destructive feedback loop of ECM stiffening and cellular differentiation. However, it is worth noting that these processes normally described occur during wound repair with strict feedback mechanisms allowing for restoration of tissue function without onset of fibrosis[3].

Within the dysregulated matrix during fibrotic onset, MPs have also been shown to be strongly upregulated and contribute to sustaining fibrotic mechanisms. CCN2 is a well-characterized pro-fibrotic MP that works by supporting the TGFβ-1 induced fibrosis mechanism in part by activation of Smad1 and Erk1/2 signaling for sustained ECM deposition and remodeling in human fibroblasts in-vitro[85]. Osteopontin (OPN) is another pro-fibrotic MP which has been shown to be upregulated in the tubules of obstructed kidneys after unilateral ureteral obstruction (UUO) in mice. Mice subjected to ureteral ligation developed hydronephrosis, tubular atrophy and interstitial inflammation and fibrosis, compared to unobstructed control animals[86]. From the tenascin family, TN-C levels are found to be significantly upregulated in fibrotic lungs in patients with idiopathic pulmonary fibrosis, compared to healthy patients[87]. Primary fibroblasts cultured from diseased lungs was found to constitutively release higher levels of TN-C, and more sensitive to TGFβ stimulation[87].

Though a large majority of MPs are pro-fibrotic, it is worth noting that there are anti-fibrotic MPs, even members amongst same family, that have been reported to attenuate the fibrotic phenotype. For example, CCN1 overexpression in a model of liver fibrosis induces ER stress and reactive oxygen specific formation to induce apoptosis of activated hepatic stellate

cells that are major producers of excessive ECM[88]. Overall, fibrosis is a powerful stimuli and context for inducing MP expression beyond the normal tissue repair and regeneration timeline.

1.4.2 The Kidney as a Model Organ to Study Injury and Maladaptive Repair

Acute kidney injury (AKI) is a pervasive clinical syndrome and is a major burden on global health, with increasing incidence rates that is directly associated with short and long term morbidity[89]. Repeated insults such as ischemia, chemical toxins, inflammation, hypoxia and physical injuries are common causes of AKI which causes cellular injury and can lead to development of chronic kidney disease (CKD)[89, 90]. Internal damage to biological structures such as the tubules, nephrons, glomeruli and blood vessels obstruct homeostatic function and triggers the pathophysiological mechanisms that underlie many clinical renal pathologies. Maladaptive repair after AKI can lead to accelerated kidney damage due to poor adaptation to the inefficient function. One possible end-stage renal disease outcome is fibrosis, which is a common cause of organ failure and outcome in chronic tissue injuries[82, 90].

The kidney is a robust model organ to study the connected mechanisms of dysregulated repair/remodeling and pathogenesis because of the extensive animal models available, convenient internal control with two kidneys, informative functional readouts through urine analysis, extensive cell lines and there still remains an large unmet need for therapeutic options to treat renal pathologies[82, 91, 92]. Moreover, as we are interested in studying MPs whose expression is context dependent, the kidney provides a safer alternative to other organs for inducible injury to follow the progression of repair and disease. Therefore, the kidney represents an all-encompassing model for our purposes in search of translatable mechanisms with MPs as the focus of our research.

1.5 Development Overview

During development, the ECM has dually important functions as both the traditional concept as a passive support scaffold for tissue growth, and as a actively remodeling structure that imparts important cues to incoming cells to direct development of tissue architecture[93].

On a macroscopic level, the ECM can act as a physical barrier between cells, tissue structures and work as a protective cushion that secure and hold organs intact[94]. The composition and delicate ratio of ECM structural proteins is a determinant for the strength, elasticity/stiffness and overall biomechanical properties and is remodeled accordingly during development to be tissue specific. For example, collagen cross-linking is a key process in ECM development in both a regulated and un-regulated manner and is crucial for stiffening of the cardiovascular system[94]. Knockout of the collagen cross-linking enzyme lysl oxidase (LOX) result in mortality at birth due to inefficient cardiac output by a weak cardiovascular system and a fragile diaphragm in murine models[94].

In the context of development, MPs also have important roles and are upregulated to participate in active remodeling. MPs within the ECM are also important in the development of bone and cartilage, though the specifics are beyond our scope. Interestingly, SMOC-2 has been reported to be responsible in the development of cranial and facial features of dogs[95]. Using genome-wide association studies, a mutation in canine SMOC-2 is linked to the development of canine brachycephaly, which causes the shortened head, nose and flat face best exemplified by breeds such as Pugs, Shih Tzus and Pekingese dogs[95].

The importance for maintaining tight regulation of the ECM and MPs during development is best highlighted by the consequences of dysregulation when this delicate balance is perturbed. To do so, we look to cases of de-regulated development where we can study its consequences.

1.5.1 Cancer – Deregulated Development

Malignant tumors are a complex structure of numerous cell types in a pool of oncogenic secretory factors all being structurally supported by a dynamically adaptive ECM. Interactions with the ECM can set off a cascade of downstream molecular signalling events that adapt the tumor microenvironment for immune suppression, communication, rapid growth and metastatic dissemination[27]. To understand the tumor stroma, comparisons have been drawn to the dysregulated ECM of chronically injured wounds that fail to heal[3, 96] but proliferating cancer cells interacting with their microenvironment can be considered a unique entity or separate tissue entirely with functional disorder[3, 97].

The biology of a developing tumorigenic ECM is strikingly similar to the phenotypes observed in fibrosis. Excessive ECM deposition of fibronectin, type I collagen and increased secretion of MPs all contribute to accelerating tumor growth[94]. Buildup of excessive matrix components can support oncogenic proliferation by interfering with cell-cell and cell-matrix interactions to amplify growth factor signals and enhance integrin signaling[98]. Though seemingly disorganized, the developing tumor ECM can undergo remodeling processes to facilitate invasion as seen with organization and alignment of collagen fibrils that act as tracks for outward movement[98].

The developing tumor ECM can also be distinguished from non-malignant ECM by the abnormalities in its biomechanical properties. ECM plasticity influences how cells perceive the external environment and subsequently how they respond[99]. Compromised tensile homeostasis in tumorigenic tissue is one of the key characteristics, and is used as a parameter when screening for breast tumors as they tend to be stiffer than the surrounding tissue[100, 101]. Within the tumor microenvironment, these elevated contractile forces can positively influence cell growth and invasion by promoting maturation of focal adhesions and matrix remodeling for rearrangement of collagen fibrils to push the invasive front of tumors outwards needed for spreading[100, 102, 103]. In contrast to tensile stress, compressive stress by the expanding tumor matrix onto surrounding tissue extracellular matrix and vasculature, effectively shrink the total interstitial space, aiding in tumor invasion into the parenchyma[100]. Moreover, changes in the interstitial pressure and

progressive increasing density of solid tumors adds yet another layer of complexity towards delivery and penetration of anti-tumor therapeutics[100, 104].

Deregulation of MP expression in the developing ECM is a hallmark of cancer as they are implicated in many pertinent processes required to sustain the malignancy. In an analogous fashion to wound healing and fibrosis, the actively transforming oncogenic ECM provides the necessary context to stimulate MP expression. For example, OPN is the best characterized member of the SIBLING family and its expression is found to be elevated in tumor ECM of different histotypes, which all report a positive correlation with tumor progression and metastasis[25]. Within the developing ECM, OPN has also been showed to induce endothelial cell migration which is important for vascularization of the growing tumor niche to supply oxygen and nutrients to proliferating malignant cells[25]. From the thrombospondin family, THBS-1 expression has also been widely used as a prognostic marker of many cancer types including, melanoma, ovarian and pancreatic carcinomas[105-107]. At sites of human primary melanoma, increased levels of THBS-1 was detected by microarray analysis compared to benign nevi and associated with melanoma cell motility[105]. Though interestingly, it was reported that THBS-1 was negatively correlated with SLUG expression, a master regulator of cell motility suggesting an alternative independent pathway[105]. Another well characterized MP is connective tissue growth factor (CTGF), or otherwise CCN2. First associated with breast and pancreatic cancer, aberrant CCN2 expression has been linked to many human cancers since its discovery[108]. Its divergent expression, whether abnormally up- or downregulated compared to normal cell counterparts is often associated with a poor clinical outcome[108]. In breast cancer, malignant cells overexpressing CCN2 gain enhanced migratory ability through activation of the ERK1/2 pathway via integrin mediated signaling[109]. Importantly, many MPs also work to transform the local matrix environment and contribute to tumor growth by influencing cell processes like invasion, migration and proliferation. In colorectal cancer, follistatin-like protein 1 (FSTL-1) from the SPARC family is reported to be significantly upregulated in cancerous tissue compared to normal and promoted invasive behavior of colorectal cancer cell lines[110]. Higher FSTL-1 expression lead to a more aggressive phenotype by activating focal adhesions for cytoskeletal rearrangement and inducing TGFβ-Smad2/3 signaling to increase cell migration[110]. Metastatic sites in the liver and spleen were also observed to develop earlier in FSTL-1 overexpressing mice

compared to control animals[110]. Figure 3 illustrates the major milestones of a primary tumor in its progression to a metastasizing cancer. Supportive or inhibitory roles of known matricellular proteins during each stage along with various stromal cells is also highlighted.

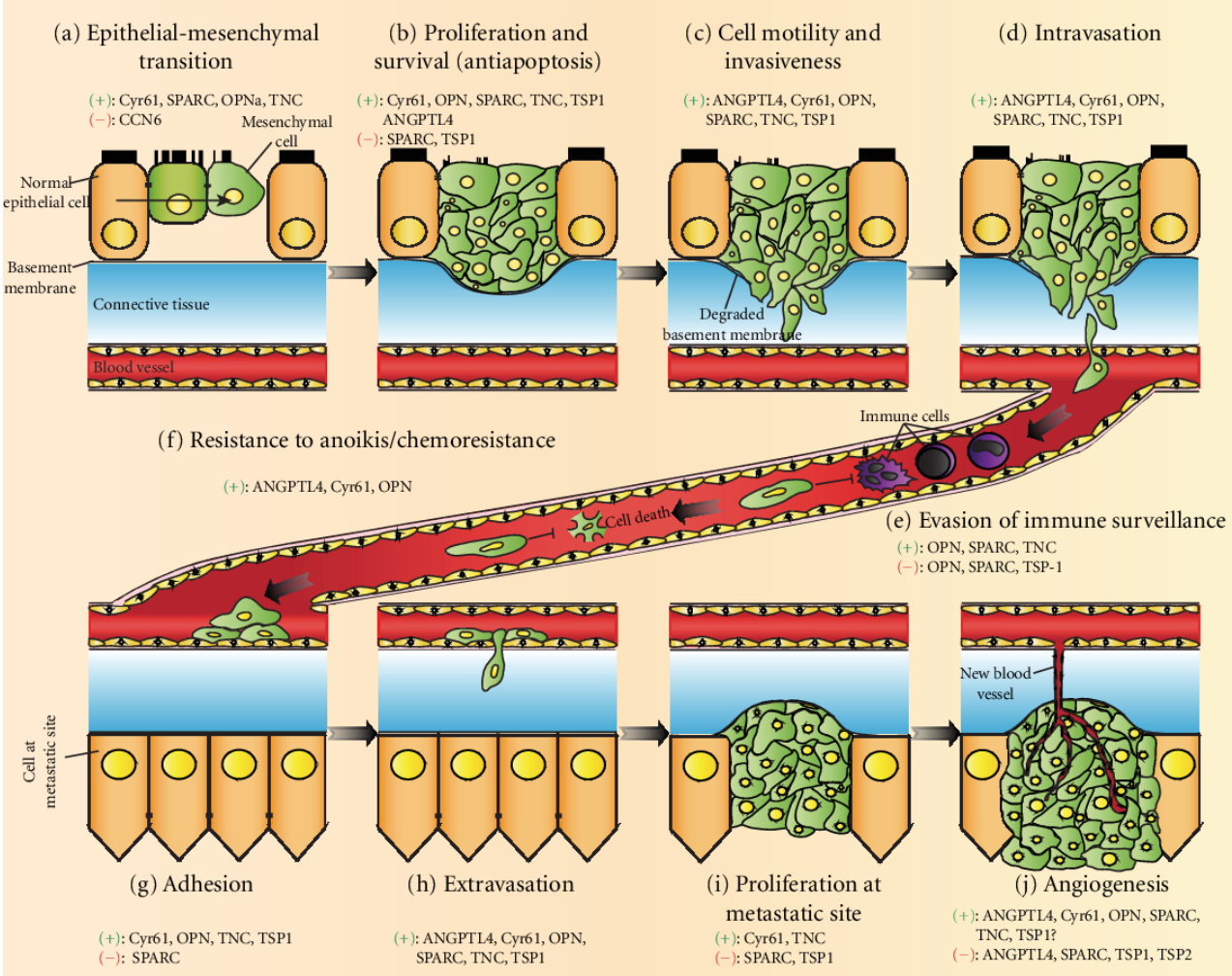

Figure 3: MPs in metastatic cancer development. Different MPs are implicated at each stage of cancer development. Action of MPs, can be pro- or anti-tumorigenic, depending on the processes involved. In the early stages of tumor initiation (a,b), MPs induce cellular and biochemical changes in vulnerable cells and promotes dysregulated survival and growth. Cell motility is aided through ECM degradation by MPs and allows tumor cells to invade into circulation (c-e) and provide protection during circulation to the metastatic site (f). Interaction with MPs to establish at the metastatic site and promote adhesion and proliferation of invaded cells (g-j). Figure is sourced from Chong et al[27].

1.6 Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a reversible biological process by which polarized epithelial cells undergo biochemical changes to transform and acquire mesenchymal, fibroblast-like properties[27, 111]. Transformed cells lose their apical-basal polarity, cuboidal morphology and cell-cell junctions in favor of a mesenchymal phenotype with fibroblast-like appearance, enhanced migratory potential, loose organization in the ECM and ability to catalyze ECM and cytoskeletal rearrangements for invasive growth[111-113]. EMT is a crucial process that has been well described and associated with embryonic development and wound healing/tissue regeneration, but our understanding of its contribution in various human pathologies remains an area of active research[111].

Considered a defining event in EMT progression is the repression and loss of E-cadherin, which in a sense opens the gateway for cells to exhibit features of a mesenchymal phenotype. E-cadherin is a tight junction protein whose function is to keep epithelial cells bound tightly to maintain their polarity and the separation between the apical epithelial layer and underlying stroma[114]. Loss of normal tissue architecture and tight junctions allows cells to move both horizontally and vertically beyond their normal imposed constraints and receive autocrine signals from diverse cell types to reinforce the EMT phenotype[114]. EMT-inducing transcription factors such as Snail, Slug, and noncoding microRNAs can act to repress E-cadherin expression while simultaneously activating Wnt/β-catenin, TGFβ and TWIST pathways to promote mesenchymal transformation by augmenting cell polarity, cell-cell contact and attachment.

Concomitant changes in the ECM and cell cytoskeleton also support the loss of E-cadherin and provide the necessary environment and tools for mesenchymal cell migration and invasion. Two important cytoskeletal markers of EMT that appear in mesenchymal cells are vimentin and α-SMA[115]. Upregulation of extracellular proteins like fibronectin and non-structural proteins such as MPs help remodel the ECM to complement and support the intracellular changes[115].

Vimentin is an intermediate filament protein expressed by various cell types including fibroblasts, endothelial cells, mesenchymal originating cells and epithelial cells undergoing EMT[116, 117]. It has functions in maintaining cell integrity, migration and invasion and when overexpressed in cancers, vimentin has been shown to drive EMT and have positive correlation tumor invasiveness and metastasis and serves as a marker of mesenchymal cells[115]. Although the exact molecular mechanisms of vimentin function remain to be elucidated, overwhelming evidence shows that its upregulation in the context of transforming epithelial or transformed mesenchymal cells have potent pro-EMT effects. Vimentin has been shown to be important in recycling endocytosed integrin receptors back to the plasma membrane to promote cell motility, which was severely attenuated with vimentin inhibition[116, 118]. Moreover, vimentin filaments are shown to be transiently concentrated and upregulated at the leading edge of sub-confluent metastatic breast cancer cells using a scratch assay to stimulate migration by generating a artificial wound gap for cells to fill in[116]. Co-operativity between actin filaments and vimentin have also been shown to regulate formation of lamellipodia and filopodia membrane protrusions that are required to propel the cell on the ECM substrate for migration[119]. Another pro-EMT function of vimentin is its ability to influence the expression of other EMT-linked genes and proteins such as receptor tyrosine kinase Axl and integrin subunit-β_4, both of which have roles in cell proliferation and migration[117].

α-SMA is a type of actin filament commonly used to identify the myofibroblast cell type but is also expressed by cancer-associated fibroblasts and mesenchymal cells in the tumor microenvironment[120, 121]. In many epithelial derived cancers, TGFβ induced EMT can trigger α-SMA expression by promoting trans-differentiation of stromal mesenchymal cells into myofibroblasts which become the effector cells[121]. Beyond just a cell marker, α-SMA is shown to be important in the formation of focal adhesions which are integrin receptors and associated protein complexes that provide a link between the cytoplasmic actin network and the ECM important for coordinating cell migration[122].

Fibronectin is a major component of the ECM and its aberrant expression has been associated with increased metastasis in numerous cancer types[115]. Fibronectin and its associated integrin, $\alpha_5\beta_1$ are upregulated in metastatic tumor niches where it can activate the ERK/MAPK

signaling cascades to promote cell proliferation and invasion. Increased fibronectin is also able to stimulate formation of focal adhesion at the leading edge of metastatic lung cancer cells, which also upregulated matrix metalloprotease mediated matrix degradation to facilitate invasion and ultimately metastasis[123, 124].

1.6.1 Epithelial-to-Mesenchymal Transition in Cancer Metastasis

From a disease perspective, EMT has been regarded as an important driver of tumor progression and metastasis because it provides tumor cells with the necessary molecular mechanisms and cellular components to migrate out of the primary site and invade secondary tissues[112]. EMT has been linked, but not limited to, cancers of the breast, neck, lung, colon, kidney and ovaries[112]. As is the case with all cancers, re-purposing and deregulation of normal cellular machinery and pathways turn EMT from a regenerative process to benefit and enable the expansion and growth of the tumor niche and propagate cancer associated cells beyond the primary lesion.

To explain the contribution of EMT in cancer metastasis, two current hypothesis exist[125]. Firstly, cancer progenitor cells do not undergo simultaneous EMT but can undergo differentiation at different times to create heterogeneity among the tumor cell population that represent the various advanced stages of the cancer[125, 126]. The cancerous cell population therefore exists at different stages of EMT which can be accelerated by other factors in the developing tumor. A second hypothesis proposes that some cancer progenitor cells initially undergo EMT to metastasize after clonal expansion, which suggests that the metastatic tumor cell population shares a cellular signature with the original transformed cancer progenitor cells[125, 126].

In human epithelial derived carcinomas, cells undergoing EMT have been shown to concentrate at the invasive front of the primary tumor and thus suggests an influence from the surrounding activated matrix environment[127, 128]. The combined loss of epithelial cell polarity and adhesive junctions result in tissue disorganization and is a hallmark seen in many epithelial

tissue carcinomas[129]. The well characterized epithelial adherent junction protein, E-cadherin, has been linked to cancer suppression mechanisms and as such, its impaired and eventual loss of function during the early stages of EMT is pivotal step in the transformation process. As mentioned previously, many of the EMT associated factors can be classified by their direct or indirect repressive actions on E-cadherin expression and un-surprisingly also have pro-oncogenic roles. Thus, combined tumorigenic stresses such as physical constraints, inflammation and metabolic stress can all lead to dysregulation in TGFβ, Wnt and TWIST pathways and create favorable conditions to trigger EMT.

Within the activated matrix environment are also MPs, which can promote EMT and initiate downstream signaling cascades that lead to invasion, migration, proliferation to promote dissemination of tumor cells to other organs by metastasis[27]. For example, the founding member of the Tenascin family, TN-C, is a well-known mediator of cell motility and invasion in various carcinomas. TN-C knockdown was shown to reduce migration of metastatic breast cancer cells measured by a Transwell assay and significantly decreased metastasis in a lung colonization assay with the same cells after inoculation into immunodeficient mice[130]. In another murine model of osteosarcoma, TN-C was shown to be crucial for driving lung metastasis that is dependent its associated integrin receptor, $\alpha_9\beta_1$[131]. In the progression of colon cancer, secreted modular calcium-binding protein-2 (SMOC-2) has been reported to promote EMT of colorectal cells. SMOC-2 overexpressing cells adopted a more elongated mesenchymal-esque morphology and did not form tight compact colonies at high cell densities compared to control cells[54]. This was coupled with a downregulation of E-cadherin, a marker of epithelial cells that is lost during EMT[111, 125]. Colorectal cancer cells overexpressing SMOC-2 also exhibited greater motility, which is hypothesized to involve integrin linked kinase activity[54].

1.7 Rationale & Research Objectives

Aim 1: To identify and analyze significant expression of MP families and their members using two mechanistically different models of murine kidney injury.

Aim 2: Analyze the role of SMOC-2 in mediating EMT induced metastasis of tubular epithelial cells in the context of Renal Cell Carcinoma.

Overall Research Objective: To study MPs in the context of tissue remodeling processes in order to understand de-regulated processes of repair in fibrosis and development of metastatic cancers.

2.0 Manuscript - Characterization of Matricellular Protein Expression Signatures in Mechanistically Diverse Mouse Models of Kidney Injury

2.1 Preface to Manuscript

The following manuscript is presented as part of my overall project completed during my training. The manuscript is published in the journal of *Scientific Reports*, with reference:

Feng, D., Ngov, C., Henley, N. *et al.* Characterization of Matricellular Protein Expression Signatures in Mechanistically Diverse Mouse Models of Kidney Injury. *Sci Rep* **9,** 16736 (2019) doi:10.1038/s41598-019-52961-5.

2.2 Manuscript

Characterization of Matricellular Protein Expression Signatures in Mechanistically Diverse Mouse Models of Kidney Injury

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Running title: Characterizing Matricellular Protein Expression during Kidney Injury

Key words: Chronic Kidney Disease, Acute Injury, Fibrosis, Animal Models, Folic Acid, Unilateral Ureteral Obstruction (UUO), RNA-Seq

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Fibrosis is the most common pathophysiological manifestation of chronic Kidney Disease (cKD). It is defined as excessive deposition of extracellular matrix (ECM) proteins. Embedded within the ECM are a family of proteins called Matricellular Proteins (MCPs), which are typically expressed during chronic pathologies for ECM processing. As such, identifying potential MCPs in the pathological secretome of a damaged kidney could serve as diagnostic/therapeutic targets of fibrosis. Using published RNA-Seq data from two kidney injury mouse models of different etiologies, Folic Acid (FA) and Unilateral Ureteral Obstruction (UUO), we compared and contrasted the expression profile of various members from well-known MCP families during the Acute and Fibrotic injury phases. As a result, we identified common and distinct MCP expression signatures between both injury models. Bioinformatic analysis of their differentially expressed MCP genes revealed similar top annotation clusters from Molecular Function and Biological Process networks, which are those commonly involved in fibrosis. Using kidney lysates from FA- and UUO-injured mice, we selected MP genes from our candidate list to confirm their mRNA expression by Western blot, which correlated with injury progression. Understanding the expressions of MCPs will provide important insight into the processes of kidney repair, and may validate MCPs as biomarkers and/or therapeutic targets of CKD.

Introduction

Chronic kidney disease (CKD) is a major health concern affecting approximately 10% of the global population[132]. Fibrosis is a maladaptive condition of repair associated with a majority of CKDs. It is characterized by aberrantly excessive accumulation and processing of stiff extracellular matrix (ECM) materials that progressively replaces the flexible parenchymal tissue[133]. In other words, the process of constructive restoration associated with the reparative ECM can shift towards a destructive remodeling (stiffening) of the tissue, leading to permanent scarring, organ malfunction and, ultimately, death[134]. While methods of intervention such as dialysis and transplantation serve as renal replacement therapies, they do not impede the progression of fibrosis. Furthermore, no treatments are currently available to effectively inhibit nor reverse the injury signals associated with maladaptive repair. Indeed, there is an urgent need to study the mechanisms of repair prior to fibrosis onset in order to identify novel biomarkers for disease monitoring as well as therapeutic targets for intervention.

The status of the ECM is a major determinant in the regulation of signaling pathways that drive repair. As such, ECM processing and cell-ECM interactions are important factors to consider in the context of fibrosis. Over the last 20 years, Matricellular Proteins (MPs) have emerged as essential regulators of these ECM events, and therefore of ECM remodeling and cellular behavior. As secreted proteins, the diverse activities of MPs are determined by multiple functional regions that interact with various ECM and cell surface molecules. The dynamic regulatory roles between the cell and its surrounding environment have distinguished MPs from the "classical" role of ECM proteins as static structural components. In contrast to the continuous presence of ECM proteins in the cellular environment, MP expression is tightly regulated and transiently expressed to exhibit context-specific effects during tissue remodeling processes (i.e. repair). However, MP expression is sustained during various injuries and chronic diseases, localizing in the extracellular space of diseased tissue and contributing to their pathologies[13, 135]. Thus, MP extracellular location and involvement during disease progression imply their potential to enter into the circulation and serve as non-invasive biomarkers of repair, as well as accessible targets to treat fibrosis with less side effects.

The majority of MPs have been previously characterized for their roles in fibrosis; however, only a few have been studied in the context of kidney fibrosis. Despite this progress from numerous experiments focusing on single MP molecules, global transcriptional profiles of multiple members from major MP families have not been compiled. Therefore, our goal was to analyze the temporal RNA expression patterns of MPs during the acute injury phase, and identify those whose expression is sustained during fibrotic pathologies. Specifically, we exploited RNA sequencing (RNA-seq) data from a toxicant and surgical mouse model of renal injury to screen all known members from the well-characterized MP families **S**ecreted **P**rotein **A**cidic and **R**ich in **C**ysteine (**SPARC**), **CCN**, Thrombospondin (**THBS**), **S**mall **I**ntegrin-**B**inding **Li**gand, **N**-linked **G**lycoprotein (**SIBLING**) and Tenascin (**TN**). We interrogated the possibility of a conserved MP transcriptome between these mechanistically different models of injury, and whether a specific type of injury can stimulate its own pattern of MP expression during the course of repair. In addition, based on MP genes with significant expression, we predict their respective functions in the two experimental models through bioinformatics analysis. Finally, we confirm the mRNA expression differences of a selection of early and late MP genes from both mouse models of kidney injury by Western Blot analysis.

Our analysis provides new insight into the plasticity of MP expression throughout the tissue remodeling process in two mechanistically different injury models, and highlights the potential of these injury-specific proteins as diagnostic/prognostic biomarkers and therapeutic targets of fibrosis, for which there is currently no approved treatment.

Results

Defining our MP Candidates and Mouse Injury Models

Over the years, many MPs have been discovered and placed within distinct families based on shared structure/function[25, 136]. The MP families SPARC, CCN, THBS, SIBLING and TN have been well-studied for their important roles in regulating repair mechanisms. We believe that the expression profiles of MP family members with known function will be indicative of the processes occurring at different stages of kidney injury repair, and/or fibrosis. Moreover, this information can be used to infer the roles of co-members with no known functions in fibrosis. Therefore, we decided to screen all 29 members belonging to these MP families. Using Pubmed and Kidney and

Urinary Pathway Knowledge Base search engines with inclusive terms "Fibrosis" and "Kidney Fibrosis", we classified our candidates into the categories of "Known in Kidney Fibrosis", "Novel to Kidney Fibrosis" or "Novel to Fibrosis" (Table 1).

We initially exploited two recently published gene expression (RNA-Seq) datasets from injured mouse kidney tissue, each representing a mechanistically different model of injury (Fig. 1), i.e. a FA toxicant model, and a UUO surgical model. As reliable kidney fibrosis models, FA is a regressive injury that permits tissue recovery, while UUO is progressive and irreversible; hence, the former has a more irregular dispersion of fibrotic tissue while the latter exhibits a more robust fibrotic response affecting the whole kidney. For the FA model, the RNA-Seq data which we analyzed was performed on total RNA from triplicate mouse kidney samples harvested at days 0, 1, 2, 3, 7 and 14 post-FA treatment (GEO# GSE65267)[137]. As for the UUO-model, RNA-Seq was performed on total RNA obtained from triplicate kidney samples after 2- and 8-days post-UUO, which was compared to 4 sham operated mice (GEO# GSE79443)[138]. Both injury models typically use 7-days post-injury as a reference point to distinguish between an acute adaptive repair response (<7-days, appearance of provisional and repaired matrix in healing process[139]) and a fibrotic outcome (≥7-days, pathological levels of repair markers[137, 140-143]), designated "Acute" and "Fibrotic" repair responses, respectively. The gene expression levels among our panel of MPs were analyzed temporally over the course of each injury stimulus, in order to compare similarities and differences between both injuries of diverse etiology.

Identification of MP Gene Expression Profiles in Folic Acid Injured Kidney

FA nephropathy is a simple injury model commonly performed on rodents using a dosage and timing with toxicokinetics comparable to those associated with human kidney diseases[140, 144]. Intraperitoneal injection (i.p.) of a high dosage of FA is known to accumulate and crystalize within the tubules of the kidney but also to have a direct toxic effect on the epithelial cells. Consequently, this results in tubular lesions and intratubular crystal-obstruction that elicit a repair response[145], accompanied by mild fibrosis into later stages. Remarkably, these same injury characteristics are found in patients transitioning into CKD, suggesting the FA-injury model to be relevant to human mechanisms[137, 146, 147]. Consistent with the FA model[53, 137, 148], the RNA-Seq dataset exhibited expected changes in gene expressions at the appropriate times for Acute (KIM-1, TGFβ at days 1-

3) and Fibrotic (collagen, fibronectin, α-sma at days 7 and 14) injury[137]. Using this time frame, we then evaluated the average fold change (relative to untreated) and absolute expression of our 29 MP candidate genes to identify those that were up- or down-regulated at Acute and Fibrotic time points of FA-induced injury (Fig. 2A-D). Absolute expression was reserved for genes whose control had no detectable expression (Fig. 2B, D).

We observed distinct dynamic and temporal trends of gene expression between MP families, but also between MP family members. Considering the trend of expression during Acute FA-induced repair (days 1-3), SIBLING and THBS genes were mostly upregulated, CCN and TN genes had mixed expression, and SPARC genes were mostly downregulated (Fig. 2A). The SIBLING gene *Spp1* was the most upregulated MP, maintaining high expression over 3-days, and peaking at over 100-fold, while the TN gene *Tnr* was the most downregulated. THBS and SIBLING were the only families with members manifesting expression exclusively after FA injury; hence, their absolute values were used to show that the THBS genes *Thbs4* and *Comp*, and the SIBLING genes *Bsp* and *Dspp*, were upregulated at some point during the 3-days of FA treatment (Fig. 2B). However, the CCN gene *Wisp2*, and SIBLING genes *Dmp1* and *Mepe*, had no detectable expression during the early stages of repair. The gene expression trend for MPs was further examined during FA-induced Fibrotic injury (days 7 and 14; Fig. 2C and D). SIBLING and THBS family members showed expression levels that were only upregulated, while CCN and SPARC family members were mostly upregulated. The TN genes tended to exhibit downregulated expression except for one member, *Tnc*, which was the second highest expressed MP next to *Spp1*. The most downregulated MP gene was from the SPARC family member *Spock1*, which was nearly 90-fold lower. Of the nondetectable MP genes from normal tissue, *Wisp2* and *Dmp1* presented a negligible increase in expression while *Mepe* still remained undetectable during late injury compared to earlier time points.

Schematically representing the similarities between Acute vs Fibrotic MP responses, gene expression levels were classified as differentially expressed (Fig. 2E and F) when they were statistically different for at least one time point using DESeq analysis with an adjusted p-value $\leq$0.05 and log2 fold-change $\geq$1.5 (Fig. 2A and C). Included in this analysis were those MP genes with a detectable expression only after injury with an absolute cut-off FPKM value of $\geq$2 (Fig. 2B

and D). Of the 29 MP genes, 13 were differentially upregulated, with none Acute, 9 Fibrotic and 4 in both phases, while 2 genes were differentially downregulated only during the Acute phase. Most of the SPARC family genes were differentially expressed during the Fibrotic MP response (*Sparcl1, Smoc2, Sparc, Fstl1*). Though upregulation of *Sparcl1* and *Smoc2* genes was classified as a Fibrotic response (Fig. 2E), their expression was differentially downregulated during the Acute response (Fig. 2F). *Thbs4, Tnc, Cyr61 and Spp1* were the only genes with at least one time point showing differential expression in both the Acute and Fibrotic phase, while no MP gene was exclusive to the Acute response. The trend of gene expression from an Acute to Fibrotic response was generally increasing for SPARC, THBS and TN families while fairly constant for the CCN family. The SIBLING family had fluctuating expression levels within both time responses, but also the only one with a member, *Mepe*, that did not have any detectable expression at any time point.

Identification of MP Gene Expression Profiles in Unilateral Ureteral Obstruction Injured Kidney

Unilateral Ureteral Obstruction (UUO) is an established mouse model of interstitial fibrosis that progressively develops from a surgical ligation of the ureter, and consequent interstitial inflammation, tubular dilation, atrophy and apoptosis[142, 143]. UUO is not a usual cause of human renal disease but a robust model of hydronephrosis[137]. Furthermore, the UUO model is well-described to recapitulate the fundamental pathological mechanisms that characterizes the various forms of CKD [149]. Gene expression changes of the repair transcriptome had the appearance of Acute injury at day 2 while Fibrosis at day 8[138]. Similar to our FA analysis, we evaluated the average fold change (relative to the control) and absolute expression of each MP family member within the UUO injury model to characterize them as Acute and/or Fibrotic repair.

Acute analysis of the MP gene expression profile from a post-UUO injury (day 2, Fig. 3A and B) indicates variability between families. Members from the SIBLING family had only upregulated gene expressions, including *Spp1*, the highest amongst all MPs. The only MPs with undetectable expression were *Tnxa, Bsp* and *Mepe*. All other MP families had expression profiles that varied in intensity among their respective members, although more MP genes were upregulated than downregulated. The most downregulated gene was the TN member, *Tnn*. Analysis of the Fibrotic phase of UUO-induced injury (day 8; Fig. 3C and D) had nearly all MP genes with upregulated

expression, while 3 genes exhibited undetectable expression (*Spock1*, *Tnr*, *Mepe*) and 6 genes had downregulated expression (*Smoc1, Spock3*, *Comp, Tnn, Tnxb, Wisp3*). In fact, 6 genes had an expression greater than a 10-fold difference (*Thbs2, Thbs4*, *Wisp1, Wisp2, Spp1*, *Dmp1*). The SIBLING family was the only one with members having undetectable mRNA levels from normal tissue (Fig. 3B and D). Of these genes, only *Dspp* showed distinct upregulation over time while *Mepe* was undetectable in both Acute and Fibrotic UUO-injury.

Differentially expressed MP genes during the progression of renal fibrosis were identified by DESeq analysis with an adjusted p-value $\leq$0.05 and log2 fold-change $\geq$1.5, or those expressed only after injury with an absolute cut-off FPKM value of $\geq$2. From the total 29 MP genes, 14 were differentially upregulated, with 11 in the Fibrotic stage and 3 in both Acute and Fibrotic stages (Fig. 3E). No genes showed a significant difference in expression that was exclusive to only the Acute stage of a UUO response. In contrast, *Tnn*, *Spock3* and *Comp* were the only MP genes found to be significantly downregulated (Fig. 3F). *Tnn* was significantly downregulated in both the Acute and Fibrotic phases of injury, whereas *Spock3* and *Comp* downregulations were restricted to the Fibrotic stage. One striking aspect of the UUO model was the number of differentially upregulated genes found only in the Fibrotic stage of injury as compared to the Acute stage, indicating distinct temporal expression. The SPARC family had the highest number of significantly upregulated genes (4 members). The expression for essentially all MP genes manifested an increasing trend from an Acute to a Fibrotic injury response, where *Tnn* was the lowest expressed gene and *Spp1* was the highest expressed gene of all MPs from both time points.

Comparing MP Expression Profiles Between Kidney Injury Mouse Models at Different Stages of Injury

To determine if MP expression is restricted to a specific mechanistic model of injury or can be generalized irrespective of the initial insult, the mRNA expression levels of MPs from the FA and UUO models were compared. Differentially expressed genes, either statistically by a DESeq adjusted p-value $\leq$0.05 or with an absolute value $\geq$2, at similar timepoints were compared between injury models: 2-days FA vs. 2-days UUO for an Acute injury analysis, and 7-days FA vs. 8-days UUO for a Fibrotic injury analysis.

In the Acute phase, we identified differential expression of 3 MP genes in the FA model (*Tnc, Sparcl1, Smoc2*), 2 in the UUO model (*Tnn, Dspp*) and 2 overlapping in both models (*Cyr61, Spp1*) (Fig. 4A). However, the Fibrotic phase had many more MP genes differentially expressed within both injury stimuli (Fig. 4B). A limited number of differentially expressed genes was found in only one of the two injury models. We identified only 2 MP genes (*Thbs1, Bsp*) restricted to FA injury, whereas 6 MP genes (*Tnn, Thbs2, Spock3, Wisp1, Wisp2, Dmp1*) were specific to the UUO model. Overall, most genes were expressed in both models (10 total; *Tnc, Thbs4, Sparcl1, Sparc, Smoc2, Fstl1, Cyr61, Ctgf, Spp1, Dspp*), indicating a conserved MP gene signature irrespective of the type of injury mechanism. All shared MP gene expressions from FA and UUO in the Fibrotic phase have the same rising trend starting from the day 2 Acute phase, except for *Spp1* which decreases in the FA model from the Acute to Fibrotic phase.

Integrated Analysis of Differentially Expressed MP Genes in Kidney Fibrosis

The Fibrotic phase experienced a larger number of differentiated MP genes than the Acute phase. So as to have a sufficient number of genes that can be organized into networks to better understand their affiliations in regulation and purpose, bioinformatics analysis was carried out on only the 29 MP genes that were differentially expressed during the Fibrotic phases of 7-day FA and 8-day UUO (Fig. 4B).

Our first level of analysis was to visualize a network of interacting relationships amongst MPs within each injury using the STRING database. A network was generated based on known protein-protein interactions with high confidence, which was set at a minimal level of 0.70 confidence (Fig. 5). Although UUO has more non-sharing MP genes than FA (Fig. 4B), the number of interactions that each gene has on average in the injury-specific network (Shared and Non-Shared) is higher in FA than UUO (higher average node number implies a higher connected network). As a result, the MP genes specific to the UUO injury were not as effective at integrating as a whole compared to the couple of genes that differed with FA.

MP genes were also associated into structured networks of Molecular Function as generated by STRING and Gene Ontology (GO). Apart from predicting the interactome between MPs for each kidney injury, our analysis also predicted the top “Molecular Function” networks from amongst

our curated MPs (Fig. 5). All predicted "Molecular Function" networks involved binding to a particular ECM molecule (i.e. heparin, fibronectin, integrin and collagen), suggesting the possibility of a binding scaffold amongst MPs when bound to their respective ECM. Although the predicted top annotations did not differ between our kidney injury models, we did reveal differences in the MP interactome that constituted each "Molecular Function" network.

As expected, our STRING and GO analyses of differentially expressed MP genes from the FA and UUO models predicted with high relevance the binding to various ECM proteins. In order to gain a generalized functional understanding of MP genes within the context of their co-expressed ECM microenvironment during the Fibrotic phase, differentially co-expressed MP (Fig. 4B) and ECM genes from the UUO or FA RNA-Seq dataset were compiled into STRING and GO analysis. For each kidney injury, the combined MP and ECM dataset permitted us to identify other potentially important interaction networks, and predict with more certainty the top "Biological Processes" from amongst the many that compose Fibrosis (Supplementary Fig. 1). Not surprisingly, the top annotation cluster (represented by node color) for both injuries was "Extracellular Structure Organization", though the UUO model had 5 MP genes (*Dmp1, Comp, Smoc2, Ctgf* and *Cyr61*)/ 55 ECM genes while FA had 4 MP genes (*Ctgf, Smoc2, Bsp2* and *Cyr61*)/ 24 ECM genes. Both injury models also shared top clusters for "Angiogenesis", "Regulation of Cell Migration" and "Regulation of Cell Adhesion" with the exception that UUO had a larger set of ECM genes. The major difference between both injuries was the additional network that FA had over UUO, which was "Regulation of Peptidase Activity". This additional network may be necessary during the remission period in the late Fibrotic FA phase when ECM deposits undergo remodeling by peptidase activity, a transition that is not achieved by UUO because of its continual injury-induced ECM production. Understandably, such top "Biological Processes" are typically involved in repair; however, our analysis also provides a cluster of MP and ECM proteins constituting each "Biological Process" network. These clusters are possibly working together within their respective intricate unit to establish their predicted "Biological Process" network. This information was combined with their interaction network (represented by interaction edges) to expose a rich source of information potentially implicated within the Fibrotic phase of their respective injury models. For instance, combining the results of our predicted "Biological Processes" with that of our various interactomes consequently reveals other probable biological processes that some molecules may

have through their association with interacting members, especially for those unclassified MP molecules (ie. Supplementary Figure 1; white nodes). Therefore, for each kidney injury we provide specific networks of MP and ECM molecules sharing various annotation clusters, and reveal the interaction networks between those statistically significant MP and ECM proteins within the "Fibrotic phase".

Overall, the differential expression of such genes may be influencing our identified pathways, leading to the predicted functional changes and disease development which are commonly observed in fibrosis.

Validating MP Expression within Acute and Fibrotic Phases and Correlating them with Renal Pathology

The RNA-Seq data from both UUO and FA injury models were validated at the level of protein expression by selecting one of the highest changes in mRNA expression profiles between models from their Acute and Fibrotic phases. As a result, we selected CYR61 and SPP1 to represent Acute MPs while SMOC2 and FSTL1 were selected to represent Fibrotic MPs. THBS2 was chosen for its novelty in kidney fibrosis, while BMP and DSPP were selected to validate novel MPs in fibrosis. We performed 250mg/kg FA or vehicle injections in C57BL/6J mice then harvested kidneys after 3-, 5- and 7-days. We also performed UUO on C57BL/6J mice and harvested their kidneys after 2- and 8-days post-UUO surgery, using the contralateral kidneys as controls. Induction of FA- and UUO-induced fibrosis in the kidney was confirmed by a significant increase in pathological tubulointerstitial fibrosis, as detected by Masson's trichrome (Fig. 6A), and the classical fibrotic marker, fibronectin (Fig. 6B). Since the ureter was tied in the UUO model, clearance from the damaged kidney to evaluate kidney function could only be performed in the FA model. Furthermore, plasma creatinine cannot measure kidney function of the UUO kidney because the unobstructed contralateral kidney compensates any loss in kidney function from the obstructed kidney[150]. Due to these limitations in the UUO model, renal function was only analyzed in the FA model, from which urine and serum were collected to confirm kidney injury by measuring serum creatinine, blood urea nitrogen (BUN) and urine protein (Fig. 6C). Our results show that the FA model had an effect on serum creatinine and BUN, implicating a deterioration in kidney function. However, we did not see a clear effect in proteinuria possibly because FA targets

the distal tubule[151], which is not involved in protein reabsorption; hence, we would not expect altered protein levels. Western blot analysis revealed that the expression of all of our candidate MP genes were significantly elevated over the course of injury, irrespective of whether it was initiated by FA administration (Fig. 7A) or UUO (Fig. 7B). Interestingly, all candidate MPs mirrored the severity of the injury, where MP expression followed the typical injury regressive and progressive patterns of FA (6A, B and C, and Fig. 7A) and UUO (Fig. 6A and B, and Fig. 7B), respectively.

Discussion

Current diagnostics and treatments for CKD are not effective for monitoring and preventing progression of the disease. Irrespective of their etiology, the majority of CKDs share the pathological characteristic of excessive ECM deposition, leading to replacement of the flexible parenchymal tissue in kidney and progressive loss of function. Although histological-based testing for the presence of ECM deposits represents the gold standard for CKD diagnosis, this painful and invasive procedure is rarely used, especially due to increased risk of complications such as hemorrhaging. Therefore, factors in the extracellular space that process ECM proteins may represent strategic targets towards impeding and/or reversing fibrosis but also allow an improved level of accessibility for therapeutic entry and detection of factors implicated in the fibrotic pathology. Since MPs meet these criteria, with the added benefit of specific expression during tissue remodeling processes (i.e. fibrosis), the current study was designed to discover the dynamic expression of commonly known MP families over the course of Acute to Fibrotic injury using RNA-Seq profiles from different fibrotic models. The presence of certain MPs in diseased tissue could serve as novel biomarkers; however, their shedding into circulation indicate their utility as non-invasive diagnostic and prognostic biomarkers. In fact, several MPs have been shown to be present in the urine of various tissue remodeling diseases, such as our selected MP candidates SMOC2, SPP1 and CYR61. We have previously shown SMOC2 within the urine of CKD patients[53], while SPP1 and CYR61 have been found within the urine of renal ischemic reperfusion injured mice[152] and prostate cancer patients[153], respectively.

We present the gene expression profiles of 29 members comprising 5 MP families. Most of these members, to the best of our knowledge, have not been previously investigated with respect to

disease progression subsequent to UUO and FA injury, and in some cases have never been studied in kidney fibrosis nor fibrosis in general (Table 1). In particular, 3 members of the SPARC family (*Sparcl1, Smoc1*), 3 from the THBS family (*Thbs2, Thbs3, Thbs4*) and 1 from the CCN family (*Wisp2*) have been studied in fibrosis but not in the context of kidney fibrosis. Furthermore, we report the expression trends of 3 SPARC members (*Spock1, Spock2, Spock3*), 4 TN members (*TnxA, TnxB, Tnr, Tnn*) and 4 SIBLING members (*Bsp, Dmp1, Dspp, Mepe*) which have not been previously described for any type of fibrotic injury.

From our list of MPs never studied in fibrosis, *Spock3, Tnn* and *Dmp1* from the UUO model, and *Bsp* and *Dspp* from the FA model were the only ones showing significant expression. *Spock3* is known for inhibiting the activity of matrix metalloproteinases[154], a large family of proteases strongly implicated in degrading a variety of ECM proteins that regulate tissue remodeling during repair. It has also been shown to be expressed by myofibroblasts, i.e. the effector cells of late stage fibrosis [155]. *Dmp1* has been studied in regulating nucleation of hydroxyapatite, which may have an effect on myofibroblast activity since hydroxyapatite drives myofibroblast activation [156, 157]. Less is known regarding *Tnn* whose functions vary with cell type. Indeed this factor inhibits proliferation and differentiation of proteoblasts [158] but stimulates tumour angiogenesis by elongation, migration and sprouting of endothelial cells [159]. It is quite conceivable that *Tnn* may have antifibrotic properties, as it is strongly downregulated over the course of fibrosis. Both *Bsp* and *Dspp* are major structural proteins of the bone matrix, but do not have any known function linking them to fibrosis and there is limited information about their tissue localization [160, 161]. Of all the MP genes that we analyzed, *Mepe* was the only one with undetectable levels in either injury model, with or without injury. These newly classified fibrotic MPs of kidney fibrosis, including those fibrotic MPs never studied in kidney fibrosis, are promising targets that merit further investigation since they complement the intricacy of repair from Acute to Fibrotic injury that has not been previously regarded.

We compared genes differentially expressed in the FA vs UUO models in order to identify MP signatures specific for each injury. FA induced a clear increase in *Thbs1* and *Bsp* expression that was not observed in UUO, and vice versa for UUO-specific *Tnn, Thbs2, Spock3, Wisp1, Wisp2* and *Dmp1*. Although *Comp* expression was differentially expressed in each injury, its upregulation

in FA and downregulation in UUO made it a part of their respective MP signature profile. Despite these genes showing significance to one type of injury, most of these genes showed a comparable trend of upregulated or downregulated expression between FA and UUO, except for *Bsp, Wisp1* and *Dmp1*. *Bsp* showed high differential expression in FA but very low expression in UUO, while both *Wisp1* and *Dmp1* showed significantly high expression in UUO but very low and negative expression in FA. As previously mentioned, *Bsp* and *Dmp1* have not been studied in fibrosis; however, *Wisp1* is known to induce collagen and fibronectin expression in podocytes[162], and its serum levels showed potential as a noninvasive biomarker of renal fibrosis in patients with CKD[163]. Our comparative analysis suggests that these injury-specific MPs could potentially serve as biomarkers that describe the etiology of fibrosis development.

FA and UUO differ considerably in their pathological mechanism, making them ideal to determine whether MP expression signatures are conserved, i.e. similar in each case, or otherwise unique to each etiology. Although the Acute phase had a low number of shared and injury specific MP signatures, these same MP genes were all found to be differentially expressed for both injury models at the Fibrotic phase. Compared to the Acute time period, it was only in the Fibrotic stages of injury where MP gene profiles were larger and could be categorized more distinctly into either UUO, FA, or both injuries; hence, a bioinformatic analysis was performed for this later phase. Our STRING analysis of differentially expressed MP and ECM genes during the Fibrotic phase identified the top predicted molecular functions and biological processes from the many that exist within the context of the ECM and repair (Fig. 5 and Supplementary Fig. 1). It also revealed a cluster of MPs for each annotation, implying a possible synergy between the combination of MPs for an effective outcome. As a whole, the bioinformatic analysis of the MP and ECM dataset is consistent with the UUO and FA fibrotic models since identified molecular functions and biological processes are well-known to be implicated in fibrosis. The shared MP expression profile between both injuries (Fig. 4B) made up most of these predicted fibrotic functions (Fig. 5 and Supplementary Fig. 1), implying their possible role in a common pathological process of kidney fibrosis. As such, it would be worthwhile to study these shared MPs as a combinatory set of therapeutic targets given the unlikelihood that targeting a single gene would lead to the complete inhibition of general fibrotic features. In fact, two of these FA and UUO overlapping MP genes, *Cyr61* and *Smoc2*, have shown therapeutic targeting potential in various models of kidney

fibrosis[53, 164, 165]. Our MP networks provide a deeper understanding of targeting a group of molecules for an effective therapeutic response in the event of a single target resistance.

Candidates for validating MP expression were selected based on their prior knowledge, and timing of expression throughout our kidney fibrotic models. SMOC2 was previously shown to be implicated in fibrosis within the FA and UUO model; hence it was used as an MP positive marker in our study. Although FSTL1, SPP1 and CYR61 were studied in other kidney injury models, herein we present their expression for the first time in the FA model. On the other hand, THBS2 have never been studied in the context of kidney fibrosis, while we present for the first time the expression of BMP and DSPP in a fibrotic setting. With respect to the timing of expression, CYR61 and SPP1 were selected to represent "Acute" MPs since their average mRNA expression between both FA and UUO were one of the highest, while that of SMOC2 and FSTL1 were higher at the Fibrotic stage. Therefore, we validated their RNA expressions and correlated them with the fibrotic pattern for each injury, which was evaluated using Masson's trichrome and the classical marker of fibrosis, fibronectin. As expected, progressive injury in UUO exhibited increasing fibronectin over time, while the regressive FA model reached peak fibronectin expression followed by a recovery period. Furthermore, kidney functional analysis on FA-treated kidneys clearly showed reduced kidney activity, with subsequent recovery. For most of our candidate MPs, protein expression levels correlated with the pattern of mRNA expression, and also with the pattern of injury progression of UUO and injury regression of FA. With the exception of the UUO model, there was a discrepancy between SMOC2 mRNA and protein expression in our FA model. Indeed, although we expected the highest expression of SMOC2 mRNA within the Fibrotic phase to translate into similar protein levels, we observed the highest protein expression in the Acute phase. This could imply a regulatory mechanism affecting protein expression, such as mechanisms of translational and/or posttranslational regulation.

The expression differences in mRNA and protein between our selected MPs may be accounted for their respective functions that collectively drive different stages of fibrosis. SMOC2 was previously shown to influence the transformation of kidney fibroblasts into myofibroblasts[53]. In the current study, SMOC2 protein expression from our UUO model gave similar higher expression in fibrosis as we previously showed using the same injury model, a time period where

myofibroblast are typically highly present[53]; However, now we provide a correlation with kidney function. In a UUO-mouse model study[166], FSTL1 has been shown to be upregulated specifically in kidney myofibroblasts after 2- and 5-days post-surgery, while our analysis was performed on the whole kidney. However, we do understand that this factor is involved in the fibrotic process in other organ models[167, 168], and in one case shown to influence the pro-fibrotic factor TGFβ in a bleomycin-induced pulmonary fibrosis model [169]. In the kidney injury models of UUO and ischemia reperfusion (I/R), CYR61 has been closely linked with proinflammatory and fibrotic roles in the early phases of injury, and its inhibition had an antifibrotic effect that could not be sustained over time[170, 171]. SPP1 has been extensively shown to be implicated in the early inflammatory phase of repair in many mouse kidney injury models, including UUO, nephrotoxic serum nephritis and diabetic nephropathy[172-174]. Taken together, each of these respective MP functions correlate with their temporal expression, as shown by our RNA and protein analysis, at which point they would be necessary for their contribution to the early repair process and eventual pathological mechanism important to the development of kidney fibrosis. In addition to validating the expression of our candidate MP genes with previous studies using the same kidney injury models, we also provide first time evidence of FST1, CYR61, SPP1 expression in the FA model, THBS2 expression in kidney-specific fibrosis, and DSPP and BMP expression in a fibrotic context, . Furthermore, we observed that such validating MP candidates had their expression correlating with the deterioration of kidney function.

In conclusion, we have provided an outline of MP gene expression patterns at various stages of kidney injury development, with predicted functional networks, that correlate and potentially mediate fibrotic progression. Such characterization of MPs in kidney fibrosis provides potential candidates to design targeted therapeutics but also identify those with early diagnostic and prognostic value.

Methods

Chemicals

Folic Acid (FA), Ponceau S, Ammonium Persulfate and TEMED were products of Sigma-Aldrich CAN. Tween20, Tris-base, Glycine, EDTA, Methanol, SDS, Acrylamide and Bis-acrylamide were obtained from VWR. All chemicals were of ACS grade or higher. Trimidox and Torbugesic were obtained from cdmv, Saint-Hyacinthe, CAN.

Animal models

C57Bl/6J mice (The Jackson Laboratory, USA), aged from 8 to 12 weeks, were housed in the animal facility at the Maisonneuve-Rosemont Hospital Research Center and were provided with Harlan Teklad rodent diet #2018 (Envigo, Lachine, CAN) and water ad libitum. All experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of laboratory animals, and under the supervision and approval of our local animal care committee, Comité de protection des animaux du CIUSSS de l'Est-de-l'ile-de-Montréal (Approved Protocol #2018-1261).

Surgery and experimental protocol

Two mouse models of kidney fibrosis were used as previously described[53]. Surgeries and injections were consistently performed at the same time of day. These models are briefly described:

FA Model. Male C57Bl/6J mice (25-29g) aged from 8 to 12 weeks received a single i.p. injection of FA (250 mg/kg) dissolved in a 0.3 M sodium bicarbonate solution. The day before sacrifice, urine was collected for 24h in metabolic cages to determine urine protein concentration. Mice were euthanized 3-, 5-, and 7-days following FA administration, for organ and blood collection. Euthanasia was performed under isoflurane anesthesia.

UUO model. Male C57Bl/6J aged from 8 to 12 weeks were anesthetized by isoflurane inhalation, and their left kidney was exposed by flank incision. The left ureter was ligated at two points proximal to the kidney with 3-0 sutures (Ethicon; Perma-hand Silk/Black Braided). The left kidney was used as a contralateral control. Mice received fluid lost replacement (1 ml normal saline, heated at 37°C, i.p.) and antibiotics immediately after surgery (Trimidox). Mice were euthanized

at 2- and 8-days following surgery for organ and blood collection. Euthanasia was performed under isoflurane anesthesia.

In both models, at the time of sacrifice, kidneys were perfused with cold PBS and immediately excised, rinsed in phosphate buffered saline (PBS) and flash-frozen in liquid nitrogen. Blood was collected for further biochemical characterization.

Biochemical parameters

Creatinine concentration in serum samples was measured with a modified enzymatic assay (CREP2, Roche Diagnostics, Canada). Briefly, samples were prepared by transferring 50µl of standard or serum to a 1.5 ml microcentrifuge tube. Proteins were precipitated and supernatant were lyophilised on a speed vac (LABCONCO freeze Dry system, VWR, Canada). Lyophilised samples were reconstituted in 25 µl deionised water and vortex mixed thoroughly. After a 30 min incubation at room temperature, samples were vortex mixed thoroughly again and then centrifuged at 11 000 x g for 5 min. 8µl of each supernatant were transferred to a half area plate (Costar #3695), in duplicate. 62µl of CREP2 R1 was added to each well. The plate was vortex mixed (MixMate, Eppendorf, Canada) at 1000 rpm 30 sec, and incubated 15 min at 37˚C to allow endogenous creatinine degradation. Readings at 405 nm and 540/630nm were performed and 31µl of CREP2 R2 was then added to each well. The plate was vortex mixed at 900rpm 30sec. Readings were performed on a kinetic mode, each minute for a 30 minutes period (ELx808, BioTek, USA).

Serum urea was measured with the Quantichrom Urea Assay Kit (BioAssay Systems, Hayward, CA) according to manufacturer's instructions. Urinary Creatinine and urine proteins were measured on an Architect c16000 clinical chemistry analyzer (Abbott Diagnostics, IL, USA), using a kinetic alkaline picrate method and a turbidimetric method respectively.

Tissue preparation for Western Blot analysis

Frozen biopsies of mice kidney were homogenized in RIPA buffer (Pierce, ThermoFisher, Canada) containing 1x protease and phosphatase inhibitor cocktail (Roche, Sigma, Canada) using an overhead stirrer (IKA). Protein concentration was determined using the BCA method (Pierce, ThermoFisher, Canada).

Western Blotting

Protein levels were assessed by Western blot analysis. Samples of 10 to 50 µg of protein were separated by electrophoresis on a 10% polyacrylamide gel containing 0.4% sodium dodecyl sulfate (SDS) and were electrophoretically transferred onto nitrocellulose membrane (Amersham Protran 0,45µm, GE Healthcare Life science, Mississauga, Canada). Membranes were saturated with 5% NFDM in Tris buffered saline (TBS) containing 0.1% Tween20 (TBST) and washed with TBST. The following primary antibodies were used to detect the specific protein: anti-SMOC2 (1:200; R&D Systems, AF5140), anti-CYR61 (1:2,000; aka CCN1 R&D Systems, AF4055), anti–FSTL1 (1:10,000; R&D Systems, AF1738), anti-SPP1 (1:10,000; aka OPN R&D Systems, AF808), anti-THBS2 (1:1000; Santa Cruz, sc-136238), anti-DSPP (1:750; Santa Cruz, sc-73632), anti-BSP (1:750; Santa Cruz, sc-73630) and anti-GAPDH (1:5,000; Abcam, ab9485). Horseradish peroxidase–conjugated secondary antibodies against sheep (Sigma, A3415), goat (Sigma, A5420) and rabbit (Santa Cruz Biotechnology, sc-2357) were used to detect the appropriate primary antibody. Bands were detected with the Clarity Max Western ECL Substrate from Bio-Rad Laboratories (Hercules, USA). Results were analyzed by computer-assisted densitometry using ImageQuant LAS-4000 system from GE Healthcare Life Sciences (Mississauga, CAN), ImageJ and FUJIFILM MultiGauge V3.0.

RNA-Seq Database and Bioinformatics analysis

We processed RNA-Seq data in the following procedure. Raw read counts from the FA (GSE65267)[137] and UUO (GSE79443)[138] mouse models were retrieved from the Gene Expression Omnibus (GEO) database repository (http://www.ncbi.nlm.nih.gov/geo/). Transcripts with a read count lower than 1 in all samples were removed. Differentially expressed (DE) transcripts were identified in each time point using DESeq2 package[175] implemented in R with default arguments. Differentially expressed MP transcripts were those containing a minimal log-fold change of $\geq$1.5 and adjusted p-value $\leq$0.05, or an absolute FPKM value of $\geq$2 when controls had no mRNA detection. To identify extracellular molecules, we used the clusterProfiler[176] R package on only differentially expressed genes (log-fold change of $\geq$1.5 and adjusted p-value $\leq$0.05) belonging to the GO term: extracellular space (GO:0005615). Ontology terms with an FDR $\leq$0.05 were considered significant.

Members of the MP families were characterized for their known involvement in fibrosis. Searches were performed using the databases PubMed, and Kidney and Urinary Pathway Knowledge Base (KUPKB) for each of our 29 MP gene candidates in combination with the inclusion key words "fibrosis" or "kidney fibrosis". Results for each MP were tabulated as being either involved in fibrosis that includes kidney fibrosis, fibrosis that excludes kidney fibrosis or not involved in any fibrosis.

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)[177] was used to visualize network interactions within our MP candidate genes based on databases of known and predicted protein interactions. GO was used as a complementary tool within STRING to identify networks of molecular functions and biological processes.

Statistical analysis

Results are expressed as mean ± standard error. Statistical significance for multiple comparisons was calculated by two-tailed Student's *t* test with a Bonferroni correction ($p \leq 0.05/n$, where n=sample size). Statistical analyzes were performed with GraphPad Prism v6.05.

Competing Interests

The authors declare no competing interests.

Contributions

C.G. conceived and designed this study. C.N., D.F. and N.H. performed the experiments and analyzed results. N.B. and C.G. conducted bioinformatics analysis of data. C.G. wrote the manuscript. All authors provided constructive comments and edits, and approved the final version of this manuscript.

Acknowledgements

Work from the Gerarduzzi laboratory is supported by the KRESCENT New Investigator Award, CIHR/KRESCENT Infrastructure Grant, Hôpital Maisonneuve Rosemont Foundation Grant and NSERC Discovery Grant.

Data Availability

The datasets analysed during the current study are available in the Genome Expression Omnibus Database (http://www.ncbi.nlm.nih.gov/geo/). Folic Acid (GEO# GSE65267) and UUO (GEO# GSE79443).

	Known in Kidney Fibrosis	Novel to Kidney Fibrosis	Novel to Fibrosis
SPARC family	Sparc Smoc2 Fstl1	Sparcl1 Smoc1	Spock1 Spock2 Spock3
Thrombospondin family	Thbs1 Comp	Thbs2 Thbs3 Thbs4	
Tenascin family	Tnc		TnxA TnxB Tnr Tnn
CCN family	Cyr61 Ctgf Nov Wisp1 Wisp3	Wisp2	
Sibling family	Spp1		Bsp Dmp1 Dspp Mepe

Table 1: Listing of candidate MP genes based on published fibrotic relevance within *in vitro* and/or *in vivo* models.

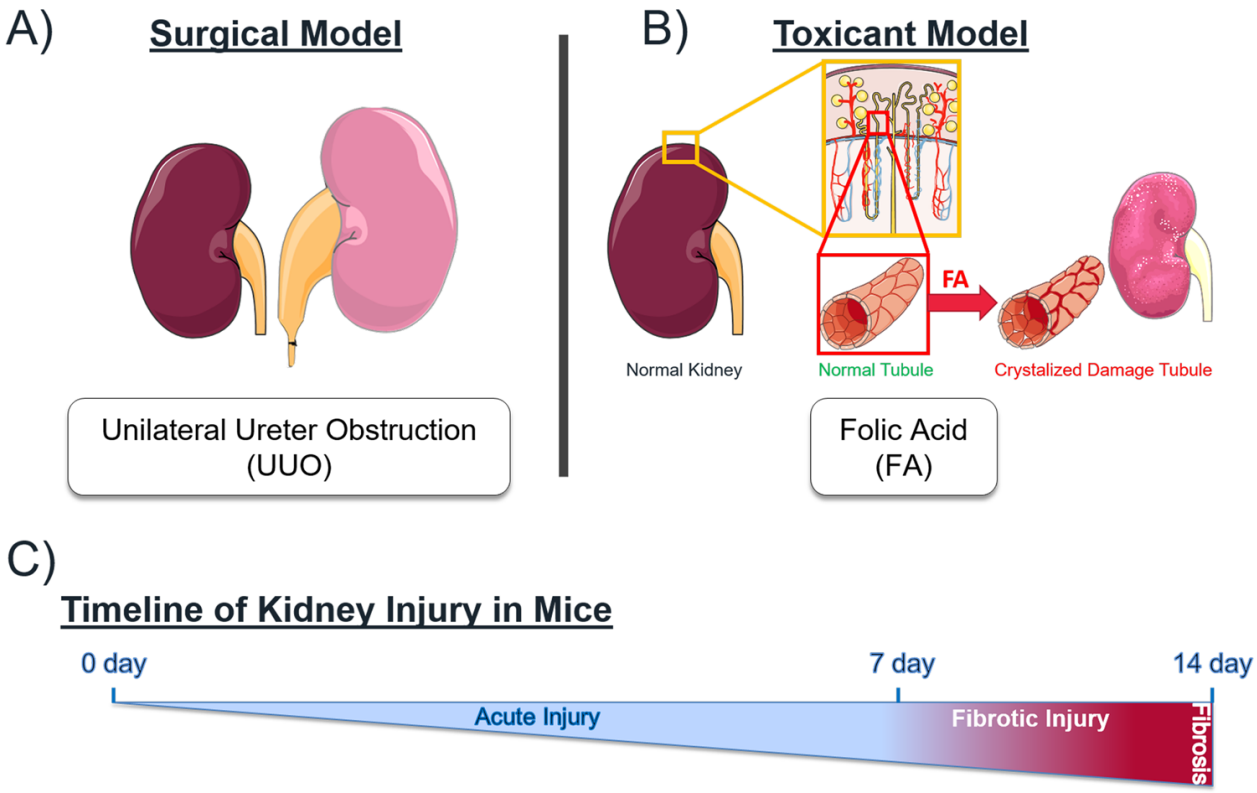


Figure 1: Mechanistically distinct mouse models of kidney injury. A) Unilateral Ureteral Obstruction by surgically tying the urinary tract leading to tubular atrophy, interstitial fibrosis and inflammation. **B)** Folic Acid administration by intraperitoneal injection results in folic acid crystal formation within the renal tubules with subsequent acute tubular necrosis/apoptosis, inflammatory cell infiltration, tubular cell proliferation, epithelial regeneration and mild fibrosis in the chronic phase. **C)** Timeline of most persistent injuries which includes an acute and fibrotic injury. Figure was produced using Servier Medical Art (http://smart.servier.com/).

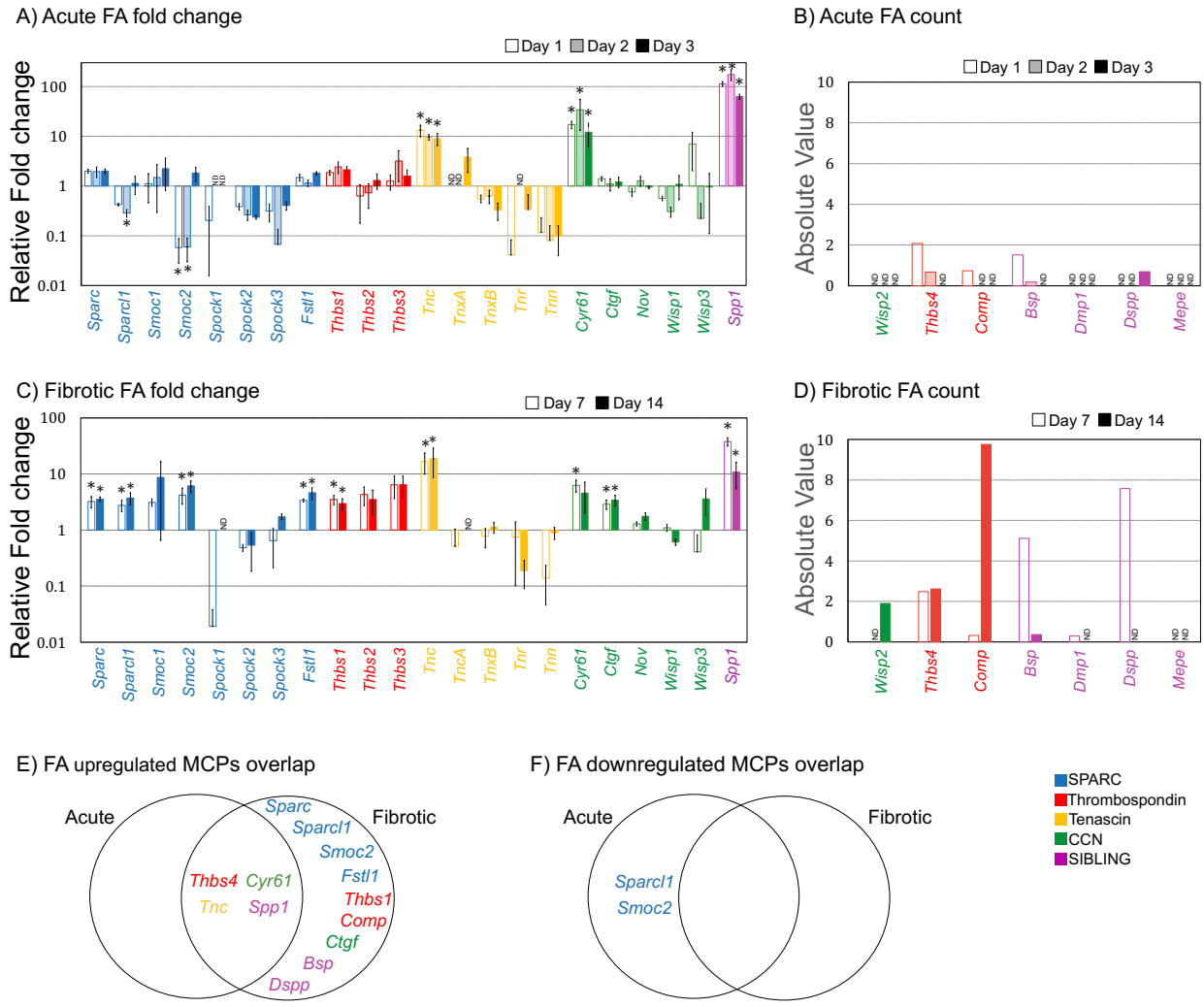


Figure 2. MP expression in mouse kidney at Acute and Fibrotic time points after FA treatment. RNA-Seq fold change (relative to untreated) and absolute value of MCP mRNA expression from mouse kidneys following a single intraperitoneal injection of 250 mg/kg FA: (**A**) Fold change and (**B**) absolute value of mRNA expression from candidate MCPs at Acute injury time points 1-, 2- and 3-days following injection. (**C**) Fold change and (**D**) absolute value of mRNA expression from candidate MCPs at Fibrotic injury time points 7- and 14-days following injection. *DESeq $p \leq 0.05$, $n = 3$ mice. Venn diagram of MCPs that are differentially (**E**) upregulated and (**F**) downregulated between Acute injury (1-, 2- and 3-days) and Fibrotic injury (7- and 14-days).

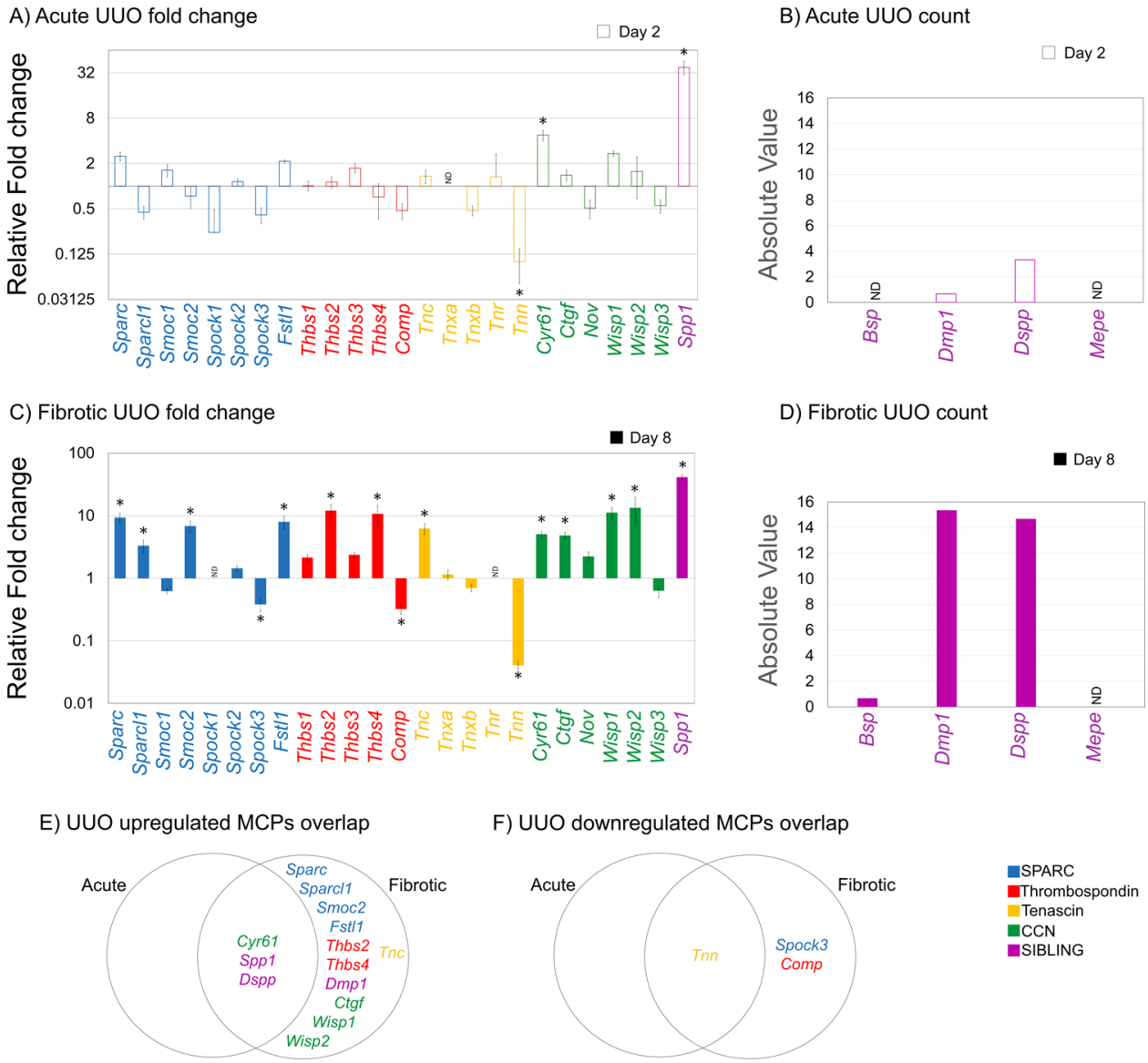


Figure 3. MCP expression in mouse kidney at Acute and Fibrotic time points after UUO surgery. RNA-Seq fold change (relative to the control) and absolute value of MCP mRNA expression from mouse kidneys following UUO surgery: (**A**) Fold change and (**B**) absolute value of mRNA expression from candidate MCPs at Acute injury time points 2-days following surgery. (**C**) Fold change and (**D**) absolute value of mRNA expression from candidate MCPs at Fibrotic injury time points 8-days following surgery. *DESeq $p \leq 0.05$, $n = 3$ mice. Venn diagram of MCPs that are differentially (**E**) upregulated and (**F**) downregulated between Acute injury (2-days) and Fibrotic injury (8-days).

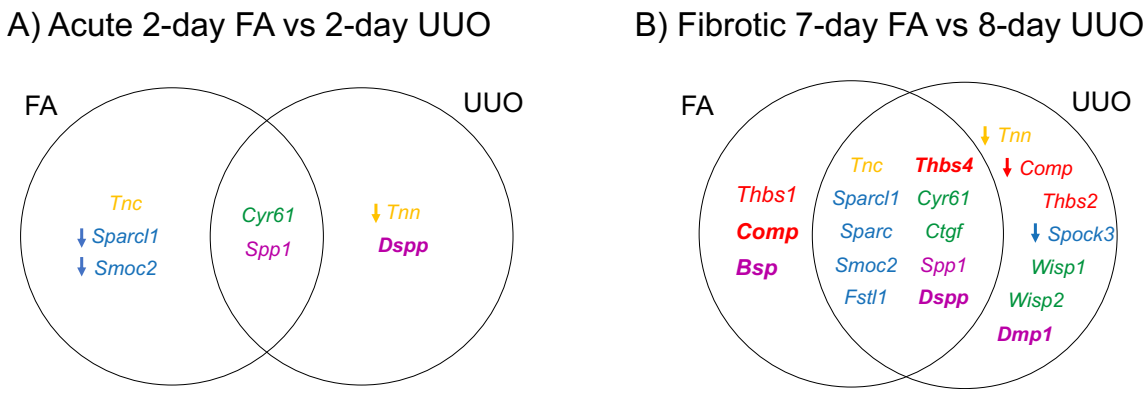
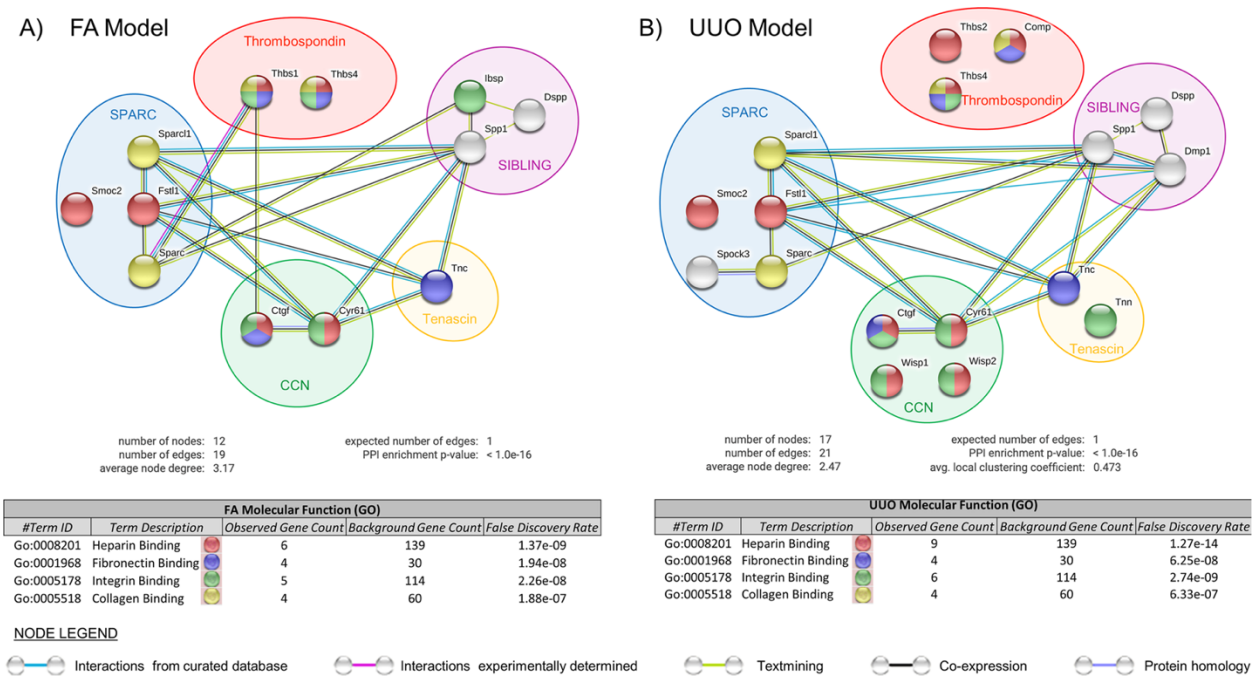


Figure 4. Differential expression of MP mRNA at different time-points between FA- and UUO-induced injuries. Candidate MP genes with significant expression found in either the **A)** Acute phase or **B)** Fibrotic phase were compared between FA and UUO injuries. Listed genes were considered to be differentially expressed if they had a DESeq analysis with an adjusted p-value $\leq$0.05 and log2 fold-change $\geq$1.5, or an absolute FPKM value $\geq$2 when controls had no detection (**bold** and *italicized*). All genes are upregulated unless specified with a downward arrow to indicate downregulation.



FA Molecular Function (GO)

#Term ID	Term Description	Observed Gene Count	Background Gene Count	False Discovery Rate
Go:0008201	Heparin Binding	6	139	1.37e-09
Go:0001968	Fibronectin Binding	4	30	1.94e-08
Go:0005178	Integrin Binding	5	114	2.26e-08
Go:0005518	Collagen Binding	4	60	1.88e-07

UUO Molecular Function (GO)

#Term ID	Term Description	Observed Gene Count	Background Gene Count	False Discovery Rate
Go:0008201	Heparin Binding	9	139	1.27e-14
Go:0001968	Fibronectin Binding	4	30	6.25e-08
Go:0005178	Integrin Binding	6	114	2.74e-09
Go:0005518	Collagen Binding	4	60	6.33e-07

Figure 5: Interaction analysis of differentially expressed MP genes within the fibrotic phase of kidney injury using the database STRING. Identification of interaction networks and top predicted molecular functions between our candidate MP genes that were differentially expressed in the fibrotic phase of the **A)** FA (7-day) and **B)** UUO injury (8-day) models, using the search tool STRING. Using the Cluster profiler R package, listed genes were considered differentially expressed since they had either a DESeq analysis with an adjusted p-value $\leq$0.05 and log2 fold-change $\geq$1.5, or an absolute FPKM value $\geq$2 when controls had no detection. Colour codes of nodes are based on the annotation term for each "Molecular Function", and interaction edges are based on a minimal confidence of 0.7 using sources of textmining, experiments, databases and co-expressions.

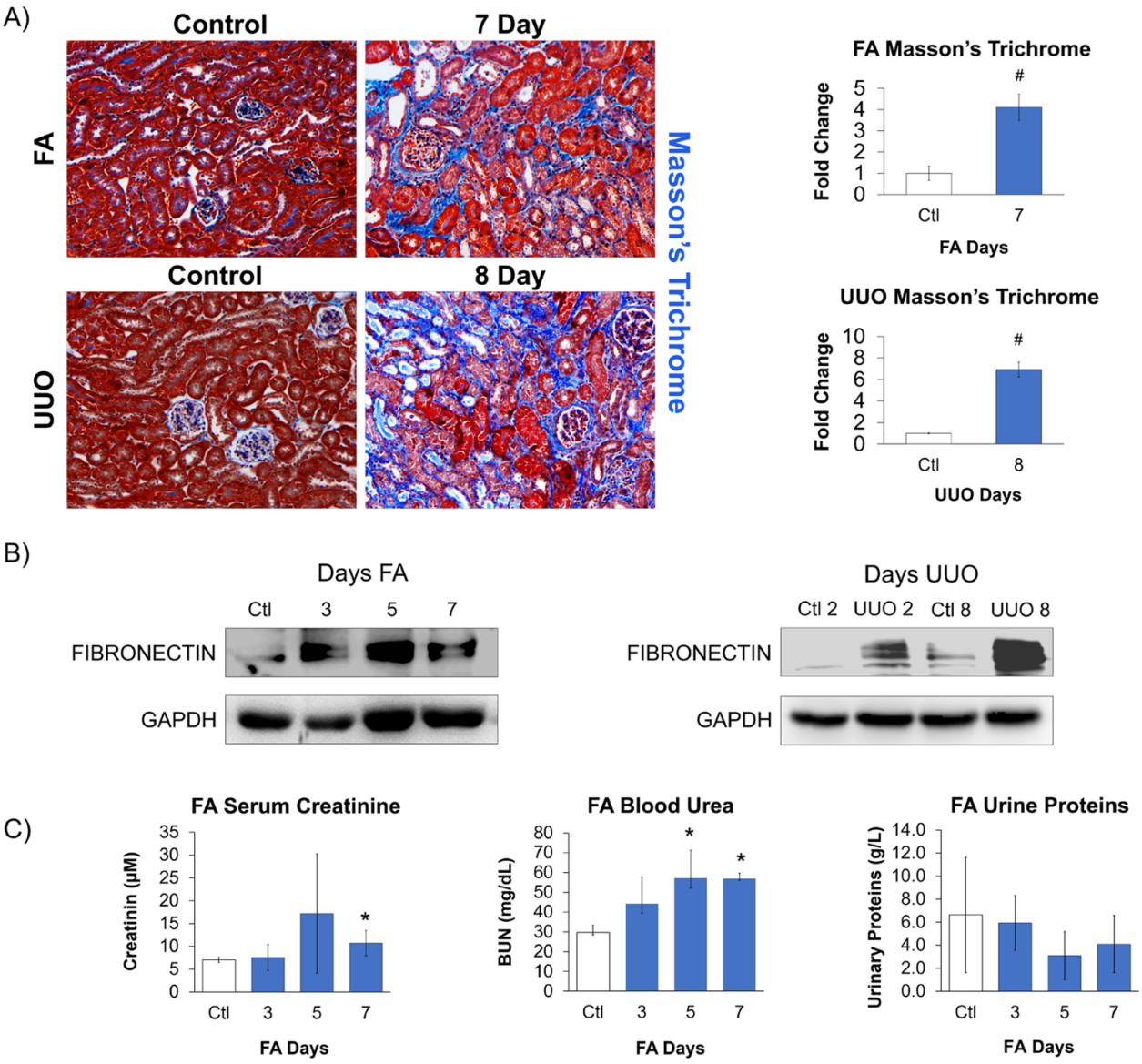


Figure 6. Confirmation of kidney injury within mouse models. Mice were subjected to FA or UUO kidney injury, and kidneys were harvested for **A)** Masson's trichrome staining and **B)** Western blot analysis for fibronectin to confirm kidney injury. Images are representative of Masson's trichrome staining (3-5 visual fields/tissue sample) and Western blots. Masson's trichrome was expressed as a fold change relative to their respective controls. **C)** Kidney function was evaluated in the FA mouse model by measuring serum creatinine, blood urea nitrogen (BUN) and urine protein at time points 3-, 5- and 7-days post FA administration. FA and UUO mice were the same used as in Figure 7. Compared to their respective controls, statistical significance was determined using a *t* test with a Bonferroni correction; # $p \leq 0.017$, n=3; and * $p \leq 0.0125$, n=4.

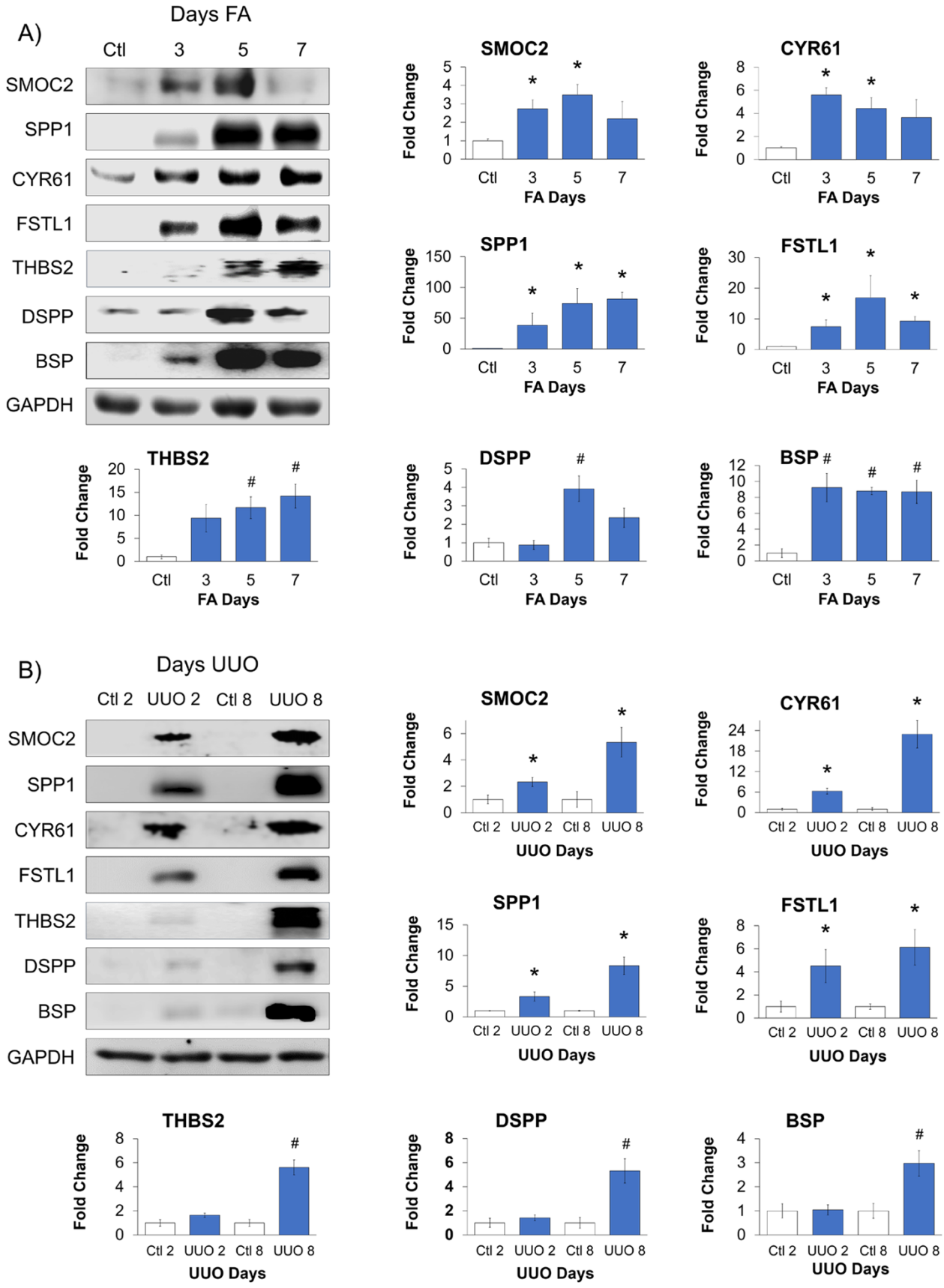
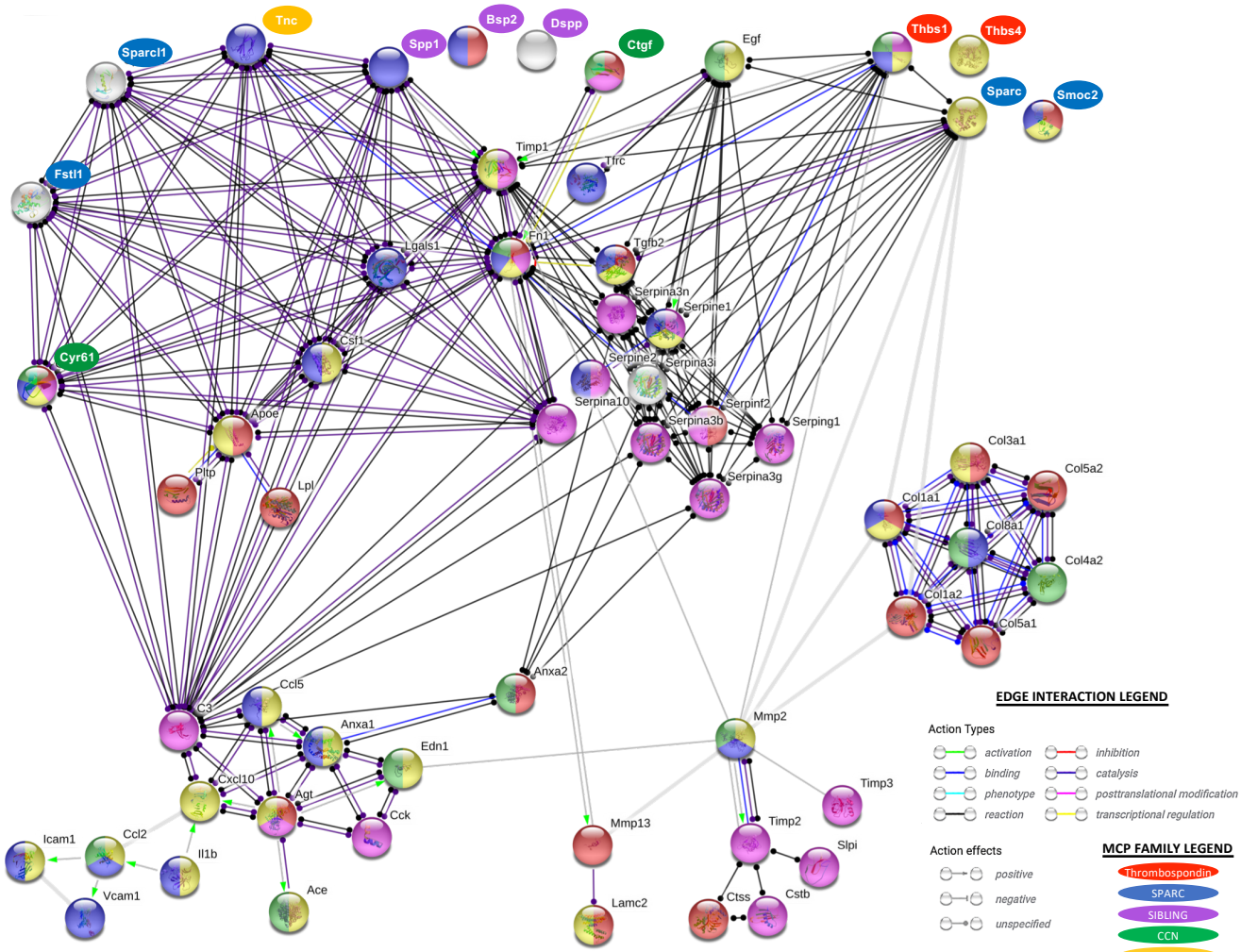

A)
Days FA
Ctl
3
5
7
SMOC2
SPP1
CYR61
FSTL1
THBS2
DSPP
BSP
GAPDH
SMOC2
CYR61
SPP1
FSTL1
THBS2
DSPP
BSP
Fold Change
FA Days
B)
Days UUO
Ctl 2
UUO 2
Ctl 8
UUO 8
UUO Days

Figure 7. Validating RNA-Seq data of selected MPs from kidney injury models. Mice were subjected to **A)** FA or **B)** UUO kidney injury and lysates were tested for selected candidate MPs SMOC2, SPP1, Cyr61, Fstl1, THBS2, DSPP and BSP. Western blots are representative images with accompanying quantification analysis of n=3-4 mice. Each blot was normalized to GAPDH and expressed as a fold change compared to **A)** FA control (Ctl), **B)** day 2 control (Ctl 2) vs day 2 UUO (UUO 2) or day 8 control (Ctl 8) vs day 8 UUO (UUO 8). Statistical significance was determined using a *t* test with a Bonferroni correction; # $p \leq 0.017$, n=3; and * $p \leq 0.0125$, n=4.

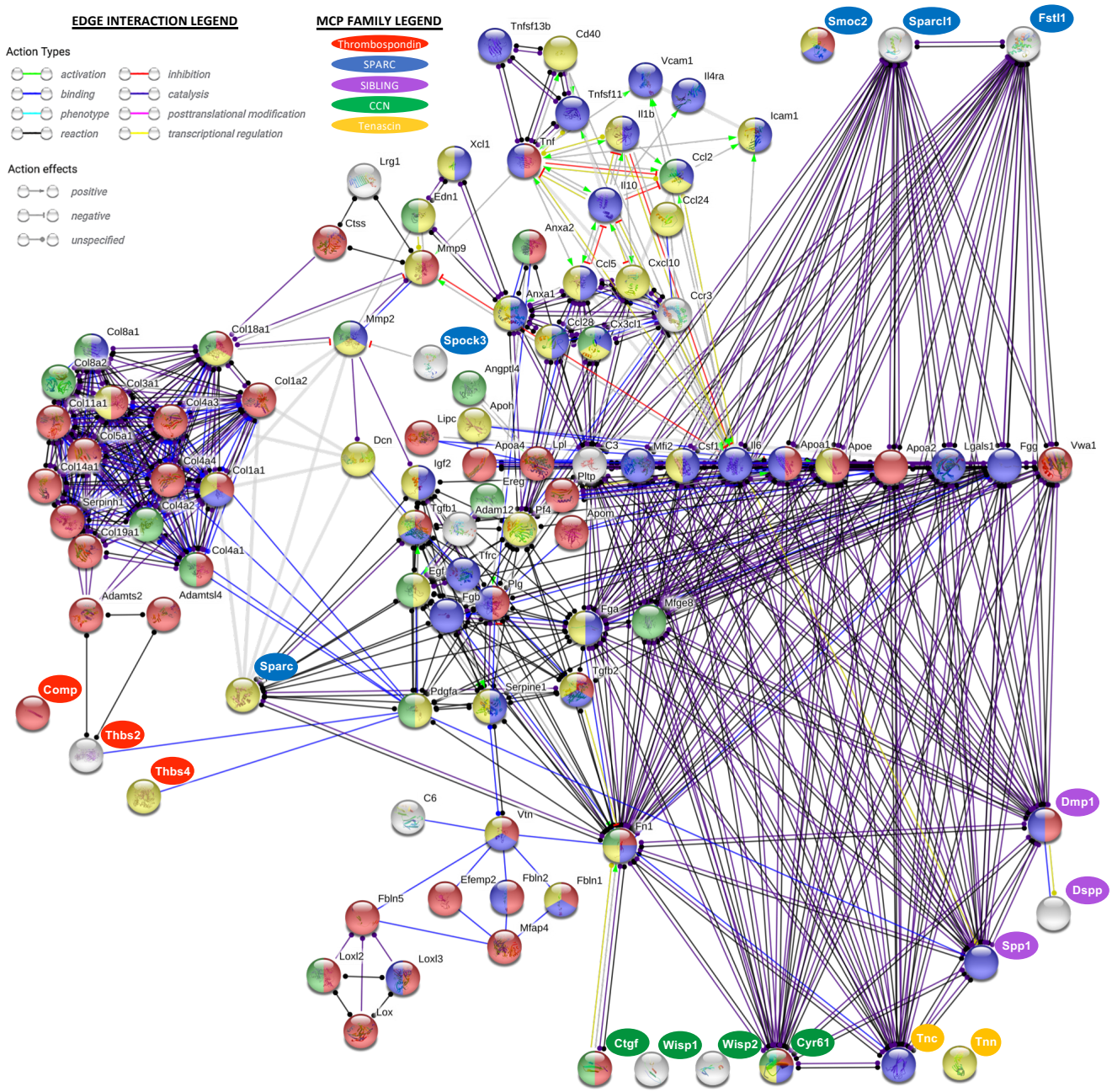
A)



Biological Process (GO)					
#term ID	*term description*	*observed gene count*	*background gene count*	*false discovery rate*	*node color*
GO:0030198	extracellular structure organization	27	214	9.73E-30	
GO:0052547	regulation of peptidase activity	27	416	4.18E-23	
GO:0030334	regulation of cell migration	32	805	4.80E-22	
GO:0030155	regulation of cell adhesion	27	624	1.34E-19	
GO:0001525	angiogenesis	17	303	8.25E-14	
	UNKNOWN				

Suppl. Figure 1. (Continued on next page)

B)



Biological Process (GO)					
#term ID	*term description*	*observed gene count*	*background gene count*	*false discovery rate*	*node color*
GO:0030198	extracellular structure organization	60	214	1.43E-75	
GO:0030155	regulation of cell adhesion	53	624	1.76E-41	
GO:0030334	regulation of cell migration	52	805	2.32E-35	
GO:0001525	angiogenesis	30	303	1.18E-24	
	UNKNOWN				

Suppl. Figure 1. Functional networks of significantly regulated MCP and ECM genes within the Fibrotic phase of kidney injury using the STRING database. The search database R was used to identify top functional networks between genes with expression values that were significantly different within the Fibrotic phase of the **A)** FA (7-day) and **B)** UUO injury (8-day) models. Listed genes were considered significantly different since expression was either statistical with a $p \leq 0.05$ or had an absolute FPKM value ≥ 2 when controls had no detection. Colour codes of nodes are based on the annotation term for each "Biological Process", and interaction edges are based on a confidence of 0.9 using sources of textmining, experiments, databases and co-expressions. Candidate MCP genes are within a coloured ellipse while ECM genes are in black font.

2.3 Discussion of Manuscript - SMOC-2 as a Candidate MP

Dysregulated tissue remodelling is a major cause and hallmark of many pathologies that ultimately perturbs the delicate biochemical and biophysical balance of the ECM and is a driving force in both fibrosis and metastatic cancer development. In tissue injury and matrix remodeling diseases, the pathological secretome is a unique signature that reflects the dysregulated systems involved. Finding patterns of expression between injuries of different etiologies is important to uncovering molecules that can serve as reliable markers for diagnosis and broad targeting therapeutics.

One relatively novel and understudied protein of the SPARC family, SMOC-2, has previously been described in the context of renal fibrosis and shown to be capable of attenuating fibrosis progression in mice[53]. In the present study, we show that SMOC-2 expression was found to be downregulated during the early acute timepoint but significantly upregulated during the fibrotic phase on injury in both the FA and UUO mice models of renal injury.

SMOC-2 expression was observed to be downregulated in the early or acute stages of repair, defined as Day 1-3 in FA (Figure 2A) and Day 2 in UUO (Figure 3A). However, by Day 7 in FA, SMOC-2 expression is drastically upregulated 6-fold and further reached nearly 10-fold at Day 14 (Figure 2B), suggesting that it is involved in the latter stages of remodeling rather than a first-responder in repair. A similar observation is made in the UUO model in which SMOC-2 expression is initially downregulated at Day 2 but increases approximately 9-fold by Day 8 (Figure 3B) compared to control. The delayed upregulation of SMOC-2 is also consistent with reports of its involvement in pathologies with chronic matrix production and remodeling such as fibrosis[53] and tumor metastasis[54] in which the accumulation of ECM proteins and persistent maladaptive signaling drive progression of such diseases. Although not done in the current study, daily tracking of SMOC-2 expression would be an informative supplement to understanding the temporal patterns on a more precise scale.

To validate the mRNA expression of SMOC-2, Western blot was used to confirm protein levels in both models. In the FA model, SMOC-2 protein expression gradually increased from

Day 3 till Day 5 but saw a downregulation at Day 7 that differed from the mRNA profile (Figure 7A). This could imply a regulatory mechanism affecting protein expression, such as mechanisms of translation and/or post-translational regulation. In the UUO model, SMOC-2 protein expression was in agreement with the trend of mRNA expression increasing from Day 2 to Day 8 (Figure 7B). The more destructive nature of injury in the UUO model may be an explanation for the sustained expression of SMOC-2 that overcomes any regulatory mechanisms as a result of irreversible damage.

In summary, work from my first manuscript confirmed expression trends for SMOC-2 in both FA and UUO kidney injury models that is consistent with the literature. Our work highlights many other novel MPs that were not previously implicated in kidney fibrosis that could warrant further characterization by a similar approach as taken with SMOC-2.

3.0 Understanding the Role of SMOC-2 in Renal Cell Carcinoma

3.1 Preface

The manuscript of Chapter 2 serves as a rationale to address Aim 2 of my thesis, presented herein as Chapter 3. This work is currently being assembled into a manuscript format for submission.

We look to Renal Cell Carcinoma (RCC) to study the involvement of related pathologies as well as being a suitable model for studying tissue injury and dysregulated matrix remodeling. Our approach to bridging the concept of aberrant ECM remodeling in fibrosis to cancer is through studying MPs, whose ability to regulate cell-cell and cell-matrix interactions make them an attractive research target to understanding parallel mechanisms. Our objectives are to evaluate if MPs from the tissue remodeling processes of repair is preserved with that found in cancer using SMOC-2 as our MP candidate.

3.2 *Manuscript in Preparation*

SMOC-2 Promotes Epithelial Mesenchymal Transition and a pro-Metastatic Phenotype in Epithelial Cells of Renal Cell Carcinoma Origin

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[2]Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Faculté de Médecine, Centre affilié à l'Université de Montréal, Montréal, Québec, Canada
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*Corresponding author

Key words: Matricellular Protein, Epithelial Mesenchymal Transition, Extracellular Matrix, Renal Cell Carcinoma, Cancer Metastasis.

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3.3 Introduction

Kidney cancers are the tenth highest occurring cancers in Canada, with Renal cell carcinoma (RCC) constituting 90% of all reported cases (reference: Canadian Cancer Society, Canadian Cancer Statistics, 2017). A higher incidence rate is observed in males versus females in developed countries worldwide (12.4/6.2 per 100,000 individuals) and is more common in males over age 60, with the median age between both genders at 65 years of age[178]. Beyond sporadic cases, the most common risk factors for developing RCC include, but are not limited to, cigarette smoking, hypertension, obesity and diabetes[179].

Upon first diagnosis, 25-30% of patients show metastatic disease most commonly affecting the lungs, bone and liver, and consequently have only an overall median survival rate of less than one year[180]. Imaging techniques such as CT, MRI and ultrasonagraphy remain the primary method of detection due to the wide range and non-specificity of symptoms presented in early stage RCC patients[181]. Thus, patients are often are mis-diagnosed and left untreated until symptoms worsen and warrant further diagnostic testing, and are often times already within an advanced stage. First line treatment options involve partial or total nephrectomy of the kidney at early stages if possible, but approximately 30% of patients relapse with an aggressive metastatic form of RCC[181]. Treatment of metastatic RCC with standard chemotherapy options has been shown to be ineffective and unresponsive but more targeted therapies such as Bevacizumab, Sunitinib and Sorafenib show improved efficacy and extend progression free survival in advanced stage metastatic RCC patients[180].

RCC tumors originate primarily from the tubules of the kidney, specifically the tubular epithelial cells which are predominately the injured cell type[182]. These injured malignant epithelial cells are the main perpetrators of metastatic disease they can undergo EMT to gain increased migratory ability and contribute to the aggressive behavior of epithelial tumor cells[183]. An important driver of cancer progression and initiator of EMT is the dynamic remodeling occurring in the surrounding tissue and tumor stroma. The disturbed tissue homeostasis in the surrounding stroma is necessary to recruit cells to the tumor site to accelerate growth and potentiate a metastatic phenotype[184].

There is an unmet need for alternative approaches to help manage and contain metastatic spreading of primary epithelial carcinomas. MPs present themselves as attractive targets because of their extracellular localization, context dependent expression and documented roles in cell proliferation, migration, invasion and ECM remodeling which together can prime the tumor niche and promote EMT to drive cancer metastasis. Therefore, they are appealing molecules to target in hopes of repressing pro-metastatic processes.

From previous studies in the lab, we had come across SMOC-2 as a potential candidate MP whose expression is found to be highly upregulated in the context of renal fibrosis. Our understanding of SMOC-2 has been steadily growing since the first reports of its function[49], yet it remains a understudied MP with a wide array of functions from regulation of cell cycle[59], development[51, 52], angiogenic growth factor activity[60], fibrosis[53] and keratinocyte cell adhesion and migration[51]. Having shown the role of SMOC-2 in repair, we wished to further extend our work and explore translatable mechanisms in multi-faceted pathologies such as cancer and tumor metastasis, as the diverse functions mediated by SMOC-2 make it an attractive target. Recent reports have shown SMOC-2 to be implicated in colon cancer metastasis[54], endometrial cancer[185] and hepatocellular carcinoma[186], however, it has never been shown in RCC. In this study, we demonstrate that SMOC-2 modulation is can promote EMT in human RCC cells (786-O, ACHN) that lead to a more invasive-metastatic phenotype.

3.4 Materials & Methods

3.4.1 Cell Culture and Media

786-O, ACHN and HK-2 cells were maintained according to ATCC protocols. Cells were sub-cultured every 2-3 days with 0.25% Trypsin-EDTA (Gibco #Cat 25200-072).

786-O cells were maintained with RPMI supplemented with 10% FBS (Gibco #Cat 11360-070), 1mM sodium pyruvate (Gibco #Cat 11360-070), 10.01mM HEPES (Calbiochem #Cat 391338), 25mM D-glucose (EMD #Cat DX0145-1) and 1% Penicillin-streptomycin (Sigma #Cat P4333-100ML).

ACHN cells were maintained with DMEM (Gibco #Cat 11995-065) and supplemented with 10% FBS (Gibco #Cat 12483-020) and 1% Penicillin-streptomycin (Sigma #Cat P4333-100ML).

HK-2 cells were maintained in DMEM/F12 (Gibco #Cat 11330-032) and supplemented with 10% FBS (Gibco #Cat 12483-020) and 1% Penicillin-streptomycin (Sigma #Cat P4333-100ML).

Cells were maintained in a humidified 5% CO_2.

3.4.2 Histology & Immunostaining

Deidentified human kidney tissue samples from patients with RCC (n=4) were obtained from the Department of Pathology at HMR. Paraffin-embedded tissues were cut into 4-6-µm sections and processed for immunofluorescence and H&E staining.

For immunofluorescence, antigen retrieval was performed in citrate solution pH=6. The sections were then labeled with anti-SMOC2 (1:100; R&D Systems, MAB5140) and anti-Vimentin 1:1000 (Sigma #HPA001762). Slides were subsequently exposed to donkey anti-mouse specific Cy3-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, 711-165-150) and donkey anti-rabbit specific AF647-conjugated (1:200 Jackson ImmunoResearch Laboratories, 711-605-152). 4,6-Diamidino-2-phenylindole (Fluoroshield with DAPI, Sigma #F6057-20ml) was used for nuclear staining. Images were acquired in the Imaging Facility at HMR. Brightness and contrast were adjusted on displayed images (identically for compared image sets) and quantified (identical threshold settings for compared image sets) using ImageJ.

3.4.3 SMOC-2 Transfection and Recombinant Protein Treatment

Overexpression

For SMOC-2 overexpression, cells were seeded overnight in a 6-well dish and transfected with 1ug of Myc-SMOC-2 (Origene, #Cat RC211979) or Myc-empty vector with JetPrime transfection reagent (Polyplus, #Cat114-15/1.5ml) following manufacturerers protocol for 24-72h. Media was replaced 4-5h post transfection. Myc-empty vector was prepared in-house using the Myc-SMOC-2 vector by restriction enzyme digestion to remove the SMOC-2 fragment.

Silencing

For SMOC-2 knockdown, cells were reverse-transfected in a 6-well dish with either 80 pmole of ssiRNA or SMOC-2 siRNA (SantaCruz, #Cat sc-63046) using JetPrime transfection reagent (Polyplus, #Cat 114-15/1.5ml) following manufacturer protocol for 6h, followed by a 5ng/mL treatment with TGFβ (Preprotech #Cat100-21C) for 15-20h.

Recombinant protein

For SMOC-2 recombinant protein treatment, cells were plated overnight in 6-well dish and treated with 10ng/mL of recombinant SMOC-2 protein (R&D Systems #Cat 5140-SM-050) or PBS vehicle diluted in fresh media. Media was replaced every 24h with fresh recombinant protein or vehicle for the duration of the experiment.

Cell Harvesting

Cells were harvested with ice-cold RIPA buffer (Thermo Fisher Scientific, #Cat 89901) containing 1X protease and phosphatase inhibitor cocktail (Roche Complete Ultra tablets #Cat 0589279100 and Roche PhosSTOP tablets #Cat 0490683700). Cell lysates were mechanically disrupted using a handheld potter and centrifuged to pellet cellular debris and protein fraction stored at -80^{o}C until use. Protein concentrations were determined by the BCA method (Pierce BCA protein assay kit, #Cat 23225) and absorbance were read at 540nm (Biotek plate reader).

3.4.4 Western Blotting

Equal protein amounts were prepared for gel electrophoresis with 3X Lameli's sample buffer and loaded onto 10% acrylamide gel for separation. Proteins were further transferred onto a 0.45µm nitrocellulose membrane (Biorad, #Cat 1620115). Proteins were revealed using the following primary antibodies: SMOC-2 (1:500, Abcam, #Cat ab56088), Myc (1:1000, Cell Signalling Technology #Cat 2276), E-cadherin (1:1000, Abcam # Cat ab15148), Fibronectin (1:5000, Abcam #ab23750), -α-Smooth Muscle Actin (1:2000, Sigma #Cat A2547), Vimentin (1:3000, Santacruz, #Cat Sc-6260), GAPDH (1:5000, Abcam #ab8485). Western blots were developed by horseradish peroxidase–conjugated secondary antibodies against mouse (SantaCruz sc-516102) and rabbit (SantaCruz sc-2357) with ECL solution (BioRad Clarity and Clarity Max#Cat 1705061 and #Cat 1705062). Bands were detected and captured using the LAS-3000 imaging system (Fujifilm Life Science).

3.4.5 Functional Assays

MTT Assay

For overexpression of SMOC-2, cells previously transfected were lysed and re-seeded into 96 adherent culture plates and let adhere, after which they were serum deprived (1% serum in respective media). For recombinant SMOC-2 treatment, cells were pre-treated for the desired time and then re-seeded into 96 well adherent culture plates and let adhere, after which they were serum deprived (1% serum in respective media). For silencing of SMOC-2, cells previously transfected were lysed and re-seeded into 24 well adherent culture plates and let adhere, after which they were serum deprived (1% serum in respective media).

After 3h of serum starvation, culture media was carefully aspirated and replaced with 100uL of fresh complete media and 20uL of MTT (5mg/mL) (Thiazolyl blue tetrazolium bromide VWR #Cat 0793-1G) in each well. The plate was incubated for 3.5h in a 37 ºC incubator. After incubation, media was carefully aspirated and replaced with 150uL of MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol). The plate was then wrapped in tin foil and further incubated at 37 ºC for 30 mins with shaking using a mini-shaker (VWR). Absorbance readings were taken at 540nm with 630nm as reference with a Biotek Plate Reader.

Migration Assay

Cells were pre-treated with SMOC-2 recombinant protein or silenced with siRNA as previously described and seeded directly into the upper chambers of 8µm Transwells inserts (Falcon, #Cat 353097) in adapted 24-well culture plates. Cells were seeded in 1% FBS containing media with 5% FBS media added to the lower chamber as a chemoattractant. Assay plates were transferred to a 37 ºC incubator and let undisturbed for 24h. Transwells were then fixed with Formalin 10%, (Hemochem, #Cat F-5010Z and stained with Crystal Violet 0,5% in 25% Methanol, (BDH Product #Cat 34024). Inserts with stained cells were imaged at 10X and quantified using ImageJ.

3.5 Results

3.5.1 Histological Confirmation of Tumorigenic Tissue in RCC Patient Biopsy Samples

To confirm the presence of RCC damage in patient biopsy samples, an H&E staining was performed (Department of Pathology, Hôpital Maisonneuve-Rosemont). In the RCC panel, a mass of condensed cells and loss of normal kidney architecture is observed, compared to the healthy control (Figure 4).

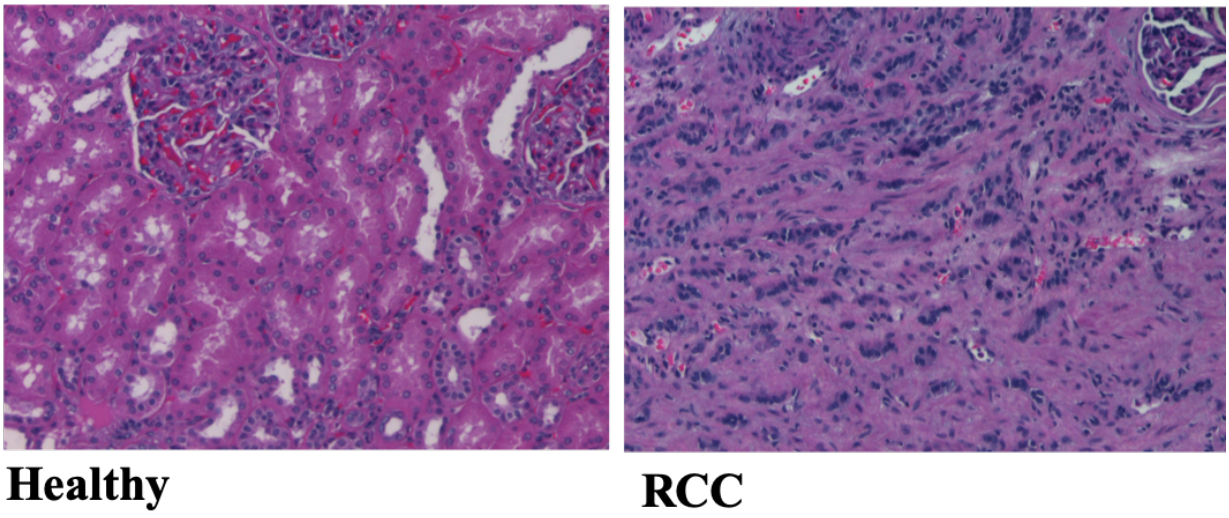


Figure 4: H&E staining of RCC patient biopsy samples. Staining performed by the Department of Pathology (Hôpital Maisonneuve-Rosemont). Images captured at 20X magnification. Figures are a representative image.

3.5.2 SMOC-2 is Highly Expressed in RCC Patient Biopsy Samples with Co-localization of Vimentin

After confirmation of tumorigenic tissue by H&E staining, we proceeded to probe for endogenous SMOC-2 and vimentin expression in RCC patient biopsies by immunofluorescence (Figure 5). SMOC-2 is shown in green and was found to concentrate along the periphery of the tubules which are distinguished by their elongated oval shape. Vimentin, shown in red was found to surround the SMOC-2 dense tubules and is a positive marker for cancerous tissue. As the tubular epithelial cells are the pre-dominate injured cell type in RCC it is unsurprising that SMOC-2 is localized to these structures[182].

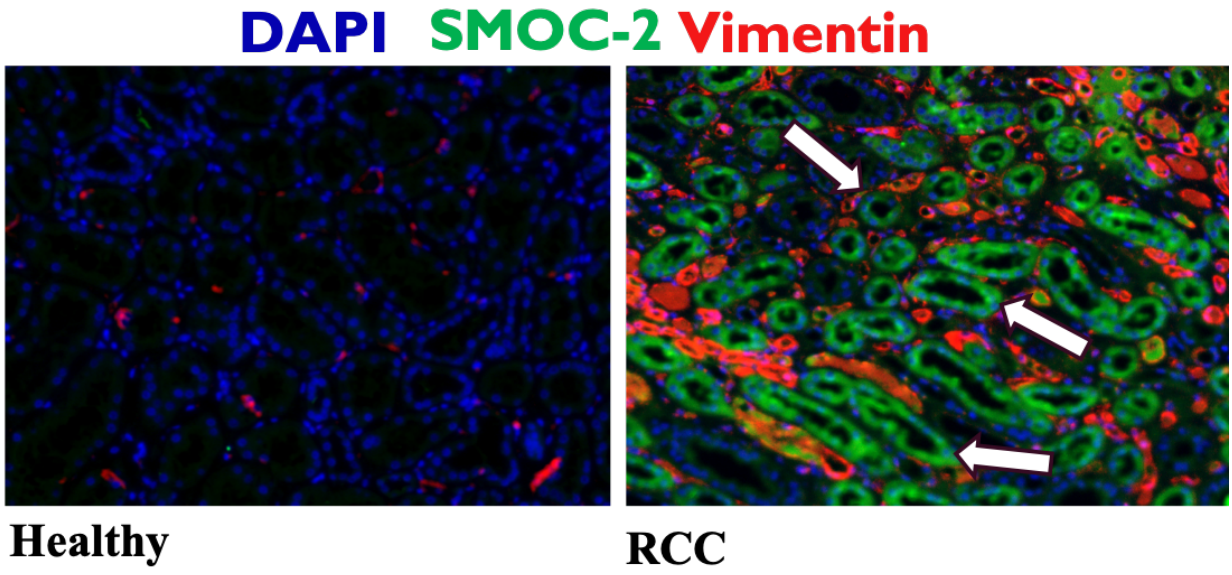


Figure 5: Immunofluorescence staining for patient biopsy samples. Staining performed by the Department of Pathology (Hôpital Maisonneuve-Rosemont). SMOC-2 (green), vimentin (red) and DAPI (blue) in RCC patient biopsy samples. White arrows indicate kidney tubules with intense SMOC-2 presence.. Images captured at 20X magnification. Figures are a representative image.

3.5.3 Endogenous SMOC-2 Expression in RCC Cell Lines

The first set of experiments was to determine the endogenous levels of SMOC-2 in the two human epithelial RCC derived cell lines, 786-O and ACHN, compared to a normal healthy kidney epithelial cell line, HK-2 (Figure 6). Cells were simply plated in culture dishes and let to adhere overnight prior to harvesting cell lysates. By Western blot, we found higher endogenous levels of SMOC-2 in the RCC specific cell lines, compared to the control. These findings support our hypothesis that SMOC-2 is upregulated in a contextual manner, in this case RCC, and is otherwise limited in expression.

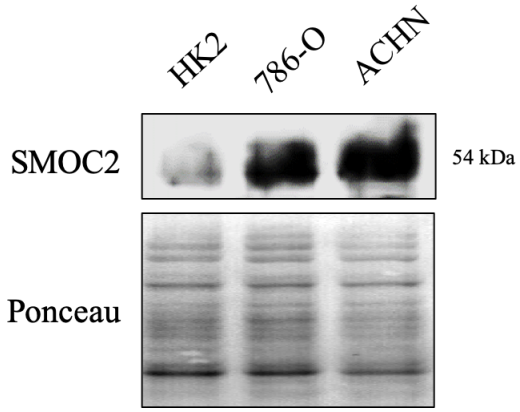


Figure 6: SMOC-2 endogenous levels detected by Western blot. Figure is a representative image.

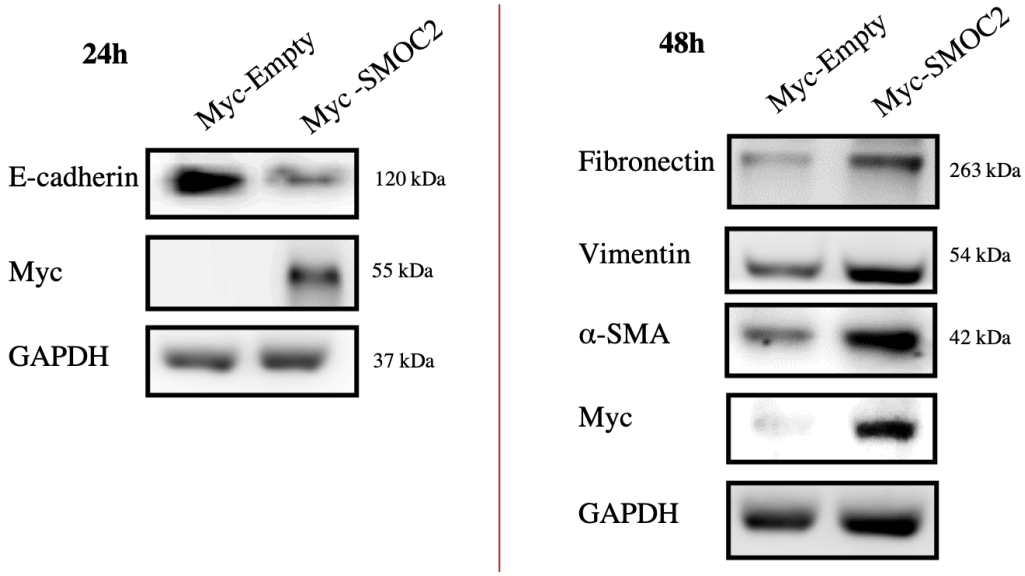
3.5.4 Activation of EMT Markers by SMOC-2 Overexpression

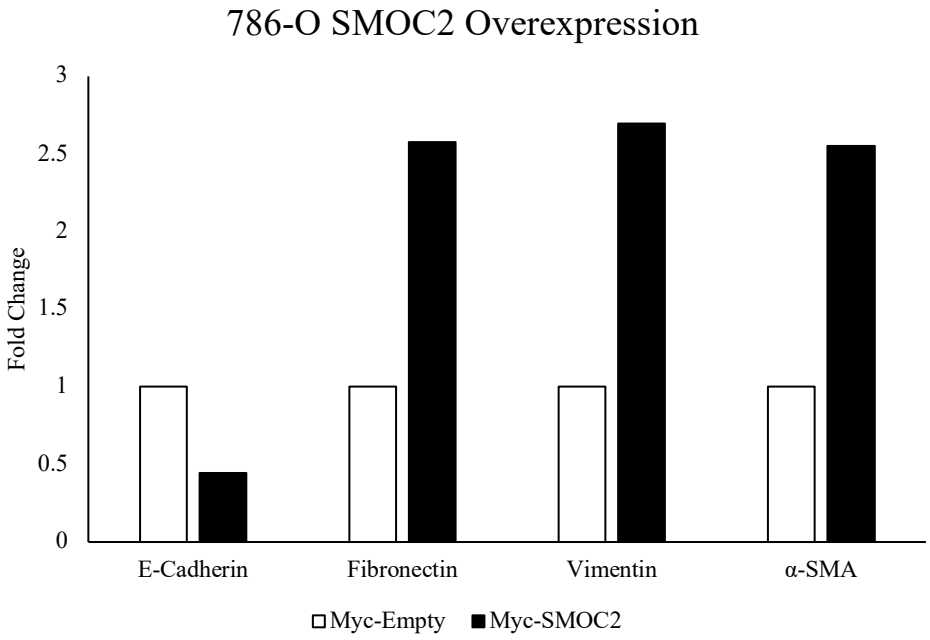
To test our hypothesis that SMOC-2 is capable of activating EMT, we sought to overexpress SMOC-2 in both cell lines and probed for changes in distinguishing markers of EMT over time. Both 786-O and ACHN cells were transfected with either Myc-Empty (control) or Myc-SMOC-2.

Beginning with 786-O cells, within 24h a downregulation of the epithelial cell marker E-cadherin is observed. By 48h, the upregulation of mesenchymal markers such as fibronectin, vimentin and α-SMA are observed (Figure 7A). As EMT begins with the loss of the epithelial cell signature, a downregulation of E-cadherin is a good indication of EMT initiation and occurs prior to the upregulation of mesenchymal markers. Not shown were the non-significant changes observed in mesenchymal markers at 24h.

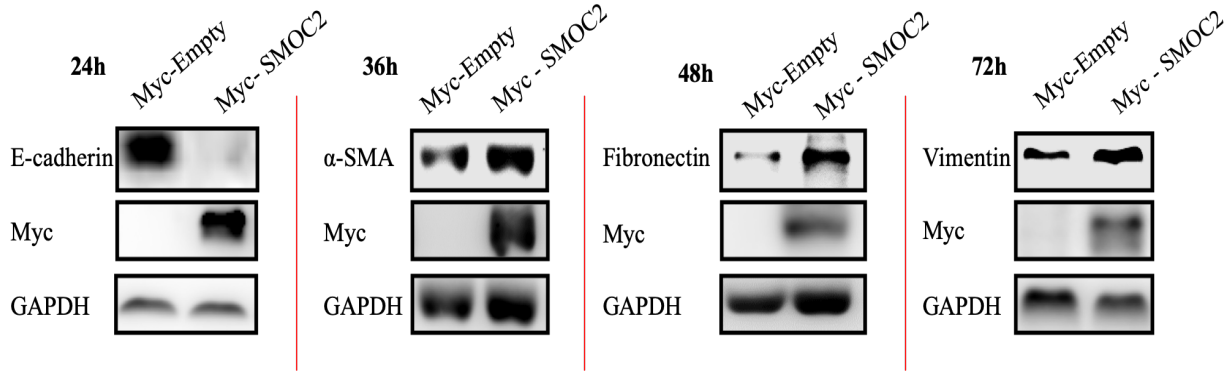
With ACHN cells, a similar shift from epithelial to mesenchymal markers was observed, starting with the initial loss of E-cadherin occurs at 24h. Differing from the 786-O cells was the upregulation of α-SMA at 36h and vimentin at 72h, though fibronectin remained strongly upregulated at 48h (Figure 7B). Although the exact timing of expression is different between the two cell types, more importantly is the conserved trend and transition from epithelial to mesenchymal that is observed.

A. 786-O


24h
Myc-Empty
Myc -SMOC2
E-cadherin
120 kDa
Myc
55 kDa
GAPDH
37 kDa
48h
Myc-Empty
Myc-SMOC2
Fibronectin
263 kDa
Vimentin
54 kDa
α-SMA
42 kDa
Myc
GAPDH


786-O SMOC2 Overexpression
Fold Change
3
2.5
2
1.5
1
0.5
0
E-Cadherin
Fibronectin
Vimentin
α-SMA
Myc-Empty
Myc-SMOC2

B. ACHN



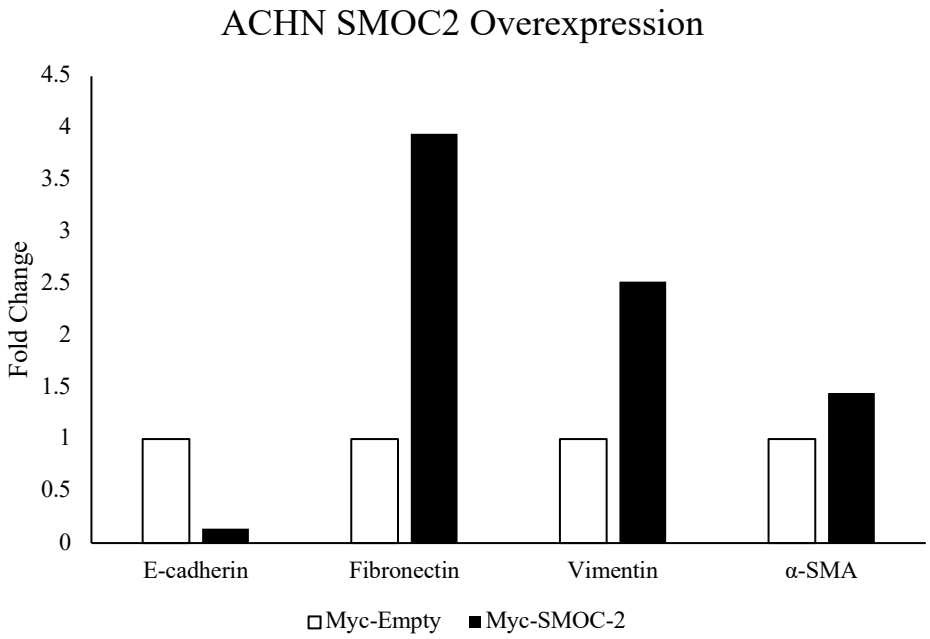


Figure 7: SMOC-2 overexpression induces EMT makers. A. 786-O cells and **B**. ACHN cells with accompanying quantification. Both cell types show loss of epithelial cell marker by 24h, followed by upregulation of mesenchymal proteins. Cells were transfected with either 1ug of Myc-Empty or Myc-SMOC-2 vector for the indicated times before harvesting. Figures are generated using a best representative image. Quantification was done using only the representative images without statistical analysis.

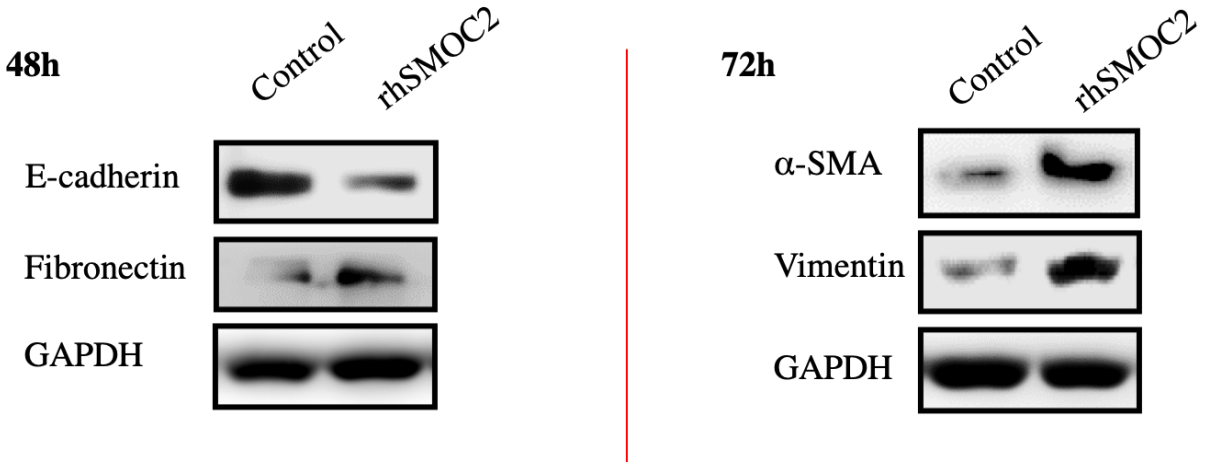
3.5.5 Activation of EMT Markers by SMOC-2 Recombinant Protein

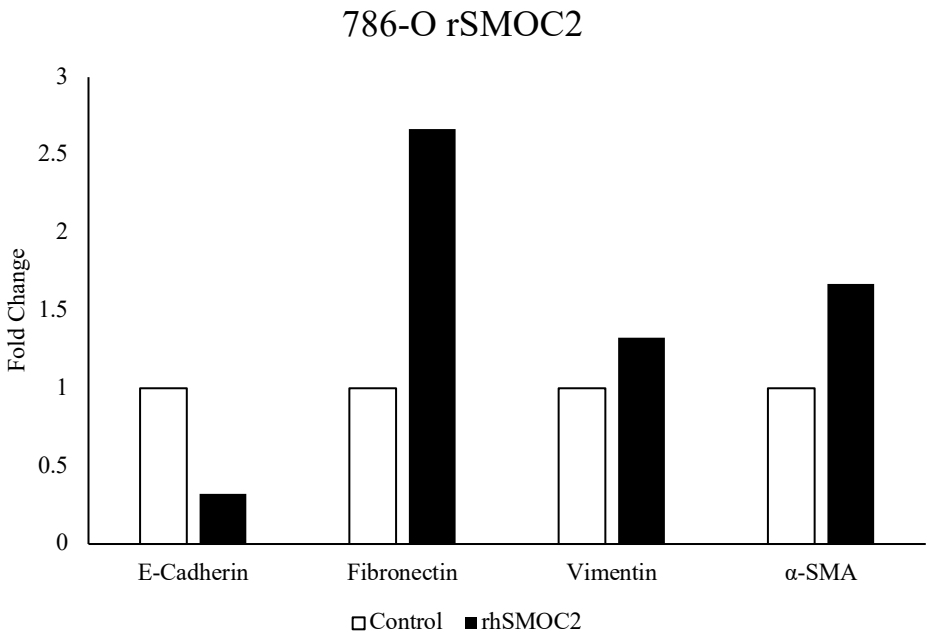
To confirm our overexpression studies, we then proceeded to treat both cell types with a soluble human recombinant SMOC-2 protein. Taking advantage of the extracellular localization and function of MPs, we simply supplemented the cell culture media with 10 ng/mL of rSMOC-2 protein and harvested cells at various timepoints for Western blot analysis. Due to the fragile stability of the recombinant protein, the cell culture media spiked with rSMOC-2 of both cell types was replenished every 24h. This was done to both recapitulate *in-vivo* scenarios where the system experiences the persistent presence of SMOC-2 and also to better mirror the overexpression studies in which there is a sustained production of protein by the cells.

EMT initiation was observed to begin by 48h as characterized by the downregulation of E-cadherin and simultaneous upregulation of fibronectin, followed by α-SMA and vimentin after 72h of exposure to rSMOC-2. This pattern of expression was observed to be the same in both 786-O (Figure 8A) and ACHN cells (Figure 8B), in contrast to the more variable profile created by SMOC-2 overexpression (Figure 7A-B). Most distinctly, it took until 48h with rSMOC-2 to observe a marked decrease in E-cadherin, which occurred an additional 24h later in comparison to SMOC-2 overexpression. With rSMOC-2 treatments, a controlled and consistent dosage of protein (10ng/mL) was introduced daily, whereas the SMOC-2 overexpression is dependent on both transfection efficiency and cell production, which may be the reason for the difference. Overall we observed the shift in key EMT proteins over a similar timeline using the two different methods.

Our rationale for overexpressing and use of recombinant SMOC-2 in RCC derived cells was to recapitulate the *in-vivo* tumor microenvironment, in which we expect the high levels of SMOC-2 to be present. The elevated expression of MPs like SMOC-2 can be triggered and sustained by persistent injury and repair signals produced by a tumor, but is difficult to mimic using in-vitro cell culture. Therefore, we chose to exaggerate SMOC-2 expression by overexpression and addition of recombinant protein to capture the effects of high levels of SMOC-2 on other EMT associated proteins like fibronectin, vimentin and α-SMA. These experiments aim to show a “proof of concept” that SMOC-2 modulation in RCC cells can activate these proteins.

A. 786-O


48h
Control
rhSMOC2
E-cadherin
Fibronectin
GAPDH
72h
Control
rhSMOC2
α-SMA
Vimentin
GAPDH


786-O rSMOC2
Fold Change
3
2.5
2
1.5
1
0.5
0
E-Cadherin
Fibronectin
Vimentin
α-SMA
Control
rhSMOC2

B. ACHN

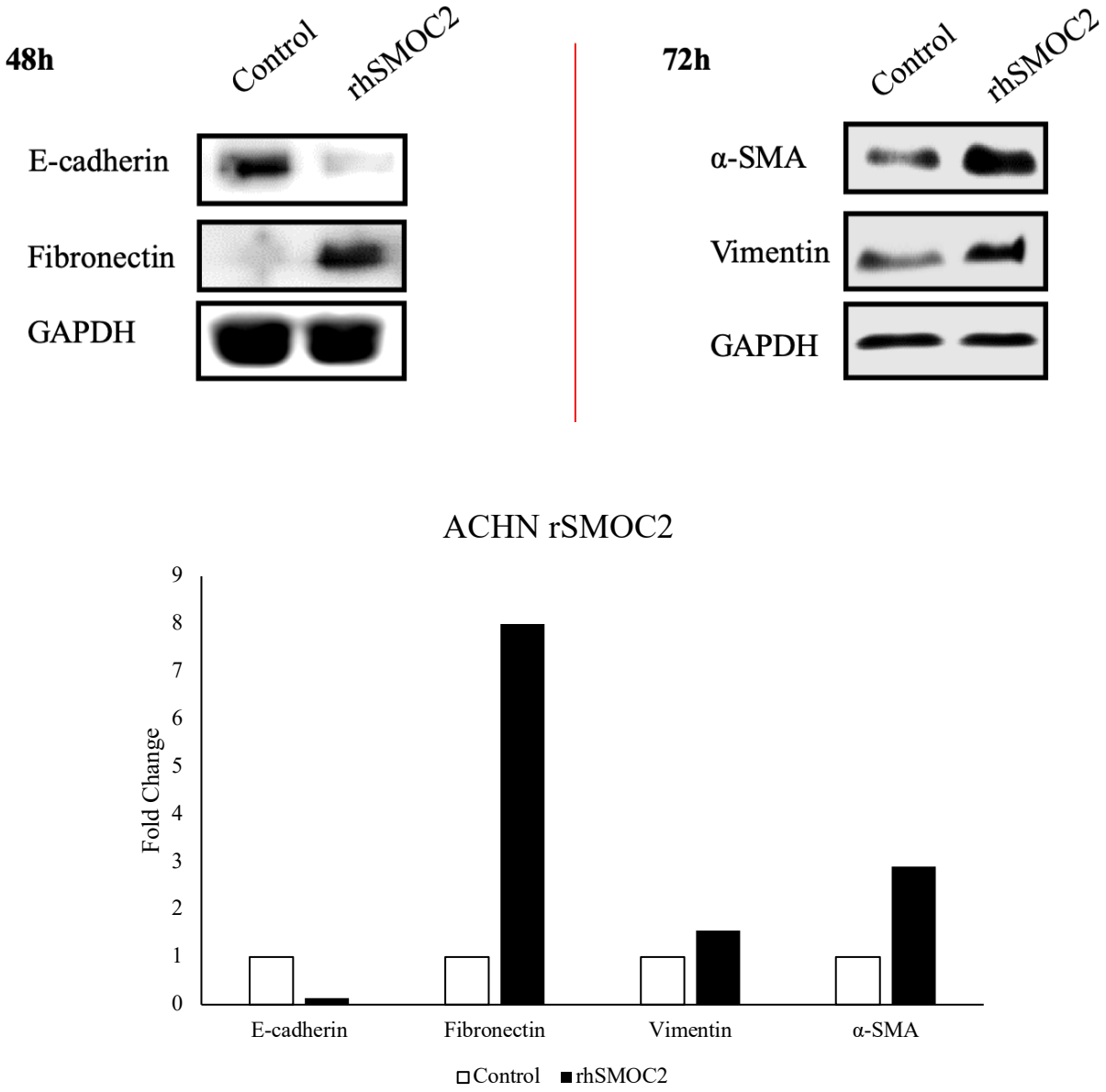


Figure 8: Recombinant SMOC-2 Protein Treatment induces EMT markers. A. 786-O cells and **B**. ACHN cells, with accompanying quantification, showing loss of epithelial cell marker by 48h, followed by upregulation of mesenchymal proteins by 72h. Both cell types were treated with 10 ng/mL rSMOC-2 or vehicle for the indicated time. Culture media with rSMOC-2 or vehicle was replenished every 24h. Figures are generated using a best representative image. Quantification was done using only the representative images without statistical analysis.

3.5.6 SMOC-2 Silencing in Stimulated RCC cells Downregulate EMT Markers.

To confirm our overexpression and recombinant studies, we next took the opposite approach with silencing SMOC-2. To address whether SMOC-2 inhibition is sufficient to limit induction of EMT, we transfected cells with SMOC-2 siRNA and assessed induction of EMT markers by stimulating with TGFβ. As TGFβ is a pro-EMT cytokine in epithelial cells, its addition is necessary for assessing the consequences of SMOC-2 silencing in EMT initiation. Due to the fleeting effects of the siRNA silencing, it was found that a total experiment time of approximately 24h best captured the window of dynamic change as the cells respond to both TGFβ stimulation and SMOC-2 silencing. By Western blot analysis, we probed for the same mesenchymal markers, fibronectin, α-SMA and vimentin, which were all downregulated in both 786-O (Figure 9A) and ACHN (Figure 9B) in SMOC-2 siRNA transfected cells compared to control.

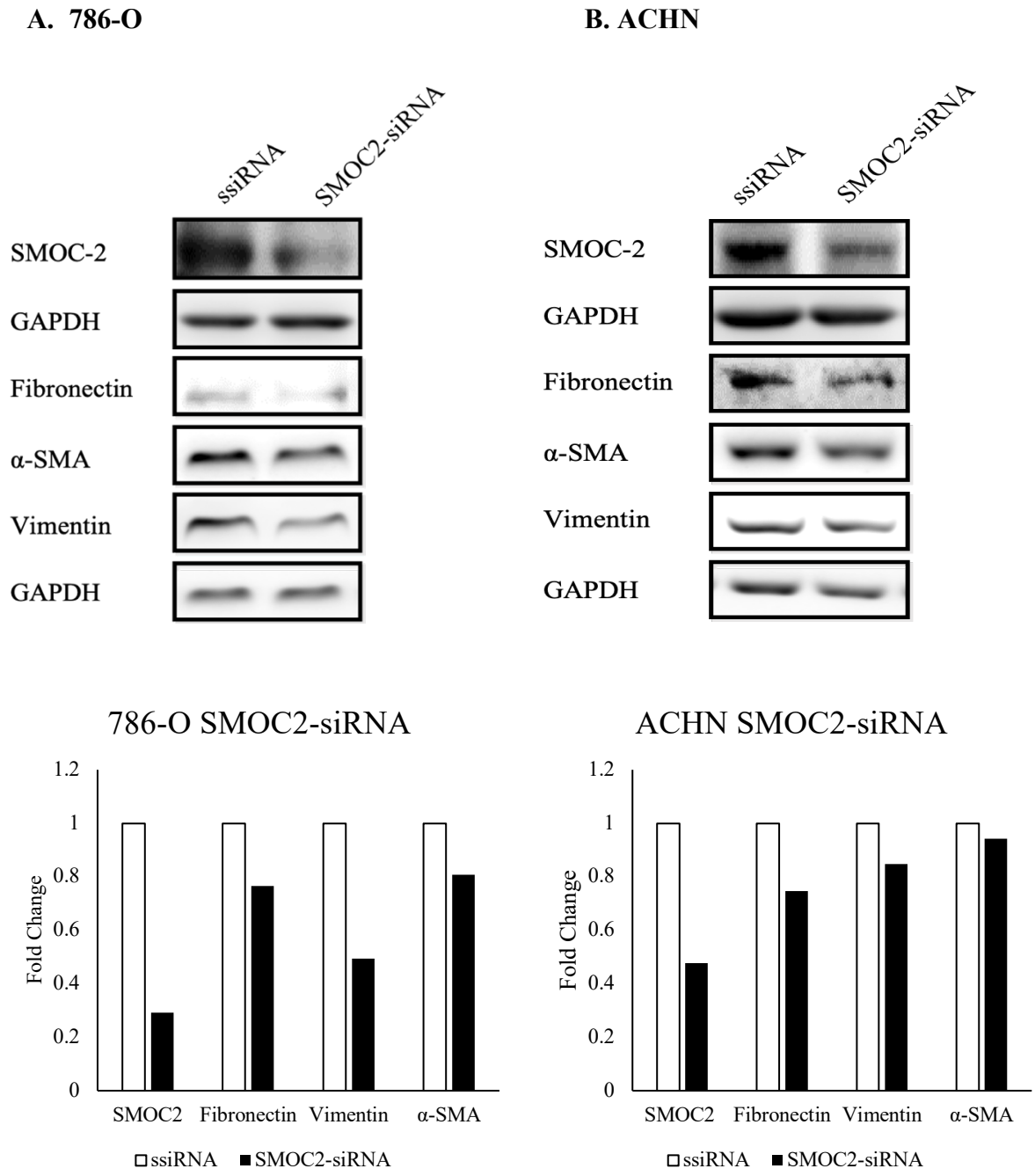


Figure 9: SMOC-2 silencing downregulates EMT markers. **A.** 786-O and **B.** ACHN, with accompanying quantification. Cells were transfected with 80 pmole of ssiRNA or SMOC-2 siRNA for 6h, followed by replacing the culture media spiked with 5ngmL of TGFβ for 15-20h before harvesting. Figures are generated using a best representative image. Quantification was done using only the representative images without statistical analysis

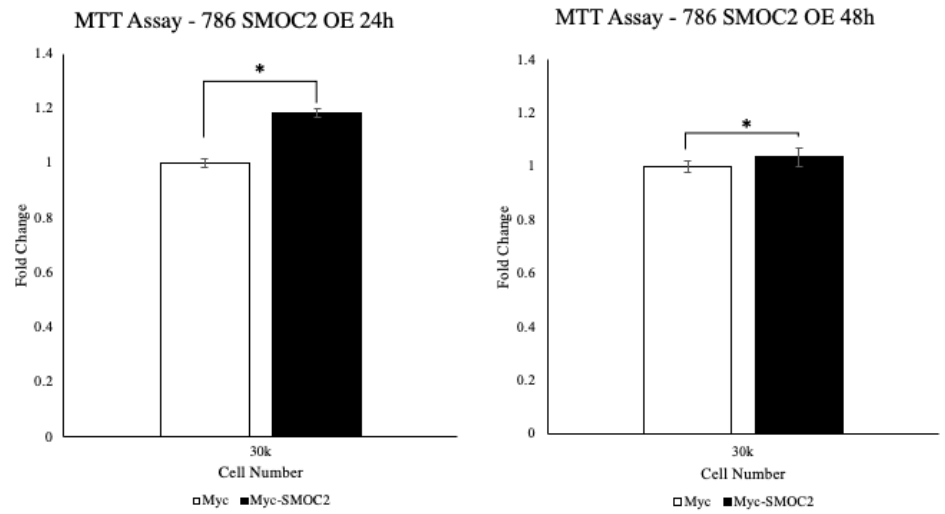
3.5.7 SMOC-2 Overexpression & Silencing Affects Cell Proliferation

To extend our findings beyond Western blots, we aimed to conduct an MTT assay to measure changes in cell proliferation in both 786-O and ACHN cells by modulating SMOC-2 using the same molecular tools of overexpression and silencing

We first found that SMOC-2 overexpressing cells showed increased proliferation at both 24h and 48h, which corresponds to the overall upregulation of EMT markers that occur within a similar time frame in both 786-O (Figure 10A) and ACHN (Figure 10B). These findings are also consistent with reports of SMOC-2 promoting proliferation in metastatic colorectal cancer cells[54] and support the idea that SMOC-2 contributes to the overall proliferative nature of cancers[60].

Next, we sought to determine if silencing of SMOC-2 by siRNA would produce the opposite effect, in which proliferation of TGFβ stimulated cells would be hindered. We found that indeed SMOC-2 silencing reduced proliferation in both 786-O (Figure 11A) and ACHN (Figure 11B) cells after an initial 24h of silencing followed by 24h of TGFβ stimulation, totaling a 48h time course. Overall, the reduced proliferation with SMOC-2 silencing implies the importance of SMOC-2 in hyperproliferative diseases such as cancer.

A. 786-O 24h and 48h



B. ACHN 24h and 48h

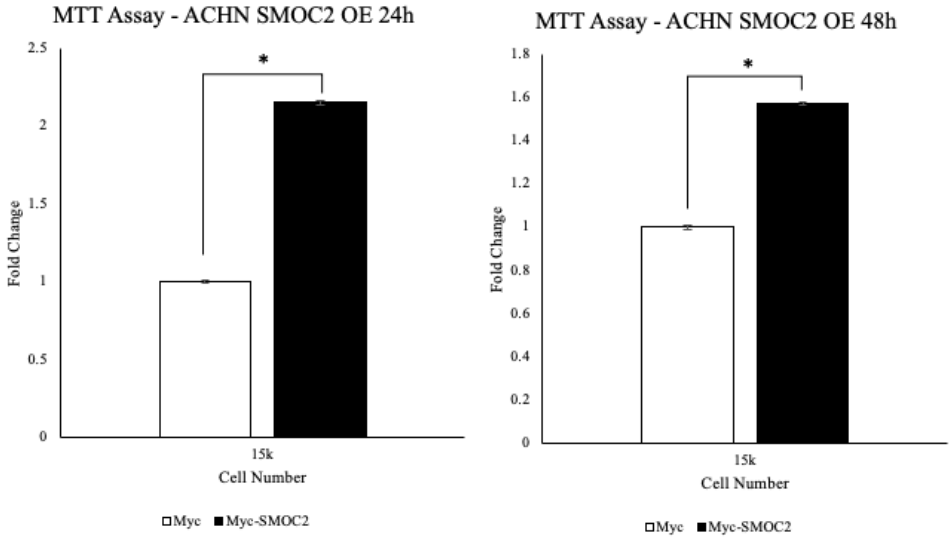


Figure 10: SMOC-2 overexpression promotes cell proliferation by MTT assay. A. 786-O and **B.** ACHN show increased cell metabolic activity measured by an MTT assay. 30k 786-O and 15k ACHN cells were transfected with Myc-Empty (Control) or Myc-SMOC2 for 24h and 48h. Cells then lysed and plated in a 96 well plate and starved with 1% FBS for 3h prior to addition of MTT reagent. Significant upregulation of cell proliferation was observed at both timepoints. 540nm absorbance read with a spectrophotometer with a 630nm reference. (n=3 per time point and cell type, $p < 0.05$ Student T-TEST).

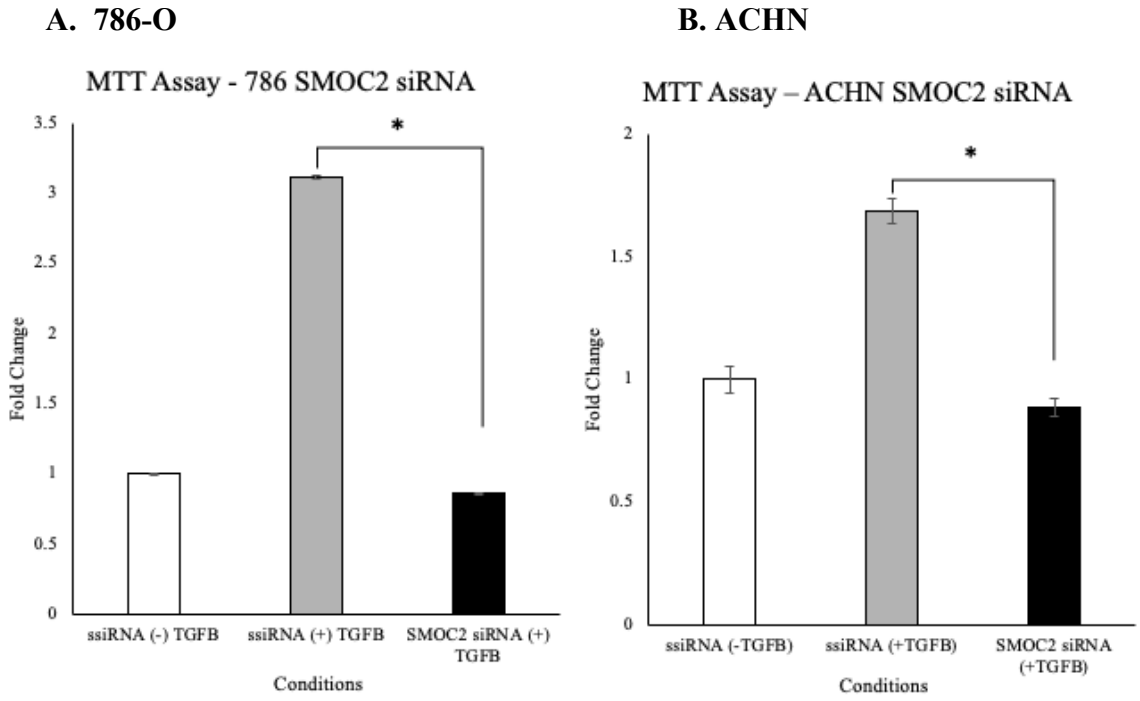


Figure 11: SMOC-2 silencing reduces cell proliferation by MTT assay. SMOC-2 silencing by siRNA transfection reduces overall cell metabolic activity measured by an MTT assay in both cell types **A.** 786-O and **B.** ACHN cells. 20k 786-O and 120k ACHN cells were transfected with either ssiRNA (Control) or SMOC-2 siRNA for a period of 24h, then treated with 5ng/mL of TGFβ for 24h. ssiRNA transfected cells were treated with or without TGFβ as an internal control. 540nm absorbance read with a spectrophotometer with a 630nm reference. (n=4 786-O and n=8 ACHN, $p < 0.05$ Student T-TEST).

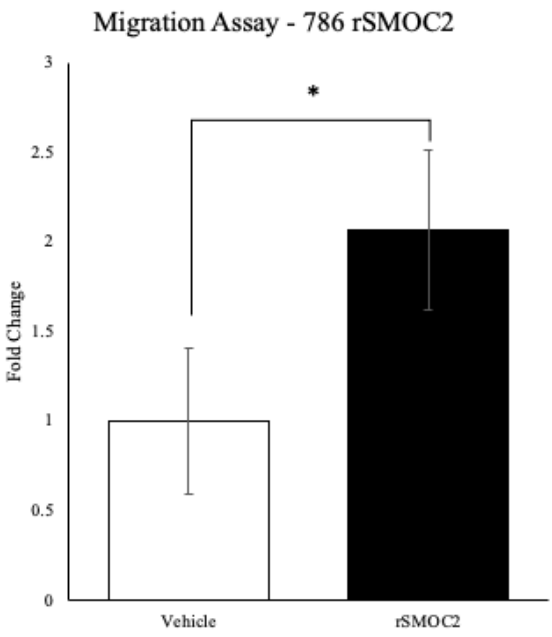
3.5.8 SMOC-2 Modulation Regulates Cell Migration

To support our hypothesis that SMOC-2 modulation impacts tumor cell metastasis we conducted a Transwell migration assay. Both 786-O and ACHN cells were treated with rSMOC-2 or transfected with SMOC-2 siRNA in separate experiments to assess the impact on cell migration through a porous membrane in three-dimensional space. The Transwell migration assay is a commonly used assay to study cancer cell metastasis due to the ease of setup and adaptability to varying cell types and sizes[187].

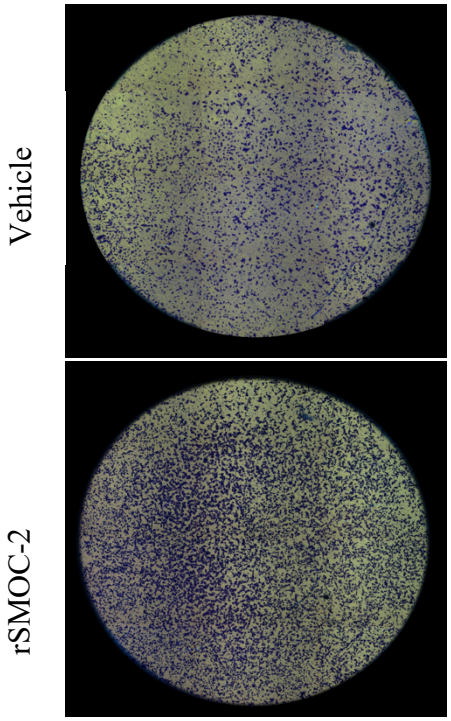
Beginning with the rSMOC-2 treatments, both 786-O (Figure 12A-B) and ACHN (Figure 12C-D) cells exhibited increased migration in 24h compared to control and both cell types showed an approximately 2-fold increase in migration. Taken together with the findings of rSMOC-2 on promoting an EMT phenotype, the increased migration provides evidence that SMOC-2 can influence metastatic behavior of RCC cells.

Next, we then asked if migration of RCC cells is decreased by silencing SMOC-2. Thus, both 786-O (Figure 13A-B) and ACHN (Figure 13C-D) cells were transfected with SMOC-2 siRNA, with addition 5 ng/mL of TGFβ. In the 786-O cells, approximately 50% decreased in migration was observed, and similarly 40% decreased in ACHN. These findings are in line with the data presented thus far and highlights the capability of SMOC-2 to mediate cell migration.

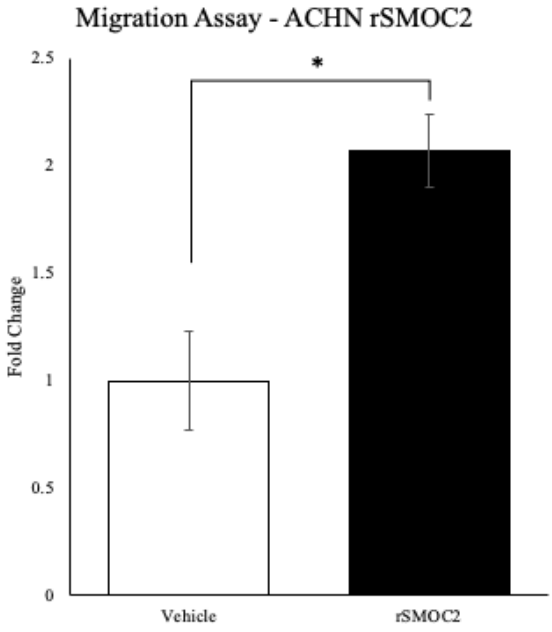
A. 786-O rSMOC-2



B. 786-O rSMOC-2 Transwell



C. ACHN rSMOC-2



D. ACHN rSMOC-2 Transwell

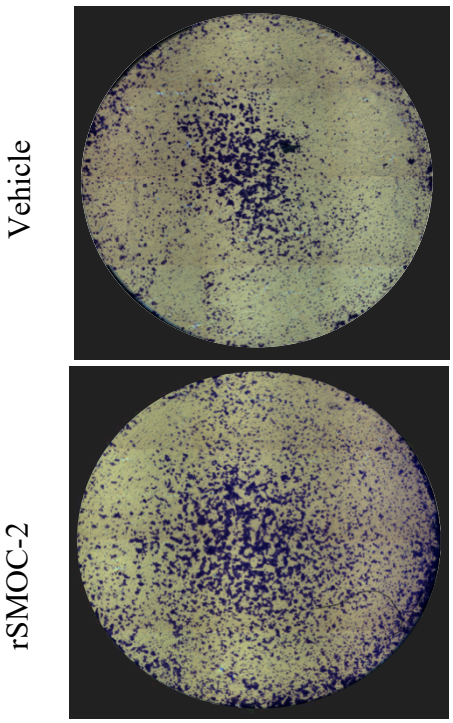
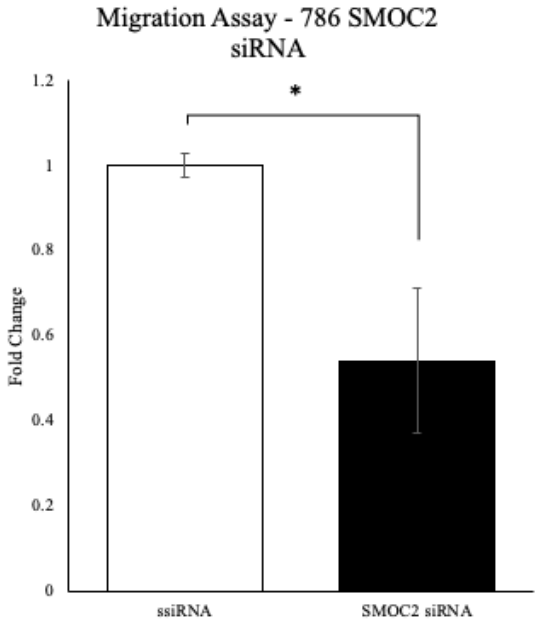
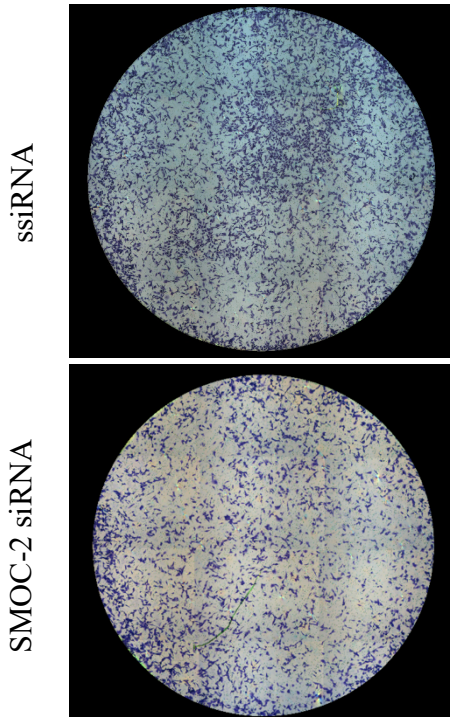


Figure 12: rSMOC-2 promotes cell migration. rSMOC-2 treated cells induces increased cell migration by Transwell migration assay in **A.** 786-O and **C.** ACHN cells. 20k 786-O and 120k ACHN cells were pre-treated 10 ng/mL of rSMOC-2 or vehicle for 24h. Cells then lysed and re-plated into 8 μm Transwells with 1% FBS in the upper chamber and 5% FBS media in the lower chamber. Cells incubated at 37 ºC and let migrate for 24h. Non-migrated cells remaining on the upper membrane were removed gently using a moist cotton swab prior to quantification. Post-migration quantification was done by crystal violet staining and imaging done by brightfield at 10X magnification (ZEISS Axiocam). Representative Transwell images used for quantification are shown for **B.** 786-O and **D.** ACHN cells. Top panel corresponds to vehicle (control) treated cells and bottom panel corresponds to rSMOC-2 treated cells. Images taken at 10X magnification and quantified using Fiji. (n=3 for both 786-O and ACHN, $p < 0.05$ Student T-TEST).

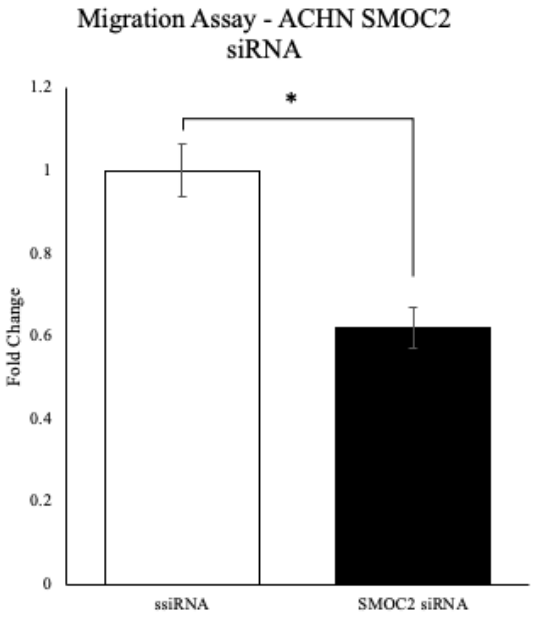
A. 786-O SMOC-2 siRNA



B. 786-O SMOC-2 siRNA Transwell



C. ACHN SMOC-2 siRNA



D. ACHN SMOC-2 siRNA Transwell

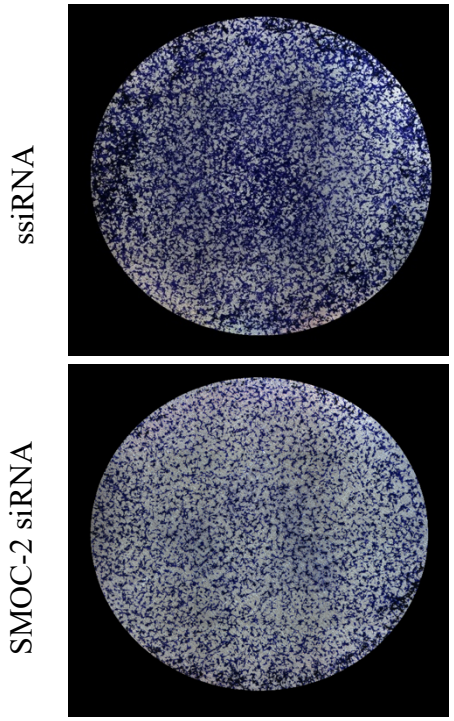


Figure 13: SMOC-2 silencing inhibits cell migration. A. 786-O and **C.** ACHN cells. 20k 786-O and 120k ACHN cells were reverse transfected with 80 pmole of SMOC-2 siRNA or ssiRNA overnight, then lysed and re-plated into 8 μm Transwells with 1% FBS in the upper chamber with 5ng/mL of TGFβ and 5% FBS without TGFβ in identical media in the lower chamber. Cells incubated at 37 ºC and let migrate for 24h. Non-migrated cells remaining on the upper membrane were removed gently using a moist cotton swab prior to quantification. Post-migration quantification was done by crystal violet staining and imaging done by brightfield at 10X magnification (ZEISS Axiocam). Representative Transwell images used for quantification are shown for **B.** 786-O and **D.** ACHN cells. Top panel corresponds to vehicle (control) treated cells and bottom panel corresponds to rSMOC-2 treated cells. Images taken at 10X magnification and quantified using Fiji. (n=3 for both 786-O and ACHN, $p < 0.05$ Student T-TEST).

3.6 Discussion

The developing tumor niche can be viewed as a wound that is unable to heal and is in a persistent state of repair that maintains the elevated expression of MPs which eventually become maladaptive and aids in potentiating metastatic behavior of cancerous cells. In order for the tumorigenic cells to disseminate from the primary site, susceptible epithelial cells can undergo EMT to gain enhanced migratory capability and the ability to remodel the ECM to favor metastasis. We hypothesize that SMOC-2 is a key MP within the tumor microenvironment that can stimulate EMT and show that controlling SMOC-2 expression can regulate mesenchymal cell markers in two human RCC cell lines, 786 and ACHN.

We first confirmed the presence of diseased tissue by an H&E staining of RCC patient biopsy samples (Figure 4). Observation of highly condensed cells and loss of normal kidney architecture distinguished cancerous tissue from the healthy controls. Next, we detected higher endogenous levels of SMOC-2 in the RCC patient biopsy samples by immunofluorescence that appeared to localize in the periphery of the tubules where tumors primarily originate from damaged epithelial structure (Figure 5). Vimentin was also found to be dispersed around the tissue and as a marker of EMT, provides evidence of mesenchymal cells residing in the tumor niche that is co-localized with SMOC-2. This preliminary and proof of concept experiment is consistent with the literature understanding that MPs are expressed in the context of tissue remodeling processes. Within the developing tumor environment, the overexpression of SMOC-2 is possibly sustained by the constant dysregulated remodeling, similar to findings in the context of fibrosis[53], but is further potentiated by oncogenic signaling in cancer. Future investigations will aim to quantify SMOC-2 levels in RCC tissue using a larger sample size to potentially identify the association of SMOC-2 expression with disease staging.

Higher endogenous levels of SMOC-2 protein was detected by Western blot in both 786-O and ACHN cells compared to normal healthy kidney cells (Figure 6), though unsurprisingly as they were originally isolated from an RCC tumor[188, 189]. Overexpression and recombinant protein treatments of SMOC-2 was able to induce the downregulation of the epithelial cell marker E-cadherin in both RCC cell lines within 24h and 48h respectively (Figure 7-8). The downregulation of E-cadherin is a defining early step in the EMT process as it signals the loss of

the epithelial phenotype as cells transition to gain mesenchymal features. The loss of E-cadherin also importantly encourages detachment and loss of cell polarity, which acts as a catalyst for EMT associated pathways such as Wnt/β-catenin, TGFβ and TWIST. Therefore, the loss of E-cadherin must be the first observation made, to which the subsequent mesenchymal markers are measured and described relative to. Interestingly, SMOC-2 overexpression triggered the loss of E-cadherin 24h earlier than with recombinant protein (Figure 8). A plausible explanation may be due to the higher concentration of SMOC-2 produced by the transfected cells in the overexpression system in comparison to the dispensed amount of 10ng/mL of rSMOC-2 protein.

After a total of 48h and 72h in 786-O and ACHN cells respectively, there was a upregulation of mesenchymal cell associated proteins such as α-SMA and vimentin as well as an increased production of fibronectin, a major fibril protein of the ECM known to be overexpressed in many cancers and shown to have pro-invasive functions in breast cancer[124] and RCC cell lines[190]. Taken together, these findings on RCC cells in an environment of high SMOC-2 show the acquisition of mesenchymal markers thru EMT.

In contrast, silencing of SMOC-2 by siRNA was sufficient to suppress vimentin, α-SMA expression and fibronectin expression in both cell types within 24h (Figure 9). Silencing was coupled with a TGFβ co-treatment post-transfection as it has been shown to induce expression of SMOC-2 in renal epithelial cells[53] and used to provide a stimulus to the cells to observe the silencing effects as knock-down of endogenous SMOC-2 was insufficient to illicit significant change.

An important observation made during our analysis was the variability in temporal expression in our panel of EMT markers using the various methods of SMOC-2 modulation. For all expression methods and both cell types, multiple timepoints ranging from 6-96h were initially screened to best capture the window of dynamic change in preliminary experiments. Timepoints were then further refined for each expression system and cell type, generating logical and reproducible results.

Although SMOC-2 was capable of upregulating key EMT associated proteins, we further investigated the functional capabilities of 786-O and ACHN cells using molecular tools to assess cell proliferation and migration. SMOC-2 overexpressing cells showed increased proliferation in an MTT assay and silencing produced the opposite result (Figure 10-11). SMOC-2 has been reported to regulate cell cycle progression by maintaining integrin-linked kinase (ILK) activity during the G1 phase in growth factor induced cell cycle progression and SMOC-2 silenced Swiss 3T3 fibroblasts led to reduced ILK activity and decreased DNA synthesis[59]. The MTT assay is a measure of the cells mitochondrial metabolic rate to process the MTT dye by mitochondrial succinate dehydrogenase and thus indirectly reflects cell proliferation as actively growing cells will have greater total mitochondrial activity[191]. Our findings are also in agreement with previous reports of SMOC-2 acting as a pro-growth molecule of colon cancer cells[54] and endometrial cancer cells[185], though measured by different means report a similar conclusion.

We further conducted a Transwell migration assay to assess the capability of cells to move physically in 3-dimensions through a porous membrane to a chemoattract while augmenting SMOC-2 expression with recombinant protein (Figure 12). For both cell types treated with rSMOC-2, there was a significant increase in cell migration compared to cells treated with vehicle, and the contrary observed with siRNA silencing (Figure 13) which is in line with all the presented data. Mechanistically, since SMOC-2 has been shown to stimulate signaling molecules of migration, namely integrin β1 and activating integrin linked kinases (ILKs) and phosphorylation of FAKs in keratinocytes[51], we hypothesize that SMOC2 is likely acting through a similar mechanism for the metastatic potential of RCC epithelial cells. Moreover, integrin β1 silencing was reported to inhibit migration of 786-O cells towards a fibronectin chemoattractant using the Transwell system[192] that is in agreement with our findings. The upregulation of ECM proteins stimulated by SMOC-2 is not only crucial for driving EMT but also promoting migration of transformed cells which is a main driver for metastasis[27].

In summary, we show that higher SMOC-2 levels are detected in regions of tumorigenic tissue and co-localizes with the cancer marker vimentin, around the periphery of the tubules in the kidney. Moreover, SMOC-2 was successful in stimulating EMT in RCC cell lines and also

promotes increased proliferation and migration that supports the metastatic phenotype of cancer cells.

4.0 Overall Discussion

Temporal changes in expression of MPs provide valuable insight for understanding their specific roles in the various steps during the tissue repair process. Using two different mechanisms of kidney injury, FA and UUO, to study MP expression patterns over the course of repair provides an informative guideline for identifying notable candidates that warrant further study in novel contexts. We chose SMOC-2 as it has been previously shown to be implicated in renal fibrosis by transforming fibroblasts to the effector cell of fibrosis, the myofibroblast[53]. Stimulation of proliferation, migration, stress fiber formation and excessive ECM production was all shown to be influenced by SMOC-2 with widespread upregulation in biopsies of fibrotic human kidneys. Chronic kidney injuries that cannot recover lead to CKD with end stage complications that include fibrosis and with time, loss of organ function and death. In diseases such as fibrosis, ECM dysregulation is the driving force for many of the pathological complications observed in which MPs play a critical role.

The status of the ECM is also an important indicator of cancer progression similar to that of identifying the extent of damage and loss of organ function in fibrosis and serves as a scaffold for propagating disease. In the context of fibrosis, buildup of fibrous proteins, primarily collagen and fibronectin, is the main driver of organ malfunction through expansion of the stiff and dense ECM materials. Similarly, cancer cells can manipulate their environment to enhance their survival, which includes adapting it to suit metastasis to spread and amplify their presence. Metastasis is driven by malignant cells, but non-cellular components of the tumor microenvironment also play an important role in forming the pre-metastatic niche in target organs. Tumor secreted fibronectin has been shown to be a pre-determinant in lung metastasis where its deposition also acts as a chemoattractant[94, 193]. In mammary cells, collagen crosslinking

which causes progressive matrix stiffening in both cancerous and fibrotic tissue, promotes focal adhesions and integrin dependent signaling to drive malignant behavior[103].

MPs are chronically expressed by tumor cells and its rearrangement of the surrounding stroma stimulate mitogenic pathways essential for tumorigenesis. SMOC-2 represents one of the many understudied and novel MPs whose functions are not entirely understood within both homeostatic and pathological contexts. Though its impact is acknowledged through correlative studies, there remains a lot of work to be done in understanding its unique contribution to disease. Our work with SMOC-2 serves as a guideline for future studies to investigate understudied MPs and uncover translatable mechanisms between dysregulated repair and cancer development.

Using SMOC-2 as our model MP to study translatable mechanisms between similar contexts under the theme of a dysregulated ECM, we look to RCC for both the novelty in the literature and familiarity of renal pathologies in our research programme. In a similar sense that the chronically dysregulated ECM in fibrosis provides the stimuli for transformation of fibroblasts to myofibroblasts, mesenchymal cells arise from epithelial cells through EMT as a consequence of the developing tumor ECM. Overexpression of MPs in the tumor ECM can come from activated fibroblasts, macrophages, surrounding stromal cells and transformed epithelial cell to alter the ECM composition to support malignancy, similar to progressive fibrotic takeover of an injured tissue. This idea provides a plausible explanation for the correlation between the upregulation of many MPs and a poor prognosis and outlook in many epithelial derived cancers.

MPs also represent another avenue of exploration in developing therapeutics to manage dysregulated ECM remodeling and pathologies that rely and thrive on such disorder. Informative disease biomarkers offer not only prognostic benefits useful for monitoring disease progression and clinical outcome but can also serve viable therapeutic targets[194]. The dormant expression of MPs beyond development present themselves as attractive context specific targets as they are highly inducible with injury or sufficient pathological triggers. Their extracellular localization is advantageous for circumventing mechanisms required for intra-cellular localization of therapeutics against target molecules. In terms of detection, MPs have been shown to be present

in circulating in blood and can be shed into the urine which strengthens their diagnostic and prognostic value[195]. For example, MPs such as SMOC-2 have been shown to be found in the urine of CKD patients[53], while OPN and CCN1 have been found within the urine of renal ischemic reperfusion injured mice[152] and prostate cancer patients[153], respectively. With regards to studying kidney related pathologies, urine proteome changes are detectable by mass spectrometry methods and provide a safer alternative to invasive biopsy methods for monitoring disease onset and progression[92, 196].

It is especially important for onset of diseases like renal fibrosis in which patients appear normal by current standards of creatinine measurement and can go undiagnosed until more severe histological changes manifest[197]. Therefore, there is a urgent need to finding unique MP biomarkers/signatures to build better screening panels to supplement and hopefully replace invasive diagnostic methods. With the emergence of contemporary therapeutic strategies that involve a multi-targeted approach for severe diseases, we have highlighted MPs as dually efficacious targets in managing renal fibrosis and RCC.

5.0 Future Perspectives

Overall, we provide evidence that supports SMOC-2 as a pro-metastatic MP that can induce EMT leading to a more proliferative and migratory phenotype in RCC cell lines *in vitro.* Next steps to further develop this hypothesis would be to determine the mechanism and major signaling pathway by which SMOC-2 confers its effects on RCC cells.

The first step will be to determine the relevant integrin receptors for SMOC-2. Integrins are important for SMOC-2 signaling as is common to many MPs, but the exact integrin subunit complex (α and β) is unknown for RCC epithelial cells. Our approach will be to screen a panel of both α and β subunits to determine the key integrins involved in mediating SMOC-2 signaling and determine the strongest interactions by immunoprecipitation. First, cells will be transfected with Myc-SMOC-2 (or Myc-Empty) to overexpress SMOC-2 and subsequent myc-pulldown, followed by Western blot using the integrin panel.

A supplementary experiment to determine the reverse effect will also be done by treating the cells with an appropriate neutralizing antibody against the determined integrin subunits prior to treatment using rSMOC-2. To verify the successful inhibition of SMOC-2 signaling, we would expect to see an attenuation in the EMT markers in the cells treated with SMOC-2 compared to the control without integrin obstruction.

Additional functional assays to strengthen our findings will also be done. The scratch or wound healing assay is a simple *in-vitro* technique to measure cell migration. An artificial wound is created by scratching a cell monolayer and can be coupled with live-cell microscopy to visualize wound closure by migrating cells. Cells overexpressing or treated with recombinant SMOC-2 protein will be compared to appropriate controls to assess the rate of migration and observations such as branching of the leading edge can be made.

Reports have been published demonstrating the impact it has on cell proliferation, morphology and something due to the augmented rigidity with or without an artificial ECM[198]. Thus, consideration for the in-vitro cell culture methods is also important with regards to the type of plastics used in the culture vessels. Strategies to pursue may be coating the culture plates with

an ECM such as Matrigels or hydrogels that resemble a basement membrane and alters the surface physical properties that cells grow on.

Lastly, long term goals for this project is to study the effects of SMOC-2 modulation *in-vivo* using murine RCC models. Previous work by the lab has demonstrated that an endotoxin-free, chemically modified SMOC-2 siRNA is able to localize in the kidneys, liver and insignificantly in the heart[53]. Proof of concept experiments demonstrated that SMOC-2 expression in the kidneys was found to be reduced by approximately 50% with application of the siRNA, following folic acid injection to induce renal injury[53]. To generate a murine model of RCC, an established method using Renca cells are introduced into the kidney by orthotopic implantation that results in reproducible primary tumor burden, in the kidneys and the lungs through metastasis[199]. Renca cells are a cell line derived from a spontaneous instance of renal cortical adenocarcinoma in Balb/C mice and has been show to mimic human RCC development and metastatic patterns[200]. Developing this *in-vivo* model in-house will be an important next step to advance our SMOC-2 research programme beyond in-vitro studies, and to translate our current molecular and functional findings. With this preliminary knowledge, we hope to pursue and refine the use of SMOC-2 siRNA as a pharmacological tool to targeting SMOC-2 in RCC, and strengthen the hypothesis that SMOC-2 is a key MP involved in RCC tumor development and metastasis.

6.0 Conclusion

Our work has shown the burgeoning role of MPs in both homeostatic and pathological matrix remodeling.

Beginning with traditional models of tissue repair, FA and UUO, we have shown that different injury stimuli trigger a unique MP signature that is dynamically changing over time. From our study, we have correlated the mRNA expression of novel MPs previously unknown in the context of fibrosis or kidney fibrosis and confirmed their protein expression by Western blot. We then proceeded to select a candidate MP, SMOC-2, that was shown be to highly upregulated during the course of repair in both models of injury that prompted us to pursue its functions in a related context of kidney injury.

As fibrosis is regarded as a pathology of dysregulated wound repair, cancers can be thought of similarly as a persistent tissue injury that does not heal. Thus, to study translatable mechanisms of kidney injury, we looked to RCC for familiarity of renal pathologies in our lab and novelty of SMOC-2 in such contexts. We first showed that expression of SMOC-2 is drastically increased in human RCC patient biopsy samples which prompted us to move into i*n-vitro* experiments. We found that SMOC-2 is capable of inducing EMT in RCC cells *in-vitro* that led to increased proliferation and migration through overexpression and recombinant protein treatments. Moreover, silencing SMOC-2 by siRNA was capable of reversing these effects.

Overall, we have contributed to the growing body of knowledge of MPs in matrix remodeling pathologies and highlighted their potential as targets for managing cancer metastasis.

7.0 References

1. Sage, E.H. and P. Bornstein, *Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin*. J Biol Chem, 1991. **266**(23): p. 14831-4.
2. Bornstein, P. and E.H. Sage, *Matricellular proteins: extracellular modulators of cell function*. Curr Opin Cell Biol, 2002. **14**(5): p. 608-16.
3. Frantz, C., K.M. Stewart, and V.M. Weaver, *The extracellular matrix at a glance*. J Cell Sci, 2010. **123**(Pt 24): p. 4195-200.
4. Schaefer, L. and R.M. Schaefer, *Proteoglycans: from structural compounds to signaling molecules*. Cell Tissue Res, 2010. **339**(1): p. 237-46.
5. Muiznieks, L.D. and F.W. Keeley, *Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective*. Biochim Biophys Acta, 2013. **1832**(7): p. 866-75.
6. Ricard-Blum, S., *The collagen family*. Cold Spring Harb Perspect Biol, 2011. **3**(1): p. a004978.
7. Singh, P., C. Carraher, and J.E. Schwarzbauer, *Assembly of fibronectin extracellular matrix*. Annu Rev Cell Dev Biol, 2010. **26**: p. 397-419.
8. Pankov, R., et al., *Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis*. J Cell Biol, 2000. **148**(5): p. 1075-90.
9. Humphrey, J.D., E.R. Dufresne, and M.A. Schwartz, *Mechanotransduction and extracellular matrix homeostasis*. Nat Rev Mol Cell Biol, 2014. **15**(12): p. 802-12.
10. Wise, S.G. and A.S. Weiss, *Tropoelastin*. Int J Biochem Cell Biol, 2009. **41**(3): p. 494-7.
11. Colognato, H. and P.D. Yurchenco, *Form and function: the laminin family of heterotrimers*. Dev Dyn, 2000. **218**(2): p. 213-34.
12. Singh, B., et al., *Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host*. FEMS Microbiol Rev, 2012. **36**(6): p. 1122-80.
13. Bedore, J., A. Leask, and C.A. Seguin, *Targeting the extracellular matrix: matricellular proteins regulate cell-extracellular matrix communication within distinct niches of the intervertebral disc*. Matrix Biol, 2014. **37**: p. 124-30.
14. Larsen, M., et al., *The matrix reorganized: extracellular matrix remodeling and integrin signaling*. Curr Opin Cell Biol, 2006. **18**(5): p. 463-71.
15. Rotstein, B., et al., *Distinct domains in the matricellular protein Lonely heart are crucial for cardiac extracellular matrix formation and heart function in Drosophila*. J Biol Chem, 2018. **293**(20): p. 7864-7879.
16. Kim, M., C.V. Carman, and T.A. Springer, *Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins*. Science, 2003. **301**(5640): p. 1720-5.
17. Qin, J., O. Vinogradova, and E.F. Plow, *Integrin bidirectional signaling: a molecular view*. PLoS Biol, 2004. **2**(6): p. e169.
18. Anderson, L.R., T.W. Owens, and M.J. Naylor, *Structural and mechanical functions of integrins*. Biophys Rev, 2014. **6**(2): p. 203-213.
19. Campbell, I.D. and M.J. Humphries, *Integrin structure, activation, and interactions*. Cold Spring Harb Perspect Biol, 2011. **3**(3).

20. Chen, J., J.S. Alexander, and A.W. Orr, *Integrins and their extracellular matrix ligands in lymphangiogenesis and lymph node metastasis*. Int J Cell Biol, 2012. **2012**: p. 853703.
21. Liu, S., et al., *Expression of integrin beta1 by fibroblasts is required for tissue repair in vivo*. J Cell Sci, 2010. **123**(Pt 21): p. 3674-82.
22. Lau, L.F., *Cell surface receptors for CCN proteins*. J Cell Commun Signal, 2016. **10**(2): p. 121-7.
23. Hastings, J.F., et al., *The extracellular matrix as a key regulator of intracellular signalling networks*. Br J Pharmacol, 2019. **176**(1): p. 82-92.
24. Lau, L.F., *CCN1/CYR61: the very model of a modern matricellular protein*. Cell Mol Life Sci, 2011. **68**(19): p. 3149-63.
25. Chiodoni, C., M.P. Colombo, and S. Sangaletti, *Matricellular proteins: from homeostasis to inflammation, cancer, and metastasis*. Cancer Metastasis Rev, 2010. **29**(2): p. 295-307.
26. Roberts, D.D., *Emerging functions of matricellular proteins*. Cell Mol Life Sci, 2011. **68**(19): p. 3133-6.
27. Chong, H.C., et al., *Matricellular proteins: a sticky affair with cancers*. J Oncol, 2012. **2012**: p. 351089.
28. Wong, G.S. and A.K. Rustgi, *Matricellular proteins: priming the tumour microenvironment for cancer development and metastasis*. Br J Cancer, 2013. **108**(4): p. 755-61.
29. Bradshaw, A.D., *Diverse biological functions of the SPARC family of proteins*. Int J Biochem Cell Biol, 2012. **44**(3): p. 480-8.
30. Murphy-Ullrich, J.E. and E.H. Sage, *Revisiting the matricellular concept*. Matrix Biol, 2014. **37**: p. 1-14.
31. Barker, T.H., et al., *SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity*. J Biol Chem, 2005. **280**(43): p. 36483-93.
32. Holbourn, K.P., K.R. Acharya, and B. Perbal, *The CCN family of proteins: structure-function relationships*. Trends Biochem Sci, 2008. **33**(10): p. 461-73.
33. Adams, J.C. and J. Lawler, *The thrombospondins*. Int J Biochem Cell Biol, 2004. **36**(6): p. 961-8.
34. Bellahcene, A., et al., *Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer*. Nat Rev Cancer, 2008. **8**(3): p. 212-26.
35. Staines, K.A., V.E. MacRae, and C. Farquharson, *The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling*. J Endocrinol, 2012. **214**(3): p. 241-55.
36. Hsia, H.C. and J.E. Schwarzbauer, *Meet the tenascins: multifunctional and mysterious*. J Biol Chem, 2005. **280**(29): p. 26641-4.
37. Midwood, K.S., et al., *Tenascin-C at a glance*. J Cell Sci, 2016. **129**(23): p. 4321-4327.
38. Jones, P.L. and F.S. Jones, *Tenascin-C in development and disease: gene regulation and cell function*. Matrix Biol, 2000. **19**(7): p. 581-96.
39. Viloria, K. and N.J. Hill, *Embracing the complexity of matricellular proteins: the functional and clinical significance of splice variation*. Biomol Concepts, 2016. **7**(2): p. 117-32.
40. Rainger, J., et al., *Loss of the BMP antagonist, SMOC-1, causes Ophthalmo-acromelic (Waardenburg Anophthalmia) syndrome in humans and mice*. PLoS Genet, 2011. **7**(7): p. e1002114.

41. Mendoza-Londono, R., et al., *Recessive osteogenesis imperfecta caused by missense mutations in SPARC*. Am J Hum Genet, 2015. **96**(6): p. 979-85.
42. Chaly, Y., et al., *FSTL1 promotes arthritis in mice by enhancing inflammatory cytokine/chemokine expression*. Arthritis Rheum, 2012. **64**(4): p. 1082-8.
43. Bornstein, P., *Thrombospondins function as regulators of angiogenesis*. J Cell Commun Signal, 2009. **3**(3-4): p. 189-200.
44. Topol, E.J., et al., *Single nucleotide polymorphisms in multiple novel thrombospondin genes may be associated with familial premature myocardial infarction*. Circulation, 2001. **104**(22): p. 2641-4.
45. Ferrand, N., et al., *Loss of WISP2/CCN5 in estrogen-dependent MCF7 human breast cancer cells promotes a stem-like cell phenotype*. PLoS One, 2014. **9**(2): p. e87878.
46. Mo, F.E. and L.F. Lau, *The matricellular protein CCN1 is essential for cardiac development*. Circ Res, 2006. **99**(9): p. 961-9.
47. Zhang, Y. and C. Wang, *Nephroblastoma overexpressed (NOV/CCN3) gene: a paired-domain-specific PAX3-FKHR transcription target that promotes survival and motility in alveolar rhabdomyosarcoma cells*. Oncogene, 2011. **30**(32): p. 3549-62.
48. Leask, A., et al., *The control of ccn2 (ctgf) gene expression in normal and scleroderma fibroblasts*. Mol Pathol, 2001. **54**(3): p. 180-3.
49. Vannahme, C., et al., *Characterization of SMOC-2, a modular extracellular calcium-binding protein*. Biochem J, 2003. **373**(Pt 3): p. 805-14.
50. Bloch-Zupan, A., et al., *Homozygosity mapping and candidate prioritization identify mutations, missed by whole-exome sequencing, in SMOC2, causing major dental developmental defects*. Am J Hum Genet, 2011. **89**(6): p. 773-81.
51. Maier, S., M. Paulsson, and U. Hartmann, *The widely expressed extracellular matrix protein SMOC-2 promotes keratinocyte attachment and migration*. Exp Cell Res, 2008. **314**(13): p. 2477-87.
52. Feng, W., et al., *Spatial and temporal analysis of gene expression during growth and fusion of the mouse facial prominences*. PLoS One, 2009. **4**(12): p. e8066.
53. Gerarduzzi, C., et al., *Silencing SMOC2 ameliorates kidney fibrosis by inhibiting fibroblast to myofibroblast transformation*. JCI Insight, 2017. **2**(8).
54. Shvab, A., et al., *Induction of the intestinal stem cell signature gene SMOC-2 is required for L1-mediated colon cancer progression*. Oncogene, 2016. **35**(5): p. 549-57.
55. Menendez, L., et al., *Identification of candidate methylation-responsive genes in ovarian cancer*. Mol Cancer, 2007. **6**: p. 10.
56. Vincent, A., et al., *Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma*. Clin Cancer Res, 2011. **17**(13): p. 4341-54.
57. Lee, C.H., et al., *MicroRNA-regulated protein-protein interaction networks and their functions in breast cancer*. Int J Mol Sci, 2013. **14**(6): p. 11560-606.
58. Finn, S.P., et al., *Expression microarray analysis of papillary thyroid carcinoma and benign thyroid tissue: emphasis on the follicular variant and potential markers of malignancy*. Virchows Arch, 2007. **450**(3): p. 249-60.
59. Liu, P., et al., *The SPARC-related factor SMOC-2 promotes growth factor-induced cyclin D1 expression and DNA synthesis via integrin-linked kinase*. Mol Biol Cell, 2008. **19**(1): p. 248-61.

60. Rocnik, E.F., et al., *The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity*. J Biol Chem, 2006. **281**(32): p. 22855-64.
61. Guezguez, A., et al., *Modulation of stemness in a human normal intestinal epithelial crypt cell line by activation of the WNT signaling pathway*. Exp Cell Res, 2014. **322**(2): p. 355-64.
62. Reed, N.I., et al., *The alphavbeta1 integrin plays a critical in vivo role in tissue fibrosis*. Sci Transl Med, 2015. **7**(288): p. 288ra79.
63. Hill, C.S., *Nucleocytoplasmic shuttling of Smad proteins*. Cell Res, 2009. **19**(1): p. 36-46.
64. Peinado, H., M. Quintanilla, and A. Cano, *Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions*. J Biol Chem, 2003. **278**(23): p. 21113-23.
65. Nagaraj, N.S. and P.K. Datta, *Targeting the transforming growth factor-beta signaling pathway in human cancer*. Expert Opin Investig Drugs, 2010. **19**(1): p. 77-91.
66. Gurtner, G.C., et al., *Wound repair and regeneration*. Nature, 2008. **453**(7193): p. 314-21.
67. Midwood, K.S., L.V. Williams, and J.E. Schwarzbauer, *Tissue repair and the dynamics of the extracellular matrix*. Int J Biochem Cell Biol, 2004. **36**(6): p. 1031-7.
68. Barrientos, S., et al., *Growth factors and cytokines in wound healing*. Wound Repair Regen, 2008. **16**(5): p. 585-601.
69. Landen, N.X., D. Li, and M. Stahle, *Transition from inflammation to proliferation: a critical step during wound healing*. Cell Mol Life Sci, 2016. **73**(20): p. 3861-85.
70. Gill, S.E. and W.C. Parks, *Metalloproteinases and their inhibitors: regulators of wound healing*. Int J Biochem Cell Biol, 2008. **40**(6-7): p. 1334-47.
71. Clark, R.A., et al., *Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization*. J Invest Dermatol, 1982. **79**(5): p. 264-9.
72. Barker, T.H. and A.J. Engler, *The provisional matrix: setting the stage for tissue repair outcomes*. Matrix Biol, 2017. **60-61**: p. 1-4.
73. Shaw, T.J. and P. Martin, *Wound repair at a glance*. J Cell Sci, 2009. **122**(Pt 18): p. 3209-13.
74. Hinz, B., *Formation and function of the myofibroblast during tissue repair*. J Invest Dermatol, 2007. **127**(3): p. 526-37.
75. Sandbo, N. and N. Dulin, *Actin cytoskeleton in myofibroblast differentiation: ultrastructure defining form and driving function*. Transl Res, 2011. **158**(4): p. 181-96.
76. Lovvorn, H.N., 3rd, et al., *Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair*. J Pediatr Surg, 1999. **34**(1): p. 218-23.
77. Schilling, J.A., *Wound healing*. Surg Clin North Am, 1976. **56**(4): p. 859-74.
78. Kyriakides, T.R. and P. Bornstein, *Matricellular proteins as modulators of wound healing and the foreign body response*. Thromb Haemost, 2003. **90**(6): p. 986-92.
79. Matsui, Y., J. Morimoto, and T. Uede, *Role of matricellular proteins in cardiac tissue remodeling after myocardial infarction*. World J Biol Chem, 2010. **1**(5): p. 69-80.
80. Wynn, T.A., *Cellular and molecular mechanisms of fibrosis*. J Pathol, 2008. **214**(2): p. 199-210.
81. Bonnans, C., J. Chou, and Z. Werb, *Remodelling the extracellular matrix in development and disease*. Nat Rev Mol Cell Biol, 2014. **15**(12): p. 786-801.

82. Zeisberg, M. and R. Kalluri, *Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis*. Am J Physiol Cell Physiol, 2013. **304**(3): p. C216-25.
83. Lu, P., et al., *Extracellular matrix degradation and remodeling in development and disease*. Cold Spring Harb Perspect Biol, 2011. **3**(12).
84. Schultz, G.S. and A. Wysocki, *Interactions between extracellular matrix and growth factors in wound healing*. Wound Repair Regen, 2009. **17**(2): p. 153-62.
85. Nakerakanti, S.S., A.M. Bujor, and M. Trojanowska, *CCN2 is required for the TGF-beta induced activation of Smad1-Erk1/2 signaling network*. PLoS One, 2011. **6**(7): p. e21911.
86. Ophascharoensuk, V., et al., *Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apoptosis*. Kidney Int, 1999. **56**(2): p. 571-80.
87. Estany, S., et al., *Lung fibrotic tenascin-C upregulation is associated with other extracellular matrix proteins and induced by TGFbeta1*. BMC Pulm Med, 2014. **14**: p. 120.
88. Borkham-Kamphorst, E., et al., *CCN1/CYR61 overexpression in hepatic stellate cells induces ER stress-related apoptosis*. Cell Signal, 2016. **28**(1): p. 34-42.
89. Yang, L., *How Acute Kidney Injury Contributes to Renal Fibrosis*. Adv Exp Med Biol, 2019. **1165**: p. 117-142.
90. Makris, K. and L. Spanou, *Acute Kidney Injury: Definition, Pathophysiology and Clinical Phenotypes*. Clin Biochem Rev, 2016. **37**(2): p. 85-98.
91. Bao, Y.W., et al., *Kidney disease models: tools to identify mechanisms and potential therapeutic targets*. Zool Res, 2018. **39**(2): p. 72-86.
92. Fliser, D., et al., *Advances in urinary proteome analysis and biomarker discovery*. J Am Soc Nephrol, 2007. **18**(4): p. 1057-71.
93. Brown, N.H., *Extracellular matrix in development: insights from mechanisms conserved between invertebrates and vertebrates*. Cold Spring Harb Perspect Biol, 2011. **3**(12).
94. Cox, T.R. and J.T. Erler, *Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer*. Dis Model Mech, 2011. **4**(2): p. 165-78.
95. Marchant, T.W., et al., *Canine Brachycephaly Is Associated with a Retrotransposon-Mediated Missplicing of SMOC2*. Curr Biol, 2017. **27**(11): p. 1573-1584 e6.
96. Schafer, M. and S. Werner, *Cancer as an overhealing wound: an old hypothesis revisited*. Nat Rev Mol Cell Biol, 2008. **9**(8): p. 628-38.
97. Bissell, M.J. and D. Radisky, *Putting tumours in context*. Nat Rev Cancer, 2001. **1**(1): p. 46-54.
98. Walker, C., E. Mojares, and A. Del Rio Hernandez, *Role of Extracellular Matrix in Development and Cancer Progression*. Int J Mol Sci, 2018. **19**(10).
99. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: a dynamic niche in cancer progression*. J Cell Biol, 2012. **196**(4): p. 395-406.
100. Butcher, D.T., T. Alliston, and V.M. Weaver, *A tense situation: forcing tumour progression*. Nat Rev Cancer, 2009. **9**(2): p. 108-22.
101. Levental, K.R., et al., *Matrix crosslinking forces tumor progression by enhancing integrin signaling*. Cell, 2009. **139**(5): p. 891-906.
102. Provenzano, P.P., et al., *Collagen reorganization at the tumor-stromal interface facilitates local invasion*. BMC Med, 2006. **4**(1): p. 38.
103. Paszek, M.J., et al., *Tensional homeostasis and the malignant phenotype*. Cancer Cell, 2005. **8**(3): p. 241-54.

104. Minchinton, A.I. and I.F. Tannock, *Drug penetration in solid tumours*. Nat Rev Cancer, 2006. **6**(8): p. 583-92.
105. Borsotti, P., et al., *Thrombospondin-1 is part of a Slug-independent motility and metastatic program in cutaneous melanoma, in association with VEGFR-1 and FGF-2*. Pigment Cell Melanoma Res, 2015. **28**(1): p. 73-81.
106. Jeanne, A., et al., *Original insights on thrombospondin-1-related antireceptor strategies in cancer*. Front Pharmacol, 2015. **6**: p. 252.
107. Lyu, T., et al., *Expression and epigenetic regulation of angiogenesis-related factors during dormancy and recurrent growth of ovarian carcinoma*. Epigenetics, 2013. **8**(12): p. 1330-46.
108. Wells, J.E., et al., *Deregulated expression of connective tissue growth factor (CTGF/CCN2) is linked to poor outcome in human cancer*. Int J Cancer, 2015. **137**(3): p. 504-11.
109. Chen, P.S., et al., *CTGF enhances the motility of breast cancer cells via an integrin-alphavbeta3-ERK1/2-dependent S100A4-upregulated pathway*. J Cell Sci, 2007. **120**(Pt 12): p. 2053-65.
110. Gu, C., et al., *FSTL1 interacts with VIM and promotes colorectal cancer metastasis via activating the focal adhesion signalling pathway*. Cell Death Dis, 2018. **9**(6): p. 654.
111. Kalluri, R. and E.G. Neilson, *Epithelial-mesenchymal transition and its implications for fibrosis*. J Clin Invest, 2003. **112**(12): p. 1776-84.
112. Roche, J., *The Epithelial-to-Mesenchymal Transition in Cancer*. Cancers (Basel), 2018. **10**(2).
113. Yilmaz, M. and G. Christofori, *EMT, the cytoskeleton, and cancer cell invasion*. Cancer Metastasis Rev, 2009. **28**(1-2): p. 15-33.
114. Wells, A., C. Yates, and C.R. Shepard, *E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas*. Clin Exp Metastasis, 2008. **25**(6): p. 621-8.
115. Zeisberg, M. and E.G. Neilson, *Biomarkers for epithelial-mesenchymal transitions*. J Clin Invest, 2009. **119**(6): p. 1429-37.
116. Vuoriluoto, K., et al., *Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer*. Oncogene, 2011. **30**(12): p. 1436-48.
117. Ivaska, J., *Vimentin: Central hub in EMT induction?* Small GTPases, 2011. **2**(1): p. 51-53.
118. Ivaska, J., et al., *PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility*. EMBO J, 2005. **24**(22): p. 3834-45.
119. Battaglia, R.A., et al., *Vimentin on the move: new developments in cell migration*. F1000Res, 2018. **7**.
120. Lee, H.W., et al., *Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma*. Clin Cancer Res, 2013. **19**(21): p. 5879-89.
121. Masszi, A., 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**(5): p. F911-24.
122. Nagano, M., et al., *Turnover of focal adhesions and cancer cell migration*. Int J Cell Biol, 2012. **2012**: p. 310616.

123. Meng, X.N., et al., *Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion*. Br J Cancer, 2009. **101**(2): p. 327-34.
124. Wang, J.P. and A. Hielscher, *Fibronectin: How Its Aberrant Expression in Tumors May Improve Therapeutic Targeting*. J Cancer, 2017. **8**(4): p. 674-682.
125. Micalizzi, D.S., S.M. Farabaugh, and H.L. Ford, *Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression*. J Mammary Gland Biol Neoplasia, 2010. **15**(2): p. 117-34.
126. Sarkar, S., et al., *Cancer development, progression, and therapy: an epigenetic overview*. Int J Mol Sci, 2013. **14**(10): p. 21087-113.
127. Brabletz, T., et al., *Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment*. Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10356-61.
128. Puisieux, A., T. Brabletz, and J. Caramel, *Oncogenic roles of EMT-inducing transcription factors*. Nat Cell Biol, 2014. **16**(6): p. 488-94.
129. Royer, C. and X. Lu, *Epithelial cell polarity: a major gatekeeper against cancer?* Cell Death Differ, 2011. **18**(9): p. 1470-7.
130. Tavazoie, S.F., et al., *Endogenous human microRNAs that suppress breast cancer metastasis*. Nature, 2008. **451**(7175): p. 147-52.
131. Sun, Z., et al., *Tenascin-C Promotes Tumor Cell Migration and Metastasis through Integrin alpha9beta1-Mediated YAP Inhibition*. Cancer Res, 2018. **78**(4): p. 950-961.
132. Jha, V., et al., *Chronic kidney disease: global dimension and perspectives*. Lancet, 2013. **382**(9888): p. 260-72.
133. Liu, Y., *Renal fibrosis: new insights into the pathogenesis and therapeutics*. Kidney Int, 2006. **69**(2): p. 213-7.
134. Wynn, T.A. and T.R. Ramalingam, *Mechanisms of fibrosis: therapeutic translation for fibrotic disease*. Nat Med, 2012. **18**(7): p. 1028-40.
135. Bornstein, P., *Matricellular proteins: an overview*. J Cell Commun Signal, 2009. **3**(3-4): p. 163-5.
136. Rienks, M. and A.P. Papageorgiou, *Novel regulators of cardiac inflammation: Matricellular proteins expand their repertoire*. J Mol Cell Cardiol, 2016. **91**: p. 172-8.
137. Craciun, F.L., et al., *RNA Sequencing Identifies Novel Translational Biomarkers of Kidney Fibrosis*. J Am Soc Nephrol, 2016. **27**(6): p. 1702-13.
138. Arvaniti, E., et al., *Whole-transcriptome analysis of UUO mouse model of renal fibrosis reveals new molecular players in kidney diseases*. Sci Rep, 2016. **6**: p. 26235.
139. Walker, J.T., et al., *Periostin as a multifunctional modulator of the wound healing response*. Cell Tissue Res, 2016. **365**(3): p. 453-65.
140. Craciun, F.L., et al., *Pharmacological and genetic depletion of fibrinogen protects from kidney fibrosis*. Am J Physiol Renal Physiol, 2014. **307**(4): p. F471-84.
141. Kang, H.M., et al., *Sox9-Positive Progenitor Cells Play a Key Role in Renal Tubule Epithelial Regeneration in Mice*. Cell Rep, 2016. **14**(4): p. 861-71.
142. Chevalier, R.L., M.S. Forbes, and B.A. Thornhill, *Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy*. Kidney Int, 2009. **75**(11): p. 1145-52.
143. Yang, L., et al., *Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury*. Nat Med, 2010. **16**(5): p. 535-43, 1p following 143.

144. Szczypka, M.S., et al., *Rare incorporation of bone marrow-derived cells into kidney after folic acid-induced injury*. Stem Cells, 2005. **23**(1): p. 44-54.
145. Sanz, A.B., et al., *The cytokine TWEAK modulates renal tubulointerstitial inflammation*. J Am Soc Nephrol, 2008. **19**(4): p. 695-703.
146. Gupta, A., et al., *Folic acid induces acute renal failure (ARF) by enhancing renal prooxidant state*. Exp Toxicol Pathol, 2012. **64**(3): p. 225-32.
147. Fu, Y., et al., *Rodent models of AKI-CKD transition*. Am J Physiol Renal Physiol, 2018. **315**(4): p. F1098-F1106.
148. Long, D.A., et al., *Increased renal angiopoietin-1 expression in folic acid-induced nephrotoxicity in mice*. J Am Soc Nephrol, 2001. **12**(12): p. 2721-31.
149. Eddy, A.A., et al., *Investigating mechanisms of chronic kidney disease in mouse models*. Pediatr Nephrol, 2012. **27**(8): p. 1233-47.
150. Hewitson, T.D., *Renal tubulointerstitial fibrosis: common but never simple*. Am J Physiol Renal Physiol, 2009. **296**(6): p. F1239-44.
151. Schubert, G.E. and G. Otten, *Chronic folic acid-nephropathy*. Res Exp Med (Berl), 1974. **162**(1): p. 17-36.
152. Muramatsu, Y., et al., *Early detection of cysteine rich protein 61 (CYR61, CCN1) in urine following renal ischemic reperfusion injury*. Kidney Int, 2002. **62**(5): p. 1601-10.
153. Mengual, L., et al., *Using gene expression from urine sediment to diagnose prostate cancer: development of a new multiplex mRNA urine test and validation of current biomarkers*. BMC Cancer, 2016. **16**: p. 76.
154. Nakada, M., et al., *Suppression of membrane-type 1 matrix metalloproteinase (MMP)-mediated MMP-2 activation and tumor invasion by testican 3 and its splicing variant gene product, N-Tes*. Cancer Res, 2001. **61**(24): p. 8896-902.
155. Scharenberg, M.A., et al., *TGF-beta-induced differentiation into myofibroblasts involves specific regulation of two MKL1 isoforms*. J Cell Sci, 2014. **127**(Pt 5): p. 1079-91.
156. Richards, J.M., et al., *Crystallinity of hydroxyapatite drives myofibroblastic activation and calcification in aortic valves*. Acta Biomater, 2018. **71**: p. 24-36.
157. He, G., et al., *Nucleation of apatite crystals in vitro by self-assembled dentin matrix protein 1*. Nat Mater, 2003. **2**(8): p. 552-8.
158. Kimura, H., et al., *Tenascin-W inhibits proliferation and differentiation of preosteoblasts during endochondral bone formation*. Biochem Biophys Res Commun, 2007. **356**(4): p. 935-41.
159. Martina, E., et al., *Tenascin-W is a specific marker of glioma-associated blood vessels and stimulates angiogenesis in vitro*. FASEB J, 2010. **24**(3): p. 778-87.
160. Bouleftour, W., et al., *The role of the SIBLING, Bone Sialoprotein in skeletal biology - Contribution of mouse experimental genetics*. Matrix Biol, 2016. **52-54**: p. 60-77.
161. Prasad, M., W.T. Butler, and C. Qin, *Dentin sialophosphoprotein in biomineralization*. Connect Tissue Res, 2010. **51**(5): p. 404-17.
162. Madne, T.H. and M.E.C. Dockrell, *CCN3, a key matricellular protein, distinctly inhibits TGFbeta1-mediated Smad1/5/8 signalling in human podocyte culture*. Cell Mol Biol (Noisy-le-grand), 2018. **64**(3): p. 5-10.
163. Zhong, X., et al., *Serum levels of WNT1-inducible signaling pathway protein-1 (WISP-1): a noninvasive biomarker of renal fibrosis in subjects with chronic kidney disease*. Am J Transl Res, 2017. **9**(6): p. 2920-2932.

164. Wang, Q., et al., *Cooperative interaction of CTGF and TGF-beta in animal models of fibrotic disease*. Fibrogenesis Tissue Repair, 2011. **4**(1): p. 4.
165. Guha, M., et al., *Specific down-regulation of connective tissue growth factor attenuates progression of nephropathy in mouse models of type 1 and type 2 diabetes*. FASEB J, 2007. **21**(12): p. 3355-68.
166. Vollmann, E.H., et al., *Identification of Novel Fibrosis Modifiers by In Vivo siRNA Silencing*. Mol Ther Nucleic Acids, 2017. **7**: p. 314-323.
167. Fang, Y., et al., *Follistatin like-1 aggravates silica-induced mouse lung injury*. Sci Rep, 2017. **7**(1): p. 399.
168. Maruyama, S., et al., *Follistatin-like 1 promotes cardiac fibroblast activation and protects the heart from rupture*. EMBO Mol Med, 2016. **8**(8): p. 949-66.
169. Dong, Y., et al., *Blocking follistatin-like 1 attenuates bleomycin-induced pulmonary fibrosis in mice*. J Exp Med, 2015. **212**(2): p. 235-52.
170. Lai, C.F., et al., *Blockade of cysteine-rich protein 61 attenuates renal inflammation and fibrosis after ischemic kidney injury*. Am J Physiol Renal Physiol, 2014. **307**(5): p. F581-92.
171. Lai, C.F., et al., *Cysteine-rich protein 61 plays a proinflammatory role in obstructive kidney fibrosis*. PLoS One, 2013. **8**(2): p. e56481.
172. Trostel, J., et al., *Different effects of global osteopontin and macrophage osteopontin in glomerular injury*. Am J Physiol Renal Physiol, 2018. **315**(4): p. F759-F768.
173. Zhu, X., et al., *Mangiferin attenuates renal fibrosis through down-regulation of osteopontin in diabetic rats*. Phytother Res, 2015. **29**(2): p. 295-302.
174. Yoo, K.H., et al., *Osteopontin regulates renal apoptosis and interstitial fibrosis in neonatal chronic unilateral ureteral obstruction*. Kidney Int, 2006. **70**(10): p. 1735-41.
175. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2*. Genome Biol, 2014. **15**(12): p. 550.
176. Yu, G., et al., *clusterProfiler: an R package for comparing biological themes among gene clusters*. OMICS, 2012. **16**(5): p. 284-7.
177. Jensen, L.J., et al., *STRING 8--a global view on proteins and their functional interactions in 630 organisms*. Nucleic Acids Res, 2009. **37**(Database issue): p. D412-6.
178. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. Int J Cancer, 2015. **136**(5): p. E359-86.
179. Nabi, S., et al., *Renal cell carcinoma: a review of biology and pathophysiology*. F1000Res, 2018. **7**: p. 307.
180. Gupta, K., et al., *Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review*. Cancer Treat Rev, 2008. **34**(3): p. 193-205.
181. Hsieh, J.J., et al., *Renal cell carcinoma*. Nat Rev Dis Primers, 2017. **3**: p. 17009.
182. Han, W.K., et al., *Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma*. J Am Soc Nephrol, 2005. **16**(4): p. 1126-34.
183. Velez-delValle, C., et al., *Epithelial cell migration requires the interaction between the vimentin and keratin intermediate filaments*. Sci Rep, 2016. **6**: p. 24389.
184. Chen, Q., et al., *Growth-induced stress enhances epithelial-mesenchymal transition induced by IL-6 in clear cell renal cell carcinoma via the Akt/GSK-3beta/beta-catenin signaling pathway*. Oncogenesis, 2017. **6**(8): p. e375.

185. Lu, H., et al., *Targeting cancer stem cell signature gene SMOC-2 Overcomes chemoresistance and inhibits cell proliferation of endometrial carcinoma*. EBioMedicine, 2019. **40**: p. 276-289.
186. Huang, X.Q., et al., *Overexpression of SMOC2 Attenuates the Tumorigenicity of Hepatocellular Carcinoma Cells and Is Associated With a Positive Postoperative Prognosis in Human Hepatocellular Carcinoma*. J Cancer, 2017. **8**(18): p. 3812-3827.
187. Justus, C.R., et al., *In vitro cell migration and invasion assays*. J Vis Exp, 2014(88).
188. Williams, R.D., et al., *In vitro cultivation of human renal cell cancer. II. Characterization of cell lines*. In Vitro, 1978. **14**(9): p. 779-86.
189. Brodaczewska, K.K., et al., *Choosing the right cell line for renal cell cancer research*. Mol Cancer, 2016. **15**(1): p. 83.
190. Murata, J., et al., *Differences in chemotaxis to fibronectin in weakly and highly metastatic tumor cells*. Jpn J Cancer Res, 1992. **83**(12): p. 1327-33.
191. Wang, P., S.M. Henning, and D. Heber, *Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols*. PLoS One, 2010. **5**(4): p. e10202.
192. Ou, Y.C., et al., *Fibronectin Promotes Cell Growth and Migration in Human Renal Cell Carcinoma Cells*. Int J Mol Sci, 2019. **20**(11).
193. Erler, J.T. and V.M. Weaver, *Three-dimensional context regulation of metastasis*. Clin Exp Metastasis, 2009. **26**(1): p. 35-49.
194. Sawyer, A.J. and T.R. Kyriakides, *Matricellular proteins in drug delivery: Therapeutic targets, active agents, and therapeutic localization*. Adv Drug Deliv Rev, 2016. **97**: p. 56-68.
195. Bulow, R.D. and P. Boor, *Extracellular Matrix in Kidney Fibrosis: More Than Just a Scaffold*. J Histochem Cytochem, 2019: p. 22155419849388.
196. Jantos-Siwy, J., et al., *Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease*. J Proteome Res, 2009. **8**(1): p. 268-81.
197. Hewitt, S.M., J. Dear, and R.A. Star, *Discovery of protein biomarkers for renal diseases*. J Am Soc Nephrol, 2004. **15**(7): p. 1677-89.
198. Caliari, S.R. and J.A. Burdick, *A practical guide to hydrogels for cell culture*. Nat Methods, 2016. **13**(5): p. 405-14.
199. Murphy, K.A., et al., *A Syngeneic Mouse Model of Metastatic Renal Cell Carcinoma for Quantitative and Longitudinal Assessment of Preclinical Therapies*. J Vis Exp, 2017(122).
200. Salup, R.R., R.B. Herberman, and R.H. Wiltrout, *Role of natural killer activity in development of spontaneous metastases in murine renal cancer*. J Urol, 1985. **134**(6): p. 1236-41.