August 2019

YAP-mediated mechanotransduction promotes fibrotic activity

Michael Racanelli
The University of Western Ontario

Supervisor
Andrew Leask
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Michael Racanelli 2019

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Recommended Citation
https://ir.lib.uwo.ca/etd/6283

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.
Abstract

Fibrosis, a phenotype associated with mortality for a multitude of diseases, has no disease-modifying treatment. We examine if Verteporfin (VP), an inhibitor of the YAP-TEAD complex, currently in wide clinical use for macular degeneration, can inhibit profibrotic gene expression in human dermal fibroblasts. Also, an *in vivo* approach was taken using a *Pten*-deficient mouse of progressive skin fibrosis for examining effects on melanoma metastasis. We found that treatment with VP reduced basal expression of a variety of profibrotic genes and prevented a myofibroblast-like phenotype in response to TGFβ1. Indeed, genome-wide expression analysis suggested that VP inhibited a cluster of profibrotic genes with and without added TGFβ1. Preliminary analysis of metastasis to the lung of melanoma tumors in *Pten*-deficient mice showed a trend towards increased metastasis the absence of *Pten* expression by fibroblasts; i.e., in a fibrotic microenvironment. My findings support targeting YAP as a potential treatment for fibrotic diseases.

Keywords

Fibrosis, mechanotransduction, extracellular matrix (ECM), Verteporfin (VP), connective tissue growth factor (CCN2/CTGF), yes-associated protein 1 (YAP), transforming growth factor beta 1 (TGFβ1), melanoma, cancer, metastasis
Summary for Lay Audience

Pathological fibrosis is the progressive stiffening of skin or other organs, ultimately interfering with organ function and resulting in death. Pathological fibrosis is involved with 45% of mortalities in the developed world and has no available treatment. Progressive stiffening of the tissue is characterized by the progressive differentiation of local cells known as fibroblasts into stiff cells known as myofibroblasts. These cells exert mechanical tension onto themselves and the scaffolding around cells known as the extracellular matrix. This effect is involved in normal processes such as wound repair, however in fibrotic disease, it becomes dysregulated. The progressive increase of tension in the tissue also changes gene expression of proteins in the cell. The most well-known protein that is produced in cases of fibrotic disease is CCN2. This protein is known to bind to the cell surface and is thought to prevent the resolution of cellular stiffness. In fact, CCN2 also functions to transition more cells into myofibroblasts and increase tissue stiffness, creating a positive-feedback loop. Increased stiffness seen in fibrotic disease highly reflects the tumor microenvironment, the scaffolding around cancerous cells, and is thought to aid the migration of tumor cells to other locations in the body. Recent research has pointed towards YAP as a central mediator of fibrotic gene expression, including CCN2. Here, we repurposed Verteporfin, a drug currently on the market for macular degeneration, as an inhibitor of YAP. Administering Verteporfin to fibroblasts prevented differentiation into myofibroblasts normally induced by TGFβ1. A mass analysis of gene expression revealed that inhibiting YAP with Verteporfin reduced expression of many known fibrosis-related genes, including CCN2. These findings support YAP as a central mediator of fibrosis, and repurposing Verteporfin to inhibit YAP could be a viable therapeutic strategy with relatively little financial investment. To test the ability of a fibrotic microenvironment to enhance cancer metastasis, we used a mouse model with silenced Pten, a cellular signaling protein upstream from Yap in the cellular signaling pathway, to induce spontaneous fibrosis. However, the induced fibrotic phenotype was not as stiff as expected, and the results were inconclusive.
Co-Authorship Statement

All experiments were performed by Michael Racanelli, except for the microarray analysis which was performed by David Carter (London Regional Genomics Center, Robarts Research Institute, London, Canada).
Acknowledgments

I would like to express thanks to my supervisor, Andrew Leask, for allowing me the opportunity and influencing my decision to pursue research. I would also like to thank James Hutchenreuther and Katherine Quesnel for training me and helping with experiments. Finally, I would like to thank my GSR and advisory committee, Tom Drysdale, Lina Dagnino and John DiGuglielmo, for the collaboration and helpful advice.
# Table of Contents

Abstract ................................................................................................................................... ii

Summary for Lay Audience ........................................................................................................ iii

Co-Authorship Statement ............................................................................................................ iv

Acknowledgments ....................................................................................................................... v

Table of Contents ....................................................................................................................... vi

List of Figures ............................................................................................................................ ix

List of Abbreviations .................................................................................................................. x

1 Introduction ............................................................................................................................. 1

1.1 Fibrosis ............................................................................................................................... 1

1.2 Systemic sclerosis (SSc) .................................................................................................... 2

1.3 The myofibroblast ............................................................................................................. 3

1.4 Integrin β1-mediated mechanotransduction ..................................................................... 5

1.5 CCN matricellular proteins .............................................................................................. 6

1.5.1 CCN2 ............................................................................................................................ 7

1.5.2 CCN2 signaling pathway .............................................................................................. 9

1.5.3 CCN2 and progressive fibrosis .................................................................................. 9

1.6 YAP/TAZ/TEAD ................................................................................................................... 11

1.7 Fibrosis and cancer metastasis ......................................................................................... 12

1.8 Phosphatase and tensin homologue ................................................................................ 16

1.9 Rationale, objective and hypothesis ............................................................................... 17

2 Materials and Methods ......................................................................................................... 19

2.1 Cell culture ....................................................................................................................... 19

2.2 RNA isolation ................................................................................................................... 20
2.3 qPCR analysis ................................................................. 20
2.4 Microarray analysis .......................................................... 22
2.5 Genotyping ...................................................................... 23
2.6 Generation of fibroblast specific *Pten* deficient mice ............... 24
2.7 Tumor studies .................................................................. 24
2.8 Skin thickness and lung metastasis analysis ................................ 25
2.9 Tissue homogenization ........................................................ 26
2.10 Immunocytochemistry analysis .......................................... 26
3 Results .............................................................................. 28
  3.1 *Cell culture-based studies* ................................................ 28
    3.1.1 VP blocks TGFβ1 induced HFF myofibroblast-like phenotype ..... 28
    3.1.2 VP significantly inhibits gene expression of *CCN2, COL1A1, COL1A2, and TGFβ1*, and does not affect expression of *PTEN or YAP*. 30
    3.1.3 VP reduces gene expression of clusters heavily enriched in ECM secretion and binding in the presence and in the absence of exogenous TGFβ1 ........................................................... 33
    3.1.4 VP reduced 28 potential key regulators of TGFβ1-induced myofibroblast differentiation ........................................................... 33
    3.1.5 VP fails to inhibit expression of *TGFβ2, TNFAIP6, and CCN2* in the presence of TGFβ1 ............................................................. 37
    3.1.6 Exogenous TGFβ1 does not interfere with inhibition of pro-fibrotic gene expression induced by inhibition of YAP with VP .......................... 37
3.2 *in vivo* mice studies of fibroblast specific *Pten*-deficiency and melanoma tumor metastasis ...................................................... 43
  3.2.1 *Pten* expression in connective ear tissue was significantly suppressed in *Pten*^fl/fl^ mice administered tamoxifen. .......................... 43
  3.2.2 *Pten* expression in skin of *Pten*^-/-^ mice is decreased, albeit non-significant ................................................................. 45
  3.2.3 Skin in *Pten*-deficient mice was significantly thicker .................. 45
3.2.4  $Pten^{fl/fl}$ and $Pten^{-/-}$ mice had no significant difference in melanoma primary tumor growth ................................................................. 48

3.2.5  Melanoma lung tumor metastasis in $Pten^{-/-}$ mice .................. 48

4  Discussion ......................................................................................................................... 51

4.1  Clinical relevance............................................................................................................. 51

4.2  YAP activation occurs in response to mechanotransduction in otherwise unstimulated fibroblasts ................................................................. 52

4.3  Significant reduction of LTBP2 and FN1 prevent myofibroblast differentiation 53

4.4  YAP selectively regulates various key pro-fibrotic genes ............................................ 54

4.5  Validation of $in vivo$ fibrotic genotype ........................................................................ 58

4.6  Loss of fibrotic phenotype .............................................................................................. 58

4.7  Limitations ....................................................................................................................... 59

4.8  Future experiments ......................................................................................................... 60

4.9  Conclusion ....................................................................................................................... 62

References .......................................................................................................................... 64

Curriculum Vitae ............................................................................................................... 80
List of Figures

Figure 1.1 Model of myofibroblast differentiation in fibrotic lesions .................................. 4

Figure 1.2 Signaling by CCN family members. ................................................................. 8

Figure 1.3 Proposed fibrotic signaling feedback loop. ..................................................... 13

Figure 1.4 YAP/TAZ activation promotes several steps of the metastatic cascade. ........ 15

Figure 3.1 VP blocks TGFβ1 induced HFF myofibroblast-like phenotype .................... 29

Figure 3.2 VP significantly inhibits gene expression of CCN2, COL1A1, COL1A2, and TGFβ1, and does not affect expression of PTEN or YAP .................................................. 32

Figure 3.3 VP reduces gene expression of clusters heavily enriched in ECM secretion and binding in the presence and in the absence of exogenous TGFβ1 .................................... 35

Figure 3.4 VP reduced 28 potential key regulators of TGFβ1-induced myofibroblast differentiation ................................................................. 36

Figure 3.5 VP fails to inhibit expression of TGFβ2, TNFAIP6, and CCN2 in the presence of TGFβ1 ................................................................. 38

Figure 3.6 Exogenous TGFβ1 does not interfere with inhibition of pro-fibrotic gene expression induced by inhibition of YAP with VP .................................................. 41

Figure 3.7 Pten expression in connective ear tissue was significantly suppressed in Pten<sup>−/−</sup> mice administered tamoxifen ................................................................. 44

Figure 3.8 Pten expression in skin of Pten<sup>−/−</sup> mice is decreased, albeit non-significant.... 46

Figure 3.9 Pten<sup>−/−</sup> had significantly increased dermal thickness compared to Pten<sup>+/+</sup> .... 47

Figure 3.10 Pten<sup>+/+</sup> and Pten<sup>−/−</sup> had no significant difference in primary tumor growth... 49
Figure 3.11 $Pten^{+\rightarrow}$ mice suggest enhanced melanoma tumor metastasis from primary tumor to the lungs, however there was no significant difference. ........................................... 50

Figure 4.1 Increased mouse group size would be required to be able to accurately measure significance................................................................. 61

List of Abbreviations

CCN (1-6) – cellular communication network (factors 1-6)
CTGF – connective tissue growth factor
DMEM – Dulbecco’s Modified Eagle's Medium
DMSO – dimethylsulfoxide
DPBS – Dulbecco’s phosphate-buffered saline
ECM – extracellular matrix
EDA – extra domain A
FA – focal adhesion
FAK – focal adhesion kinase
FBS – fetal bovine serum
HFF – human foreskin fibroblast
PI3K – phosphatidylinositol 3 kinase
PIP2 – phosphatidylinositol (4,5)-bisphosphate
PIP3 – phosphatidylinositol (3,4,5)-trisphosphate
PTEN – phosphatase and tensin homologue, deleted on chromosome ten
$\alpha$-SMA – alpha-smooth muscle actin
SSc – systemic sclerosis
TEAD – TEA domain
TGFβ (1) – transforming growth factor beta (1)

VP – verteporfin

YAP – yes-associated protein 1
1 Introduction

1.1 Fibrosis

Fibrosis, the final pathological outcome of many chronic inflammatory diseases, involved with nearly 45% of all deaths in the developed world. There is no available anti-fibrotic treatment that blocks and/or reverses disease progression (Wynn, 2007; Wynn et al., 2012). In normal tissue repair, inflammation occurs in response to injury in three distinct phases. The first is an early proinflammatory step, in which the release of cytokines recruits neutrophils, monocytes, and other innate immune cells to the site of injury. In the second phase, the proinflammatory response begins to subside and key inflammatory cells such as macrophages switch to a reparative phenotype. In the final step, the inflammatory response is resolved with inflammatory cells exiting the site of lesion or are eliminated through apoptosis (Eming et al., 2017). Wound repair macrophages are characterized by the enhanced production of numerous growth factors, including platelet derived (PDGF), insulin-like (IGF-1), and vascular endothelial (VEGF-α), which promote cell proliferation and blood vessel development (Shimokado et al., 1985; Rappolee et al., 1988; Berse et al., 1992; Chujo et al., 2009; Willenborg et al., 2012). They also produced transforming growth factor beta one (TGFβ1), which stimulates local and recruited fibroblasts into myofibroblasts that facilitate wound contraction and closure, as well as the synthesis of extracellular matrix (ECM) components (Akhurst et al., 2012). In normal tissue repair, myofibroblasts are resolved through apoptosis (Leight et al., 2012), and recruited cells are returned to their original state. Production of the ECM is balanced with degradation of the ECM through the action
of matrix metalloproteinases (MMPs) (Gill et al., 2008). Pathological fibrosis is characterized by the excess deposition of extracellular matrix (ECM) components by myofibroblasts, such as collagen and fibronectin, in an organ or tissue. Normal tissue repair can turn into an irreversible fibrotic response in cases of severe or repetitive injury, or in cases where the wound healing response becomes dysregulated (Wynn et al., 2012). Progressive fibrosis results in gradual loss of organ function due to the replacement of functional tissue with scar tissue, and is the major cause of morbidity in individuals with systemic sclerosis (SSc) (Ho et al., 2014).

1.2 Systemic sclerosis (SSc)

Systemic sclerosis (SSc), also referred to as scleroderma, is an auto-immune rheumatic disease that is characterized by progressive fibrosis of the skin, internal organs and vasculature. Although uncommon, it has high morbidity and mortality. As the etiology of SSc fibrosis is unknown, treatment options are limited to those that target disease symptoms (Denton et al., 2017). Genome-wide expression profiling of SSc skin revealed that the disease is highly heterogeneous, with patients subdivided into either “fibroproliferative” or “inflammation/innate immunity” cohorts based on the overexpression of genes compared to individuals without SSc (Milano et al., 2008). Altered expression of genes associated with transforming growth factor beta (TGFβ) and Wnt signaling are commonly seen in fibroblasts cultured from SSc skin biopsy samples; these genes appear to be overexpressed in the fibroproliferative cohort of SSc patients (Gardner et al., 2006). Sustained, localized inflammation, vascular damage and the resultant presence of local and systemic profibrotic growth factors/cytokines facilitate
the differentiation of resident fibroblasts (and possibly recruited cells, including pericytes, fibrocytes and endothelial/adipogenic progenitor cells) into α-smooth muscle actin (SMA)-expressing myofibroblasts. The latter are considered the effector cells of fibrosis, which deposit and remodel large quantities of ECM components (Trojanowska, 2010) (Figure 1.1). Thus, induction of myofibroblasts is also promoted by the increased ECM stiffness inherent to the scar tissue, although it is unclear the extent to which ECM stiffness acts upstream or in parallel to the effect of growth factors and cytokines on fibroblasts.

### 1.3 The myofibroblast

Myofibroblasts, the primary type of cells that produce ECM in response to injury in every organ of the body, produce high levels of α-smooth muscle actin (SMA)-containing stress fibers, which are highly contractile. These fibers are linked to the surrounding ECM through focal adhesions, and transduce mechanical tension from the ECM to the myofibroblast (Bissell, 1998; Koivisto et al., 2014; Hinz, 2016). In normal wound repair, TGFβ-induced myofibroblasts are resolved through apoptosis or deactivation after wound repair is completed. However, should this program persist or be aberrantly activated, fibrotic responses result. For example, when endothelial cells are injured, an extra-
Profibrotic growth factors such as TGFβ1 stimulate differentiation of resident fibroblasts into α-SMA expressing myofibroblasts, which display elevated integrin/focal adhesion kinase (FAK) mediated extracellular matrix (ECM) contraction. Myofibroblasts excessively deposit collagen and other pro-fibrotic components, such as cellular communication network factor 2 (CCN2), which stiffen the ECM. Some pro-fibrotic components signal back on other resident fibroblasts to stimulate further myofibroblast differentiation and ECM stiffness, thus creating a pro-fibrotic autocrine signaling loop. If the production of these mechano-sensitive factors is regulated by YAP, then intervention with verteporfin (VP) may break the autocrine pro-fibrotic loop and provide a therapeutic benefit.
domain A (EDA)-containing isoform of fibronectin is released, which recruits and activates progenitor cells that differentiate into myofibroblasts, failing to senesce after the initial stimulus is removed (Hutchenreuther et al., 2016). These myofibroblasts then excessively deposit and remodel collagen and other ECM components, resulting in increased ECM stiffness, which further acts on myofibroblasts. Thus, myofibroblasts are maintained by an autocrine, pro-adhesive signaling loop. Supporting this notion, myofibroblasts cultured from lesion areas of SSc patients, compared to fibroblasts from unaffected areas of SSc patients or with healthy individuals, excessively adhere to and contract the ECM (Thannickal et al., 2003; Leask, 2012). Overall, the available data suggest that mechanical tension from the ECM acting on the fibroblast may initiate and perpetuate the fibrotic phenotype through a process known as mechanotransduction.

1.4 Integrin β1-mediated mechanotransduction

Fibroblasts involved in fibrotic lesions develop stronger attachments to the ECM through structures called focal adhesions (FAs). These attachments signal through clusters of specialized cell-surface receptors called integrins, which stimulate differentiation of fibroblast into myofibroblasts (Eckes et al., 2000; Gabbiani, 2003). Focal adhesion kinase (FAK) mediated mechanotransduction was shown to be required for TGFβ- induced myofibroblast differentiation. This was demonstrated using FAK-deficient fibroblasts, or using the FAK/src inhibitor PP2 (Liu et al., 2007). We found that FAK contributes to the persistent fibrotic phenotype of SSc fibroblasts (Shi-wen et al., 2012). Indeed, cell attachment to the ECM and consequent activation of FAK is required for myofibroblast differentiation in response to TGFβ1 (Thannickal et al., 2003; Liu et
Mechanical tension from the ECM is communicated through integrins to the nucleus through SMA-containing stress fibers. Integrins are heterodimers composed of one α- and one β- subunit. The main integrins that are involved in adhesion to type I collagen include: α1β1, α2β1, and α11β1 (Schiro et al., 1991; Pozzi et al., 1998; Carracedo et al., 2010). The integrin subunit β1 is as a key, central mediator of profibrotic mechanotransduction (Asano et al., 2006; Zhou et al., 2010). Moreover, integrin αvβ1 mediates activation of latent TGFβ in response to strain and ECM contraction (Leask et al., 2014; Reed et al., 2015). Partial inhibition of integrin α1β6 prevented murine pulmonary fibrosis without exacerbating inflammation (Horan et al., 2008). Thus integrin-mediated adhesion may act, depending on the context, in both parallel and upstream fashions to promote myofibroblast activity. Mice with fibroblasts that are deficient in the integrin β1 subunit are resistant to bleomycin-induced skin fibrosis; however, they also show impaired cutaneous tissue repair and progressive skin thinning, indicating an essential role for this subunit in dermal homeostasis (Liu et al., 2009, 2010 a).

1.5 CCN matricellular proteins

The cellular communication network (CCN) family of matricellular proteins nomenclature was officially designated by the HUGO Gene Nomenclature Committee in October 2018 (Perbal et al., 2018). The family contains six matricellular proteins (CCN1 through 6), each consisting of four conserved cysteine rich domains; an insulin-like growth factor-binding domain, a Von Willebrand factor type C domain, a thrombospondin type 1 domain and a CT-terminal domain containing a cysteine knot
motif (except for CCN5 which lacks a CT domain) (Figure 1.2). These matricellular proteins are secreted into the ECM where they have a wide variety of regulatory roles. These roles are just recently being elucidated, and are proposed to interact in a dynamic spatio-temporal relationship between multiple members of the CCN family and each of their four domains (Perbal, 2018). The tetra modular structure of the CCN proteins potentially allows for a wide variety of interactions and functions. Either these functions overlap or are activated by individual CCN proteins based on the cytokines expressed in the specific tissue (Perbal, 2018).

1.5.1 CCN2

Formerly known as connective tissue growth factor (CTGF), cellular communication network factor 2 (CCN2) was first identified as a protein secreted from human umbilical vein endothelial cells (Bradham et al., 1991). Although not usually expressed under normal conditions by mesenchymal cells, CCN2 is rapidly induced in fibroblasts in response to tissue injury (Igarashi et al., 1993; Kapoor et al., 2008). In an in vivo rabbit model of hypertrophic scarring, antisense-mediated degradation of CCN2 mRNA reduced excess scarring without significantly affecting wound healing (Sisco et al., 2008). In healthy tissues, CCN2 is essentially absent, however in bleomycin-induced skin fibrosis and SSc skin lesions, CCN2 is overexpressed. This suggests CCN2 may be a key indicator and mediator of fibrotic injury (Igarashi et al., 1996; Liu et al., 2010 b).
Figure 1.2 Signaling by CCN family members.

CCN1, CCN2 and CCN3 bind TGFβ, fibronectin, integrins, LRP1 and HSPGs as indicated. CCN proteins appear to signal principally through the C-terminal quarter (domain IV) to activate adhesive signaling pathways and hence amplify responses to TGFβ or fibronectin. (Reproduced with permission from: All in the CCN family: essential matricellular signaling modulators emerge from the bunker (Leask et al., 2006). doi: 10.1242/jcs.03270)
1.5.2 CCN2 signaling pathway

In response to TGFβ, fibroblasts not only differentiate into myofibroblasts but also begin to express CCN2 (Igarashi et al., 1993). TGFβ-induced CCN2 expression occurs via the canonical Smad3 pathway; however, the maintenance of the SSc phenotype is independent of Smad signaling, as the Smad-binding element in the CCN2 promoter is required for TGFβ-induced CCN2 promoter activation, but not for the heightened CCN2 promoter activity observed in fibroblasts isolated from fibrotic lesions of SSc patients (Holmes et al., 2001). CCN2 is expressed in cultured activated myofibroblasts. In a three-dimensional co-culture system, SSc epidermal cells also increase CCN2 expression through interleukin 1 alpha (IL-1α) production and endothelin-1 induced TGFβ expression (Aden et al., 2010). CCN2 exists in the ECM where it binds to fibronectin and the fibronectin receptor integrins α4, α5, β1, as well as syndecan-4. Fibroblast attachment to CCN2 also requires MEK/ERK activity (Holmes et al., 2001). CCN2-deficient myofibroblasts showed reduced activation via FAK/Akt signaling pathways (Shi-wen et al., 2006), suggesting that CCN2 may be a fibrogenic marker or mediator.

1.5.3 CCN2 and progressive fibrosis

Use of immunohistochemical and specific ELISA assays has shown that CCN2 protein expression levels correlate with the progression of SSc and pulmonary fibrosis; possibly indicating that CCN2 is a key regulator in the un-resolving progressive nature of fibrotic disease (Sato et al., 2000). More specifically, levels of the amino-terminal half of CCN2 in plasma and dermal interstitial fluid of SSc patients correlate with the severity of the disease (Dziadzio et al., 2005). Even more importantly, in a transient fibrotic mouse
model induced by TGFβ, addition of CCN2 caused the fibrotic lesion to persist. More specifically, although subcutaneous injection of TGFβ resulted in the formation of granulation tissue, this fibrotic response was transient, and disappeared after cessation of application of ligand, despite 7 days of consecutive injections. However injection of TGFβ1 combined with CCN2 produced a sustained fibrotic response that persisted for at least 14 days and after cessation of ligand injection (Mori et al., 1999). Thus, CCN2 presents itself as a possible target for therapeutic intervention to interfere with the progressiveness of SSc, without harming normal fibrogenesis.

Recently, we generated a conditional knockout model that allowed for the direct testing of whether expression of CCN2 is required for fibrosis. We used a transient bleomycin model and a progressive fibrosis model involving a fibroblast-specific deletion of the adhesive signaling suppressor Pten; the fibrotic phenotype seen in both models of skin fibrosis were rescued by a fibroblast-specific deletion of Ccn2 (Liu et al., 2011, 2013a). Moreover, targeting CCN2 using a monoclonal antibody partially blocked TGFβ/CCN2-induced skin fibrosis in mice (Ikawa et al., 2008). Similarly, CCN2 expression is essential for fibrotic models in other organs (Morales et al., 2013; Dorn et al., 2018; Petrosino et al., 2019). FG-3019, a humanized monoclonal anti-CCN2 targeting antibody which has been under development for ~15 years, has been tested on mice and showed to block angiotensin II-induced skin fibrosis. (Makino et al., 2017). The anti-fibrotic effects of FG-3019 was also beneficial in three other murine fibrotic models; a multiorgan fibrosis model through intraperitoneal injections of CCN2 and TGFβ2, the unilateral uretal obstruction renal fibrosis model, and an intratracheal bleomycin-induced pulmonary fibrosis (Wang et al., 2011). When tested in phase 2 human clinical trials on
idiopathic pulmonary fibrosis (IPF), results on a small number of patients suggested that FG-3019 could slow disease progression (Raghu et al., 2016). Investigation into the control of CCN2 expression may yield an alternative strategy to blocking CCN2 expression. A small molecule inhibitor may be able to penetrate tissue better than a large antibody and therefore may prove to be an effective therapy.

1.6 YAP/TAZ/TEAD

Yes-associated protein 1 (YAP) and TAZ are mechanosensitive transcriptional co-activators known for regulating the Hippo signalling pathway to control organ size and tumorigenesis (Huang et al., 2005). YAP/TAZ bind to the TEA domain (TEAD) family of transcription factors to co-activate gene transcription (Vassilev et al., 2001). Recently, YAP has been shown to be a downstream regulator of fibroblast activation and mechanotransduction in physiological and pathological fibrosis (Liu et al., 2015; Piersma et al., 2015; Mohri et al., 2017; Rinschen et al., 2017). Furthermore, YAP regulation of mechanotransduction was shown to involve integrins (Elbediwy et al., 2016).

Visudyne© (VP for injection) is an approved drug currently used in the treatment of macular degeneration. The drug is injected intravenously and photoactivated in the eye. Interestingly, the non-photoinduced molecular form of VP has been shown to inhibit YAP activation, with no significant cytotoxicity in human trabecular meshwork cells below 2 µM (Chen et al., 2015). Thus, YAP presents itself as a possible target for therapeutic intervention. CCN2 expression is stimulated by an active YAP/TAZ/TEAD transcription factor complex (Quan et al., 2014). Indeed, we were the first group to demonstrate the involvement of YAP/TEAD in promoting CCN gene expression (Leask
et al., 2003). Chromatin immunoprecipitation experiments confirmed that TEAD and YAP bound the CCN2 promoter (Zhao et al., 2008). Indeed, CCN2 expression can be used as a readout of activation of the YAP/Hippo pathway (Yang et al., 2018). In human keratinocytes, knockdown of YAP significantly reduced expression of both CCN1 and CCN2 (Quan et al., 2014). Thus, a potential non-canonical mechanotransduction signalling loop emerges, where YAP-mediated CCN2 expression induces stiffening of the ECM, which further stimulates expression of CCN2 (Figure 1.3). Overexpression of YAP-mediated CCN2 through the pro-adhesive signaling loop can have severe effects not only in fibrotic disease, but other diseases as well, as seen in many types of cancer.

1.7 Fibrosis and cancer metastasis

As early as 1998, the CCN family of proteins was linked to cancer and its progression, as recombinant CCN1 protein was found to promote angiogenesis and tumor growth (Babic et al., 1998). Later, both CCN1 and CCN2 were discovered to be expressed in primary tumors and the stromal fibroblasts within the fibrotic tumor stroma (Rachfal et al., 2004). In fact, CCN1 and CCN2 are overexpressed in many types of cancer, and have been linked to tumor progression and metastasis (Xie et al., 2004; Pickles et al., 2007). Indeed, formation of a fibrotic tumor microenvironment is induced by CCN2, reflecting stiffening of the ECM in fibrosis. Integrin-mediated ECM stiffening, collagen crosslinking, and formation of FAs were found to enhance breast tumorigenesis (Levental et al., 2009). Interestingly, there is also evidence that CCN2 and YAP/TAZ are drivers of cancer metastasis (Shimo et al., 2006; Deng et al., 2007; Warren et al., 2018). We found that CCN2 expression by tumor stroma is required for melanoma metastasis (Hutchenreuther et al., 2015). An in vitro YAP or TAZ knockdown strongly reduced the
Membrane bound integrin heterodimers composed of $\alpha_5/\beta_1$ subunits bind mechanically active ECM components such as CCN2, which activates Src/FAK signaling and PI3K. This activates YAP downstream and translocates it to the nucleus, stimulating expression of pro-fibrotic genes. ECM components are then secreted from the cell, causing further stiffening of the ECM and further activation of integrin receptors.

**Figure 1.3 Proposed fibrotic signaling feedback loop.**
expression of \textit{CCN2}, and the ability of these transfected melanoma cells to form lung metastases in mice following tail injection (Nallet-Staub et al., 2014).

In addition to the involvement of a fibrotic microenvironment in tumorigenesis and tumor metastasis, there is also evidence that it favors chemotherapy resistance. Activation of integrin $\beta_4$ on mammary malignant epithelial cells is required for resistance to apoptosis (Weaver et al., 2002). Melanoma caused by the BRAF (V600E) mutation, initially susceptible to targeted therapy, commonly develops chemotherapy resistance within 6-8 months by a variety of mechanisms, including stimulation of ECM production and remodeling via integrin-$\beta_1$/Src/FAK signaling (Frame et al., 2015; Hirata et al., 2015; Taylor et al., 2018). Indeed, cancer associated fibroblasts (CAFs) are thought to provide a safe-haven for melanoma through formation of a stiff ECM. It may be possible that migration of cancer cells through the ECM is aided by the stiffness of a fibrotic tumor stroma. Primary tumors possibly prime their microenvironment for increased cell motility through YAP/TAZ mediated expression of \textit{CCN2} to increase matrix stiffness and through formation of focal adhesions. Through this it may be possible for cancer cells to invade past the basement membrane and move into the blood stream, where they can spread to other organs (Figure 1.4).

Further support for the notion that activated mechanotransduction promotes tumor growth and metastasis comes from recent studies showing that YAP/TAZ/TEAD promote metastasis for numerous types of cancer, including melanoma, lung cancer, breast cancer, cholangiocarcinoma, gastric cancer, ovarian cancer, colorectal cancer, and oral squamous cell carcinoma. (Warren et al., 2018). In other regards, invasiveness of melanoma cells with an activating BRAF mutation was linked to a loss of adhesive signaling suppressor
Figure 1.4 YAP/TAZ activation promotes several steps of the metastatic cascade.

Depicted are the critical steps in the metastatic cascade. To spread tumor cancer cells (brown) undergo an EMT and then must invade through basement membranes and the surrounding tissue until they encounter a blood or lymphatic vessel. To intravasate they must then invade between endothelial cells (yellow). While in circulation, tumor cells interact with immune cells (blue) and platelets (orange). To seed distant organs, cells must arrest either by becoming lodged in small capillaries, or through active adhesion to the vessel wall, and then successfully exit the vessel (extravasation). To form a metastatic tumor, the cancer cell must survive and proliferate in a new microenvironment. Whether increased YAP/TAZ activity or decreased Hippo pathway activity promotes each of these steps is indicated. The direction of blood flow is indicated by red dotted arrows. (Used with open access from Warren et al., 2018 doi: [10.3390/cancers10040115](https://doi.org/10.3390/cancers10040115). Copyright © 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article)
phosphatase and tensin homologue, deleted on chromosome ten (PTEN) (Dankort et al., 2009).

1.8 Phosphatase and tensin homologue

PTEN was first identified as a tumor suppressor frequently lost in a variety of cancers including brain, breast, and prostate (Li et al., 1997; Steck et al., 1997) PTEN is a dual specificity protein and lipid phosphatase whose primary target is the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which it hydrolyzes to phosphatidylinositol (4,5)-bisphosphate (PIP2) (Maehama et al., 1998). PTEN also blocks phosphatidylinositol 3 kinase (PI3K) by preventing recruitment of AKT (Stambolic et al., 1998), which directly interferes with the aforementioned mechanotransduction pathway. In glioma cells, PTEN activity is required for cellular migration, and there is evidence that this migration is regulated through suppression of Src (Raftopoulou et al., 2004; Dey et al., 2008). Furthermore, PTEN has also been reported to dephosphorylate FAK, leading to reduced cell migration and focal adhesion formation (Tamura et al., 1998).

PTEN protein expression was notably reduced in diffuse cutaneous SSc fibroblasts, consequently phosphorylation of Akt was also elevated (Parapuram et al., 2011). An in vivo deletion of Pten, specific to fibroblasts, resulted in spontaneous development of progressive dermal fibrosis. Loss of Pten resulted in a 3-fold increase in dermal thickness.
due to excess deposition of collagen (Parapuram et al., 2011). Furthermore, fibrosis caused by loss of Pten in mouse fibroblasts was dependent on Ccn2 expression (Liu et al., 2013 a; Parapuram et al., 2014). In these experiments, knockout of Pten or Ccn2 was induced specifically in fibroblasts through expression of Cre-ER(T) under a fibroblast-specific mouse proa2(I)collagen promoter, along with a 6-kb transcriptional enhancer located far upstream from the promoter (Zheng et al., 2002; Ramirez et al., 2006). To investigate the ability of a fibrotic microenvironment to exacerbate melanoma tumor metastasis, we could use the aforementioned Pten-deficient mouse model to induce a spontaneous, pathological fibrotic tumor microenvironment, and assess its effect on tumor metastasis.

1.9 Rationale, objective and hypothesis

Activated mechanotransduction is common in both progressive fibrosis and cancer metastasis. It is possible the activated tumor stroma reflects the fibrotic microenvironment seen in SSc, and that a stiffened ECM may aid in primary tumor metastasis through enhanced pro-adhesive signaling. Verteporfin (VP) has been shown to suppress the formation of the YAP-TEAD complex (Feng et al., 2016). This allows us to investigate the role of YAP in pro-fibrotic gene regulation and mechanotransduction, both in the presence and absence of TGFβ1. I hypothesize that YAP inhibition with VP in fibroblasts will decrease expression of pro-fibrotic gene clusters both in the presence and absence of TGFβ1, and that an increased fibrotic microenvironment will enhance the metastasis of melanoma in vivo. Thus, the objectives for this experiment are as follows:
1. Assess the alterations to the transcriptome of a fibroblast due to YAP inhibition during TGFβ-induced fibroblast activation.

2. Assess the effect of an exacerbated fibrotic microenvironment on melanoma metastasis using a Pten-deficient in vivo fibrotic model.
2 Materials and Methods

2.1 Cell culture

Human foreskin fibroblasts (HFFs) were purchased from the ATCC and stored in liquid nitrogen. HFFs were thawed and grown in T75 flasks (Fisher Scientific Cat#07000229). HFFs were cultured at 37.5 °C and 5% CO2 in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (ThermoFisher Cat#11995-073), 10% fetal bovine serum (FBS) (Life Technologies Cat#12483-020) and 1% antibiotic/antimycotic solution (ThermoFisher Cat#15240-062). Culture medium was replaced every 2-3 days, and cells were passaged when reaching 80% confluency. They were rinsed with Dulbecco’s phosphate-buffered saline (DPBS) (ThermoFisher Cat#14040182), detached using trypsin-EDTA 0.25% (ThermoFisher Cat#25200072) for two minutes, and sub cultured onto new flasks with fresh medium. After the tenth passage (P10), HFFs were sub cultured again onto 6-well plates and once they reached 80% confluency, they were serum-starved by replacing the medium with low glucose DMEM (ThermoFisher Cat#11885092) with 0.5% FBS and 1% antibiotic/antimycotic.

HFFs (P10) were then treated with Verteporfin (VP) (Sigma-Aldrich CAS#129497-78-5). VP was diluted in dimethylsulfoxide (DMSO) (ThermoFisher Cat#TS-20688) to 0.5 µg/ml, and cells were treated with either DMSO as a vehicle control or VP (volumes of each treatment were equal). Cells were also treated with either VP or DMSO in parallel with recombinant human TGFβ1 (R&D Systems Cat#240-B-002) at 1 µg/ml. Total treatment time was 6 hours, with TGFβ1 being added 30 minutes after VP. After
treatment, the cells were washed with 1 ml DPBS and Trizol was added to begin RNA extraction.

2.2 RNA isolation

Trizol (Invitrogen cat#15596018) was added, 1 ml per well, plates were scrapped into tubes and incubated at room temperature for 5 min. Chloroform (Sigma cat#C2432) was added, 0.2 ml for every 1 ml of Trizol. Samples were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 min, then were centrifuged at 12,000 RCF for 15 min at 4°C. The upper aqueous layer was transferred to a separate tube (~450 µl per 1 ml Trizol), and 0.5 ml of isopropyl alcohol (Sigma cat#I9516) was added to each tube. Tubes were inverted 2-4 times and incubated at room temperature for 10 min, followed by centrifugation at 12,000 RCF for 15 min at 4°C. Supernatant was discarded and the RNA pellet was washed by adding 1 ml ethanol (100%). Tubes were inverted 2 times and centrifuged again 12,000 RCF for 7 min at 4°C. The ethanol was removed, and the washing step was repeated one more time. Following centrifugation, the pellet was dried by removing the ethanol and allowed to air dry. The RNA pellet was dissolved in 30 µl UltraPure™ DNase/RNase-Free Distilled Water (cat#10977-023), and frozen at -22°C.

2.3 qPCR analysis

RNA concentrations were determined using a NanoDrop 2000/2000c Spectrophotometer. To synthesize cDNA, 1 µg of RNA was loaded into 16 µl UltraPure™ DNase/RNase-Free Distilled Water, in addition to 4 µl Quanta cDNA 5X qScript supermix (VWR cat#CA101414-112). The mixture was inserted into a
thermocycler to undergo a polymerase chain reaction (5 min @25°C, 30 min @ 42°C, 5 min @ 85°C, held at 4°C) to synthesize cDNA.

Quanta SYBR master mix (VWR cat#CA101414-284) were made for various genes of interest (0.7 µl UltraPure™ DNase/RNase-Free Distilled Water, 5 µl SYBR mix, 0.3 µl primer, for every 4 µl cDNA diluted 1:30, plated on a 384-well plate). Primers were selected using Primer-BLAST and purchased from Life Technologies for the following targets:

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-actin</td>
<td>CCTCGCCTTTGCGGATCC</td>
<td>CGCGGCGATATCATCAGTCG</td>
</tr>
<tr>
<td>CTGF/CCN2</td>
<td>GAGGAGTGCTGTGTGACG</td>
<td>TCTCCAGTCGGTAACCAGC</td>
</tr>
<tr>
<td>CCN1</td>
<td>CGGCTCCCTGTGTTGGAAT</td>
<td>TTGAGCAGTGGGACCATGAA</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CCCCGAGGCTCTGAAGGTC</td>
<td>GGAGCACCATTGGCACCTTT</td>
</tr>
<tr>
<td>COL1A2</td>
<td>CCCCGGTCTCTGCTGGAAGT</td>
<td>GCCAGGGGACCACTGCAC</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>GCCGTTGGAGGGAAATTGAG</td>
<td>TGAACCCGTGGATGTCACTT</td>
</tr>
<tr>
<td>FN1</td>
<td>CTGGCCAGTCTCATAAACAG</td>
<td>CGGGAATCTTCTCTGTCAGCC</td>
</tr>
<tr>
<td>aSMA</td>
<td>CCGGGACTAAGACCGGATC</td>
<td>TTGTCACACACCAAGGATC</td>
</tr>
<tr>
<td>ITGA5</td>
<td>TGCCGAGTTACCGAAGACTG</td>
<td>TGCAATCTGCTCTGAGTGG</td>
</tr>
<tr>
<td>ITGA11</td>
<td>CTGTGCGAGGCTGTACAGC</td>
<td>TGTAGCCAAAGAAGGCGTGC</td>
</tr>
<tr>
<td>LRP1</td>
<td>GAGTCTGCTTCGTTGCCTA</td>
<td>CAGTCATTGTCTTTGTCATCT</td>
</tr>
<tr>
<td>EDN1</td>
<td>AGAACAGTCTTACAGCGTGA</td>
<td>TGGACTGGGAGGTGGTTTCT</td>
</tr>
<tr>
<td>POSTN</td>
<td>TGCCCAGGCTTTTGCCCATT</td>
<td>CGTTGCTCTCCAAACCTCTA</td>
</tr>
<tr>
<td>TNC</td>
<td>GCCAATCATTTGAACAAAGCGG</td>
<td>CCCCTCCACTGACCACTA</td>
</tr>
<tr>
<td>PTEN</td>
<td>TGTAAGCTGGAGGGACGAGA</td>
<td>GGAATAGTTACTCCCTTTGTCTC</td>
</tr>
<tr>
<td>YAP</td>
<td>GAACCTCGCTTCAGGTCCCT</td>
<td>AGGGTCAGGCTTTGGTCTA</td>
</tr>
</tbody>
</table>
PCR amplification was conducted with the following heat cycles in a real-time thermocycler using ABI7900 software (95°C for 2:00min, [95°C for 0:10min, 60°C for 0:30min]x40). β-actin was used for reference, CT values from this housekeeping gene were used to calculate ∆Ct. Delta-delta CT analysis (ΔΔCt = ΔCt (treatment) – ΔCt (Control average)) yielded fold-change (Fold gene expression = 2^-(ΔΔCt)) for the treatment groups. The data sets were tested using an unpaired t-test/ordinary one-way ANOVA (P<0.05) (GraphPad Prism™ 8).

2.4 Microarray analysis

Two separate 6-hour treatments of VP (0.5 µg/ml) on HFFs ± TGFβ1 (1µg/ml) were performed as described in section 2.1 (n=2). All sample labeling and GeneChip processing was performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (RIN≥7) (Caliper Life Sciences, Mountain View, CA). Single stranded complementary DNA (sscDNA) was synthesized from 100 ng of total RNA, as per the Affymetrix GeneChip WT PLUS Reagent Kit (http://media.affymetrix.com/support/downloads/manuals/wtplus_reagentkit_assay_manual.pdf, Affymetrix, Santa Clara, CA). Total RNA was first converted to cDNA, followed by in vitro transcription to make cRNA. This cRNA was used to synthesize single stranded cDNA, and the cRNA was hydrolyzed. 5.5 ug of single stranded cDNA was synthesized, end labeled and hybridized, for 16 hours at 45°C to a Human Gene 2.0 ST array. All liquid handling steps were performed by a GeneChip Fluidics Station 450 and
GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) using Command Console v3.2.4.

Probe level (.CEL file) data were generated using Affymetrix Command Console v3.2.4. Probes were summarized to gene level data in Partek Genomics Suite v6.6 (Partek, St. Louis, MO) using the RMA algorithm (Irizarry et al., 2003). Partek software was used to determine gene level ANOVA p-values and fold changes. A Fisher’s exact test was used to create p-values for GO and KEGG Pathway enrichment. Gene level ANOVA data input into Transcriptome Analysis Console 4.0.1 software (ThermoFisher) were filtered by fold-change values to compare gene lists between different treatments.

2.5 Genotyping

DNA was extracted from an ear notch boiled in an acidic solution (25mM NaOH/0.2mM EDTA) and then neutralized (40mM Tris-HCL). Genotype was determined through polymerase chain reaction followed by gel electrophoresis. Primers and amplification conditions were as follows: Cre700 forward: ATCCGAAAAGAAAACGTTGA reverse: ATCCAGGTTACGGATATAGT (94.0/5:00, [94.0/0:45, 55.0/0:30, 72/1:00]x35 cycles, 72/7:00), yielding amplicons 500kb in size. Pten forward: ACTCAAGGCAGGGATGAGC reverse 1: AATCTAGGGCCTCTTGTGCC reverse 2: GCTTGATATCGAATTCCTGCAGC (94.0/1:00, [94.0/0:30, 55.0/0:30, 72.0/1:40]x35, 72/7:00)(Lesche et al., 2002). Fragments were resolved on 1% agarose gels and visualized with ethidium bromide. Further qPCR analysis to compare Pten expression between Pten^{fl/fl} + corn oil and Pten^{fl/fl} + tamoxifen groups was performed as described in section 2.3 using the following Pten
primer (F: ACCAGAGACAAAAAGGGAGTCACAA R: CCGGTCTGTAATCCAGGTGATTTT).

2.6 Generation of fibroblast specific Pten deficient mice

Mice hemizygous for tamoxifen-dependent Cre recombinase under the control of a fibroblast-specific promoter-enhancer cassette from the proα2(I) collagen gene (Zheng et al., 2002) were bred with mice homozygous for floxed Pten alleles to generate mice heterozygous for floxed Pten and hemizygous Cre. These mice were bred another generation to generate the desired genotype: hemizygous Cre and homozygous for floxed Pten. Male mice at 3 weeks of age with the desired genotype were subjected to intraperitoneal injections of tamoxifen suspended in corn oil (0.1 ml of 10 mg/ml 4-hydroxytamoxifen, Sigma) daily for 5 days to induce a gene knockout specific to dermal fibroblasts (Pten\textsuperscript{flox}). Control mice were also generated by injecting a cohort of Pten\textsuperscript{fl/fl} mice with only corn oil, the vehicle for the tamoxifen. Mice required 60 days following tamoxifen treatment to develop a fibrotic phenotype.

2.7 Tumor studies

B16F10 murine melanoma cells (ATCC) were cultured in the same high glucose DMEM medium with 10% FBS, and 1% antibiotic/antimycotic solution as described in section 2.1, detached using 0.25% trypsin/0.02% EDTA solution, and resuspended in the same low glucose DMEM, serum-free, and with 1% antibiotic/antimycotic solution as described in section 2.1 at a density of 3300 cells/µl. Injection of 330,000 cells
subcutaneously into the right flank of each C57BL/6J mouse (Jackson Laboratory) was performed 60 days post tamoxifen injection date. Once a palpable tumor was detected, tumor volume measurements were obtained for all mice for 14 days using a digital caliper and the following equation: Tumor Volume (mm$^3$) = $\frac{1}{2}$(Length of Longest Tumor Dimension) x (Length of Narrowest Tumor Dimension)$^2$. After 14 days, the mice were sacrificed, administering heparin before ketamine overdose and cervical dislocation. All animal protocols were approved by the animal care committee at the University of Western Ontario. Post-euthanasia, the lungs were flushed with PBS and then inflated by administering 4% paraformaldehyde (PFA) through the trachea. Lung, tumor with surrounding skin, and normal skin from the left flank were harvested and fixed in 4% paraformaldehyde dissolved in PBS (PFA) for 24 hours. Afterwards, tissues were sent to Robarts Research Institute in 70% ethanol to be processed. Tissues were then embedded in paraffin, sectioned at 5 µm on a microtome and collected on Superfrost Plus (Fisher Scientific) slides.

### 2.8 Skin thickness and lung metastasis analysis

Lung and skin sections were dewaxed in xylene and rehydrated in four descending concentrations of ethanol (100%, 95%, 80%, 70%), stained with hematoxylin for 1 min and eosin-y for 7 min. Bright-field images of skin were obtained at 3 varying depths through the tissue. Skin thickness was then measured using Eclipse software at 6 points along the skin, from the surface of the epidermis down to the bottom of the dermis, for all 3 depths. Lung sections were imaged, and the area of dense purple metastatic foci was
assessed as the percentage of the total area of the lung using imageJ software. The two groups were then compared using an unpaired T-test, and significance was set at P<0.05.

2.9 Tissue homogenization

Skin tissues harvested from the opposite flank of the injected tumor were placed inside prefilled zirconium bead microtubes (MIDSCI Cat#D1032-30) along with 1 ml of Trizol. The tissues were then homogenized using a Beadbug Microtube Homogenizer (MIDSCI Cat#D1030) for 3 individual basic cycles, or until the tissues were completely homogenized. The Trizol was then transferred to a separate tube where RNA was extracted as described in section 2.2.

2.10 Immunocytochemistry analysis

A 24-well plate with glass slide inserts was coated with 500µl of collagen type 1 bovine overnight (10µg/ml) (Invitrogen Cat#A1064401). HFFs were then plated (8000 cells/well) and allowed to attach overnight. Cells were then serum starved and treated with VP or DMSO ± TGFβ as described in section 2.1 for 24 hours, administering TGFβ1 30 min after VP. Cells were fixed in 4% paraformaldehyde (Sigma), washed in PBS two times for 2 minutes each, and blocked for 45 minutes in a solution containing 0.1% TritonX-100 in PBS and 10% donkey serum (Jackson Immunoresearch, West Grove, PA, USA). Cells were then incubated with rhodamine-conjugated phalloidin (ThermoFisher #R415) diluted 1:1000 in blocking solution for 45 min in the dark. After washing in PBS two times for 2 minutes, the glass slides were mounted on coverslips using DAPI fluorescent mounting medium (Vectashield #H-1200). Fluorescence images
were acquired with a Zeiss Imager M1 fluorescence microscope (Toronto, ON, Canada), using 360nm to visualize DAPI in blue and 540nm to visualize rho-phalloidin in red. Images were overlaid using Northern Eclipse software (Empix, Mississauga, ON, Canada).
3 Results

3.1 Cell culture-based studies

3.1.1 VP blocks TGFβ1 induced HFF myofibroblast-like phenotype

As an initial proof-of-concept approach to assess if VP could block profibrotic activity of fibroblasts, I examined changes in fibroblast morphology and differentiation into myofibroblasts that result in response to treatment with VP and TGFβ1. HFFs were treated with VP for 24 hours in the presence or absence of TGFβ1. HFFs that were incubated only with TGFβ1 demonstrated increased formation of stress fibers consistent with myofibroblast differentiation, as well as increased cell growth. HFFs treated VP alone demonstrated seemingly fewer formations of stress fibers, and when combined with TGFβ1, completely inhibited the ability of TGFβ to induce a myofibroblast-like phenotype in HFFs (Figure 3.1).
Figure 3.1 VP blocks TGFβ1 induced HFF myofibroblast-like phenotype

HFFs (passage 10) were plated at approximately 8000 cells/well in a 24-well plate, precoated with 10µg/ml collagen on glass slides. Cells were treated with either DMSO or VP and ± TGFβ1 in triplicate for 24 hours. Cells were then fixed with 4% PFA and blocked in solution and incubated in rho-phalloidin diluted 1:1000 in blocking solution. Afterwards slides containing the cells were mounted on larger glass slides with DAPI fluorescent mounting media. Images were taken under fluorescent microscopy. An image that best represented the average in each condition are provided above. HFFs treated with TGFβ1 alone displayed increased cellular growth as well as increased differentiation into myofibroblasts, indicated by formation of SMA stress fibers visualized in red. Treatment with VP 30 min prior to TGFβ1 completely inhibited this effect (Bar, 100 µm).
3.1.2 VP significantly inhibits gene expression of *CCN2, COL1A1, COL1A2, and TGFB1*, and does not affect expression of *PTEN or YAP*

Accumulation of collagen within scar tissue is a characteristic feature of and leading contributing factor to pathological fibrosis (Hinz et al., 2012; Borthwick et al., 2013). Over-secretion of collagen in fibrotic lesions by myofibroblasts is not induced by TGFβ1, at least at the mRNA level (Liu et al., 2014). Therefore, when assessing the ability of VPs to inhibit collagen expression, we first determined the effect of VP on gene expression in unstimulated cells (fibroblasts treated with DMSO vehicle control). Otherwise, VP treatment was administered on HFFs for 6 hours, and a delta-delta CT qPCR analysis was performed. Data sets were analyzed using a standard unpaired t-test. Baseline expression of *CCN2, COL1A1, COL1A2, and TGFB1* were significantly inhibited by VP treatment, while expression of *PTEN* and *YAP* were not (Figure 3.2).

VP treatment reduced levels of TGFβ1 mRNA, suggesting the intriguing possibility that the inhibition of a fibrotic phenotype in cells treated with VP could be caused by an inhibition of TGFβ expression.
Figure 3.2 VP significantly inhibits gene expression of *CCN2*, *COLIA1*, *COLIA2*, and *TGFB1*, and does not affect expression of *PTEN* or *YAP*

HFFs were treated with only DMSO or VP for 6 hours and analyzed using qPCR delta-delta CT analysis. All graphs are plots of mean mRNA fold-change ± standard deviation. Means of the two treatment groups were compared using an unpaired t-test (GraphPad Prism 8.1). Baseline expression of *CCN2*, *COLIA1*, *COLIA2*, and *TGFB1* were inhibited by VP treatment, while expression of *PTEN* and *YAP* were not. (A) *CCN2* (P<0.05) (n=16) (B) *COLIA1* (P<0.05) (n=16) (C) *COLIA2* (P<0.05) (n=16) (D) *TGFB1* (P<0.05) (n=14) (E) *PTEN* (P=0.990) (n=8) (F) *YAP* (P=0.686) (n=10)
3.1.3 VP reduces gene expression of clusters heavily enriched in ECM secretion and binding in the presence and in the absence of exogenous TGFβ1

To investigate whether downregulation of pro-fibrotic genes was due to inhibition of YAP or to decreased expression of TGFβ1, gene level ANOVA fold-change values from the microarray analysis were filtered to identify downregulated (<-1.5-fold change) in VP treatments compared with DMSO, as well as VP+TGFβ1 compared with TGFβ1 (<-1.5-fold change). The two lists of genes were examined using DAVID’s Gene functional annotation tool to give insight on alterations in gene expression induced by VP treatment, with and without TGFβ1. Between the two lists of 900+ genes each, 45.4% were shared. Both lists contained clusters of genes heavily enriched in ECM secretion and binding (Figure 3.3).

3.1.4 VP reduced 28 potential key regulators of TGFβ1- induced myofibroblast differentiation

To investigate specific gene targets that were upregulated by TGFβ1 and that were inhibited by VP, two gene lists were compared. One list contained genes that were upregulated by TGFβ1 (> 1.5-fold), and the other contained all the genes that were kept at or below baseline expression by VP in the presence of TGFβ1 compared to DMSO (<1). On this basis, 28 potential key regulators of myofibroblast differentiation were found in both lists. Of these 28 targets, the only targets whose mRNA expression was also reduced by VP treatment in the presence of exogenously added TGFβ1 were ENPP1 and LTBP2 (Figure 3.4).
(A) Total: 1,258

Down in VP vs DMSO 908

Down in VP+TGFB1 vs TGFB1 921

(B) | Gene cluster               | Enrichment score |
---|----------------------------|------------------|
| Transmembrane              | 66.91            |
| Cell adhesion              | 31.66            |
| Endoplasmic reticulum      | 28.43            |
| Secreted/ECM               | 22.51            |
| ECM/collagen organization  | 17.67            |
| Cell-cell adhesion         | 16.91            |
| Cell migration             | 8.46             |

(C) | Gene cluster               | Enrichment score |
---|----------------------------|------------------|
| Transmembrane              | 74.22            |
| Cell adhesion              | 24.59            |
| Endoplasmic reticulum      | 23.92            |
| Secreted/ECM               | 21.3             |
| Cell migration             | 10.65            |
| Focal adhesion             | 9.63             |
| Vasculature                | 8.0              |
Figure 3.3 VP reduces gene expression of clusters heavily enriched in ECM secretion and binding in the presence and in the absence of exogenous TGFβ1.

Gene expression data obtained by microarray analysis were input into Transcriptome Analysis Console and two genes lists were generated. One list contained genes that were downregulated by VP compared to DMSO (< -1.5-fold change). The other list contained genes that were downregulated by VP in the presence of TGFβ1, compared to TGFβ1 alone (< -1.5-fold change). (A) When the two lists were compared to each other, 45.4% of the genes were in common. (B) The first list of 908 genes downregulated by VP alone (< -1.5-fold) were input into DAVID Gene functional annotation tool. Returned clusters contained genes heavily enriched in transmembrane, cell adhesion, endoplasmic reticulum, secreted/ECM, ECM/collagen organization, cell-cell adhesion, and cell migration. (C) The second list of 921 genes downregulated by VP in the presence of TGFβ1 compared to TGFβ1 alone expression levels (< -1.5-fold) was input into DAVID Gene functional annotation tool. Returned clusters were enriched in clusters consistent with the previous list.
Microarray results were filtered to create lists of genes altered in each treatment. (A) A list of genes that were upregulated by TGFβ1 (>1.5-fold) was compared to a list of genes in VP+TGFβ1 that were expressed at baseline or below. Between these lists, 41 similarities were found, 28 of which were encoding genes. (B) A list of these similarities with their fold-change values indicating down regulation with VP+TGFβ1 vs. DMSO. (C) The list of 28 genes was input into DAVID Gene functional annotation tool. Returned clusters that were most enriched, similar to clusters in Figure 3.3, involve the ECM and cell migration.

**Figure 3.4** VP reduced 28 potential key regulators of TGFβ1- induced myofibroblast differentiation.
3.1.5 VP fails to inhibit expression of \( \text{TGF}\beta 2 \), \( \text{TNFAIP6} \), and \( \text{CCN2} \) in the presence of TGF\( \beta 1 \)

To give further insight on VP’s inhibition of myofibroblast differentiation, and to assess VP as a potential candidate for therapeutic intervention in fibrosis, more gene lists were compared as described previously. One list contained genes that were downregulated by VP compared to DMSO (<-1.5-fold change), and the other contained a list of genes that were upregulated in VP+TGF\( \beta 1 \) treatment compared to DMSO. Three genes were common to both lists: \( \text{TGFB2} \), \( \text{TNFAIP6} \), and \( \text{CCN2} \) (Figure 3.5).

3.1.6 Exogenous TGF\( \beta 1 \) does not interfere with inhibition of pro-fibrotic gene expression induced by inhibition of YAP with VP

To ensure the results from the microarray was not due to deficiency of endogenous TGF\( \beta 1 \) induced by treatment with VP, additional trials of VP treatment on HFFs were performed both in the presence and absence of exogenous TGF\( \beta 1 \) (1 µg/ml). VP treatment ± TGF\( \beta 1 \) was administered on HFFs for 6 hours, and a delta-delta CT qPCR analysis was performed. Data sets were analyzed using an ordinary one-way ANOVA. Expression of \( \text{CCN2} \), \( \text{FN1} \), \( \text{ITGA5} \), \( \text{LRP1} \), and \( \text{EDN1} \) were significantly inhibited in the presence of VP, irrespective of the presence or absence of exogenous TGF\( \beta 1 \) (Figure 3.6).

In summary, treatment of fibroblasts with VP blocked formation of a fibrosis-like phenotype, through reduction in expression of ECM secreting and binding gene clusters, irrespective of the presence or absence of endogenous or exogenous TGF\( \beta 1 \).
Figure 3.5 VP fails to inhibit expression of *TGFβ2*, *TNFAIP6*, and *CCN2* in the presence of TGFβ1

Microarray ANOVA results were input into Transcriptome analysis console 4.0.1 and filtered to create lists of genes altered in each treatment. (A) A list of 908 genes that were downregulated by VP (<1.5-fold) was compared to a list of 739 genes that were upregulated by VP+TGFβ1 compared to DMSO (>1.5-fold). Between these lists, 6 similarities were found, of which 3 were encoding genes. (B) A list of these genes with their fold change values (VP+TGFβ1 vs DMSO).
(A) CCN2

(B) CCN1

(C) FN1

(D) aSMA

mRNA Fold-Change

DMSO  TGFB1  VP  VP + TGFB1

0  5  10  15

0  1  2  3

0.0  0.5  1.0  1.5  2.0  2.5

(DMSO) (TGFB1) (VP) (VP + TGFB1)
HFFs were treated with either VP or DMSO ± TGFβ1 (1 µg/ml) for 6 hours and RNA was extracted. A SYBR qPCR was performed and analyzed using the delta-delta CT method. The mean for each treatment was plotted ± standard deviation. Data sets were tested for significance using an ordinary one-way ANOVA (GraphPad Prism 8.1).

(A) CCN2 (P<0.05) (n=13)  (B) CCN1 (P=0.105) (n=7)  (C) FN1 (P<0.05) (n=17)  (D) aSMA (P=0.124) (n=7)  (E) ITGA5 test (P<0.05) (n=16)  (F) ITGA11 (P=0.578) (n=5)  (G) LRP1
(P<0.05) (n=17) **(H)** *EDN1* (P<0.05) (n=7) **(I)** *POSTN* (P<0.05) (n=5) **(J)** *TNC* (P=0.062) (n=17) **(K)** A chart comparing the fold change values of the replicated qPCR trials to their microarray ANOVA counterparts for validation of gene expression.
3.2  *in vivo* mice studies of fibroblast specific *Pten*-deficiency and melanoma tumor metastasis

3.2.1  *Pten* expression in connective ear tissue was significantly suppressed in *Pten*\textsuperscript{fl/fl} mice administered tamoxifen.

Indeed, inhibition of YAP with VP *in vitro* successfully prevented a fibrotic phenotype induced by TGFβ1. To assess the importance of the pro-adhesive signaling pathway *in vivo*, we must first ensure that our fibroblast specific *Pten*-deficient fibrotic mouse model has the correct genotype and phenotype.

To confirm that *Pten* was inactivated upon tamoxifen treatment in our mouse model of fibrosis, we used ear notches as a tissue source from mice in two *Pten*\textsuperscript{fl/fl} groups; one group was treated with corn oil (vehicle control) and the other with tamoxifen. DNA was extracted from ear tissues, which contained *Colla2*-expressing fibroblasts. DNA from both groups was amplified using the same *Pten* primer sets. PCR amplification of DNA from *Pten*\textsuperscript{fl/fl} mice administered corn oil returned a single band at just above 1000bp, which is the size for the expected fragment for intact *Pten*. PCR amplification of DNA from *Pten*\textsuperscript{fl/fl} mice administered tamoxifen returned two bands, one faint band at just above 1000bp and another much stronger band at 450bp, which is the size of the expected excised fragment of *Pten*, indicating a successful inactivation of *Pten* in *Colla2* expressing fibroblasts (*Figure 3.7*). Hereafter, the two groups will be termed *Pten*\textsuperscript{fl/fl}, indicating the group of mice treated only with corn oil; and *Pten*\textsuperscript{−/−}, indicating the group of mice that received tamoxifen.
Figure 3.7 *Pten* expression in connective ear tissue was significantly suppressed in *Pten*<sup>n/m</sup> mice administered tamoxifen.

Suppression of *Pten* was supported using DNA extracted from ear notches. Post tamoxifen injections, ear notches were taken, and DNA was extracted. DNA from the ear notches reflects mostly fibroblasts, but also other cell types (for e.g., epithelial cells). The DNA was primed for *Pten* using the same primer for both groups and amplified in a PCR. Amplicons were resolved on a 1% agarose gel and visualized with ethidium bromide. *Pten<sup>fl/fl</sup>* + corn oil produced amplicons just above 1000kb, the expected size for floxed *Pten*. *Pten<sup>fl/fl</sup>* + tamoxifen produced strong bands at around 450kb, the expected size for the broken fragment of *Pten*, as well as faint bands at just above 1000kb, intact *Pten* (n=3).
3.2.2 *Pten* expression in skin of *Pten*<sup>−/−</sup> mice is decreased, albeit non-significant

To further support the suppression of *Pten* gene transcription, skin tissue from *Pten<sup>fl/fl</sup>* and *Pten<sup>−/−</sup>* was homogenized, and RNA was extracted using the TRIzol method. Later, cDNA was synthesized and primed for murine *Pten* primers in a SYBR qPCR. Delta-delta CT analysis showed a decrease, albeit non-significant, in the amount of *Pten* mRNA in the *Pten*<sup>−/−</sup> group compared to *Pten<sup>fl/fl</sup>* ([Figure 3.8](#)). This result may have achieved significance if a larger sample size was used.

3.2.3 Skin in *Pten*-deficient mice was significantly thicker

To support the desired phenotype was acquired by the *Pten*<sup>−/−</sup> group in our fibrotic mouse model, skin sections from both groups were analyzed using H&E staining. Skin thickness was quantified by measuring the distance from the epidermis to the bottom of the dermal layer. Measurements were taken for each subject at six points along the image and repeated for two additional skin sections per subject. Measurements were averaged, and the two groups were compared using a standard t-test. Dermal thickness was significantly greater in the *Pten*<sup>−/−</sup> group compared to *Pten<sup>fl/fl</sup>* mice, consistent with the notion that tamoxifen administration induced fibrosis in our model ([Figure 3.9](#)).
Figure 3.8 *Pten* expression in skin of *Pten*⁻/⁻ mice is decreased, albeit non-significant.

Skin tissue from both groups was homogenized in microtubes with TRIzol and zirconium beads. RNA was then extracted using chloroform/TRIzol and amplified into cDNA. Delta-delta CT analysis using *Pten*⁻⁻/⁻ as a control yielded fold-changes that show a trend towards significant reduction of *Pten* expressed RNA in *Pten*⁻⁻/⁻ group, analyzed using a standard unpaired t-test (GraphPad Prism 8.1) (P=0.079) (n=3). Plots are mean mRNA fold-change ± standard deviation. *Pten*⁻⁻/⁻ had 4.27-fold less *Pten* mRNA compared to *Pten*⁻⁻/⁻.
Figure 3.9 Pten<sup>−/−</sup> had significantly increased dermal thickness compared to Pten<sup>fl/fl</sup>.

Skin sections from both groups were stained with hematoxylin and eosin-y (H&E) and visualized under bright-field microscopy. Dermal thickness was measured from the top of the epidermis to the top of the adipose layer for both groups using ImageJ software. Data set was tested for a normal distribution using a Shapiro-Wilk test. Thickness mean of both groups were compared using a standard unpaired t-test and plotted ± standard deviation. The dermal thickness was significantly greater in the Pten<sup>−/−</sup> compared to Pten<sup>fl/fl</sup> (P=0.0083) (n=3) Bar, 250 µm.
3.2.4  *Pten*^{fl/fl} and Pten^/- mice had no significant difference in melanoma primary tumor growth

To assess the effects of a fibrotic environment on melanoma metastasis, we first assessed whether there were differences in tumor growth. Increases in the size of the primary tumor were monitored through a two-week period using digital calipers and an ellipsoidal equation was used to estimate tumor volumes. Over the entire period of tumor growth, there was no significant difference in tumor volume between *Pten*^{fl/fl} and Pten^/- (Figure 3.10).

3.2.5  Melanoma lung tumor metastasis in Pten^/- mice

Based on previous results, it appears that Pten^/- mice exhibit a pro-fibrotic dermal phenotype, as reported by others (Parapuram et al., 2011, 2014; Liu et al., 2013 b). It was also predicted that melanoma metastasis might be more prevalent in a fibrotic microenvironment. To assess this, lung sections from both groups were stained with hematoxylin and eosin to visualize the presence of dark purple metastatic foci, indicated by the red arrows (Figure 3.11). Visual inspection suggested the presence of more metastatic lesions in Pten^/-, but due to the low animal numbers, there was no significant difference detected between the groups.
Figure 3.10  *Pten*<sup>fl/fl</sup> and *Pten*<sup>−/−</sup> had no significant difference in primary tumor growth.

Syngeneic B16F10 murine melanoma cells (330,000) were injected subcutaneously in the right flank of either *Pten*<sup>fl/fl</sup> or *Pten*<sup>−/−</sup> C57BL/6 mice 60 days after being administered corn oil or tamoxifen respectively. On detection of a palpable tumor, volume measurements using calipers were taken daily for 14 days. Following this the mice were sacrificed via ketamine overdose and cervical dislocation. Means of tumor sizes were plotted ± standard deviation and compared at each time point between *Pten*<sup>fl/fl</sup> and *Pten*<sup>−/−</sup> mice, and no significant difference was found between them at each time point using a Mann-Whitney test (P=0.264).
Figure 3.11 *Pten*–/– mice suggest enhanced melanoma tumor metastasis from primary tumor to the lungs, however there was no significant difference.

Sections were stained with hematoxylin and eosin-y (H&E) and visualized under bright-field microscopy. The images of the entire lung sections were taken and the percent area that metastases take up was measured using imageJ software. Means for the two groups are plotted ± standard deviation and are presented as a fraction of metastasis area/lung area (mm³), compared using an unpaired standard t-test (P=0.081) (n=3) Bar, 125 µm.
4 Discussion

4.1 Clinical relevance

Fibrosis is a phenotype seen in many different diseases, with no treatment available on the market. Almost every organ in the body can undergo physiologic and pathologic fibrotic reactions. The problem arises when normal tissue repair becomes unregulated and disrupts tissue homeostasis. This excess deposition of ECM components ultimately leads to failure of the afflicted organ, involved with 45% of the mortality rates in the Western hemisphere (Wynn, 2008; Rosenbloom et al., 2010). Furthermore, fibrosis may also cause mortality through exacerbation of other diseases, such as the metastasis of cancer, although further investigation in this interaction is required. The key effector cell of fibrosis, the myofibroblast, is irreversibly differentiated from fibroblasts and continuously secretes pro-fibrotic proteins and ECM components that stiffen the ECM and stimulates further differentiation of local cells into myofibroblasts (Gabbiani, 2003; Leask et al., 2014; Hutchenreuther et al., 2016). This positive feedback loop seen in pathological fibrosis has no cure and intervention of this process would provide a large therapeutic benefit to many diseases.

ECM stiffness seen in fibrotic disease can influence gene transcription in a process known as mechanotransduction. Pro-fibrotic ECM components bind to mechanosensitive integrin receptors and translate mechanical tension on the cell surface into nuclear signaling to alter gene expression and drive myofibroblast differentiation (Zhou et al., 2010). The nuclear effector of this pathway is YAP/TAZ, a heterodimer that activates the TEAD. Persistent activation and differentiation of myofibroblasts is a key characteristic
of fibrosis (Gabbiani, 2003; Lagares et al., 2012; Hutchenreuther et al., 2016), which is perpetuated by heightened integrin-mediated mechanotransduction in a stiff ECM environment (Asano et al., 2006; Carracedo et al., 2010; Zhou et al., 2010). This mechanotransduction has been shown to be mediated by YAP (Liu et al., 2015), therefore interruption of mechanotransduction may provide therapeutic benefit in fibrotic disease.

VP is a drug already approved for macular degeneration. If VP can disrupt pathological fibrosis through inhibition of YAP, then cross-purposing this drug could be used to treat a large variety of disease without the need for clinical trials. Drug discovery for a specific molecular target is a long development process that involves trial and error and significant investment. Furthermore, completion of clinical trials for a compound can be an almost decade-long process with even more capital investment. Circumventing this process by cross-purposing VP for fibrotic treatment could provide a large therapeutic benefit with very little investment.

4.2 YAP activation occurs in response to mechanotransduction in otherwise unstimulated fibroblasts

As expected, TGFβ1 alone induced a myofibroblast-like phenotype, characterized by increased focal adhesions and actin stress fiber formation. Treatment with VP completely inhibited this effect (Figure 3.1). Interestingly, SMA fiber recruitment seen in the TGFβ1 treatment was not reflected by changes in mRNA (Figure 3.6). Given SMA’s previously shown requirement of matrix stiffness (Arora et al., 1994), it is likely that SMA
expression was not induced due to a lack of a matrix substrate in our cell culture conditions. The SMA fibers induced by TGFβ1 (Figure 3.1) likely arose as a consequence of formation of FAs, but without changes in α-SMA mRNA expression.

To give insight into how VP blocks a TGFβ1 induced myofibroblast-like phenotype, changes in gene transcription induced by VP both in the presence and absence of TGFβ1 were analyzed. Two list of genes downregulated by VP, one with and one without TGFβ1, were compared to each other, in addition to being input into a DAVID cluster analysis (Figure 3.3). It seems VP could potentially block TGFβ induced myofibroblast differentiation through inhibition of genes whose expression positively influences ECM modulation, and cell binding to other cells and the ECM. However it is unknown which of these genes were directly influenced by TGFβ1 to elicit this response. VP inhibition of fibrotic gene clusters in the absence of TGFβ1 compared to baseline tells us in normal conditions, there is a baseline expression of fibrotic gene clusters, which is inhibited by VP. If VP blocks activation of YAP mechanotransduction which is required for basal expression of pro-fibrotic genes, then YAP activation may be required for the ability of TGFβ1 signalling to induce gene expression.

4.3 Significant reduction of LTBP2 and FN1 prevent myofibroblast differentiation

To investigate specific gene targets that were induced by TGFβ1 and affected by YAP inhibition, genes that were upregulated by TGFβ1 were compared to genes VP kept at baseline or below in the presence of TGFβ (Figure 3.4). DAVID cluster analysis of these 28 genes returned gene clusters that modulate the ECM, like clusters found
previously (Figure 3.3). One of the most inhibited genes, latent transforming growth factor-β binding protein 2 (LTBP2), has recently had its protein product become a clinical biomarker for IPF progression, which also reflects the level of differentiation of lung fibroblasts into myofibroblasts (Enomoto et al., 2018). LTBP2 has been shown to interact with FN1, more specifically LTBP2 failed to integrate into the ECM in the absence of FN1 (Vehvilainen et al., 2009). FN1 was significantly inhibited by VP both in the presence and absence of TGFβ1 (Figure 3.6). Thus, it is possible that VP prevents a TGFβ1 induced HFF myofibroblast-like phenotype through reduction of both LTBP2 and FN1 mRNA expression, compromising ECM stability and subsequent reduction in mechanotransduction.

4.4 YAP selectively regulates various key pro-fibrotic genes

Various pro-fibrotic gene targets were selected to be analyzed through qPCR analysis. This served to replicate the results from the microarray ANOVA for validation (Figure 3.6). The selection of these important pro-fibrotic gene targets was based on previous research in the field.

A biomarker of progressive fibrosis in SSc (Sato et al., 2000; Leask et al., 2009; Morales et al., 2013; Dorn et al., 2018), CCN2 is induced by TGFβ1 signaling and reduced by treatment with VP. CCN1, another matricellular protein, known for its involvement in angiogenesis, inflammation, and wound repair, has also been shown to enhance TGFβ1/SMAD3 signaling and contribute to the fibrogenic response (Kurundkar et al., 2016). Here, CCN1 is not significantly affected by either TGFβ1 or VP. Both
CCN1 and CCN2 are thought to be downstream of YAP signaling in basal cell carcinoma (Quan et al., 2014). Here it seems that although CCN2 is reduced through VP inhibition of YAP, it is not completely inhibited by it. This is possibly due to the very low level of basal expression of CCN2 in normal, resting fibroblasts, skewing the relative expression of VP +TGFβ1 compared to DMSO. In Figure 3.6, CCN2 expression was not significantly increased by VP +TGFβ1 compared to DMSO.

Fibronectin (FN1) is a fibrillar protein that gives stiffness to the ECM and binds with integrins to transmit mechanical tension from the ECM through the cell’s cytoskeleton, αSMA (Cao et al., 2017). FN1 inhibition has also been shown to reduced collagen expression and improve liver function in liver fibrosis (Altrock et al., 2015). FN1 expression was significantly inhibited by VP both in the presence and absence of TGFβ1, however SMA was unaffected. Recent findings have shown that α-SMA mRNA expression is induced by matrix stiffness (Avery et al., 2018).

Integrins, as discussed previously, are the membrane bound receptor that interact with fibronectin, collagen, CCN2, and other ECM substrates to form focal adhesions and transmit mechanical tension from the ECM through the cellular cytoskeleton. Heterodimers of integrins α5β1 bind to fibronectin, collagen and laminin (Chen et al., 2016 a). Heterodimer α11β1 binds to collagen and α11 transcription has been shown to be increased by TGFβ1 signaling in fibrosarcoma cell line as well as in primary fibroblasts (Lu et al., 2010). Here, both integrin subunits α5 and α11 are shown to be unaffected in the presence of VP and TGFβ1.
Low-density lipoprotein receptor-related protein 1 (LRP1) is a receptor that has been shown to be involved with signal transmission and endocytosis. More importantly, recent studies have shown LRP1 to mediate transcytosis of CCN2 in chondrocytes (Kawata et al., 2012). Furthermore, a smooth muscle cell targeted gene deletion of LRP1 in mice (smLRP1−/−) resulted in protection of vasculature through mediation of the ECM. More specifically, smLRP1−/− mice showed increased protein levels of collagen and CCN2 in the ECM compared to smLRP+/+ (Muratoglu et al., 2013). In myofibroblasts, production of microvesicles (MV) are stimulated by serum. Inhibition of LRP1 in myofibroblasts resulted in a significant reduction in MV secretion (Laberge et al., 2018). In HFFs, inhibition of YAP by VP significantly inhibited expression of LRP1 both in the presence and absence of TGFβ1. It’s possible that excretion of CCN2 and other pro-fibrotic proteins into the ECM through MVs could be reduced through VP inhibition of LRP1.

Endothelin-1 (EDN1), one of the three human isoforms typically involved with vasoconstriction, may also play a role in fibrogenesis. Inhibition of the highly specific EDN1 receptor A (ETA) decreased collagen deposition and improved lung function in bleomycin-induced lung fibrosis in mice (Manitsopoulos et al., 2018). As previously mentioned, TGFβ1 induces endothelial-to-mesenchymal transition (EndoMT), providing a source of activated myofibroblasts. EDN1 has been shown to potentiate EndoMT while also inducing expression of TGFβ receptor 1 and 2 genes, suggesting a possible autocrine mechanism of TGFβ1 induced fibrogenesis (Wermuth et al., 2016). In HFFs, TGFβ1 significantly induced EDN1 expression, which was significantly reduced but not inhibited by VP in the presence of TGFβ1.
Periostin (POSTN) is a secreted matricellular protein first found in epithelial ovarian carcinoma, acting as a ligand on integrins αVβ3 and αVβ5, promoting cell motility (Gillan et al., 2002). Our previous findings show that CCN2-deficient B16(F10) melanoma cells demonstrated decreased expression of periostin and decreased the cell’s ability to migrate through collagen. This was also reflected in vivo, where CCN2-deficient mice demonstrated a loss of periostin and coincided with decreased metastases of melanoma from the skin to the lungs (Hutchenreuther et al., 2015). With treatment of VP on HFFs, expression of periostin is significantly inhibited in both the presence and absence of TGFβ.

Tenascin-C (TNC) is an ECM glycoprotein which is expressed rapidly in kidney fibrotic lesions. The mitogenic activity of TNC has also been shown to require the integrin-FAK signaling pathway (Fu et al., 2017). TNC was also identified as a promoter of lung adenocarcinoma metastasis (Gocheva et al., 2017). Treatment with VP on HFFs significantly inhibited TNC expression from TGFβ1 induced expression levels, both in the presence and absence of TGFβ1.

Most importantly, expression of collagen type 1 alpha 1 and 2 were significantly inhibited by VP. Excess deposition of collagen into the ECM is a hallmark of fibrotic disease (Gabbiani, 2003; Wynn, 2008). Administering VP to patients with SSc or other fibrotic diseases may provide therapeutic benefit through reduction of collagen expression.

Thus, inhibition of YAP-regulated mechanotransduction with VP has elucidated the regulation of many important pro-fibrotic targets that all may play a part in influencing
ECM stiffness. However, it is unclear how much impact each specific target has. It is most likely that all the affected targets contribute to the fibrotic phenotype in some way, and that future analysis of ECM modulation should incorporate a multitude of targets.

4.5 Validation of in vivo fibrotic genotype

The genotype of our in vivo mouse model of progressive fibrosis was first assessed by extracting DNA from ear notches taken post tamoxifen injections. Extracted DNA from both conditions were primed using the same Pten primer, performed in parallel. Bands from Pten^{fl/fl} were intact, while bands from Pten^{-/-} included both intact and fragmented Pten. The ear notch tissue used to extract DNA contains mainly fibroblasts, but also other cell types, which may explain the presence of the intact band since our KO model is targeted towards fibroblasts exclusively. However, this discrepancy may also be due to the tamoxifen-induced gene KO not being 100% efficient. Later, skin was homogenized, and this time extracted RNA was primed for Pten. Delta-delta CT analysis of the qPCR showed a trend towards decrease in Pten mRNA, Pten^{-/-} skin had 4.27-fold less Pten mRNA than the control group. As before, these results do not reflect only fibroblast cultures, but other cell types as well. Regardless, the Pten^{-/-} group is verified to have the intended genotype.

4.6 Loss of fibrotic phenotype

Previously this progressive fibrotic model targeting Pten has increased dermal thickness 3-fold (Parapuram et al., 2011). However, the mean fold-change of the Pten^{-/-} group’s dermal thickness compared to the mean of Pten^{fl/fl} was only 1.25-fold greater than the control group. Even more interestingly, our progressive model was given more
time to progress compared to previous trials, 60 days compared to 45 days. The phenotype of our $Pten^{-/-}$ skin was not as fibrotic as it should be, even though the mice had the desired genotype. Our colonies of mice were bred for over 10 generations, therefore one reasonable explanation for this discrepancy could be genetic drift, the accumulation of random mutations in the colony. Some of these mutations may affect the desired phenotype, which would help to explain the large variability in the skin adipose layer and dermal layer’s thickness, as well as the underwhelming increase in skin thickness in $Pten^{-/-}$. It is reasonable to expect that the reduced impact on skin thickness would reduce any expected impact on increasing melanoma metastasis.

4.7 Limitations

Previous analysis of filtered gene lists from the microarray ANOVA were generated with the criteria that the gene targets were induced by TGFβ1 by at least 1.5-fold. It is possible that key gene targets regulated by YAP are not induced by TGFβ1 significantly enough to reach the set threshold and may have been excluded in the analysis. One example is FN1, which was not induced enough to be included in Figure 3.4 alongside LTBP2. Furthermore, DAVID gene clusters analysis performed well to divide the list of genes into functional groups but is not ideal for functional classification of relatively unknown gene targets. Most notably FN1 and COL1A1 were lacking from notable fibrotic gene clusters.

Also, it is worth considering the possible inefficiency of VP as an inhibitor of YAP. The lack of inhibition of various pro-fibrotic gene target expression (Figure 3.5) could be
due to incomplete inactivation of YAP. Another recent study concluded that VP did not seem ideal as an pharmacological inhibitor of YAP-TEAD (Gibault et al., 2016).

Finally, the relatively small sample size for multiple experiments severely limited the analysis for multiple experiments. All the in vivo experiments had a relatively sample size (n=3), and results such as Figure 3.8 and 3.11 could have achieved significance if a larger sample size was used. The sample size for the microarray was also low (n=2). Changes in the transcriptome may not accurately represent average changes in the transcriptome for all HFFs. This may describe the small discrepancies in fold-change values between the microarray and replicated trials in Figure 3.6.

4.8 Future experiments

After comparing the average area melanoma metastasis occupies of the lung, it was revealed there was no significant difference between the two, however our results suggest that there might be an increase and a larger number of N values might uncover this fact. To determine the required group size to achieve a significant difference, a power analysis was performed using G*Power 3 software. To achieve significance, three more replicates with similar results per group would be needed (Figure 4.1). This trend combined with the underwhelming effect of Pten suppression on progressive fibrosis can supports that further experiments with much larger sample sizes are required to further elucidate the effect of a fibrotic microenvironment on cancer metastasis.

Furthermore, future experiments could verify YAP as a positive mediator of fibrosis through a double knockout mouse model of both Pten and Yap. If blocking Yap activity rescues the fibrotic phenotype induced by Pten knockout, then it further supports a role
Figure 4.1 Increased mouse group size would be required to be able to accurately measure significance.

G*Power 3.1.9.2 was used to perform a power analysis on a standard two-tailed T-test between $Pten^{fl/fl}$ and $Pten^{-/-}$. The type of power analysis “A priori” was selected. Effect size ($d=2.32$) was calculated and input using the built-in tool using the means and standard deviation of lung metastasis area for both groups. Values for $\alpha$ and Power were $P=0.05$ and $P=0.95$ respectively, with an allocation ratio $N2/N1=1$. Output results yielded required sample size to achieve desired $P=0.95$ would require three more animal subjects per group, for a total $n=12$. 
for YAP as a key regulator of fibrogenesis. Alternatively, VP could be administered in
drug form along with bleomycin or a Pten<sup>−/−</sup> mouse model to see if Yap inhibition with
VP could prevent induced fibrosis.

Further analysis of YAP activation as a pro-mediator of fibrotic gene transcription
should also be performed. Specifically, analysis of gene expression profiles induced by
inhibition of YAP could be investigated using tools such as IBM’s Watson machine
learning, or artificial intelligence (AI). Advanced mass-data analysis looking for patterns
in data or literature could provide insights into modulation of the ECM in fibrotic disease
that humans can’t perceive (Chen et al., 2016b).

4.9 Conclusion

Here we confirm our hypothesis that VP inhibition of YAP reduces expression of
fibrogenic gene clusters. Furthermore, VP treatment completely inhibited a TGFβ1-
induced fibrosis-like phenotype in HFFs. Investigation using microarray analysis
elucidated LTBP2 expression reduced by YAP inhibition both in the presence and
absence of exogenous TGFβ1 and perhaps a key mediator of ECM induced fibroblast
activation. Induced progressive fibrosis in vivo using Pten-deficient mice didn’t induce
the fibrotic phenotype or exacerbate melanoma tumor metastasis as expected, however
due to the lackluster fibrotic phenotype we induced, further trials should be completed. A
more recent study has shown YAP/TAZ activation as a key target for metastatic cancer
(Warren et al., 2018). Perhaps YAP activation in myofibroblast differentiation,
profibrotic gene expression, and cancer metastasis are all linked through increased
mechanotransduction in a fibrotic microenvironment, thus therapeutic strategies to
prevent over-activation of YAP via verteporfin could be beneficial to a multitude of diseases.
References


gastroenterology., 33, 295–302.


Gibault, F.; Corvaisier, M.; Bailly, F.; Huet, G.; Melnyk, P.; Cotelle, P., 2016: Non-


Irizarry, R. A.; Bolstad, B. M.; Collin, F.; Cope, L. M.; Hobbs, B.; Speed, T. P., 2003:


Perbal, B.; Tweedie, S.; Bruford, E., 2018, December: The official unified nomenclature adopted by the HGNC calls for the use of the acronyms, CCN1-6, and discontinuation in the use of CYR61, CTGF, NOV and WISP 1-3 respectively. *Journal of cell communication and signaling.*


Reed, N. I.; Jo, H.; Chen, C.; Tsujino, K.; Arnold, T. D.; DeGrado, W. F.; Sheppard, D.,


Thannickal, V. J.; Lee, D. Y.; White, E. S.; Cui, Z.; Larios, J. M.; Chacon, R.; Horowitz, J. C.; Day, R. M.; Thomas, P. E., 2003: Myofibroblast differentiation by transforming growth factor-betal is dependent on cell adhesion and integrin signaling via focal...


# Curriculum Vitae

<table>
<thead>
<tr>
<th><strong>Name:</strong></th>
<th>Michael Racanelli</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post-secondary Education and Degrees:</strong></td>
<td>University of Western Ontario London, Ontario, Canada 2016-present MSc</td>
</tr>
<tr>
<td></td>
<td>University of Western Ontario London, Ontario, Canada 2011-2015 B.MSc</td>
</tr>
<tr>
<td><strong>Honors and Awards:</strong></td>
<td>Dean’s Honor List 2015, 2012</td>
</tr>
<tr>
<td><strong>Related Work Experience:</strong></td>
<td>Teaching Assistant University of Western Ontario 2017-2018</td>
</tr>
</tbody>
</table>

**Publications:**