4-21-2014 12:00 AM

TAK1 Mediates TGF beta-1 Responses in Gingival Fibroblasts

Hanna Kuk
The University of Western Ontario

Supervisor
Dr. A. Leask
The University of Western Ontario

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

TAK1 Mediates TGF beta-1 Responses in Gingival Fibroblasts

(Thesis format: Monograph)

by

Hanna Kuk

Graduate Program in Physiology and Pharmacology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

ABSTRACT

In fibroblasts, transforming growth factor β-1 (TGFβ1) signals *via* canonical and non-canonical pathways to promote wound healing or hyper-contractile responses resulting in scars. The oral cavity however does not scar and fibrotic responses such as gingival hyperplasia are characterized by a hyper-proliferative response. The involvement of non-canonical, TGFβ-activated kinase-1 (TAK1) - mediated TGFβ1 signaling in gingival fibroblasts has not been previously examined. Here I show that TAK1 selective inhibitor (5Z)-7-Oxozeaenol blocks TGFβ1-induced expression of wound healing and fibrotic marker CCN2 (connective tissue growth factor) in gingival fibroblasts. Genome-wide expression profiling revealed that essentially all TGFβ1 induced genes were in fact sensitive to TAK1 inhibition in gingival fibroblasts (139/147) including those involved in proliferative and wound healing responses. This data was confirmed using RT-qPCR to detect mRNA expression as well as functional cell proliferation assay. (5Z)-7-Oxozeaenol might be investigated in the treatment of gingival hyperplasia in the future.

KEYWORDS: TGFβ1; TAK1; (5Z)-7-Oxozeanol; fibrosis; wound healing; CCN2; gingival fibroblasts; proliferation

CO-AUTHORSHIP STATEMENT

All segments of this work are submitted for publication in:

Kuk H., Carter D., Leask A. 2014. 5Z-7-Oxozeanol inhibits the effects of TGFβ1 on human gingival fibroblasts. Wound Repair Regen…*Review Pending*…

Hanna Kuk performed all the experiments except:

Gene Expression Profiling which was conducted independently by David Carter, London Regional Genomics Centre, Robarts Research Institute, London, Canada

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to all those who provided me with the possibility of undertaking and completing this project as well as my home department of Physiology and Pharmacology. This has been a rewarding experience that has helped me in shaping my character and opening new avenues into the future. I am grateful to my supervisor Dr. A. Leask for his guidance, time and advice through the course of my research. I am also grateful to my committee members Dr. C.A Seguin, Dr. J. DiGuglielmo as well as my Graduate Studies Committee Representative Dr. M. Prado for their valuable feedback and guidance.

I also thank David Carter for his help with the gene expression profiling section of my work as well as for access to the ViiA™ 7 Real-Time PCR System and Software used in RT-qPCR component of my research. I also thank Dr. C.A. Seguin for access to the Nanodrop 2000 program used in the assessment of RNA samples, the department of Dentistry at the University of Western Ontario for access to the western blot film development as well as Dr. Siqueira for access to the iMark™ microplate absorbance reader for the proliferation assay component.

I thank my lab members Shangxi, Katherine and James for their patience with my questions and easy access to reagents in the lab. I am also grateful to Fen Guo, a former post-doctoral fellow in our lab for her encouragement at the beginning of my Master's which helped me to persevere in times of uncertainty.

Lastly, my thanks go to my family and friends for their best wishes and loving support and encouragement.

TABLE OF CONTENTS

Abstract…… ii
Co-Authorship Statement …… iii
Acknowledgments…… iv
Table of Contents…… v
List of Tables…… vii
List of Figures…… viii
List of Abbreviations…… ix

Chapter 1. Introduction…… 1
1.1 Overview of physiological wound healing …… 2
1.2 Comparative anatomy: structure of skin and gingiva…… 3
1.2.1 Structural anatomy of skin…… 3
1.2.2 Structural anatomy of gingiva…… 5
1.3 Pathological wound healing: overview of fibrosis…… 6
1.3.1 Fibrotic lesions contain abundant myofibroblasts…… 7
1.3.2 Origin of myofibroblasts…… 8
1.4 Fibroblast cells…… 10
1.4.1 Fibroblast to myofibroblast differentiation…… 11
1.5 The role of TGFβ1 in wound healing and fibrosis…… 11
1.5.1 Canonical TGFβ1 signaling in fibroblasts…… 13
1.5.2 Targeting canonical TGFβ1 signaling in fibrosis disease treatment…… 15
1.5.3 Non-canonical TGFβ1 signaling in fibroblasts…… 18
1.6 TAK1…… 19
1.6.1 TAK1-mediated non-canonical TGFβ1 signaling in fibroblasts…… 20
1.6.2 Role of TAK1 in dermal homeostasis and wound repair…… 21
1.6.3 (5Z)-7-Oxozeaenol: a TAK1 selective inhibitor…… 22
1.7 TGFβ1 signaling: role of CCN2…… 23
1.7.1 CCN2 mediates wound healing and fibrosis…… 23
1.8 Gingival hyperplasia…… 26
1.9 Human gingival fibroblasts *vs* dermal fibroblasts. What do we know?…… 28
1.9.1 A comparison between gingival and dermal fibroblasts: differences in function …… 29
1.10 Rationale, Objectives and Hypothesis…… 31

Chapter 2. Methods…… 33
2.1 Human gingival fibroblast culture…… 34
2.2 RNA extraction …… 34
2.3 RT-qPCR analysis…… 35
2.4 Gene expression profiling …… 35
2.5 Western Blot analysis …… 37
2.6 Indirect immunofluorescence procedure …… 38
2.7 Proliferation assay …… 39
2.8 Statistical analysis …… 40

Chapter 3. Results……... 41
3.1 TAK1 mediates TGFβ1 induced gingival fibroblast CCN2 expression……...……. 42
3.2 Inhibition of TAK1 blocks TGFβ1 induced mRNA expression in human gingival fibroblasts ……. 47
3.3 TAK1 mediates the induction of genes involved with proliferation ……. 48
3.4 TAK1 inhibition blocks TGFβ1 induced proliferation in gingival fibroblasts ……..…..……...……..…… 53
3.5 TAK1 mediates TGFβ1- induced expression of wound healing cluster mRNAs in gingival fibroblasts ……....……..…...…….. 56

Chapter 4. Discussion ……...…. 59
4.1 Summary of results ……..……...……. 60
4.2 Research significance ……..……... 62
4.3 Limitations and future research ……...……..…….. 68

Chapter 5. References ……...…. 74

Appendix……...….. 94
Supplemental Tables…….…….……..95
Supplemental Table 3.3…….……..……95
Supplemental Table 3.4 ……..99
Permission to Reprint Figures ……..…….…….……..… 103
Curriculum Vitae ……..…...…….…….…….. 113

LIST OF TABLES

3.1 Cluster analysis of TAK1 dependent mRNAs with over 1.7 fold induction in response to TGFβ-1 treatment. Cell cycle/Proliferation cluster 50

3.2 Cluster analysis of TAK1 dependent mRNAs with over 1.7 fold induction in response to TGFβ-1 treatment. Wound healing clusters 51

Supplemental Table 3.3. List of genes sensitive to TAK1 inhibition with over 1.7 fold induction in response to TGFβ-1 treatment ……..……..……95

Supplemental Table 3.4. List of genes with over 1.7 fold induction in response to TGFβ-1 treatment ……....……..…….……..……99

LIST OF FIGURES

1.1 Comparative anatomy: Structure of skin and gingival masticatory mucosa in cross section…… 4

1.2 Myofibroblast characterization and possible origins……..……. 9

1.3 TGFβ1 signaling in fibroblasts…….. 14

1.4 The diverse, multifunctional, and pleiotropic effects of TGFβ……..… 17

1.5 CCN2 protein structure (signal peptide and Modules I-IV) and signaling in fibroblasts…….……..…… 24

1.6 Hereditary gingival overgrowth …..……..…… 27

3.1 TAK1 mediates TGFβ1- induced *CCN2* gene expression…….…...…… 43

3.2 TAK1 mediates TGFβ1- induced CCN2 protein expression: Western blot data…..… 44

3.3 TAK1 mediates TGFβ1- induced CCN2 protein expression: indirect immunofluorescence analysis……...…… 45

3.4 (5Z)-7-oxozeaenol inhibits TGFβ1-induced mRNA expression in human gingival fibroblasts……..…...… 49

3.5 Inhibition of TAK1 reduces TGFβ1-induced *Endothelin 1* and *JunB* mRNA expression …….…….……..……..…...… 54

3.6 TAK1 mediates TGFβ1- induced gingival fibroblast proliferation……...… 55

3.7 Inhibition of TAK1 reduces the expression of TGFβ1-induced wound healing genes.…….....…….……..…… 57

LIST OF ABBREVIATIONS

Abbreviation	Full Name
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid Assay
BrdU	5-Bromo-2'-Deoxyuridine
CCN2	Cysteine-rich 61 (**C**yr61), Connective Tissue Growth Factor (**C**TGF), Nephroblastoma over-expressed (**N**ov) 2
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EDN1	Endothelin 1
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
HPK1	Hematopoietic progenitor kinase-1
HRP	Horseradish Peroxidase
IGFBP3	Insulin-Like Growth Factor Binding Protein 3
IL11	Interleukin 11
JUNB	Transcription Factor Jun-B
NP40	Nonyl phenoxypolyethoxylethanol
PBS	Phosphate Buffered Saline

PBST	Phosphate Buffered Saline and Tween
PDGFA	Platelet-Derived Growth Factor Alpha Chain Gene
RIPA Buffer	Radioimmunoprecipitation Assay Buffer
RMA Algorithm	Robust Multi-Array Average Algorithm
RT-qPCR	Reverse Transcription Real Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
TAK1	Transforming Growth Factor β-Activated Kinase 1
TAB1	TAK1-binding protein-1
TAB2	TAK1-binding protein-2
TBST	Tris-Buffered Saline and Tween
TGFβ1	Transforming Growth Factor Beta 1
α-SMA	α-Smooth Muscle Actin

1. Introduction

1.1 Overview of physiological wound healing

Disruption or damage of the anatomical structure and function of a tissue or organ is characterized as a wound (Lazarus et al., 1994; Robson et al., 2001). Wound healing is an orderly physiological process in all tissues and organs of the body in response to injury, with the goal of anatomical and functional restoration of that tissue or organ (Guo and DiPietro, 2010; Velnar et al., 2009). Aside from the liver, eye and skeletal tissues, this process of repair is generally conserved across various tissues and organs and has been described as having four phases: hemostasis/coagulation, inflammation, proliferation and resolution/remodeling (Guo and DiPietro, 2010; Hunt et al., 2000; Robson et al., 2001; Velnar et al., 2009; Wynn 2008). It has been hypothesized that failure to terminate the repair program leads to excessive deposition of connective tissue in place of the normal parenchyma resulting in the development of fibrotic scars (Guo and DiPietro, 2010; Leask 2008; Wynn 2008). Interestingly, in contrast to every other adult tissue, the oral cavity does not scar in response to injury (Egusa et al., 2010; Guo et al., 2011a, 2011b; Schor et al., 1996; Wikesjo et al., 1992). Understanding the differences in wound healing responses among different tissue types is of importance for future clinical benefit (Ebisawa et al., 2011; Eslami et al., 2009; Guo et al., 2011a, 2011b; Schor et al., 1996). At present however, relatively little is known about the fundamental mechanisms underlying scarless repair in the gingiva, thus the discussion of wound healing and fibrotic mechanisms in the gingiva are best considered in the context of dermal repair. What is currently known about cutaneous and gingival tissue repair, fibrosis and structural anatomy are thus compared and discussed below.

1.2 Comparative anatomy: structure of skin and gingiva

1.2.1 Structural anatomy of skin

Skin, the largest organ of the body is comprised of three layers: the epidermis, dermis and hypodermis (Wolff et al., 2007) (**Figure. 1.1**). The epidermis, composed of keratinized stratified squamous epithelium is of ectodermal origin (Wolff et al., 2007). Depending on the thickness of the skin, the epidermis consists of five (thick skin: palms, soles) or four (thin skin) outermost to innermost layers: stratum corneum, stratum lucidum (in thick skin only), stratum granulosum, stratum spinosum and stratum basale (Vi 2010; Wolff et al., 2007). Analogous to the skin, the superficial layer of the gingiva is comprised primarily of keratinocytes with occasional melanocytes, Langerhans' cells (antigen presenting cells) and neurosensory cells. It is further classified into four sub-layers similarly to skin: stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Nanci, 2013; Squier and Kremer, 2001). Differentiation of keratinocytes as they move from the deep (stratum basale) to superficial (stratum granulosum) layers constitutes the squamous appearance of the layer, resulting in keratin squames which are shed in the stratum corneum (Nanci, 2013; Squier and Kremer, 2001; Thomson, 2012).

Dermis, mesodermal in origin, is a strong and flexible connective tissue layer below the epidermis and above the hypodermis which is composed of loose connective and fatty tissues (Gosain et al., 2004; Harper and Grove, 1979; Montagna and Parakkal, 1974; Wolff et al., 2007). Fibroblasts, macrophages, mast cells, extracellular matrix (ECM) (types I and III collagen, elastin and fibronectin, proteoglycans), glycoproteins, nerve fibers and blood vessels are found within the dermis (Gosain et al., 2004; Palaiologou et al., 2001). The dermis is further subdivided into papillary and reticular

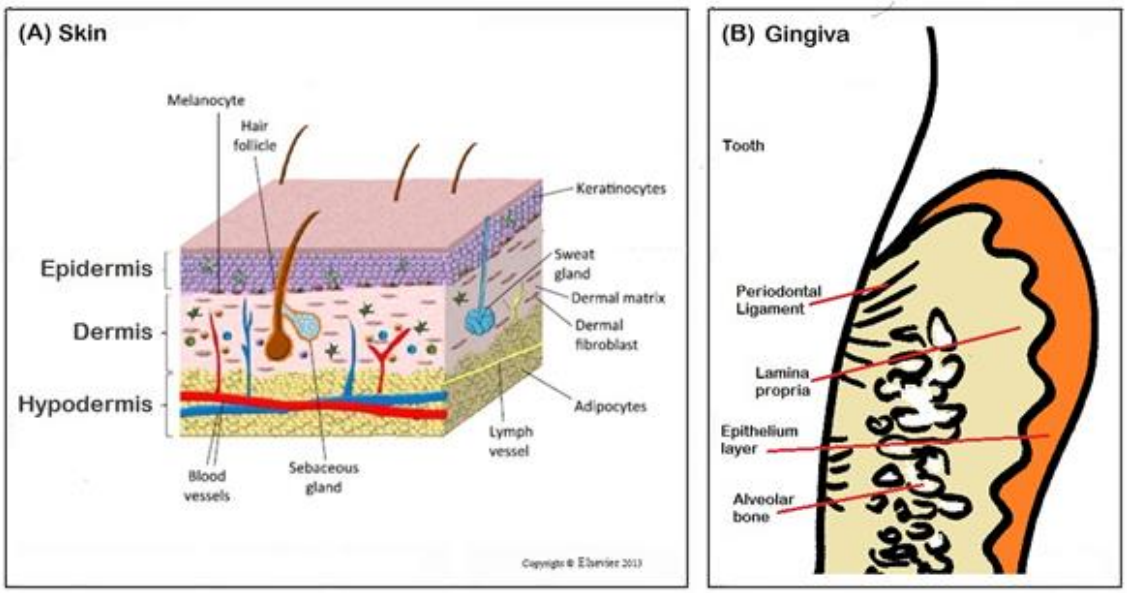


Figure 1.1. Comparative anatomy: Structure of (A) skin and (B) gingival masticatory mucosa in cross section. Epithelium layer is the outermost layer of the gingival masticatory mucosa while the outermost layer in the skin is known as the epidermis. The dermis (skin) or lamina propria (gingiva) are the connective tissue layers below the outermost layer. (A) Reprinted from: Kendall AC, Nicolaou A. 2013. Bioactive lipid mediators in skin inflammation and immunity. Progress in Lipid Research. Volume 52, Issue 1, pages 141–164 with permission from Elsevier. http://dx.doi.org/10.1016/j.plipres.2012.10.003

sub-layers in the superficial to deep order. The former forms ridges at the point of contact with the epidermis and contains thin, poorly organized collagen fibers and higher collagen type III to type I ratio as well as many more fibroblasts, which show higher proliferation and enzymatic activity (Gosain et al., 2004; Harper and Grove, 1979; Montagna and Parakkal, 1974; Vi 2010; Weber et al., 1984). The reticular region, located above the hypodermis, is characterized by a decreased collagen type III to type I ratio, thick and organized collagen fibers and fibroblasts that show reduced proliferation capacity yet increased contractile responses (Harper and Grove, 1979; Vi 2010; Weber et al., 1984). Hair follicles as well as sweat and sebaceous glands are also found within the dermis.

1.2.2 Structural anatomy of gingiva

Gingival tissue is classified as the oral mucosa surrounding the tooth which acts as a protective barrier (mechanical damage and environmental factors) for the underlying tissues (Nanci, 2013; Seoane Leston et al., 2002; Squier and Kremer, 2001). Gingival mucosa which faces the oral cavity is further known as masticatory mucosa. The epithelial and connective tissue layers of gingiva are organized in a similar fashion to that of the skin. Masticatory mucosa is comprised of the superficial keratinized stratified squamous epithelium layer which is supported by the underlying lamina propria layer, analogous to the dermis layer of the skin (Nanci, 2013; Squier and Kremer, 2001; Thomson, 2012). The basal lamina further forms the interface between the two layers (Nanci, 2013; Squier and Kremer, 2001; Thomson, 2012) (**Figure 1.1**). Located below the lamina propria, the submucosal region in the gingiva is analogous to the hypodermis layer of the skin (Nanci, 2013; Squier and Kremer, 2001; Thomson, 2012). The lamina

propria is organized in a similar fashion to the dermis. Conversely, unlike skin, gingival masticatory mucosa lacks hair follicles as well as sweat and sebaceous glands (Seoane Leston et al., 2002).

1.3 Pathological wound healing: overview of fibrosis

Wound healing can be affected by multiple factors, generally characterized as local (pertaining to the wound itself) or systemic (pertaining to the overall health of the organism) (Guo and DiPietro, 2010). Simultaneous processes of inflammation, tissue remodeling and regeneration serve as indicators of fibrotic disease, loosely defined by a time-dependent increase of total collagen deposition which exceeds the rate of collagen degradation (Wynn, 2008). The excessive production, remodeling and contraction of the ECM further leads to excessive scarring, the hallmark feature of fibrotic diseases (Krieg et al., 2007; Leask 2008). The resulting fibrotic scars exhibit a reduction in original elasticity of the tissue, are fairly acellular and replace normal tissue architecture while diminishing its function (Singer and Clark, 1999; Toriseva et al., 2007). It is estimated that close to 45% of deaths in developed countries are attributed to some form of the disease condition which affects the function of multiple organs (Pinzani 2008; Wynn 2008). Fibrotic diseases are also the largest group of diseases to which there is no established therapy (Leask 2008; Pinzani 2008; Wynn 2008). Diabetic nephropathy, pulmonary fibrosis, liver cirrhosis, atherosclerosis, systemic sclerosis/scleroderma, gingival hyperplasia and even hypertension are some of the numerous examples of fibrotic diseases affecting individual organs as well as the entire organism (Leask 2008, 2011; Pinzani 2008).

Although fibrosis is a complex disease involving many mediators, it is thought that myofibroblasts, fibroblast-like contractile cells, as well as three main extracellular factors TGFβ1, endothelin-1 and CCN2 (connective tissue growth factor) represent a common mechanism underlying the onset and progression of fibrosis (Krieg, et al., 2007).

1.3.1 Fibrotic lesions contain abundant myofibroblasts

The cell type responsible for persistent fibrosis is the myofibroblast, a contractile and secretory cell which is characterized by the increased intracellular staining for α-Smooth Muscle Actin (α-SMA), incorporated into stress fibers that span the length of the cell (Gabbiani 2003). α-SMA is a contractile protein which attaches to the ECM through fibronexus/focal adhesion structures (the *in vivo* and *in vitro* junction respectively) on the cell surface and promotes the excessive adhesion and mechanical force generation at the site of wounding, thus promoting wound closure and scar formation (Hinz, 2006, 2007, 2010; Hinz and Gabbiani, 2003; Hinz et al., 2007; Krieg et al., 2007; Shi-wen et al., 2012). Focal adhesions, composed of actin filaments, act as a link between the contractile α-SMA protein inside the cell and the ECM outside (Hinz and Gabbiani, 2003; Liu et al., 2009; Parsons, 2003, Tomasek, et al., 2002). They contain two other important components: integrins (the cell surface receptors for ECM) and focal adhesion kinase which promotes adhesive signaling responses and subsequent fibrosis (Chen et al., 2005; Hinz and Gabbiani, 2003; Liu et al., 2009; Mimura et al., 2005; Parsons 2003; Tomasek, et al., 2002). Essentially, the myofibroblast phenotype *in vitro* and *in vivo* is characterized by the appearance of a contractile cytoskeleton accompanied by the *de novo* expression of α-SMA positive stress fibers, integrin attachments to the ECM, replacement of N-

cadherin adhesions with the formation of bigger OB-cadherin (cadherin-11)-type cell-cell adhesions as well as the formation of large, so–called "supermature" focal adhesions (**Figure 1.2**) (Hinz 2007, 2010; Hinz et al., 2004).

Increased myofibroblast numbers in the dermis correlate with the excessive production of collagen and other ECM components, contraction of ECM and granulation tissue formation (Hinz and Gabbiani, 2003; Hinz et al., 2007; Krieg et al., 2007; Tomasek, et al., 2002). Increased myofibroblast numbers in the skin of systemic sclerosis patients also directly correlates with the extent of local collagen deposition and the Rodnan skin score — which measures the extent of scarring and disease progression (Hinz et al., 2012; Kissin, et al., 2006; Krieg et al., 2007).

In physiological wound healing, myofibroblasts begin their regression and undergo apoptosis with the onset of wound re-epithelialization. In contrast, during fibrosis myofibroblasts persist, resulting in the excessive production and remodeling of the ECM (Desmoulière et al., 1995; Hinz et al., 2012; Krieg et al., 2007).

1.3.2 Origin of myofibroblasts

Several theories have been proposed relating to the origin of myofibroblasts, possibly the earliest point of control in fibrotic disease progression and most accurate indication of the onset of fibrosis (**Figure 1.2**) (Hinz et al., 2007; 2012; Krieg et al., 2007). For example, the dermal myofibroblasts that are detected in response to wound healing and/or scleroderma have been purported to arise as a result of: 1) differentiation of resident dermal fibroblasts *via* signaling in response to growth factors such as TGFβ1; 2) recruitment of circulating fibrocytes, bone marrow (BM)-derived circulating cells or

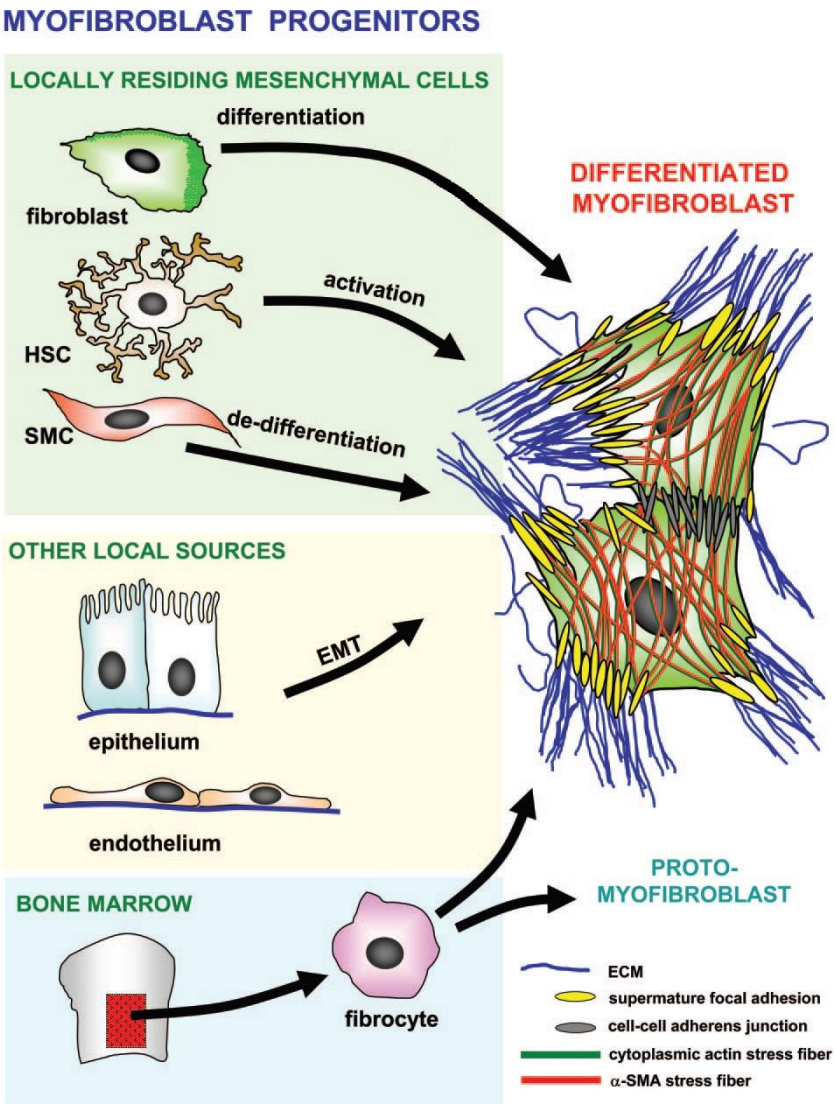


Figure 1.2. Myofibroblast characterization and possible origins. Although several theories have been proposed relating to possible myofibroblast progenitors, fibroblast to myofibroblast differentiation (driven by TGFβ1) is considered to be a predominant source of myofibroblasts in wound healing and fibrosis. Expression of adherens junctions, super-mature focal adhesions and α-SMA incorporation into stress fibers are indicative of myofibroblast phenotype. (Adapted with permission from *Figure 2. One cell, multiple origins***.** Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. 2007. The myofibroblast: one function, multiple origins. Am J Pathol. 2007 Jun;170(6):1807-16. DOI: 10.2353/ajpath.2007.070112; http://www.elsevier.com)

resting mesenchymal precursor cells to the specific tissue site and subsequent differentiation into myofibroblasts *via* induction by cell-ECM contacts or cytokines; 3) activation of vascular pericytes and smooth muscle cells concomitant with the repair of damaged vasculature in response to injury or 4) clonal selection of resting myofibroblasts (Abe et al., 2001; Direkze et al., 2003; Hinz et al., 2001, 2007; Krieg et al., 2007; Rajkumar et al., 2005).

Of interest still is the traditional view of fibroblast to myofibroblast differentiation, likely to be the predominant process in physiological wound healing and repair. This connective tissue repair process is primarily driven by fibroblasts which are resident connective tissue cells. These cells differentiate into a contractile myofibroblast phenotype in response to mechanical tension and growth factors such as TGFβ1 (Hinz 2007, 2010; Krieg et al., 2007; Leask 2008).

1.4 Fibroblast cells

Fibroblasts are connective tissue cells of mesodermal origin, loosely defined as cells of "spindle shape" capable of forming an extracellular matrix rich in type I and/or type III collagen, adhering to ECM and lacking markers for other cell lineages (Alberts et al., 1994; Chang, et al., 2002; Ebisawa et al., 2011). They are the most versatile connective tissue cell type which can be classified as Loose (in adipose tissue), Fibrous (in tendons, ligaments and cartilage) and Specialized (in bone and blood) that together create a structural framework of body architecture (Alberts et al., 1994). Certain so-called "immature" fibroblasts (sometimes referred to as mesenchymal cells) from various body regions are capable of differentiating into other connective tissue cell types, often co-

existing in a mixed population with the mature fibroblasts (Alberts et al., 1994; Egusa et al., 2010).

Fibroblasts are considered essential for the wound healing process (Abe et al., 2001). Deficiency in fibroblast proliferation and wound closure leads to ulceration. Conversely, abnormal proliferation and/or differentiation into myofibroblasts lead to the excessive production of collagen and other ECM proteins as well as contraction of ECM resulting in fibrosis (Krieg et al., 2007; Leask, 2008).

1.4.1 Fibroblast to myofibroblast differentiation

Growth factors such as TGFβ1, biomechanical tension and matrix signaling are three known factors required for fibroblast to myofibroblast differentiation (Gabbiani 2003; Lagares et al., 2012; Tomasek et al., 2002). Importantly, the disruption of the architecture and composition of ECM, which normally acts as a stress shield for the resident fibroblasts, leads to fibroblast activation and migration into the wound site (Hinz 2007; Hinz et al., 2007; Tomasek et al., 2002; Werner and Grose, 2003).

1.5 The role of TGFβ1 in wound healing and fibrosis

TGFβ1 was first identified in 1983 and has been characterized as a member of TGFβ superfamily which also includes Nodals, Inhibins, Activins, Bone Morphogenetic Protein (BMP), Growth and Differentiation Factor (GDF), Mullerian inhibitory substance (MIF) as well as the two remaining TGFβ isoforms: TGFβ2 and TGFβ3 (Assoian et al., 1983; Barrientos et al., 2008; Kingsley 1994; Rahimi and Leof 2007). The three TGFβ isoforms are predominantly expressed by macrophages, endothelial, fibroblasts and epithelial cells at the site of the injured skin (Barrientos et al., 2008; Rahimi and Leof, 2007). Both covalent and non-covalent interactions of mature TGFβ polypeptide with the

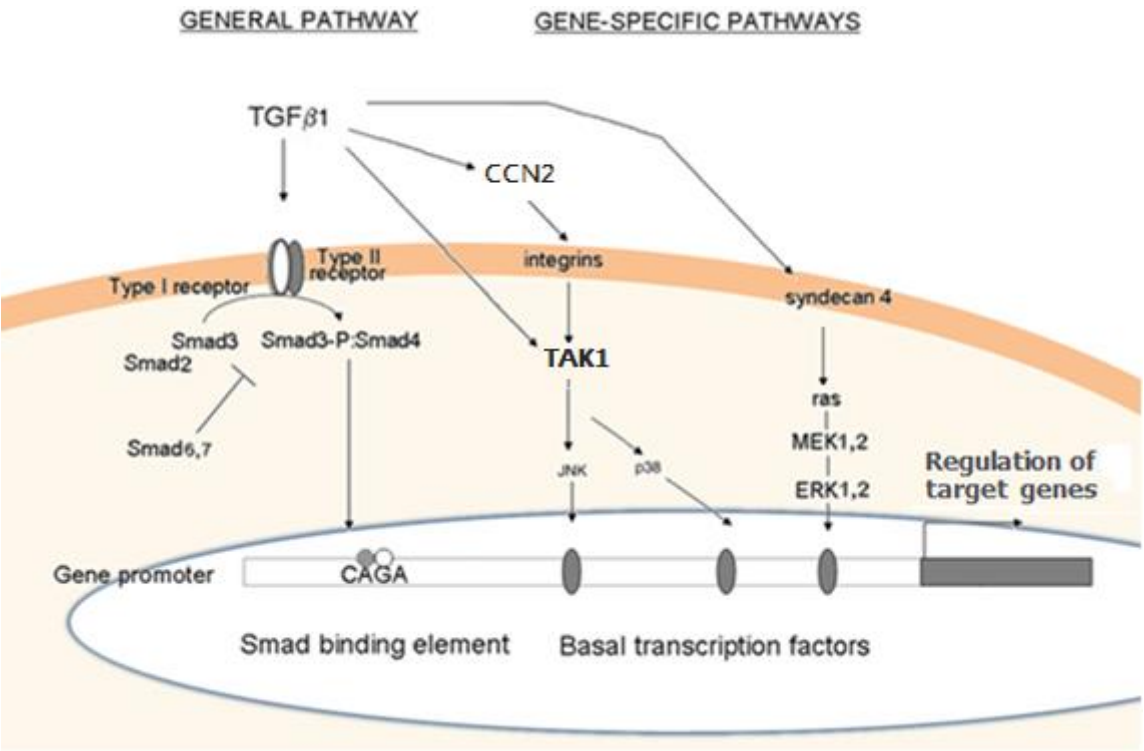
latency associated peptide (LAP) or other latent TGFβ-binding proteins renders it inactive (Leask 2008; Munger et al., 1997; Rahimi and Leof, 2007). Upon cellular secretion, these latent TGFβ-binding proteins and/or LAP are removed by proteolysis, allowing for the formation of an active 25kDa TGFβ dimer which initiates signaling responses by interacting with the type II/type I (activin receptor-like kinase, ALK)/TGFβ heteromeric receptor complex comprised of one of the five and seven distinct receptor types respectively (Leask 2008; Rahimi and Leof, 2007). Type III (betaglycan) and type V (IGFBP-3R/LRP-1) receptors may be further utilized in conjunction to the type I and type II receptor activation (Blobe et al., 2001; Eickelberg et al., 2002; Huang et al., 2003; Leal et al., 1997, 1999; Liu et al., 1994, 1997; O'Grady et al., 1991, 1992). ALK5 however is the most frequently utilized TGFβ type I receptor in fibroblast cells (Leask, 2008; Rahimi and Leof, 2007).

TGFβ1 participates in all stages of the physiological wound healing with increases in its expression shortly after the onset of injury (Barrientos et al., 2008). During the remodeling phase of wound healing, increases in the expression of tissue inhibitor of metalloproteinases (TIMPs), collagen type I and III as well as decreases in the expression of matrix metalloproteinases (MMP) MMP-1, MMP-3, and MMP-9 are mediated by TGFβ1 signaling (Barrientos et al., 2008). Additional responses include facilitation of wound contraction and closure by myofibroblasts, up-regulation of fibronectin, collagen, fibronectin and integrin receptors and protease inhibitors as well as recruitment of inflammatory cells to the site of injury during the earlier stages of wound healing (Barrientos et al., 2008; Clark 1996; Mauviel et al., 1996; Meckmongkol et al., 2007; Papakonstantinou et al., 2003; Zeng et al., 1996).

Fibroblast activation in response to TGFβ1 involves two separate, Smad-dependent and Smad-independent pathways, also known as the canonical and non-canonical pathways, respectively (Bhattacharyya et al., 2008; Leask 2006, 2008; Leask and Abraham, 2004; Massague 1998; Mehra and Wrana, 2002; Rahimi and Leof, 2007) (**Figure 1.3**).

1.5.1 Canonical TGFβ1 signaling in fibroblasts

Canonical, Smad-dependent TGFβ1 signaling in cells such as fibroblasts involves binding of TGFβ1 ligand to the type II/type I TGFβ receptor heteromer and activation of downstream Smad complexes, transcription factors which mediate the expression of collagen and other pro-fibrotic genes (Leask 2008; Massague 1998; Rahimi and Leof, 2007) (**Figure 1.3**). The Smad family of transcriptional co-regulators includes eight different Smad isoforms distributed among three sub-classes and utilized by different members of the TGFβ family (Rahimi and Leof 2007). Smad 2 and 3 generally are activated by TGFβ, Activin and Nodal members of the TGFβ superfamily; Smad 1, 5 and 8 are activated by MIS, BMP and GDF whereas the inhibitory Smads; Smad 6 and 7 act as negative regulators of BMP and TGFβ/Activin/BMP pathways, respectively (Goumans et al., 2002; Rahimi and Leof 2007; Trojanowska, 2009). Interestingly, the negative regulators Smad 6 and Smad 7 are similarly transcribed *via* SBE activity upon TGFβ stimulation, thus regulating its own expression and TGFβ signaling in a negative feedback loop (Leask, 2008; Lin et al., 2003). Finally, in scleroderma fibroblasts, Smad 1 is involved in TGFβ-mediated signaling where the presence of both TGFβ and CCN2 drive Smad 1 phosphorylation and the subsequent increase in CCN2 production in a positive feedback loop (Pannu et al., 2007; Trojanowska, 2009).



BioMed Central is the original publisher

Figure 1.3. TGFβ1 signaling in fibroblasts. Canonical and non-canonical signaling pathways are outlined (General and Gene-Specific pathways respectively). TGFβ1 initiates signaling responses by interacting with the type II/type I TGFβ heteromeric receptor complex, resulting in Smad3/Smad4 activation, translocation to the nucleus and transcription of target genes by binding to the CAGA consensus sequence of Smad-binding DNA elements (SBE). Alternatively, TGFβ1 signals *via* integrin/syndecan4 receptors synergistically with CCN2 for additional, tissue and context specific regulation. (Adapted with permission from *Figure 2.* Leask A. 2006. Scar wars: is TGFβ the phantom menace in scleroderma? Arthritis Res Ther. 2006; 8(4): 213. Doi: 10.1186/ar1976. BioMed Central is the original publisher).

Considering the canonical signaling pathway, TGFβ1 binds to the type II TGFβ receptor leading to the recruitment of type I (ALK5) TGFβ1 receptor. ALK5 becomes phosphorylated at its glycine-serine rich domain, and phosphorylates Smad 2/3 complex at the C terminal Ser-Ser-X-Ser (SSXS) motif leading to dissociation of Smad 2/3 complex from the receptor and its subsequent association with the common mediator Smad 4 (Derynck and Zhang, 2003; Leask 2008, Massague et al., 2005; Rahimi and Leof, 2007; Sporn and Jakowlew, 2010). Additional adaptor proteins including SARA (Smad anchor for receptor activation), HRS/HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) and cPML may be recruited to facilitate ALK5-dependent phosphorylation of Smads (Lin et al., 2004; Miura et al., 2000; Rahimi and Leof 2007; Tsukazaki et al., 1998). p300/CBP co-activators may further assist in the Smad-dependent gene activation and Fast-1 nuclear DNA binding protein is necessary for Smad2 DNA interaction (Ghosh and Varga 2007; Leask, 2008; Liu et al., 1999; Zhou et al., 1998).

Nuclear translocation of Smad 2/3-Smad 4 trimer complex and direct interaction of Smad 3/4 with the GC rich sequences of certain promoters as well as the GTCT/CAGA consensus sequence of Smad-binding DNA elements (SBE) leads to expression of collagen alpha-2(I) chain and other profibrotic genes (Bhattacharyya et al., 2008; Derynck and Zhang, 2003; Massague et al., 2005; Rahimi and Leof, 2007)

1.5.2 Targeting canonical TGFβ1 signaling in fibrosis disease treatment

TGFβ1 signaling in fibroblasts induces both fibroblast proliferation and differentiation into myofibroblast as well as ECM synthesis, connective tissue deposition and contraction (Grotendorst et al., 2004; Guo et al., 2012; Leask 2008; Rahimi and Leof,

2007). However, signaling *via* members of the TGFβ superfamily is known to be involved with multiple physiological and cellular processes including immunosuppression, cell differentiation, proliferation (mesenchymal cells), migration, growth inhibition (epithelial, endothelial, hematopoietic and neuronal cells, murine embryonic fibroblasts) or apoptosis (**Figure 1.4**). (Datto et al., 1999; Grotendorst et al., 2004; Guo et al., 2012; Leask 2008; Rahimi and Leof, 2007; Roberts et al.,1986). Interestingly, a great number of these distinct cellular responses are mediated *via* the canonical signaling pathway, often involving identical TGFβ-Smad family signaling participants (Rahimi and Leof, 2007). However, the canonical TGFβ1 signaling pathway does not mediate CCN2/CTGF or α-SMA expression in scleroderma fibroblasts (Chen et al., 2006; Holmes et al, 2001). Aside from fibroblasts, TGFβ1 is also produced and locally secreted by macrophages and other cell types and exhibits a profibrotic or suppressive role depending on the cell type (Rahimi and Leof, 2007; Wynn 2008).

The fibrogenic phenotype arising from fibroblast exposure to TGFβ1 is non-heritable and persists only in the presence of TGFβ1 polypeptides, suggesting the removal of "excess" TGFβ1 is sufficient to revert cells to their previous physiologic state (Leask, 2008; McWhirte et al., 1994). TGFβ-deficient mouse models exhibit systemic tissue necrosis, organ failure and death (Kulkarni et al., 1993). In contrast, murine models with *TGFBR1* (ALK5) deletion die in utero, probably as a result of vascular defects incompatible with life (Carvalho et al., 2007; Larsson et al., 2001). Similarly, Smad3 knock-out murine models develop emphysema, chronic inflammation and colorectal adenocarcinomas among other morbidities (Bonniaud et al., 2004a; Datto et al., 1999; Yang et al., 1999; Zhu et al., 1988).

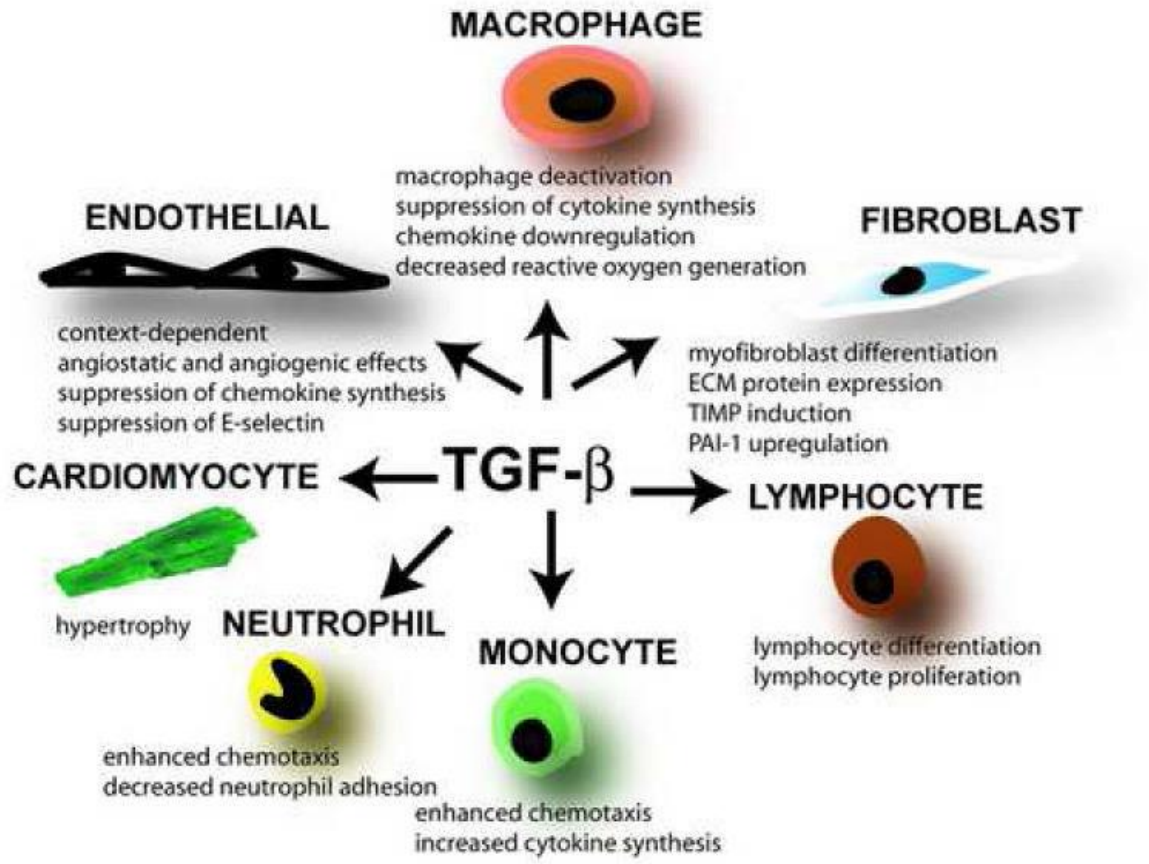


Figure 1.4. The diverse, multifunctional, and pleiotropic effects of TGFβ. Signaling *via* TGFβ affects multiple cell types and cellular processes making it difficult to target TGFβ1 directly as a potential fibrotic disease treatment (Reproduced with permission of Oxford University Press from *Figure 2*. Bujak M, Frangogiannis NG. 2007. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res. May 1;74(2):184-95. Epub 2006 Oct 7; Doi: 10.1016/j.cardiores.2006.10.002)

Not surprisingly, studies that manipulate canonical TGFβ1 signaling pathways do not provide a specific point of interference which can be applied in future therapy of fibrotic diseases (Bonniaud et al., 2004; Denton et al., 2007; Evans, et al., 2003; Hu et al., 2003; Kulkarni et al., 1993; Leask et at., 2008; Shull et al., 1992). Further, the use of a recombinant human anti-TGFβ1 antibody (CAT-192) in a clinical trial for the treatment of early-stage diffuse cutaneous systemic sclerosis resulted in adverse side-effects including death without evidence of efficacy (Denton et al., 2007). Collectively, these observations suggest that examining non-canonical TGFβ signaling pathways, which may mediate specific cellular responses to TGFβ1, might be more target specific for the treatment of fibrosis.

1.5.3 Non-canonical TGFβ1 signaling in fibroblasts

Several Smad-independent, non-canonical TGFβ1 signaling pathways have been previously identified in fibroblasts (Leask 2008; Massague 1998; Rahimi and Leof, 2007; Verrecchia and Mauviel, 2007; Wynn 2008; Zhang 2009). These pathways cooperate with canonical TGFβ1 signaling, and tend to be gene and tissue specific (**Figure 1.3**). Non-canonical TGFβ1 signaling can involve the MEK/ERK, p38 or JNK MAP kinase (MAPK) pathways. For example, TGFβ-dependent activation of Ras-MEK1-ERK1/2-Elk-1 occurs *via* heparan sulfate-containing proteoglycan (HSPG) syndecan 4 as a co-receptor (Barrientos et al., 2008; Chen et al., 2005; Massague et al., 2005; Verrecchia and Mauviel, 2007; Zhang 2009). Additional non-canonical signaling may involve Rho-like GTPase signaling pathways as well as phosphatidylinositol-3-kinase (PI3K)/AKT pathways (Zhang 2009). One example of the latter includes TGFβ-mediated activation of

p21 activated kinase 2 (PAK2) and Akt *via* PI3K intermediate (also an independent regulator) in fibroblasts (Rahimi and Leof, 2007; Wilkes et al., 2003; 2005).

1.6 TAK1

TGF-β-activated kinase 1 (TAK1) mediates the ability of TGFβ1 to activate the p38 and JNK MAP kinase cascades (Guo et al., 2012; Shim et al., 2005, Shi-Wen et al., 2009; Yamaguchi et al., 1995; Yamashita et al., 2008). This protein belongs to the mitogen-activated protein kinase kinase kinase (MAPKKK) family and was first identified in 1995 based on a screen of a murine cell line cDNA expression library (Shim et al., 2005; Yamaguchi et al., 1995). Activation of ectopic TAK1 in the MC3T3-E1 cell line following TGFβ treatment implicated this kinase in TGFβ1-mediated MAPK signaling, and established its name as TGFβ-activated kinase 1 (Yamaguchi et al., 1995).

In humans, the 606 amino acid TAK1 protein sequence contains a unique 300 amino acid C terminal region, as well as a putative N terminal domain resembling that of Raf-1 and MEKK kinases (30% identity) (Wu et al., 2013; Yamaguchi et al., 1995). An ATP-driven protein kinase catalytic region lies at the junction between the two domains (Wu et al., 2013).

TAK1-mediated signaling is activated in a number of cellular responses, including inflammation, apoptosis as well as certain cancers (Sakurai, 2012; Shim et al., 2005; Yamashita et al., 2008). Aside from TGFβ signaling, tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β) and Toll-Like receptor ligands use TAK1 as an intermediate in the activation of JNK/p38 and I-kappa B kinases (IKK), as well as downstream transcription factors including Activator protein 1 (AP-1) and NF-kappaB (Guo et al., 2012; Shim et al., 2005; Shi-wen et al., 2009; Wang et al., 1997,2001;

Yamashita et al., 2008). TAK1 is therefore an important mediator of inflammatory responses (*via* NF-kappaB and AP-1 activation) (Shim et al., 2005; Shi-wen et al., 2009; Yamashita, et al., 2008).

TAK1 has organ-specific effects. For example, TAK1 is essential for hepatic homeostasis. Loss of TAK1 from the liver using hepatocyte-specific *Tak1*-deficient (Tak1ΔHEP) mice resulted in inflammation, spontaneous hepatocyte death and perisinusoidal fibrosis developing at one month of age and eventually resulting in cancer (Inokuchi et al., 2010). Interestingly, tamoxifen-driven, global TAK1 deletion in mice conferred resistance to the development of renal fibrosis upon unilateral ureteric obstruction, resulting in reduced myofibroblast counts, collagen and pro-fibrotic gene expression due to abolished IL-1, TNF-α-induced JNK, p38, and NF-κB signaling attributed to TAK1 loss (Ma et al., 2011).

1.6.1 TAK1- mediated non-canonical TGFβ1 signaling in fibroblasts

In human and mouse fibroblasts, TGFβ1 activation of TAK1 results in the phosphorylation of stress-activated MAP kinase members JNK (*via* MKK4 intermediate) and Stress Activated Kinase p38 (*via* MKK6 or MKK3 intermediates) (Guo et al., 2012; Liu et al., 2007; Lovgren et al., 2011; Rahimi and Leof, 2007; Shibuya, et al., 1996; Thannickal et al., 2003; Wang et al., 1997, 2001; Yamaguchi et al., 1995; Yamashita et al., 2008). TGFβ receptor-dependent phosphorylation of TAK1 is also known to involve HPK1 and/or TAB1/TAB2 adaptor proteins (Rahimi and Leof 2007; Shibuya et al., 1996; Wang, et al., 1997, 2001; Yamashita et al., 2008). Interestingly, TAB2 was found to be dispensable in regards to TGFβ induced gene expression and TAK1-mediated JNK signaling (Shim, et al., 2005). A study by Yamashita and colleagues in 2008 also

identified TNF-receptor-associated factor 6 (TRAF6) ubiquitin ligase as a direct link between the TGFβ receptor complex and TAK1, where the activated receptor complex leads to K63 polyubiquitination of TRAF6 which then mediates TAK1 activation by a yet unknown mechanism, possibly including TAK1/TAB2 complex formation and/or activation (Shim et al., 2005; Wang, et al., 2001; Yamashita et al., 2008). Activation of TAK1 through TGFβ1 receptor signaling is further thought to involve focal adhesion kinase: phosphorylation of TAK1 upon TGFβ1 stimulation was reduced either by pre-treatment of wild-type fibroblasts with the FAK/Src inhibitor PP2, or in fibroblasts with FAK deletion (Shi-wen et al., 2009). Canonical, Smad-mediated signaling was unaltered upon TAK1 deletion, consistent with the idea that TAK1 is a mediator of non-canonical TGFβ1 signaling (Shi-wen et al., 2009; Shim et al., 2005; Yamashita et al., 2008).

1.6.2 Role of TAK1 in dermal homeostasis and wound repair

The TAK1-JNK/p38 pathway has been previously shown to play an important role in adhesive, migratory, proliferative and contractile responses of dermal fibroblasts, as well as in fibroblast to myofibroblast differentiation in response to TGFβ1 (Guo et al., 2012). Much of what is known about the role of TAK1 in fibrosis comes from studies of dermal fibroblasts and cutaneous disease. Fibroblasts isolated from the skin of scleroderma patients exhibited elevated levels of constitutively active TAK1 protein (Shi-wen et al., 2009). Decreased expression of collagen and other pro-fibrotic genes in the skin was observed in the fibroblast-specific TAK1 knock-out (TAK1-KO) mouse model, which also showed reduced skin thickness, delayed wound closure and decreased collagen deposition (Guo et al., 2012). TAK1-KO dermal fibroblasts exhibited reduced levels of proliferation (Guo et al., 2012). Inhibition of TAK1 in human skin fibroblasts

using (5Z)-7-Oxozeaenol, a TAK1 selective inhibitor, also led to reduced expression of ~ 66% of pro-fibrotic genes following *in vitro* treatment with TGFβ1 (Guo et at., 2012; Ninomiya-Tsuji et al., 2003). Thus, previous studies suggest that TAK1 plays an important role in maintaining dermal homeostasis as well as in mediating some of the pathways expected to play a role in fibrotic disease onset and progression.

1.6.3 (5Z)-7-Oxozeaenol: a TAK1 selective inhibitor

(5Z)-7-Oxozeaenol (also known as C 292, F 152, FR148083, L 783279, LL-Z1640-2) is a natural resorcyclic lactone first isolated in 1978 from an unidentified fungus and later in 2003 from the f6024 fungal strain culture broth in a quest for identifying an inhibitor of TAK1 to combat pro-inflammatory signaling (Ellestad et al., 1978; Murray, 2009; Ninomiya-Tsuji et al., 2003). (5Z)-7-Oxozeaenol acts at the level of the ATP binding site of TAK1 to irreversibly inhibit TAK1 catalytic activity by forming a covalent bond with the C8' of the cis-enone and Cys174 residue of TAK1 (Ninomiya-Tsuji et al., 2003; Wu et al., 2013). A TAK1-specific IC50 of 8 nM and 65 nM was previously reported based on the *in vitro* and *in vivo* kinase assays respectively (Ninomiya-Tsuji et al., 2003). In comparison, the *in vitro* IC50 for MEKK1 was 268 nM and the activities of MEKK4 (another MAPKKK family representative) and ERK were not inhibited at 500 nM (5Z)-7-Oxozeaenol (Ninomiya-Tsuji et al., 2003). Downstream targets of TAK1 such as JNK and p38 were also not inhibited by (5Z)-7-Oxozeaenol (Ninomiya-Tsuji et al., 2003).

1.7 TGFβ1 signaling: role of CCN2

In dermal and gingival fibroblasts, TGFβ1 potently induces the expression of the pro-fibrotic marker and mediator CCN2 (connective tissue growth factor), a member of

the CCN family of matricellular proteins (named for the first three members: **c**yr61, **c**tgf and **n**ov) (Black and Trackman 2008; Brigstock 2010; Leask 2008; Leask et al, 2009; Thompson et al., 2010). Matricellular proteins are known for their regulatory, as opposed to structural roles in modifying responses to growth factors and the microenvironment (Leask 2013a, 2013b.) Aside from CCN2, the CCN family in humans is comprised of 5 other members classified together based on a predicted four-modular structure and presence of N-terminal secretory signal: cysteine-rich 61 (Cyr61/CCN1), nephroblastoma over-expressed (Nov/CCN3), and the Wnt-induced secreted proteins (WISP-1, CCN4), -2 (WISP-2, CCN5) and 3 (WISP-3, CCN6) (Brigstock, 2003). CCN2 was identified over 20 years ago by Bradham and others as a 38-kDa, cysteine-rich protein secreted in endothelial cell culture capable of inducing fibroblast chemotaxis and cell division (Bradham et al., 1991; Leask 2013a, 2013b). The mature CCN2 protein is comprised of four modular domains: an insulin-like growth factor binding protein (IGFBP) domain (module I), a Von Willebrand factor domain (module II), a thrombospondin-homology domain (module III), cysteine knot heparin-binding domain (module IV) and possibly an un-cleaved signal peptide sequence. A "hinge region" between modules II and III allows for differential cleavage and fragmentation of the CCN2 protein which may have an effect on CCN2 function (**Figure 1.5**) (Bork 1993; Brigstock, 2003; Leask 2013a, 2013b; Perbal 2004).

1.7.1 CCN2 mediates wound healing and fibrosis

As observed with TGFβ1 expression, the levels of CCN2 increase rapidly following injury, and both the proliferative and remodeling phases of wound healing are

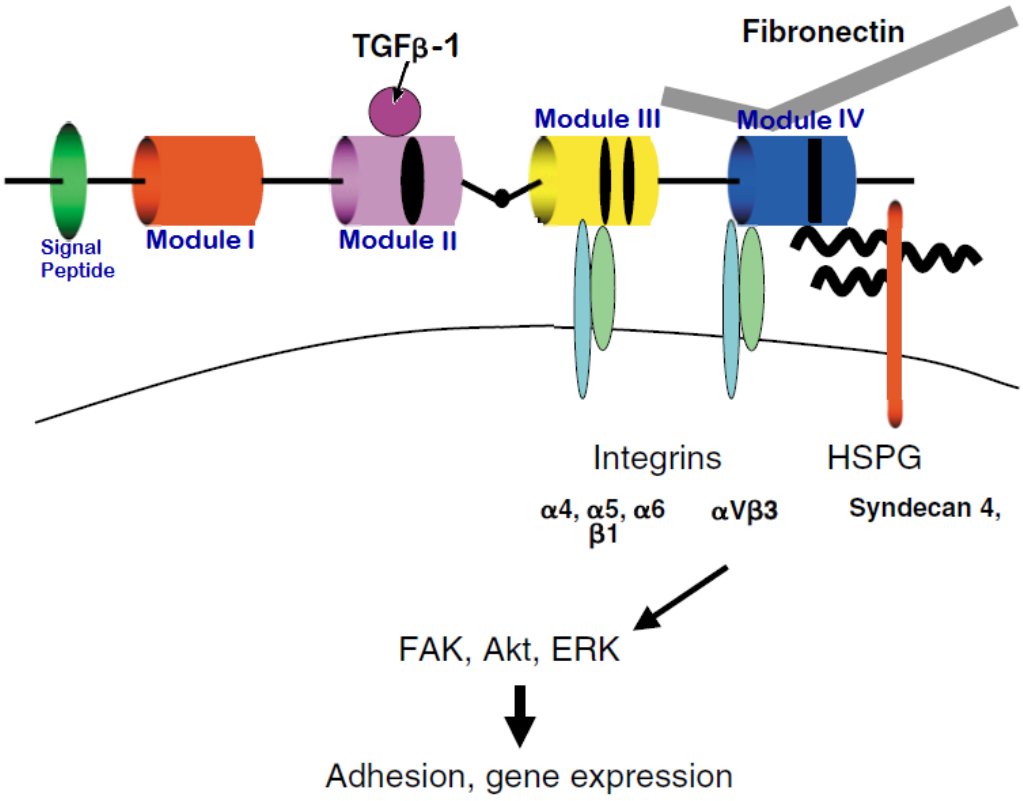


Figure 1.5. CCN2 protein structure (signal peptide and Modules I-IV) and signaling in fibroblasts. CCN2 binds integrins and heparan sulfate proteoglycan (HSPG) receptors on fibroblast surface. Binding of fibronectin and synergistic binding of TGFβ1 by CCN2 transduces the signals from the ECM inside the fibroblast. (Adapted from: *Figure 2.* Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci. 2006 Dec 1;119 (Pt 23):4803-10. Doi: 10.1242/jcs.03270; jcs.biologists.org)

regulated by CCN2-mediated response (Barrientos, et al., 2008). CCN2 expression is elevated in fibrotic conditions affecting all organs including atherosclerosis, scleroderma, keloids, hypertrophic scars, gingival hyperplasia, muscular dystrophy, Dupuytren's disease, idiopathic pulmonary fibrosis as well as diabetic retinopathy and nephropathy (Barrientos et al., 2008; Holmes et al., 2001; Kantarci et al., 2006; Leask 2013a, 2013b; Leask et al., 2009; Ponticos et al., 2009; Sonnylal et al., 2010; Thompson et al., 2010; Uzel et al., 2001). Elevated levels of CCN2 expression also serve as indicators of the severity of systemic fibrosis and correlate well with the presence of myofibroblasts and elevated ECM production by fibroblasts (Leask and Abraham, 2006; Ponticos et al., 2009). Loss of CCN2 expression in fibroblasts results in resistance to skin fibrosis, suggestive of the requirement of CCN2 for the development of skin fibrosis *in vivo* (Liu et al., 2011). CCN2 expression is induced by TGFβ1, yet it also behaves as a co-factor promoting TGFβ1-mediated ECM adhesion and fibrogenesis (Mori et al., 1998; Shi-wen et al., 2006; Wang et al., 2011) (**Figure 1.5**). CCN2 promotes fibroblast adhesion *via* association with HSPG and integrins, the specific identity of which tend to be cell type specific (Leask and Abraham, 2006). CCN2 potentiates other responses including: fibroblast growth, proliferation and chemotaxis, increase expression and deposition of collagen, fibronectin and other matrix proteins, up-regulation of integrin receptors, angiogenesis, endothelial migration, adhesion and proliferation (Barrientos et al., 2008; Holmes et al., 2001; Krieg et al., 2007; Leask 2008, 2013a, 2013b; Ponticos et al., 2009). The heparin binding domain also mediates the ability of CCN2 to bind low-density lipoprotein receptor-related protein 1 (LRP1) (Leask and Abraham, 2006; Segarini et al.,

2001). Thus, understanding how CCN2 is regulated in fibroblasts is likely to yield valuable insights into the treatment of fibroproliferative disorders.

1.8 Gingival hyperplasia

In contrast to every other adult tissue, the oral cavity does not scar in response to injury (Egusa et al., 2010; Guo et al., 2011a, 2011b; Schor et al., 1996, Sciubba et al., 1978). Unlike skin wounds, gingival wounds heal faster and without scarring, although the stages of the healing process and their sequence remain similar between the two tissue types (Egusa et al., 2010; Guo et al., 2011a, 2011b; Schor et al., 1996, Sciubba et al., 1978; Wikesjo et al., 1992). One example of a fibrotic condition in the oral cavity is gingival hyperplasia, characterized by an excessive gingival overgrowth in susceptible patients as a side effect to antiepileptic medications, calcium channel blockers and immunosuppressant drugs or as a result of other factors including disease and genetic predisposition (Clocheret et al., 2003; Nakib and Ashrafi, 2011) (**Figure 1.6**). In contrast to the typical, hyper-contractile response observed in fibrotic diseases, gingival hyperplasia is characterized by a hyper-proliferative response, likely due to the inherent tissue-specific differences in resident fibroblast cells (Guo et al., 2011a, 2011b). *In vivo*, fibrotic responses in the oral cavity are not generally characterized by the presence of myofibroblasts but instead are characterized by hyper-proliferation of fibroblast cells (Bitu et al., 2006; Damasceno et al., 2012; Sobral et al., 2010). This feature could arise due to a reduced or altered sensitivity of gingival fibroblasts, relative to dermal fibroblasts to mechanical strain, TGFβ1 or the use of alternative TGFβ1 signaling pathways (Guo et al., 2011a,b).

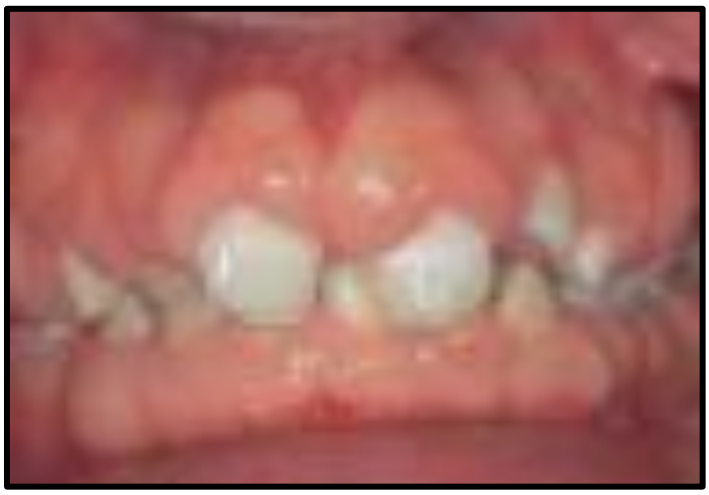


Figure 1.6. Hereditary gingival overgrowth. Unlike skin, gingiva does not scar. In contrast to the hyper-contractile fibrotic disease presentation associated with myofibroblast differentiation, gingival hyperplasia is characterized by a fibroblast proliferation and relative lack of myofibroblasts in the oral cavity. Taken directly from: Fig. 1b. Orthodontic records after periodontal surgery. Clocheret K1, Dekeyser C, Carels C, Willems G., 'Idiopathic gingival hyperplasia and orthodontic treatment: a case report.', Journal of Orthodontics, 2003 Mar;30(1):13-9. http://jorthod.maneyjournals.org/

1.9 Human gingival fibroblasts *vs* dermal fibroblasts. What do we know?

Studies analyzing transcriptional and functional differences among fibroblasts resident in various tissue niches are gaining importance with increasing evidence of inter-tissue fibroblast heterogeneity (Chang et al., 2002; Chipev et al., 2002; Ebisawa et al., 2011; Guo et al., 2011a, 2011b; Yasuda et al., 2006). Although dermal and gingival fibroblasts appear to be morphologically identical in culture, differences at the level of gene expression have been previously reported (Ebisawa et al., 2011; Guo et al., 2011a, 2011b). A study by Ebisawa and colleagues (2011) recently investigated differential gene expression in primary cultures (passages 4-5) of human gingival and dermal fibroblasts using DNA microarray, to gain a better insight into the existing functional differences of the two cell types (Ebisawa et al., 2011). From the 5284 analyzed genes, the expression of 5% (278) of genes was found to be significantly different; 114 genes were only expressed in dermal fibroblasts whereas 164 genes were only expression in gingival fibroblasts (Ebisawa et al., 2011). Differences in the extracellular matrix-related genes, oxidoreductase activity-related genes, cytokine activity-related genes and growth factor-related genes were prominent between the two cell types (Ebisawa et al., 2011). A similar study using DNA microarray gene expression profiling identified close to 800 genes which were found to be enriched in the human dermal fibroblast cell line as compared to human gingival fibroblasts (Guo et al., 2011b). These included the pro-adhesive genes such as paxillin, integrins α2 and α4 and FAK (Guo, et al., 2011b). In a different study, expression of integrins β3, a11, α2 and α5 exhibited 15-, 10-, 5- and 5- fold increased expression, respectively, in human dermal fibroblasts as compared to human gingival fibroblasts (Palaiologou et al., 2001). In contrast, expression of integrin subunit α3, α8

and β6 was found to be increased in gingival fibroblasts as compared to dermal fibroblasts by 50-, 15- and 5- fold, respectively (Palaiologou et al., 2001). Interestingly, no correlation was found between the expression pattern of heterodimeric integrin receptors and the fibroblast attachment profile to ECM (Palaiologou, et al., 2001).

1.9.1 A comparison between gingival and dermal fibroblasts: differences in function

The relative differences in wound healing and fibrotic responses between skin and gingiva may be further precipitated when fibroblasts are subjected to the wound healing/fibrogenic contexts and fibrogenic stimuli. Three dimensional fibrin lattices have been used to culture fibroblasts to mimic cellular environment during the early phases of wound healing, including inflammation and formation of fibrin clot (Bell et al., 1983; Lorimier et al., 1996, 1998; Niewiaro et al., 1972). Gingival fibroblasts seeded onto a fibrin lattice resulted in a rapid retraction and lysis of the lattice and complete disappearance after one week, suggesting a higher degree of fibrinolytic activity in gingival fibroblast as compared to dermal fibroblasts (Lorimier et al., 1998). Interestingly, the expression of matrix metalloproteinases (MMP2, MMP9) and tissue inhibitors of metalloproteinases (TIMP2) was more rapid and robust by the gingival fibroblast, suggesting a greater capacity of matrix remodeling by the gingival as compared to dermal fibroblasts (Chaussain Miller et al., 2002)

Further, the shielding properties normally exhibited by the ECM matrix as a protective barrier from the external environment and mechanical forces may be lost as a result of injury and are thus thought to expose the fibroblasts to the forces of mechanical tension, followed by the differential adaptation of the two cell types to the new, “stress unshielded” environment, likely promoting fibroblast to myofibroblast differentiation

(Guo et al., 2011a; 2011b, Hinz and Gabbiani, 2003; Kessler et al., 2001). Mechanical strain induced a number of pro-proliferative genes (*CDC6, cdk6*) as well as *TGFβ1, ET-1* (albeit at relatively low levels) and *CCN2* gene expression in donor-derived human gingival fibroblasts whereas potent induction of collagen type I and αSMA did not occur (Guo et al., 2011a; 2011b). Further, the fibrotic gene cluster was not identified using microarray analysis of the gingival fibroblasts post 72 hour strain while genetic cluster involved in cellular proliferation (cdc6,cdk6) was induced in response to strain, consistent with the notion that gingiva possess a hyper-proliferative as opposed to hyper-contractile fibrotic response (Guo et al., 2011a; 2011b).

Similarly to strain, a six hour induction of human dermal fibroblasts with TGFβ1 ligand (4ng/ml) potently increased the levels of CCN2, collagen I and α-SMA mRNAs, whereas only CCN2 expression was induced in human gingival fibroblasts in response to TGFβ1 albeit to a lesser extent than in dermal fibroblasts (Guo et al 2011a). The effect was attributed to the fact that gingival fibroblasts possess reduced ET-1 mRNA and protein as compared to human dermal fibroblasts; the difference in the αSMA and collagen I induction between the two cell types was rescued upon co-incubation of gingival fibroblasts with both TGFβ and ET-1 (Guo, et al., 2011a).

Further of interest is the observation that gingival wounds heal faster and with less scar formation as compared to skin wounds, although the stages of the healing process and their sequence remain similar between the two tissues (Egusa, et al., 2010; Guo et al., 2011a, 2011b; Schor, et al., 1996, Wikesjo, et al., 1992). Therefore it is possible that TGFβ1 signals through different pathways in dermal *vs* gingival fibroblasts.

In this regard, the role of non-canonical TGFβ1 signaling in gingival fibroblast remains to be explored. (Guo et al, 2011a, 2011b).

1.10 Rationale, Objectives and Hypothesis

Canonical TGFβ1 signaling is involved in multiple intracellular signaling pathways and in a number of physiological and cellular processes (Datto et al., 1999; Grotendorst et al., 2004; Guo et al., 2012; Leask 2008; Mehra and Wrana 2002; Rahimi and Leof, 2007; Roberts et al., 1986; Yamashita et al., 2008). Thus, broad targeting of the TGFβ1- mediated pathways may lead to deleterious side effects; previous studies which tried to interfere with the downstream targets of the canonical TGFβ1 signaling yielded no promising results (Bonniaud et al., 2004a; Datto et al., 1999; Evans et al., 2003; Grotendorst, et al., 2004; Hu et al., 2003; Leask et at., 2008; Mehra and Wrana, 2002; Rahimi and Leof, 2007; Roberts et al., 1986; Wang, et al., 1997; Yamashita, et al., 2008). Indeed, the use of CAT-192, a recombinant human anti-TGFβ1 antibody, led to adverse side-effects including death and no evidence of efficacy in the clinical trials of early-stage diffuse cutaneous systemic sclerosis treatment (Denton et al., 2007). Previous studies have identified the non-canonical, TAK1/JNK mediated signaling to play a role in physiological and pathological fibroblast function in the skin (Guo et al., 2012). Targeting the non-canonical pathways may thus represent a new and promising field in the development of novel therapeutics for the fibrotic disease treatment.

The involvement of non-canonical, TAK1-mediated signaling in TGFβ1 driven, pro-fibrotic or hyper-proliferative responses in gingival fibroblasts has not been previously examined. Whether TAK1 mediates TGFβ1-driven fibro-proliferative responses in gingival fibroblasts is also unknown. In addition, the role of TAK1-mediated

CCN2 expression in gingival fibroblasts has not been previously investigated, although the known involvement of CCN2 signaling in fibrotic conditions including gingival hyperplasia render CCN2 as a promising target for future anti-fibrotic therapies (Black and Trackman, 2008; Blom et al., 2002). Therefore, my overall objective was to use the TAK1 selective inhibitor (5*Z*)-7-Oxozeaenol to assess the role of TAK1 in human gingival fibroblast responses to TGFβ1. My hypothesis was that TAK1 mediates the ability of TGFβ1 to a) induce CCN2 expression and b) induce transcriptional responses in gingival fibroblasts. My thesis described data supporting the hypothesis that targeting the non-canonical TAK-mediated TGFβ1 signaling pathway may be useful to combat gingival hyperplasia in the future.

2. Materials and Methods

2.1 Human gingival fibroblast culture

Gingival fibroblasts from one healthy human donor were used for this study and are described elsewhere (Thompson et al., 2010). Cells were grown as monolayers in high glucose DMEM growth media (Gibco) supplemented with 10% FBS (Gibco) and 1% Antibiotic-Antimycotic mix (Gibco) at 37°C, 5% CO_2 (SANYO Scientific laboratory incubator). At 40 -60% confluence, cultures were serum starved (low glucose DMEM growth media supplemented with 0.5% FBS and 1% Antibiotic-Antimycotic antibiotic mix) overnight and pre-treated with DMSO (vehicle control; Sigma-Aldrich) or 400 nM (5*Z*)-7-Oxozeaenol (Tocris) for 45 minutes followed by treatment with or without recombinant human TGFβ1 (4 ng/ml; 90 pM; R&D systems) for 6, 24, 48 and 72 hours as indicated (Guo et al., 2012). Cultures of passages 8 through 10 were utilized for this study.

2.2 RNA extraction

Cell monolayers were subjected to the total RNA extraction adapting the Trizol method as described previously (Ambion, Life-Technologies; Chomczynski and Sacchi, 1987). Briefly, cell monolayers were lysed in TRIzol® (Ambion, Life Technologies) followed by the addition of 0.2 volumes chloroform (Sigma-Aldrich). The samples were centrifuged to allow for phase separation and ~70% of the supernatant was collected. Isopropanol (Sigma-Aldrich) was added in a 1:1 isopropanol to supernatant fraction volume ratio respectively and the samples were centrifuged to pellet the precipitated RNA. The latter was washed with 80% ethanol (GreenField Ethanol Inc), pelleted, air-dried and re-dissolved in nuclease-free water (Thermo Fisher Scientific). The concentration and integrity of the extracted RNA sample was measured using the Nucleic

Acid, Nanodrop program (Thermo) and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) and RNA 6000 Nano kit (Caliper Life Sciences). The latter procedure was conducted at the London Regional Genomics Centre (David Carter, Robarts Research Institute, London, Ontario, Canada).

2.3 RT-qPCR analysis

Extracted RNA samples (50 ng) were reversed transcribed and amplified using TaqMan Human gene Expression assays (Applied Biosystems) in a 15-µL reaction containing qScript™ XLT One-Step RT-qPCR ToughMix (Quanta Biosciences), TaqMan Human gene specific target primers (Applied Biosystems) and FAM (6-carboxyfluroscein labeled) gene specific TaqMan MGB probes (Assays on demand; Applied Biosystems) as per previously established protocols (Thompson et al., 2010). ViiA™ 7 Real-Time PCR System and ViiA™ 7 Software were used for detection and analysis of the amplified signal according to manufacturer's instructions (Applied Biosystems). Relative gene expression was obtained for *CCN2, IL11, PDGFA, EDN1, JUNB, IGFBP3* and *SERPINE1* genes and standardized to the 18S expression as the internal control using the ΔΔCt method (Livak et al., 2001). Triplicate samples were examined and the experiments were repeated on three independent occasions with averages +/- SEM (N=3) calculated. Two-Way ANOVA and Tukey's Post Hoc analysis were used for statistical analysis (GraphPad Prism software).

2.4 Genome-wide expression profiling

All sample labeling and GeneChip processing was conducted by the London Regional Genomics Centre (David Carter, Robarts Research Institute, London, Ontario,

Canada) for two independent HGF treatment sets (DMSO, TGFβ1, (5Z)-7-Oxo as well as TGFβ1 + (5Z)-7-Oxo treated; Passages 8 and 9)**.** Briefly, single stranded complementary DNA (sscDNA) was prepared from 200 ng of total RNA as per the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays and the Affymetrix GeneChip WT Terminal Labeling kit and Hybridization User Manual. Total RNA was first converted to cDNA, followed by *in vitro* transcription to make cRNA. Five and a half micrograms of single stranded cDNA was synthesized, end labeled and hybridized, for 16 hours at 45°C, to Human Gene 1.0 ST arrays (Affymetrix). All liquid handling steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G using Command Console v1.1 (Affymetrix). Probe level (.CEL file) data was generated using Affymetrix Command Console v1.1. Probes were summarized to gene level data in Partek Genomics Suite v6.6 (Partek) using the RMA algorithm (Irizarry et al., 2003). Partek was used to determine gene level ANOVA p-values and fold changes. Gene lists were created using a filter of 1.7 fold change and p-value of < 0.05. Genes showing 1.7 fold induction and 1.7 fold down regulation in the TGFβ1 + (5*Z*)-7-Oxozeaenol group as compared to the (5*Z*)-7-Oxozeaenol group alone were subtracted from the pool of TGFβ1 (1.7 fold) induced genes as compared to the DMSO control group. This resulting pool of (5*Z*)-7-Oxozeaenol-dependent mRNAs with over 1.7 fold induction in response to TGFβ-1 treatment was subject to Functional Annotation clustering (DAVID Functional Annotation Software). The 1.7 fold cut-off value was selected based on the previous analysis of TGFβ1 treated human dermal fibroblasts carried out in a separate study in our lab (Guo et al., 2012).

2.5 Western Blot analysis

Cells were serum starved overnight, pre-treated with (5*Z*)-7-Oxozeaenol or DMSO followed by the treatment with or without recombinant human TGFβ1 as described previously. Cell monolayers were lysed 24 hours later in RIPA buffer (100 mM Tris HCl, pH 7.4; 150 mM NaCl; 1% NP40; 0.1% SDS; 5 mM EDTA) supplemented with protease inhibitor mini complete tablet (Roche) and sonicated twice for 5 sec with concomitant 10 min incubation periods on ice. Samples were then centrifuged at 13,700 x g for 12 min at 4°C and the supernatant fraction collected. Total protein concentration of the lysates was established using the BCA assay microplate procedure (Pierce) based on the standard curve absorption values and the total protein content was equalized to 50 µg among all samples with Laemmli (63 mM Tris-Cl, pH 6.8; 10% glycerol; 5% β-mercaptoethanol, 2% SDS, 0.01% bromophenol blue) and RIPA Lysis buffers as per manufacturer's instructions (Pierce). The lysates were then incubated at 99°C for 5 min and loaded onto the 10% SDS gel matrix subjected to 130 V for ~2 hours in the presence of 1X Running buffer (5 mM Tris; 40 mM Glycine; 0.02% SDS) required for protein separation. The 10% SDS gel was transferred onto nitrocellulose membrane using iBlot dry transfer kit and iBlot dry transfer machine (Invitrogen). The membranes were incubated for 1 hour in Blocking buffer solution (50 mM Tris, 150 mM NaCl; 0.05% Tween®-20; 5% skim milk) followed by the addition of CCN2 (L-20) goat polyclonal primary antibody (Santa Cruz Biotechnology; sc-14939) in a 1:250 dilution and subsequent overnight incubation at 4°C. The following day, membranes were washed three times in TBST buffer (50 mM Tris, 150 mM NaCl; 0.05% Tween®-20) at room temperature for 10 min. Specific anti-goat secondary HRP conjugate polyclonal antibody

(Jackson Immunoresearch, Cat. 705-036-147) was added to the membrane in a 1:2000 dilution in the blocking buffer and incubated for one hour at room temperature. Washing procedure was repeated and membranes imaged on X-ray film (Kodak) with the use of Chemiluminescent Substance (1:1 Lumino/Enhancer solution to Stable Peroxide Solution ratio; Thermo Scientific; Department of Dentistry, University of Western Ontario). Membranes were then washed in TBST and stripped with the Restore western blot stripping buffered (Thermo) for 20 min at room temperature, blocked and re-probed with β-actin mouse monoclonal primary antibody (Sigma, A1978-200UL; 1:8000 dilution) overnight at 4°C in TBST. Appropriate anti-mouse HRP conjugated antibody was chosen (Jackson Immunoresearch; 1:8000 dilution) to obtain β-actin signal intensity at the 1.5 sec exposure time period. Densitometry measures for CCN2 band intensities for TGFβ1 and TGFβ1 + (5*Z*)-7-Oxozeaenol treatment groups were obtained using ImageJ program (NIH) and standardized to the respective β-actin levels. Student's t-test was used for statistical analysis (GraphPad Prism software).

2.6 Indirect immunofluorescence procedure

Cells cultured on glass coverslips (VWR) were fixed in 4% paraformaldehyde in PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl; pH 7.4), permeabilized with 0.2% TritonX100 (Sigma-Aldrich) in PBS and blocked with 5% Donkey serum in PBST (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl ,0.05% Tween® 20; pH 7.4) for 30 min at room temperature followed by the addition of CCN2 (L-20) goat polyclonal antibody (Santa Cruz Biotechnology; sc-14939) in 1:100 dilution. Following one hour incubation at room temperature, the samples were washed three times with PBS for 5 min and incubated with a DyLight 594 anti-goat IgG secondary antibody in a 1:1000 dilution

in 5% Donkey serum in PBST for 45 min at room temperature in the dark (Jackson ImmunoResearch Laboratories; Cat. 705-516-147). The coverslips were washed with PBS (three times for 5 min) and mounted on the slides using VECTASHIELD® Mounting Media (Vector Laboratories). Images were taken with a Zeiss Axiophot microscope using Northern Eclipse software. Total fluorescence intensity was obtained using the Northern Eclipse software for each individual image and divided by the representative cell number obtained by using DAPI stain as a guide for the number of cells captured in each individual image. Relative fluorescence intensity ratio was further obtained using DMSO treatment group as a standard. Two-Way ANOVA and Tukey's Post Hoc analysis were used for statistical analysis (GraphPad Prism software).

2.7 Proliferation Assay

For the cell proliferation assay, cells were seeded in 96-well plates (Greiner Bio-One) at 500 cells per well and cultured for one day in 10% FBS, high glucose DMEM media as described above. A “no-cell control” group was also incorporated involving only the addition of cell culture media. Cultures were then serum starved (0.5% FBS, low glucose DMEM) overnight and were pre-treated with DMSO (vehicle control) or 400 nM (5*Z*)-7-Oxozeaenol for 45 minutes followed by subsequent treatment with or without recombinant human TGFβ1 (4 ng/m; 90 pM) in quadruplicates. BrdU reagent (1X; Cell Signalling) was also added to all treatment groups at this time. The latter incorporates into the newly synthesised DNA. Cultures were incubated for zero, 24, 48 and 72 hours and subject to the BrdU proliferation assay using a kit as per manufacturer’s instructions (Cell Signalling). Briefly, cells were fixed and DNA denatured using a fixing/denaturing solution. Primary BrdU mouse mAb was added to bind the incorporated BrdU followed

by the addition of an anti-mouse IgG, HRP-linked antibody to bind the primary antibody. HRP substrate TMB was added to develop color and the plate incubated for 30 min followed by the addition of Stop solution. Proliferation was then determined from the absorbance values reading obtained at 450 nm (iMark™ microplate absorbance reader) and this corresponds to the quantity of BrdU incorporated into the cells. The raw data was processed for the detection and removal of outliers using the Interquartile Range calculation and was further noise adjusted by subtracting the average "no-cell control" reading from each individual well. Experiments were performed thrice.

2.8 Statistical Analysis

Student's t-test, two-way ANOVA and Tukey's Post Hoc analysis were used for statistical analysis (GraphPad Prism software) where applicable.

3. Results

3.1 TAK1 mediates TGFβ1- induced gingival fibroblast CCN2 expression

CCN2 is a matricellular protein of the CCN family implicated in fibro-proliferative responses in fibroblasts (Chen and Lau, 2009; Leask and Abraham, 2006). CCN2 is up-regulated in response to TGFβ1 *via* canonical and non-canonical signaling pathways and acts to enhance TGFβ1-mediated fibroblast signaling responses (Grotendorst et al., 2006; Holmes et al., 2001; Mori et al., 1998; Thompson et al., 2010; Wang et al., 2011). Moreover, CCN2 is required for skin fibrosis *in vivo* (Liu et al., 2011). In dermal fibroblasts, TGFβ1 induces *CCN2* expression *via* TAK1 (Guo et al., 2012). I thus investigated whether TAK1 mediates TGFβ1-induced *CCN2* mRNA and protein expression using Real Time PCR analysis as well as Western blot and indirect immunofluorescence analysis, respectively. At the transcriptional level, TGFβ1 significantly up-regulated *CCN2* expression in gingival fibroblasts resulting in 7- fold increase in *CCN2* mRNA levels similar to previously published results (**Figure 3.1**) (Thompson et al., 2010). Pre-treatment of gingival fibroblasts with (5*Z*)-7-Oxozeaenol, a TAK1 selective inhibitor (Ninomiya-Tsuji et al., 2003) 45 minutes prior to the addition of TGFβ1 resulted in a significant reduction of TGFβ1-induced *CCN2* mRNA expression (**Figure 3.1**). Similar results were seen when protein expression was examined. CCN2 protein levels were not readily detectable in unstimulated gingival fibroblasts using Western blot and indirect immunofluorescence analyses as anticipated from previously published data reporting low baseline *CCN2* expression in gingival fibroblast cells (Thompson et al., 2010) (**Figures 3.2** and **3.3**).

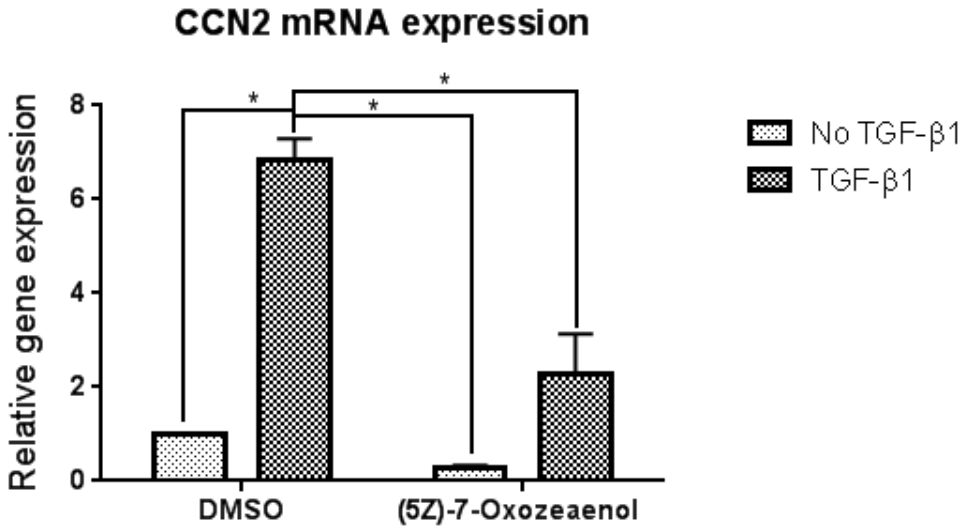


Figure 3.1. TAK1 mediates TGFβ1 induced *CCN2* gene expression. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4 ng/ml) ligand or left untreated. Total RNA was harvested six hours later and subjected to TaqMan RT-qPCR analysis using the indicated probe/primer set. 18S RNA was used as the internal control. Note: the relative gene expression between DMSO and (5Z)-7-oxozeaenol in the absence of TGFβ1 was not statistically significant. (N=3; averages+/-SEM are shown; *$p<0.05$, Two-Way ANOVA followed by Tukey's Post Hoc analysis).

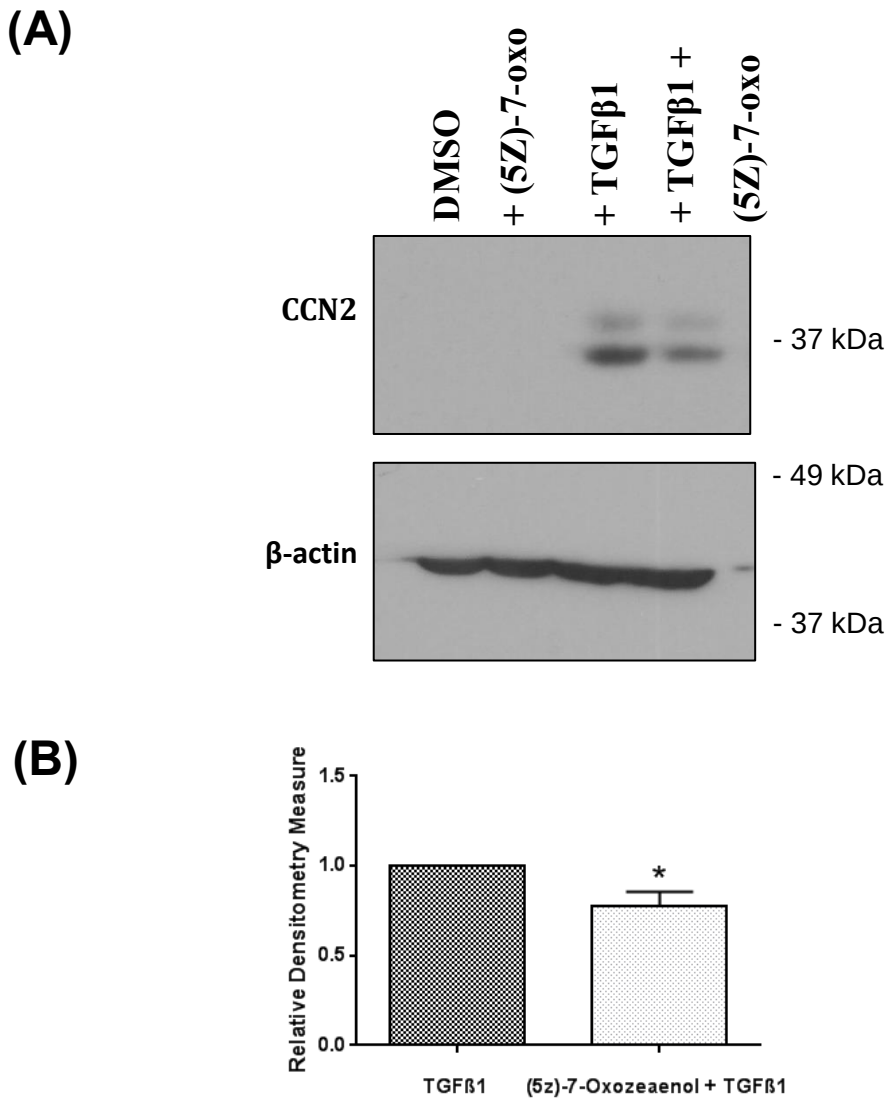


Figure 3.2. TAK1 mediates TGFβ1 induced CCN2 protein expression: Western blot data. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4ng/ml) or left untreated for 24 hours. Proteins were harvested and subject to Western blot analysis with anti-CCN2 and anti-β-actin antibodies, as indicated. Experiments were performed on 4 separate occasions. (A) Representative blot is shown. (B) Relative fold decrease in CCN2 expression in response to (5Z)-7-oxozeaenol pre-treatment was calculated using densitometry for TGFβ1 induced groups (N=4, averages +/ SEM are shown; * $p<0.05$; Student's t-test)

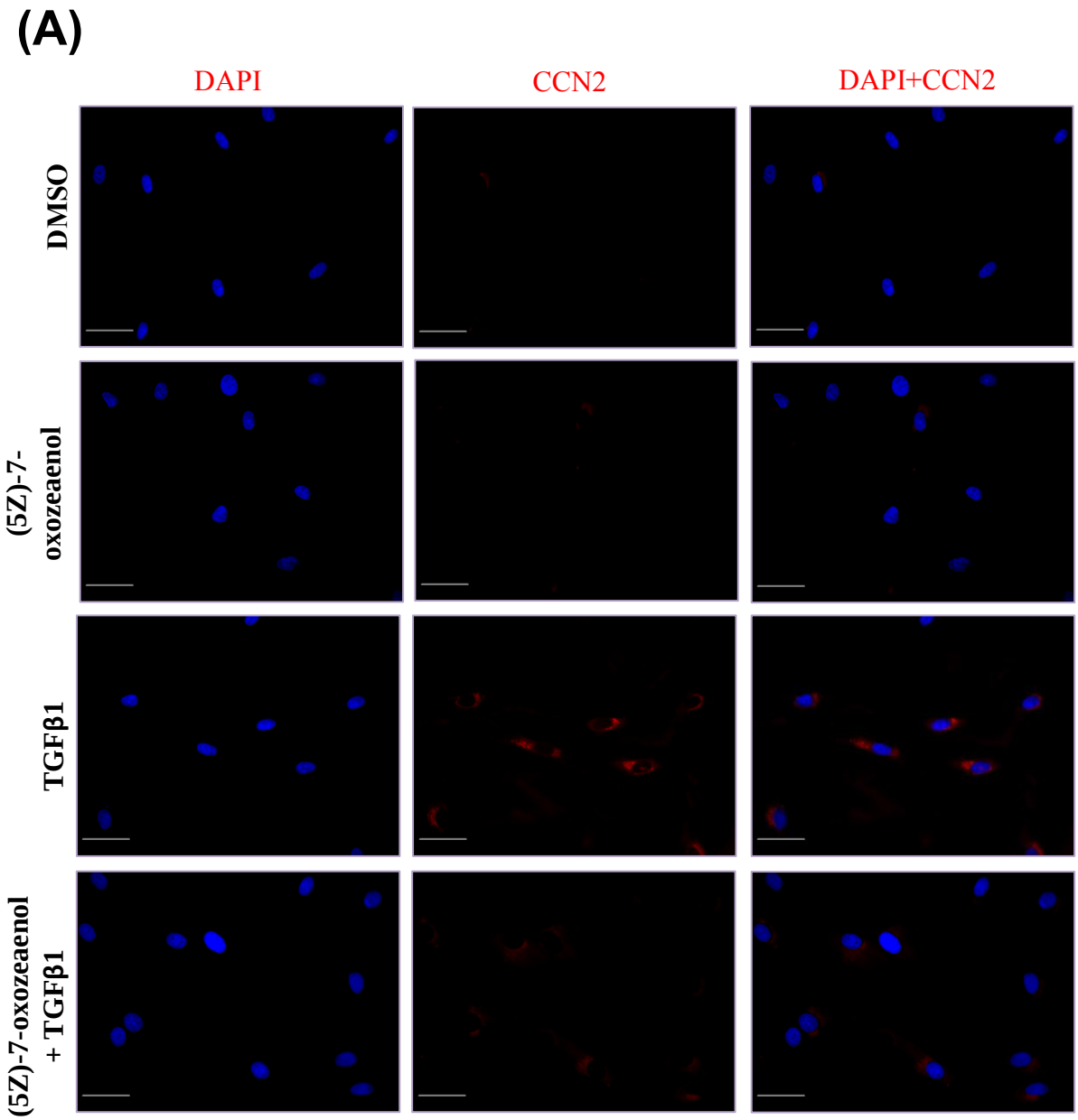

(A)
DAPI
CCN2
DAPI+CCN2
DMSO
(5Z)-7-oxozeaenol
TGFβ1
(5Z)-7-oxozeaenol + TGFβ1
(B)
Relative Fluorescence Intensity
10
8
6
4
2
0
*
*
*
DMSO
(5Z)-7-Oxozeaenol
no TGFß1
TGFß1

Figure 3.3. TAK1 mediates TGFβ1-induced CCN2 protein expression: indirect immunofluorescence analysis. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment for 24 hours with or without TGFβ1 (4 n/gml). Cells were fixed and stained with an anti-CCN2 antibody and DyLight 594 conjugated secondary antibody. Cells were counterstained with DAPI to detect nuclei. Experiments were conducted four times. (A) Representative micrographs are shown. White bar represents 50 μm. (B) Total fluorescence intensity was obtained using the Northern Eclipse software for each individual image and divided by the representative cell number obtained by using DAPI stain as a guide for the number of cells captured in each individual image. Relative fluorescence intensity ratios were subsequently calculated using DMSO as a standard (N=4, averages +/ SEM are shown; * $p<0.05$, Two-Way ANOVA followed by Tukey's Post Hoc analysis).

Induction of cells with TGFβ1 increased CCN2 protein expression in both Western blot and immunofluorescence microscopy assays while the pre-treatment with (5*Z*)-7-Oxozeaenol resulted in a significant reduction of TGFβ1-induced CCN2 protein expression paralleling the results obtained by examining *CCN2* mRNA expression (**Figures 3.2** and **3.3**). Indirect immunofluorescence analysis further revealed that induction of gingival fibroblasts cells with TGFβ1 resulted in the accumulation of CCN2 in the cytoplasm, possibly Golgi apparatus as was previously reported for dermal fibroblasts (Chen et al., 2001); this accumulation was reduced in the presence of (5*Z*)-7-Oxozeaenol (**Figure 3.3**). Given that CCN2 expression is a potent marker of TGFβ's fibrogenic actions (Leask and Abraham, 2006) and that TGFβ1-induced CCN2 expression in a TAK1-dependent manner, I thought that TAK1 may participate in mediating TGFβ1 fibrogenic responses in gingival fibroblasts.

3.2 Inhibition of TAK1 blocks TGFβ1-induced mRNA expression in human gingival fibroblasts

In order to further investigate a role of TAK1 in TGFβ1 signaling in gingival fibroblasts, cells were treated with or without TGFβ1 in the presence or absence of (5*Z*)-7-Oxozeaenol inhibitor and subjected to gene expression profiling using GeneChip Human Gene 1.0 ST arrays. The use of gene expression profiling at the beginning of my study allowed me to (i) assess the extent of (5*Z*)-7- Oxozeaenol effect on the ability of TGFβ1 to induce genome-wide transcription and (ii) to identify functional categories of genes affected by TAK1 inhibition. Of the 28,869 genes provided on the Human GeneChip Gene 1.0 ST Arrays, 147 genes were up-regulated in response to TGFβ1 (1.7 fold induction compared to DMSO control group), 139 of which were found to be

sensitive to (5*Z*)-7-Oxozeaenol inhibition (**Figure 3.4**). These results suggested that TAK1 mediates the majority (95%) of TGFβ1 signaling responses in gingival fibroblast cells (Ninomiya-Tsuji et al., 2003). Functional annotation of the 139 genes included clusters for cell cycle/proliferation as well as wound healing (including migratory) responses which became the focus of my subsequent analysis (**Tables 3.1** and **3.2** respectively).

3.3 TAK1 mediates the induction of genes involved with proliferation

Based on the expression profiling data, a proliferation cluster consisting of eight genes was identified (**Table 3.1**). *Endothelin 1* and the *Jun-B* proto-oncogene were chosen as representative genes from this cluster based on their known involvement in scleroderma disease and pro-fibrotic programming in response to TGFβ respectively (Chung et al., 1996; Gervasi et al., 2012; Leask, 2008; Mauviel et al., 1993, Morelli et al, 1995; Vancheeswaran et al., 1994; Yamane et al, 1992; 1996). Endothelin 1 *(END1)* is a secreted protein and a downstream target of TGFβ1 with increased levels observed in patients with fibrosis (Abraham et al., 1997; Kawaguchi et al., 1994; Leask, 2008; Miyauchi et al., 1992; Morelli et al, 1995; Shi-Wen et al., 2006; Vancheeswaran et al., 1994; Yamane et al, 1992) while transcription factor Jun-B (*JUNB*) is induced by TGFβ1 signaling in fibroblasts, mesenchymal and epithelial cells and is an important positive mediator of epithelial mesenchymal transition (Chung et al., 1996; Gervasi et al., 2012; Mauviel et al., 1993, 1996). To confirm the microarray data and to establish whether the treatment of gingival fibroblasts with TAK1 inhibitor may hinder TGFβ1- induced proliferation at the transcriptional level, I cultured gingival fibroblasts in the presence or

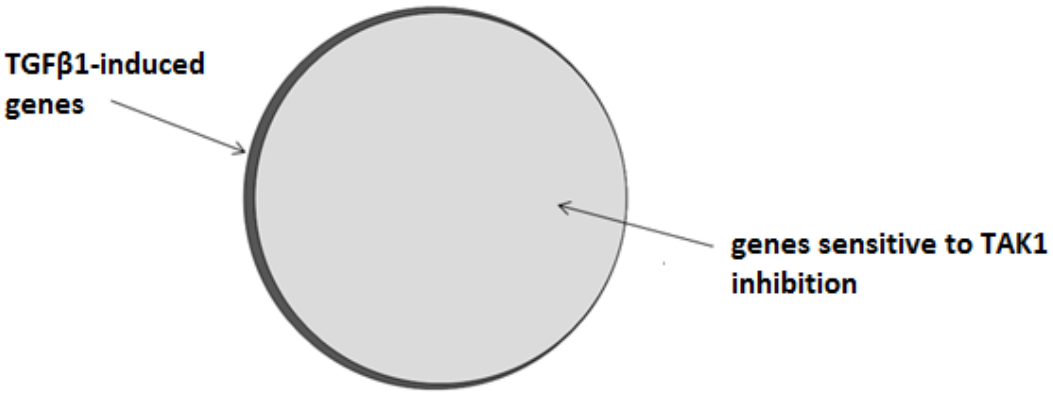


Figure 3.4. (5Z)-7-oxozeaenol inhibits TGFβ1-induced mRNA expression in human gingival fibroblasts. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4 ng/ml) ligand or left untreated. Total RNA was harvested six hours later and subjected to gene expression profiling using GeneChip Human Gene 1.0 ST arrays (N=2). 147 genes were up-regulated in response to TGFβ1 (1.7 fold induction compared to DMSO control group) and 139 genes of the latter group were found to be sensitive to (5*Z*)-7-Oxozeaenol inhibition.

Table 3.1. Cluster analysis of mRNAs sensitive to TAK1 inhibition with over 1.7 fold induction in response to TGFβ-1 treatment. (N=2; DAVID Functional Annotation Software). Cell cycle/Proliferation cluster

Affymetrix ID	GENBANK ACCESSION	Gene name		Fold Increase
8116921	AK291838	endothelin 1	*EDN1*	6.7818
8024485	AF078077	growth arrest and DNA-damage-inducible, beta	*GADD45B*	2.39958
8139207	AK290584	inhibin, beta A	*INHBA*	2.04306
8026047	BC004250	jun B proto-oncogene	*JUNB*	1.87672
7922976	AK292167	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	*PTGS2*	1.92186
8147012	BC022265	protein kinase (cAMP-dependent, catalytic) inhibitor alpha	*PKIA*	2.41012
8040473	AF498971	ras homolog gene family, member B	*RHOB*	1.88318
8010061	AF200328	sphingosine kinase 1	*SPHK1*	1.72518

Table 3.2. Cluster analysis of mRNAs sensitive to TAK1 inhibition with over 1.7 fold induction in response to TGFβ-1 treatment. (N=2; DAVID Functional Annotation Software).

(A) Wound Healing cluster

Affymetrix ID	GENBANK ACCESSION	Gene name		Fold Increase
7950933	AB041035	NADPH oxidase 4	*NOX4*	8.49418
8160637	AK297541	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	*B4GALT1*	2.12002
8072678	BC001491	heme oxygenase (decycling) 1	*HMOX1*	2.05866
8114572	BC033097	heparin-binding EGF-like growth factor	*HBEGF*	4.0607
8056184	AK290300	integrin, beta 6	*ITGB6*	2.41065
8039484	AK290572	interleukin 11	*IL11*	1.84543
8137670	AK292217	platelet-derived growth factor alpha polypeptide	*PDGFA*	2.07886
8135069	AK293248	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	*SERPINE1*	2.17387

(B) Cell motion, migration and adhesion cluster

Affymetrix ID	GENBANK ACCESSION	Gene name		Fold Increase
8023220	AF010193	SMAD family member 7	*SMAD7*	2.841
8160637	AK297541	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	*B4GALT1*	2.12002
8116921	AK291838	endothelin 1	*EDN1*	6.7818
8072678	BC001491	heme oxygenase (decycling) 1	*HMOX1*	2.05866
8114572	BC033097	heparin-binding EGF-like growth factor	*HBEGF*	4.0607
8139488	AK298143	insulin-like growth factor binding protein 3	*IGFBP3*	1.88748
8137670	AK292217	platelet-derived growth factor alpha polypeptide	*PDGFA*	2.07886
8010061	AF200328	sphingosine kinase 1	*SPHK1*	1.72518
8148304	AF205437	tribbles homolog 1 (Drosophila)	*TRIB1*	2.96456
7962579	AY454159	adhesion molecule with Ig-like domain 2	*AMIGO2*	3.45511
8022674	BC036470	cadherin 2, type 1, N-cadherin (neuronal)	*CDH2*	2.04821
8035517	AK074508	cartilage oligomeric matrix protein	*COMP*	3.02008
8121685	AJ420528	discoidin, CUB and LCCL domain containing 1	*DCBLD1*	1.85226
8056184	AK290300	integrin, beta 6	*ITGB6*	2.41065
8102232	AF198532	lymphoid enhancer-binding factor 1	*LEF1*	1.90975
8123936	AK292682	neural precursor cell expressed, developmentally down-regulated	*NEDD9*	2.93624
8047738	AL833606	neuropilin 2	*NRP2*	1.73487
8040473	AF498971	ras homolog gene family, member B	*RHOB*	1.88318

absence of (5*Z*)-7-Oxozeaenol and with and without TGFβ1 treatment. Six hours later RNAs were extracted and subjected to RT-qPCR analysis. Confirming the gene array data, both *EDN1* and *JUNB* mRNA were found to be significantly induced by TGFβ1 ligand and application of the TAK1 selective inhibitor (5*Z*)-7-Oxozeaenol inhibited this response (**Figure 3.5**). Indeed, in the presence of TGFβ1 and (5*Z*)-7-Oxozeaenol, *EDN1* and *JUNB* mRNA expression were reduced to basal levels seen in DMSO treated fibroblasts. Addition of (5*Z*)-7-Oxozeaenol did not have a significant effect on *EDN1* and *JUNB* mRNA expression (**Figure 3.5**).

3.4 TAK1 inhibition blocks TGFβ1-induced proliferation in gingival fibroblasts.

To further elucidate gingival fibroblast proliferation at the functional level, I cultured gingival fibroblasts under the four treatment conditions described previously in the presence of BrdU, a synthetic thymidine analogue which is incorporated into the newly-synthesized DNA (**Figure 3.6**). Presence of incorporated BrdU was assessed quantitatively by absorbance readings at zero, 24, 48 and 72 hours. Addition of TGFβ1 significantly increased human gingival fibroblast proliferation at 48 and 72 hours as compared to DMSO treated cells. Addition of (5*Z*)-7-Oxozeaenol resulted in a significant reduction in cell proliferation responses measured at 72 hours to the levels below the DMSO control group in the presence of added TGFβ1 (**Figure 3.6**). Consistent with the gene cluster analysis, TAK1 inhibition blocked the ability of TGFβ1 to induce proliferation in human gingival fibroblasts.

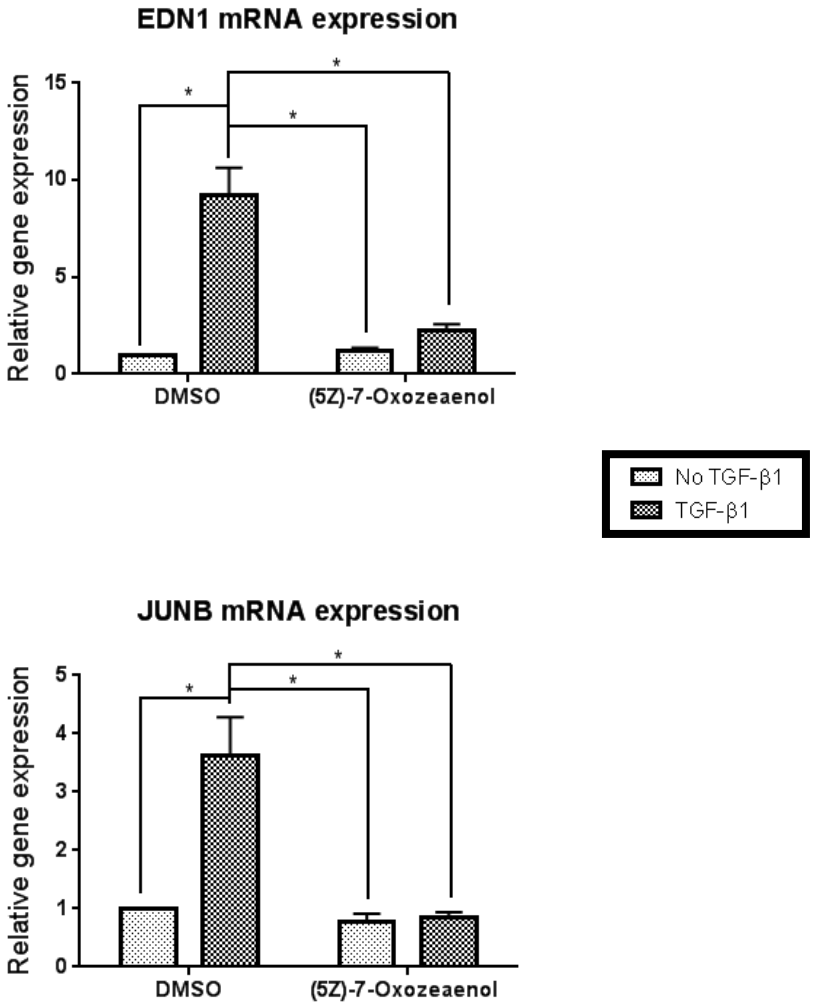


Figure 3.5. Inhibition of TAK1 reduces TGFβ1-induced *Endothelin 1* and *Jun B* mRNA expression. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4 ng/ml) ligand or left untreated. Total RNA was harvested six hours later and subjected to TaqMan RT-qPCR analysis using the indicated probe/primer sets. 18S RNA was used as the internal control. Note: the relative gene expression between DMSO and (5Z)-7-oxozeaenol in the absence of TGFβ1 was not statistically significant. (N=3; averages+/-SEM are shown. * $p<0.05$; Two-Way ANOVA followed by Tukey's Post Hoc analysis).

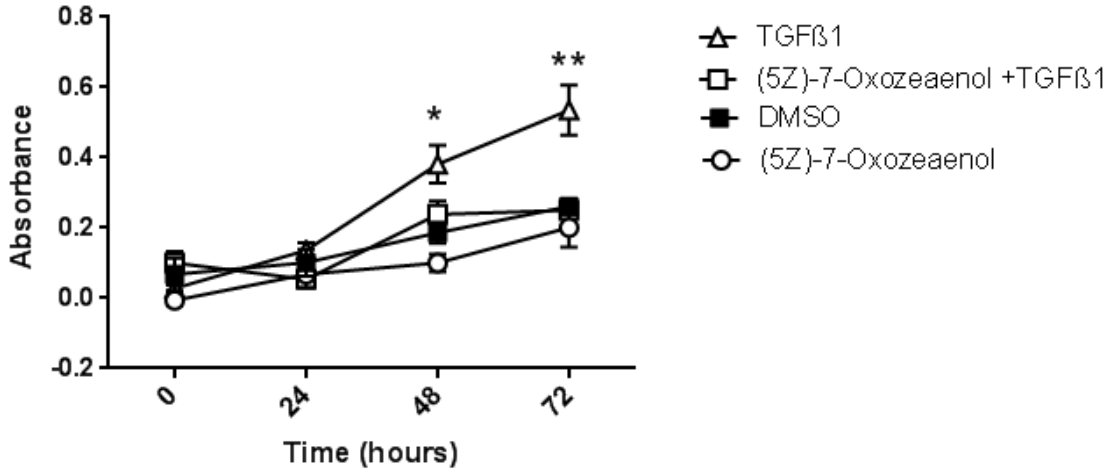


Figure 3.6. TAK1 mediates TGFβ1 induced gingival fibroblast proliferation. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol ((5Z)-7-oxo; 400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4 ng/ml) ligand or left untreated. Cultures were grown in the presence of BrdU for up to 72 hours as described in the methods. One of three representative experiments is shown; (N=4; averages+/-SEM are shown * $p<0.05$ for: DMSO *vs* TGFβ1; (5Z)-7-oxo *vs* TGFβ1, TGFβ1 *vs* (5Z)-7-oxo+TGFβ1; ** $p<0.05$ for: DMSO *vs* TGFβ1; (5Z)-7-oxo *vs* TGFβ1, TGFβ1 *vs* (5Z)-7-oxo+TGFβ1. Two-Way ANOVA followed by Tukey's Post Hoc analysis.

3.5 TAK1 mediates TGFβ1- induced expression of wound healing cluster mRNAs in gingival fibroblasts

Based on the expression profiling data, a wound healing cluster of eight genes as well as a migration cluster consisting of eighteen genes were also identified (**Table 3.2**). I decided to use RT-qPCR analysis to validate these observations and to further investigate the role of TAK1 inhibition in the fibrotic component of the TGFβ1-dependent fibroproliferative responses of fibroblast cells. Based on their known involvement with fibrogenic responses, insulin-like growth factor binding protein 3 (*IGFBP3*) was chosen from the migration/adhesion cluster group and platelet-derived growth factor-α-polypeptide (*PDGFA*), *Interleukin-11* and *SERPINE-1* genes were chosen from the wound healing cluster group for the subsequent RT-qPCR analysis (**Figure 3.7**). *IL11* was previously implicated as an anti-apoptotic mediator in lung fibroblasts derived from Idiopathic Pulmonary Fibrosis patients and increased murine pulmonary myofibroblast counts upon *IL11* overexpression were also reported (Moodley et al., 2003). *PDGFA* is a secreted protein and mitogen belonging to the Platelet-derived growth factor family found to be overexpressed in a number of fibrotic conditions including pulmonary fibrosis and scleroderma at the level of fibroblasts/myofibroblasts (Trojanowska 2008). *IGFBP3* is another secreted protein associated with increased ECM production and ECM binding in a TGFβ-dependent manner. Increased expression of *IGFBP3* was observed in dermal fibroblasts from scleroderma patients and lung fibroblasts from idiopathic pulmonary fibrosis patients (Pilewski et al., 2005). Finally, elevated levels of *SERPINE1*, a pro-fibrogenic factor, were observed in the dermal fibroblasts isolated from the lesions of scleroderma patients (Dong et al., 2002; Ghosh

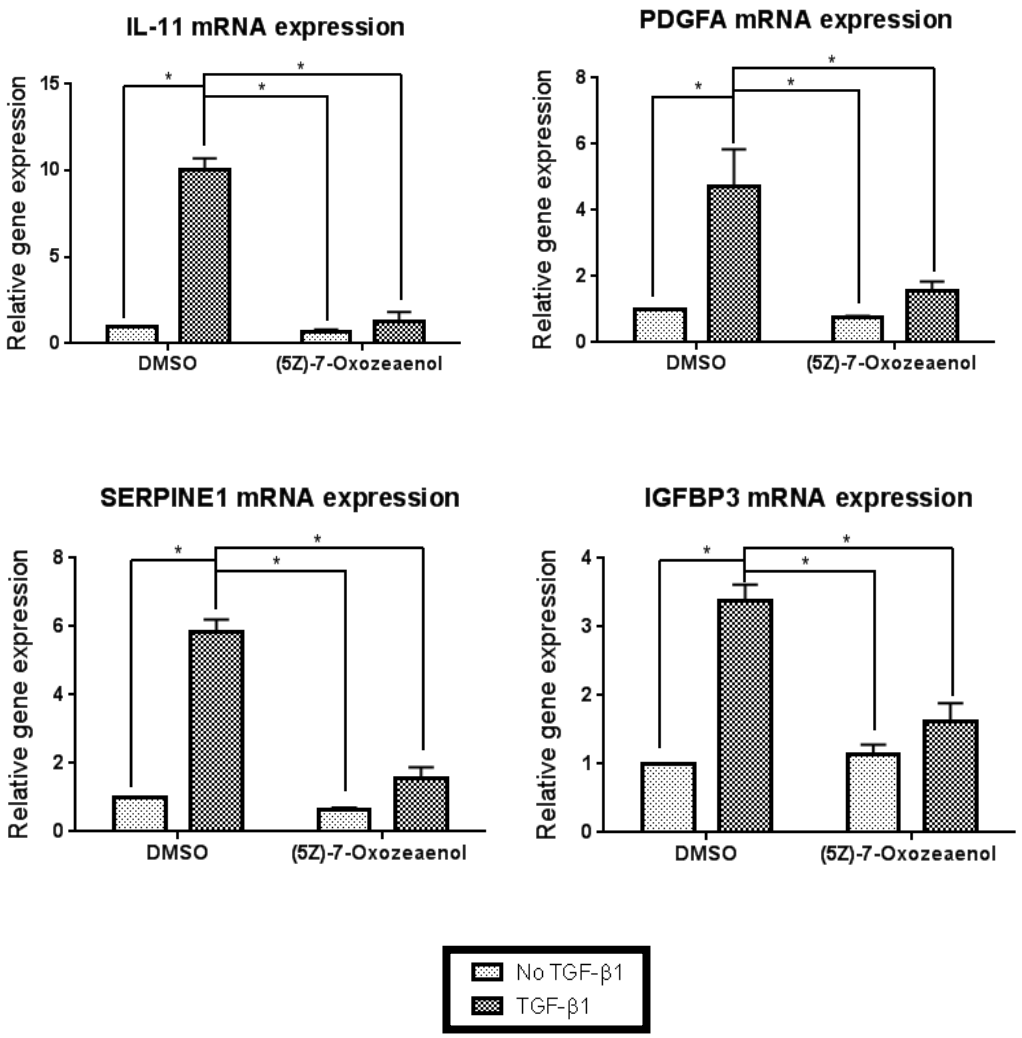


Figure 3.7. Inhibition of TAK1 reduces the expression of TGFβ1 induced wound healing genes. Human gingival fibroblasts were serum starved overnight and pre-treated with (5Z)-7-oxozeaenol (400 nM) or DMSO for 45 min followed by treatment with TGFβ1 (4 ng/ml) ligand or left untreated. Total RNA was harvested six hours later and subjected to TaqMan RT-qPCR analysis using the indicated probe/primer sets. 18S RNA was used as the internal control. Note: the relative gene expression between DMSO and (5Z)-7-oxozeaenol in the absence of TGFβ1 was not statistically significant. (N=3; averages+/-SEM are shown. * p <0.05, Two-Way ANOVA followed by Tukey's Post Hoc analysis).

and Vaughan, 2012). RT-qPCR analysis confirmed the gene array data, showing that *IL11*, *PDGFA*, *IGFBP3* and *SERPINE1* mRNAs were significantly induced by TGFβ1 in a fashion that was sensitive to TAK1 inhibition (**Figure 3.7**). Baseline expression of all of the chosen genes was not significantly affected with the addition of (5*Z*)-7-Oxozeaenol.

Collectively, these data suggest that TAK1 mediates TGFβ1 responses in gingival fibroblasts including proliferation, wound healing as well as CCN2 expression.

4. Discussion

4.1 Summary of results

Although previous studies have shown that the non-canonical, TAK1-mediated signaling pathway plays a role in dermal fibroblasts and in physiological wound healing in the skin, the involvement of non-canonical TAK1 signaling in TGFβ1 driven, pro-fibrotic or hyper-proliferative responses in gingival fibroblasts has not been previously examined (Guo et al., 2012). I thus thought that, as in dermal fibroblasts, TAK1 may mediate wound healing and proliferative responses in gingival fibroblasts in response to TGFβ1. Throughout my studies I have used a TAK1 selective inhibitor (5Z)-7-Oxozeanol to test this hypothesis in the context of TGFβ1 signaling in gingival fibroblasts using concentrations and treatment conditions previously established for fibroblasts in culture (Guo et al., 2012; Renzoni et al., 2004). As an initial screen, I asked if (5Z)-7-Oxozeanol could block TGFβ1- induced CCN2 expression, given that CCN2 is a known marker and mediator of fibrosis (Brigstock 2010; Chen and Lau, 2009; Leask, 2008, 2009; 2011; 2013a, 2013b, Thompson et al., 2010). Indeed, (5Z)-7-Oxozeanol significantly reduced TGFβ1-induced CCN2 expression at both transcriptional and translational levels as evidenced by the RT-qPCR and indirect immunofluorescence microscopy/Western blot respectively (**Figures 3.1**, **3.2** and **3.3**). Genome wide expression profiling allowed me to further identify functional clusters of genes grouped based on their involvement in a given functional outcome. I have identified a number of TGFβ1-induced genes sensitive to TAK1 inhibition from the proliferative and wound healing (including migratory) clusters (**Tables 3.1** and **3.2**, respectively). Moreover, the majority of the genes induced with TGFβ1 were in fact sensitive to TAK1 inhibition, suggesting that the non-canonical TAK1 pathway is of critical importance in the gingival

fibroblasts in response to TGFβ1. I have also confirmed this pattern of regulation for a number of representative genes from each functional cluster at the transcriptional level using RT-qPCR (**Figures 3.5** and **3.7**). Of interest also was the observation that unlike skin wounds, gingival wounds heal faster and without scarring, possibly due to the fact that unlike fibroblasts from the dermis layer of the skin, gingival fibroblasts are also less able to differentiate into α-SMA-expressing myofibroblasts *in vitro* and *in vivo:* gingival hyperplasia overgrowths are not populated by myofibroblast and with respect to fibrotic conditions, such as gingival hyperplasia, a hyperproliferative phenotype is observed (Egusa et al., 2010; Guo et al., 2011a, 2011b; Schor et al., 1996, Wikesjo et al., 1992). I thus became interested whether the TGFβ1-induced proliferation in gingival fibroblasts may be mediated by TAK1 and whether TAK1 inhibition using an exogenously administered inhibitor could in theory be applied to alleviate the fibro-proliferative responses in gingival hyperplasia in the future. Indeed, increased proliferative responses of gingival fibroblasts upon TGFβ1 treatment were reduced to baseline levels with the application of TAK1 selective inhibitor (5Z)-7-Oxozeanol (**Figure 3.6**). Collectively, my work suggests that TAK1 mediates proliferation and wound healing responses to TGFβ1 in gingival fibroblasts. The results are also consistent with my long-term hypothesis that TAK1 inhibition might be used to treat gingival hyperplasia and that further investigation of TAK1-mediated TGFβ1 signaling in gingival fibroblast cells may lead to a better understanding of a) the basis of gingival scarless repair and b) the role of the non-canonical, TAK1- mediated TGFβ1 signaling as it pertains to the hyper-proliferative response associated with this fibroblast cell type.

4.2 Research significance

My results are consistent with the overall hypothesis that TAK1 inhibitors such as (5Z)-7-Oxozeanol may be considered in the future for gingival hyperplasia treatment. Unlike other areas of the body which scar in response to injury, gingival wounds heal faster and without scarring, although the stages of the healing process and their sequence remain similar between the two tissue types (Egusa et al., 2010; Guo et al., 2011a, 2011b; Schor et al., 1996, Wikesjo et al., 1992). One of the possible explanations for this differential phenotype is that fibrotic responses in the gingiva are hyper-proliferative as opposed to hypercontractile (Bitu et al., 2006; Damasceno et al., 2012; Sobral et al., 2010). The basis of this differential phenotype could be that gingival fibroblasts differ from dermal fibroblasts in their response to endogenous and/or exogenous stimuli, resulting in gingival overgrowth and hyperplasia (Guo et al., 2011a, 2011b). Gingival hyperplasia commonly arises as a side-effect to a number of drugs including antiepileptic medications, immunosuppressant drugs and as well as calcium channel blockers (Clocheret et al., 2003; Nakib and Ashrafi, 2011). Prevalence of gingival hyperplasia associated with Diltiazem (calcium channel blocker) reaches 20% while Cyclosporine (immunosuppressant) or Phenytoin (antiepileptic treatment) are associated with 30% and 40-50% prevalence rate respectively (Boltchi et al., 1999; Casetta et al., 1997; Ciancio et al., 1991; Dahlof et al., 1993; Fu et al., 1998; Myers and Newton, 1991; Steinberg 1981). As of 1995, over 20 prescription medications were associated with gingival enlargement in the United States (Rees et al., 1995). Withdrawal from drug therapy or substitution for a different drug serves as the most effective treatment for gingival hyperplasia however gingivectomy (surgical debridement) may be necessary in cases where

withdrawal/substitution are not possible or in all other cases of gingival hyperplasia (including inflammation, idiopathic gingival hyperplasia, gingivitis, genetic predisposition) (Khocht et al., 1997; Marshall et al., 1999; Somacarrera et al., 1997).

TGFβ1, a member of TGFβ superfamily, promotes wound healing and fibrotic responses both *in vitro* and *in vivo* (Leask 2008). Fibroblasts are the cells present in the connective tissue that mediate tissue repair and fibrosis. Fibroblast activation in response to TGFβ1 involves both canonical Smad-dependent and non-canonical Smad-independent pathways (Leask 2006, 2008; Leask and Abraham, 2004; Massague 1998; Rahimi and Leof, 2007). At the level of transcriptome, close to 95% of genes were up-regulated in response to TGFβ1 in gingival fibroblasts were found to be sensitive to TAK1 inhibition. In contrast, only 67% of genes were not induced by TGFβ1 in the presence of (5*Z*)-7-Oxozeaenol in dermal fibroblasts in a study using comparable fold cut-off value (1.7 fold) and treatment conditions (400 nM (5*Z*)-7-Oxozeaenol; 4 ng/ml TGFβ1) (Guo et at., 2012). Thus, compared to dermal fibroblasts, the vast majority of TGFβ1 signaling responses in gingival fibroblasts are mediated *via* the non-canonical TAK1 pathways. In dermal fibroblasts, wound healing and ECM gene clusters were identified for the TGFβ1 induced genes sensitive to TAK1 inhibition (Guo et at., 2012). Similarly, the wound healing cluster under TAK1 regulation was also identified in gingival fibroblasts in the current study, as well as a cluster cell proliferation.

CCN2, a member of the CCN family of matricellular proteins is both a marker and a mediator of fibrosis (Blom et al., 2002; Brigstock 2010; Chen and Lau, 2009; Leask, 2008, 2009; 2011; 2013a, 2013b, Thompson et al., 2010). CCN2 expression is also known to be induced by TGFβ1 in gingival and dermal fibroblasts and expression of

this protein correlates well with tissue fibrosis and fibrotic cells (Brigstock, 2010; Chen and Lau, 2009; Guo et al., 2011a, 2011b; Leask, 2008, 2011). In gingival and dermal fibroblasts, TGFβ1-induced CCN2 expression is known to involve canonical signaling pathways (Guo et al., 2011a; Holmes et al., 2001; Thompson et al., 2010). Previously, mRNA and protein expression of CCN2 in dermal fibroblasts were found to be reduced with the (5Z)-7-Oxozeanol, a selective inhibitor of the non-canonical TAK1 signaling in response to TGFβ1 treatment. Here I show for the first time that (5Z)-7-Oxozeanol suppresses the ability of TGFβ1 to induce *CCN2* mRNA as well as protein expression in gingival fibroblasts. CCN2 expression in response to TGFβ1 may thus be subject (at least in part) to the non-canonical regulation in both dermal and gingival fibroblasts.

Previous studies demonstrated that CCN2 showed increased expression in fibrotic tissues where it promoted fibrosis by activating adhesive signaling (Brigstock, 2010; Chen and Lau, 2009; Leask, 2008, 2011). Adhesive properties of gingival and dermal fibroblasts were also previously assessed using dermal or gingival fibroblast monolayers cultured on collagen type I and III, fibronectin and vitronectin substrates. Dermal fibroblasts were more spread out and displayed increased number of focal adhesions and actin stress fibers as compared to gingival fibroblasts up to 24 hours post adhesion on either collagen type I or fibronectin substrates (Guo et al., 2011a; 2011b). Adhesion *via* FAK is essential for TGFβ1-induced human fetal lung myofibroblast differentiation (Thannickal et al., 2003). FAK is further involved in driving myofibroblast differentiation *via* the non-canonical TGFβ signaling pathway involving FAK-mediated JNK activation *via* MEKK1 (Shi-wen et al., 2009). FAK forms a complex with integrin receptors is and activated upon integrin mediated fibroblast attachment to the substrate (Lagares et al.,

2012; Michael et al., 2009; Shi-wen at al., 2012). Addition of FAK inhibitor or deletion of FAK in fibroblasts decreased the expression of fibrogenic genes and blocked fibrosis *in vivo* (Lagares et al., 2012; Shi-wen et al., 2012). Inhibitors of FAK and integrins (β1) have been proposed as potential therapeutic agents in alleviating the symptoms of scleroderma disease (Shi-wen et al., 2012). Interestingly, the endogenous levels of FAK protein are also reduced in gingival fibroblasts as compared to dermal fibroblasts (Guo et al., 2011a, 2011b) perhaps accounting (at least in part) for the impaired myofibroblast differentiation potential and impaired adhesion kinetics of gingival fibroblasts as compared to dermal fibroblasts. Induction of TAK1 upon TGFβ stimulation was further reduced upon pre-treatment of wild type mouse embryonic fibroblasts with the FAK/Src inhibitor PP2, or with the use of FAK -/- fibroblasts, suggestive of FAK-dependent induction of TAK1 in TGFβ signaling (Shi-wen et al., 2009). As TAK1-dependent TGFβ1 signaling operates in both gingival and dermal fibroblasts, additional signaling pathways must be responsible for the fibro-contractile phenotype observed in fibrotic tissue in other parts of the body. Indeed, Chen and others report TGFβ1-induced contraction of dermal fibroblasts to be syndecan 4 and MEK/ERK dependent; notably, ERK was required for assembly of α-SMA stress fibers and hence ECM contraction (Chen et al., 2005). Alternatively, TGFβ1 signaling may be subject to epigenetic regulation in gingival versus dermal fibroblasts, accounting for scarless repair; indeed, recent results from our laboratory suggest that expression of microRNA MIR218 is altered between gingival and dermal fibroblasts (Guo et al., 2014).

Endothelin-1 was also chosen as a representative gene from the proliferative cluster showing decreased TGFβ1-mediated mRNA expression levels with (5*Z*)-7-

Oxozeaenol pre-treatment. ET-1 belongs to a family of peptides originally recognized for their role as vasoconstrictors and section from vascular endothelial cells (Horstmeyer et al., 2005; Kawaguchi et al., 1994; Shi-wen et al., 2006). Levels of ET-1 expression were found to be increased in a number of fibrotic disease conditions including drug induced gingival overgrowth and gingival fibrosis (Abraham et al., 1997; Kawaguchi et al., 1994; Leask, 2008; Miyauchi et al., 1992; Morelli et al, 1995; Shi-Wen et al., 2006; Subramani et al., 2013; Tamilselvan et al., 2007; Vancheeswaran et al., 1994; Yamane et al, 1992). ET-1 is a 21-amino acid ligand produced by fibroblasts and other cell types (epithelial and endothelial cells, cardiomyocytes, smooth muscle and immune cells) in a form of an inactive prepro-ET1 which is further processed by endopeptidase and endothelin-converting enzyme (ECE) into a proET-1 and active ET-1 respectively (Horstmeyer et al., 2005; Kawaguchi et al., 1994; Leask 2008; Shi-wen et al., 2001, 2006; Tamilselvan et al., 2007). ET-1 acts synergistically with TGFβ to promote differentiation of fibroblasts into myofibroblast and is also induced by, and mediates downstream signaling in response to TGFβ (Galie et al., 2004; Horstmeyer et al., 2005; Krieg et al., 2007; Rahimi and Leof, 2007; Rodriguez et al., 2003; Shephard et al., 2004). Stimulation of dermal fibroblasts with ET-1 alone did not lead to sufficient up-regulation of α-SMA, a marker of myofibroblast differentiation while a synergistic increase in α-SMA expression was observed upon co-stimulation with ET-1 and TGFβ1(Shephard et al., 2004). Similarly, inhibition of endogenous TGFβ1 activity using a neutralizing antibody as well as co-inhibition of ET-1 activity were required to completely prevent α-SMA expression in keratinocyte-fibroblast co-cultures (Shephard et al., 2004). Interestingly, gingival fibroblasts possess reduced *EDN1* mRNA and ET-1 protein expression as compared to

human dermal fibroblasts. Subjecting gingival fibroblasts to mechanical strain or a six hour induction with TGFβ1 (4ng/ml) did not result in up-regulation of collagen type I or αSMA, as was the case with dermal fibroblasts under the same culture conditions (Guo et al., 2011a,b). These differences in the αSMA and collagen I induction between the two cell types were rescued upon co-incubation of gingival fibroblasts with both TGFβ and ET-1. That myofibroblast differentiation is relatively absent in fibrotic conditions such as gingival hyperplasia may thus be partially explained by the reduced gingival fibroblast responsiveness (αSMA and collagen expression) to mechanical strain and TGFβ1 induction as a result of a reduced production of ET-1, as compared to dermal fibroblast cells (Bitu et al., 2006; Damasceno et al., 2012; Guo et al., 2011a,b; Sobral et al., 2010).

Aside from the active role in ECM remodeling and myofibroblast differentiation, ET-1 exhibits mitogenic properties as well as production of PDGF and TNF-α inflammatory cytokines and TGFβ1 (Chamnez et al., 1996; Gandhi et al., 2000; Horstmeyer et al., 2005; Nakamura et al., 1995; Shi-wen et al., 2001). Expression of ET-1 upon TGFβ1 treatment was found to be subject to canonical as well as non-canonical JNK regulation in human dermal and mouse embryonic fibroblasts (Lagares et al., 2010). Furthermore, ET-1 expression in response to TGFβ1 is Smad-independent in the pulmonary fibroblast cells subject to comparable TGFβ1 induction (4 ng/ml) and is instead mediated *via* the JNK/c-Jun/activator protein-1(AP-1) (Krieg at al., 2007; Shi-Wen et al., 2006). As these data suggest that TAK1 regulates JNK activation, the non-canonical TAK1 signaling pathways may mediate TGFβ1 induced ET-1 expression in lung fibroblasts (Rahimi and Leof, 2007). Based on the results I present here, TGFβ1-induced *EDN1* mRNA expression was blocked by TAK1 selective inhibitor, suggesting

that ET-1 may be regulated in a similar fashion in both pulmonary and gingival fibroblasts. Inhibition of TAK1 in gingival fibroblasts may lead to inhibition of the JNK/c-Jun/activator protein-1(AP-1) signaling similar to what was reported in pulmonary fibroblasts (Rahimi and Leof, 2007). In fibroblasts, TGFβ1 and ET-1 synergize to promote myofibroblast differentiation (Shephard et al., 2004). It also is interesting to note that basal ET-1 expression in human gingival fibroblasts is lower than that in dermal fibroblasts and this lower level of expression may explain why TGFβ1 is relatively unable to induce myofibroblast differentiation in gingival fibroblasts (Guo et al., 2011a, 2001b)

4.3 Limitations and future research

The results suggest that TAK1 pathway operates in gingival fibroblasts and mediates essentially all responses to TGFβ1, including proliferation and CCN2 induction. These results must nevertheless be interpreted with caution since the mRNA expression levels of individual genes should not be examined in the context of gene expression profiling alone, but needs independent verification through other procedures. In this thesis, the results obtained using gene array profiling were verified using real time PCR analysis and functional assay.

A confounding factor may be equally present among the various treatment conditions in this study, as I cultured gingival fibroblasts on rigid, stiff plastic tissue culture plates. Progressive increases in the matrix stiffness during normal wound healing is thought to correlate with fibroblast-myofibroblast differentiation as mechanical tension encourages the formation of focal adhesion and stress fibers (Hinz, 2010; Hinz et al., 2001; Balestrini et al., 2012). In theory, this finding is not surprising given the current

knowledge of the role of ECM in sheltering the residing fibroblasts from the outside mechanical forces as well as the observation of a loss of fibroblast phenotype and emergence of myofibroblasts when the "stress-shielding" capacity of ECM is lost upon injury (Guo et al., 2011a, 2011b; Hinz and Gabbiani 2003). In addition, the later stages of wound healing response are characterized by the ECM remodeling and increases in the ECM stiffness. Compliant, two-dimensional matrices were therefore developed in the past with polyacrylamide, polyvinyl alcohol or silicone based elastomers (Hinz, 2010).

Although soft, two dimensional matrices are thought to serve as better models of true tissue compliance, the use of such compliant two dimensional matrices is also not without limitations. First, the true tissue compliance is complex and not homogenous (Hinz, 2010). Second, matrices of polyacrylamide or silicone cannot be remodeled by the attached fibroblast cells as these synthetic substances are not biologically degradable (Hinz, 2010). This creates several important limitations in terms of experimental design. Increases in the deposition of collagen and other factors by the cultured cells may overcome the effects of the underlying soft matrix, effectively increasing its stiffness (Hinz 2010). The degree of culture confluence may further overturn the effect of soft matrix when the increased formation of cell-cell contacts leads to increased mechanical tension exerted on the cell culture by cells (Hinz 2010). In contrast, the three-dimensional matrices of collagen or fibronectin completely surround cells in culture and may better represent the microenvironment encountered by the fibroblast in the lamina propria layer (Grinnell, 2003). Three dimensional culture matrices have been also previously employed to better recreate the physiological conditions experienced by the fibroblasts in the dermis or lamina propria layers, including the presence of cell-cell and

cell-matrix adhesions, regulation of cellular proliferation and growth as well as *in vivo* wound healing and remodeling responses (Chaussain Miller et al., 2002).

Consequently, although it might have been interesting to compare the responses of gingival fibroblast cultured on more compliant and elastic substrates or in using three dimensional matrices, that TGFβ1 still potently induced gene expression and proliferation of cells cultured on plastic suggests that alteration in fibroblast phenotype due to culturing cells on plastic is not sufficient to cause a complete differentiation of fibroblasts to myofibroblast. CCN2 is both a marker and a mediator of fibrosis, and expression of this protein correlates well with tissue fibrosis and fibrotic cells (Brigstock, 2010; Chen and Lau, 2009; (Guo et al., 2011a, 2011b; Leask, 2008, 2011). I did not detect basal CCN2 protein expression (DMSO treatment group) using two separate methods of analysis: western blot and immunofluorescence microscopy and should gingival fibroblast cells undergo this process of differentiation in response to the stiff, plastic substrate, high basal expression of CCN2 in cultured gingival fibroblasts would have been apparent. Further, induction of gingival fibroblast monolayers with TGFβ1 lead to robust responses not only in CCN2 expression (mRNA and protein) but also in the expression of wound healing and proliferative genes as well as measures of gingival fibroblast proliferation using a functional assay. Had gingival fibroblast cells undergone the process of differentiation in response to the stiff substrate, additional induction with TGFβ1 would likely have not caused significant effects. That fibrotic conditions such as gingival hyperplasia are characterized by increased proliferation of fibroblast cells, as opposed to differentiation into myofibroblasts, further supports the notion that gingival fibroblasts respond differently to the fibrogenic stimuli such as TGFβ1 induction or

"stress-unshielded" environment provided by the rigid plastic culture vessel (Bitu et al., 2006; Damasceno et al., 2012; Sobral et al., 2010). Three-dimensional collagen or fibronectin lattices nevertheless may be utilized in the future studies of gingival fibroblast ECM remodeling in the presence and absence of (5Z)-7-oxozeaenol.

The use of an animal model to study the role of TAK1 in gingival fibroblasts should be considered for the future. Full body *MAP3K7*-KO (deletion of TAK1) mice die *in utero* during early embryogenesis (E10) likely due to impaired neural tube development (Shim et al., 2005). Thus, in order to study TAK1 *in vivo,* a number of mice strains were generated allowing for conditional *MAP3K7* (TAK1) deletion from various cell types such as: mouse embryonic fibroblast (MEF) cells, myocardium, hepatocytes, intestinal epithelial cells, keratinocytes, cartilage and fibroblast cells (Inokuchi et al., 2010; Kajino-Sakamoto et al. 2008; Liu et al., 2006; Ma et al., 2011; Omori et al., 2006; Sato et al., 2005; Shim et al. 2009; Xie et al., 2006; Zhang et al., 2000; Zheng et al., 2002). In particular, a fibroblast-specific *MAP3K7*-KO mouse model may be considered for subsequent studies of TAK1-mediated signaling in gingival fibroblasts, wound healing functional assessment as well as nifedipine-induced gingival hyperplasia in mice as was previously reported using rat models (Kataoka et al., 2001). In the former, fibroblast explants from gingiva of control (C57/BL6) as well as fibroblast specific TAK1-KO mice may be studied in greater detail in response to TGFβ1 induction or mechanical strain in culture, with subsequent analysis of the canonical and non-canonical signaling pathways. A similar study has been conducted using an established fibroblast specific *MAP3K7*-KO mouse model where the loss of TAK1 resulted in impaired proliferative, adhesive and migratory properties of cultured dermal fibroblasts (Guo et al.,

2012). Further, the phenotypic differences in gingival tissues of the control and fibroblast specific *MAP3K7*-KO mice may be monitored histologically in response to oral administration of nifedipine (Nalini et al., 1990) or in response to the administration of other drugs which are known to be associated with gingival hyperplasia induction.

Compared to studies relying on the genetic knock-out of *MAP3K7*, studies using a small molecule may be better suited for the translational, bench-to-bedside research approach. (5Z)-7-Oxozeaenol has been previously administered to mice and rats. A two week IP injection of (5Z)-7-Oxozeaenol into female nude mice at 15 mg/kg resulted in neuroblastoma sensitization to doxorubicin (Dox) and etoposide (VP-16) chemotherapeutic drugs using orthotopic neuroblastoma mouse model (Fan et al., 2013). Intracerebroventricular as well as IP injection of (5Z)-7-Oxozeaenol into male mice at various doses accounted for a promising neuroprotective therapeutic approach in stroke treatment (White et al, 2012). Further, adult male Sprague–Dawley rats injected intracerebroventricularly with (5Z)-7-oxozeaenol exhibited improved neurological function and neuronal survival after induced traumatic brain injury (Zhang et al., 2013). Unfortunately, studies primarily focusing on the safety of systemic (5Z)-7-oxozeaenol administration as well as pharmacokinetic and pharmacodynamic properties still remain to be conducted in the future, including pre-clinical trials and Phase I clinical trials in humans. Should local application of (5Z)-7-oxozeaenol be considered for gingival hyperplasia treatment, the overall effect of this ligand on the oral mucosa and underlining tissues as well as the effect on gastrointestinal tract must also be established.

A number of additional TAK1 inhibitors including Pyrazolo[4,3-c]isoquinolines and 5Z-7-oxozeaenol analogues have been recently identified and may be considered for

treatment of gingival hyperplasia and/or study of TAK1-mediated signaling responses (Buglio et al., 2012, Lockman et al., 2011, and Mortier et al., 2010). Oral administration of TAK1 inhibitors may also be considered in the future where a study using orally administered LYTAK1, a small molecule inhibitor of TAK1, showed a reduction of TAK1-mediated resistance to chemotherapeutic drugs for pancreatic adenocarcinoma treatment in orthotopic nude mouse model (Melisi et al., 2011).

Collectively, my results suggest that TAK1 mediates the ability of TGFβ1 to induce CCN2 expression (mRNA and protein) and transcriptional responses in gingival fibroblasts. Notably, TAK1 inhibition blocks the ability of TGFβ1 to elicit proliferative responses as well as the expression of wound healing genes in gingival fibroblasts. My thesis described data supporting the long-term hypothesis that targeting the non-canonical TAK1-mediated TGFβ1 signaling pathway may be useful to combat gingival hyperplasia in the future.

5. REFERENCES

Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. 2001.Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol; 166:7556–7562.

Abraham, D. J., R. Vancheeswaran, M. R. Dashwood, P. Pantelides, W. Shi-Wen, R. M. du Bois, and C. M. Black. 1997. Increased levels of endothelin-1 and differential endothelin type A and B receptor expression in scleroderma-associated fibrotic lung disease. Am. J. Pathol. 151:831–841

Alberts B, Bray D, Lewis J, et al. 1994. Fibroblasts and Their Transformations: The Connective-Tissue Cell Family. In "Molecular Biology of the Cell." New York; Garland Science. 3rd edition.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. 1983. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem. 258(11):7155-60.

Balestrini JL, Chaudhry S, Sarrazy V, Koehler A, Hinz B. 2012. The mechanical memory of lung myofibroblasts. Integr Biol (Camb). 4(4):410-21.

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. 2008. Growth factors and cytokines in wound healing. Wound Repair and Regeneration 16:585-601.

Bell E, Sher S, Hull B, Merrill C, Rosen S, Chamson A, Asselineau D, Dubertret L, Coulomb B, Lapiere C, Nusgens B, Neveux Y. 1983. The Reconstitution of Living Skin. J Invest Dermatol 81

Bhattacharyya S, Chen S, Wu M, Warner-Blankenship M, Ning H, Lakos G, Mori Y, Chang E, Nihijima C, Takehara K, Feghali-Bostwick C, Varga J. 2008. Smad-independent transforming growth factor-beta regulation of early growth response-1 and sustained expression in fibrosis - Implications for scleroderma. Am J Pathol 173:1085-1099.

Bitu CC, Sobral LM, Kellermann MG, Martelli-Junior H, Zecchin KG, Graner E, Coletta RD. Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. 2006. J Clin Periodontol. 33:393-400

Black SA Jr, Trackman PC. 2008. Transforming growth factor-beta1 (TGFbeta1) stimulates connective tissue growth factor (CCN2/CTGF) expression in human gingival fibroblasts through a RhoA-independent, Rac1/Cdc42-dependent mechanism: statins with forskolin block TGFbeta1-induced CCN2/CTGF expression. J Biol Chem 283:10835-47

Blobe GC, Schiemann WP, Pepin MC, Beauchemin M, Moustakas A, Lodish HF, O'Connor-McCourt MD. 2001. Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627–24637.

Blom IE, Goldschmeding R, Leask A. 2002. Review. Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? Matrix Biol;21(6):473-82.

Boltchi FE, Rees TD, Iacopino AM. Cyclosporine Ainduced gingival overgrowth: A comprehensive review. Quintessence Int 1999;30:775-783.

Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M, Lavery C, Margetts P, Roberts A, Gauldie J. 2004a. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. Journal of Immunology 173:2099-2108.

Bonniaud P, Martin G, Margetts PJ, et al. 2004b. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/ c mouse lungs. Am J Respir Cell Mol Biol 31:510-16

Bork, P. 1993. The modular architecture of a new family of growth regulators relatedto connective tissue growth factor. FEBS Lett. 327, 125-130.

Bradham DM, Igarashi A, Potter RL, Grotendorst GR. 1991. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol. 114(6):1285-94.

Brigstock DR. 2010. Connective tissue growth factor (CCN2, CTGF) and organ fibrosis: lessons from transgenic animals. Journal of Cell Communication and Signaling 4:1-4.

Brigstock, D. R., Goldschmeding, R., Katsube, K. I., Lam, S. C., Lau, L. F., Lyons, K., Naus, C., Perbal, B., Riser, B., Takigawa, M. et al. 2003. Proposal for a unified CCN nomenclature. Mol. Pathol. 56, 127-128.

Buglio, D., Palakurthi, S., Byth, K., Vega, F., Toader, D., Saeh, J., Neelapu, S.S., Younes, A., 2012. Essential role of TAK1 in regulatingmantle cell lymphoma survival. Blood 120, 347–355.

Bujak M, Frangogiannis NG. 2007. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res. May 1;74(2):184-95.

Carvalho RLC, Itoh F, Goumans MJ, et al. Compensatory signalling induced in the yolk sac vasculature by deletion of TGFb receptors in mice.2007. J Cell Sci; 20:4269–4277.

Casetta I, Granieri E, Desidera M, et al. Phenytoin-induced gingival overgrowth: A community-based cross-sectional study in Ferrara, Italy. Neuroepidemiology 1997;16:296-303.

Chamnez P, Vignola AM, Albat B, Springall DR, Polak JM, Godard P & Bousquet J.1996. Involvement of endothelin in mononuclear phagocyte inflammation in asthma. J Allergy Clin Immunol 98, 412–420.

Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO. 2002. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci USA 99

Chaussain Miller C, Septier D, Bonnefoix M, Lecolle S, Lebreton-Decoster C, Coulomb B, Pellat B, Godeau G. 2002. Human dermal and gingival fibroblasts in a three-dimensional culture: a comparative study on matrix remodeling. Clin Oral Investig 6

Chen C, Lau LF. 2009. Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol 41:771-783.

Chen S, Ning H, Ishida W, Sodin-Semrl S, Takagawa S, Mori Y, Varga J. 2006. The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression. J Biol Chem 281:21183-21197.

Chen Y, Segarini P, Raoufi F, Bradham D, Leask A. 2001. Connective tissue growth factor is secreted through the Golgi and is degraded in the endosome. Exp Cell Res 271:109-117.

Chen Y, Shi-Wen X, van Beek J, Kennedy L, McLeod M, Renzoni EA, Bou-Gharios G, Wilcox-Adelman S, Goetinck PF, Eastwood M, Black CM, Abraham DJ, Leask A. 2005. Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK - Insights into pathological scarring in chronic fibrotic disease. Am J Pathol 167

Chipev CC, Simon M. 2002. Phenotypic differences between dermal fibroblasts from different body sites determine their responses to tension and TGFβ1. BMC Dermatology 2:13.

Chomczynski P, Sacchi N. 1987. Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. Analytical Biochemistry 162:156- 159.

Chung, K.Y., A. Agarwal, J. Uitto, and A. Mauviel. 1996. An AP-1 binding sequence is essential for regulation of the human alpha2(I) collagen (COL1A2) promoter activity by transforming growth factor-beta. J Biol Chem 271:3272–3278.

Ciancio S, Bartz N, Lauciello F. Cyclosporine-induced gingival hyperplasia and chlorhexidine: A case report. Int J Periodontics Restorative Dent 1991;11:241-245.

Clark RAF. 1996. In: Clark RAF, editor. The molecular and cellular biology of wound repair. 2nd ed. New York: Plenum Press.

Clocheret K, Dekeyser C, Carels C, Willems G. 2003. Idiopathic gingival hyperplasia and orthodontic treatment: a case report. J Orthod, Vol. 30 13–19

Dahlof G, Preber H, Eliasson S, et al. Periodontal condition of epileptic adults treated long-term with phenytoin or carbamazepine. Epilepsia 1993;34:960-964.

Damasceno LS, Gonçalves Fda S, Costa e Silva E, Zenóbio EG, Souza PE, Horta MC. 2012. Stromal myofibroblasts in focal reactive overgrowths of the gingiva. Braz Oral Res. 26:373-7

Datto M, Frederick J, Pan L, Borton A, Zhuang Y, Wang X. 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol 19:2495-2504.

Denton CP, Merkel PA, Furst DE, Khanna D, Emery P, Hsu VM, Silliman N, Streisand J, Powell J, Akesson A, Coppock J, Hoogen Fv, Herrick A, Mayes MD, Veale D, Haas J, Ledbetter S, Korn JH, Black CM, Seibold JR. 2007. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. Arthritis Rheum 56(1):323-333.

Derynck R, Zhang Y. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577-584.

Desmoulière A, Redard M, Darby I, Gabbiani G. 1995. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol;146:56–66

Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, Poulsom R, Hodivala-Dilke K, Alison MR, Wright NA. 2003. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-tranplanted mice. Stem Cells 21

Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, ten Dijke P, White B, Wigley FM, Goldschmidt-Clermont PJ. 2002. Deficient Smad7 expression:A putative molecular defect in scleroderma. Proc Natl Acad Sci USA 99:3908–3913.

Ebisawa K, Kato R, Okada M, Sugimura T, Latif MA, Hori Y, Narita Y, Ueda M, Honda H, Kagami H. 2011. Gingival and dermal fibroblasts: Their similarities and differences revealed from gene expression. Journal of Bioscience and Bioengineering 111

Egusa H, Okita K, Kayashima H, Yu G, Fukuyasu S, Saeki M, Matsumoto T, Yamanaka S, Yatani H. 2010. Gingival Fibroblasts as a Promising Source of Induced Pluripotent Stem Cells. Plos One 5:e12743

Eickelberg O, Centrella M, Reiss M, Kashgarian M, Wells RG. 2002. Betaglycan inhibits TGF-b signaling by preventing type I-type II receptor complex formation. J Biol Chem 277:823–829.

Ellestad GA, Lovell FM, Perkinson NA, Hargreaves RT and McGahren WJ. 1978. New zearalenone related macrolides and isocoumarins from an unidentified fungus. Journal of Organic Chemistry 43: 2339-2343.

Eslami A, Gallant-Behm CL, Hart DA, Wiebe C, Honardoust D, Gardner H, Haekkinen L, Larjava HS. 2009. Expression of Integrin alpha v beta 6 and TGF-beta in Scarless vs Scar-forming Wound Healing. Journal of Histochemistry & Cytochemistry 57

Evans R, Tian Y, Steadman R, Phillips A. 2003. TGF-beta 1-mediated fibroblast-myofibroblast terminal differentiation - the role of Smad proteins. Exp Cell Res 282:90-100.

Fan Y, Cheng J, Vasudevan SA, Patel RH, Liang L, Xu X, Zhao Y, Jia W, Lu F, Zhang H, Nuchtern JG, Kim ES, Yang J. 2013.TAK1 inhibitor 5Z-7-oxozeaenol sensitizes neuroblastoma to chemotherapy. Apoptosis. 18(10):1224-34. doi: 10.1007/s10495-013-0864-0.

Fu E, Nieh S, Hsiao CT, Hsieh YD, Wikesjö UM, Shen EC. 1998.Nifedipine-induced gingival overgrowth in rats: Brief review and experimental study. J Periodontol 69:765-771

Gabbiani G. 2003. The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200

Galié N, Manes A, Branzi A.2004. The endothelin system in pulmonary arterial hypertension. Cardiovasc Res;61(2):227-37.

Gandhi CR, Kuddus RH, Uemura T & Rao AS.2000. Endothelin stimulates transforming growth factor-beta1 and collagen synthesis in stellate cells from control but not cirrhotic rat liver. Eur J Pharmacol 406, 311–318.

Gervasi M, Bianchi-Smiraglia A, Cummings M, Zheng Q, Wang D, Liu S, Bakin AV. 2012. JunB contributes to Id2 repression and the epithelial–mesenchymal transition in response to transforming growth factor–β. J Cell Biol 196(5):589–603.

Ghosh AK, Varga J. 2007.The transcriptional coactivator and acetyltransferase p300 in fibroblast biology and fibrosis. J Cell Physiol. 213(3):663-71.

Ghosh AK, Vaughan DE. 2012. PAI-1 in tissue fibrosis. J Cell Physiol 227:493-507.

Gosain A, Luisa MD, DiPietro A. 2004. Aging and wound healing. World J Surg 28

Goumans M, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. 2002. Balancing the activation state of the endothelium *via* two distinct TGF-beta type I receptors. EMBO J 21:1743-1753.

Grinnell F.2003.Fibroblast biology in three-dimensional collagen matrices. Trends Cell Biol;13(5):264-9.

Grotendorst G, Rahmanie H, Duncan M. 2004. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. Faseb Journal 18:469-479

Guo F, Carter DE, Leask A. 2011a. Mechanical Tension Increases CCN2/CTGF Expression and Proliferation in Gingival Fibroblasts via a TGF beta-Dependent Mechanism. Plos One 6:e19756.

Guo F, Carter DE, Mukhopadhyay A, Leask A. 2011b. Gingival Fibroblasts Display Reduced Adhesion and Spreading on Extracellular Matrix: A Possible Basis for Scarless Tissue Repair?. Plos One 6:e27097.

Guo F, Xu Shiwen, Hutchenreuther J, Khan K, Denton C, Abraham D, Leask A. 2012. Tak1 Expression by Fibroblasts is Required for Fibrogenic Responses in Vivo and in Vitro. Wound Repair and Regeneration 20:A100-A100.

Guo S, DiPietro LA. 2010. Factors Affecting Wound Healing. J Dent Res 89(3):219-29

Harper RA, Grove G. 1979. Human-Skin Fibroblasts Derived from Papillary and Reticular Dermis - Differences in Growth-Potential Invitro. Science 204

Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. 2001. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. Mol Biol Cell 12

Hinz B, Gabbiani G. 2003. Mechanisms of force generation and transmission by myofibroblasts. Curr Opin Biotechnol 14

Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G. 2001. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Pathol 159

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

Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmouliere A, Varga J, De Wever O, Mareel M, Gabbiani G. 2012. Recent Developments in Myofibroblast Biology Paradigms for Connective Tissue Remodeling. Am J Pathol 180

Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ. 2004. Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell 15

Hinz B. 2006. Masters and servants of the force: The role of matrix adhesions in myofibroblast force perception and transmission. Eur J Cell Biol 85

Hinz B. 2007. Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 127

Hinz B. 2010. The myofibroblast: Paradigm for a mechanically active cell. J Biomech 43

Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. 2001. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. J Biol Chem 276:10594–10601

Horstmeyer A, Licht C, Scherr G, Eckes B, Krieg T.2005. Signalling and regulation of collagen I synthesis by ET-1 and TGF-beta1. FEBS J; 272(24):6297-309.

Hu B, Wu Z, Phan SH. 2003. Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. Am J Respir Cell Mol Biol. 29(3 Pt 1):397-404. Epub 2003 Apr 17.

Huang SS, Ling TY, Tseng WF, Huang YH, Tang FM, Leal SM, Huang JS. 2003. Cellular growth inhibition by IGFBP-3 and TGF-beta1 requires LRP-1. FASEB J 17:2068–2081.

Hunt TK, Hopf H, Hussain Z. 2000. Physiology of wound healing.Adv Skin Wound Care13:6-11.

Inokuchi S, Aoyama T, Miura K et al. 2010. Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. Proc Natl Acad Sci USA 107:844–9

Irizarry R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., Terence, P., &Speed, T.P. (2003), Summaries of Affymetrix GeneChip probe level data Nucleic Acids Research 31(4):e15.

Kajino-Sakamoto R, et al. 2008. Enterocyte-derived TAK1 signaling prevents epithelium apoptosis and the development of ileitis and colitis. J Immunol 181:1143–1152.

Kantarci A, Black SA, Xydas CE, Murawel P, Uchida Y, Yucekal-Tuncer B, Atilla G, Emingil G, Uzel MI, Lee A, Firatli E, Sheff M, Hasturk H, Van Dyke TE, Trackman PC. 2006.Epithelial and connective tissue cell CTGF/CCN2 expression in gingival fibrosis. J Pathol. 210(1):59-66.

Kataoka M, Shimizu Y, Kunikiyo K, Asahara Y, Azuma H, Sawa T, Kido J, Nagata T. 2001.Nifedipine induces gingival overgrowth in rats through a reduction in collagen phagocytosis by gingival fibroblasts. J Periodontol. 72(8):1078-83.

Kawaguchi, Y., K. Suzuki, M. Hara, T. Hidaka, T. Ishizuka, M. Kawagoe, and H. Nakamura. 1994. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. Ann. Rheum. Dis. 53:506–510.

Kendall AC, Nicolaou A. 2013. Bioactive lipid mediators in skin inflammation and immunity. Prog Lipid Res.52(1):141-64

Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, Eckes B. 2001. Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. J Biol Chem 276

Khocht A, Schneider LC. Periodontal management of gingival overgrowth in the heart transplant patient: A case report. J Periodontol 1997;68:1140-1146.

Kingsley D. 1994. The TGF-Beta Superfamily - New Members, New Receptors, and New Genetic Tests of Function in Different Organisms. Genes Dev 8:133-146.

Kissin EY, Merkel PA, Lafyatis R. 2006. Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. Arthritis Rheum 54

Krieg T, Abraham D, Lafyatis R. 2007. Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast-epithelial cell interactions. Arthritis Research & Therapy 9:S4.

Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders. KC, Roberts AB, Scorn MB, Ward JM, Karlsson S: 1993. Transforming Growth Factor Beta Null Mutation In Mice Causes Excessive Inflammatory Response And Early Death. Proc Natlacad Sci Usa 90:770—774.

Lagares D, Busnadiego O, Garcia-Fernandez RA, Kapoor M, Liu S, Carter DE, Abraham D, Shi-Wen X, Carreira P, Fontaine BA, Shea BS, Tager AM, Leask A, Lamas S, Rodriguez-Pascual F. 2012. Inhibition of focal adhesion kinase prevents experimental lung fibrosis and myofibroblast formation. Arthritis Rheum 64:1653-1664.

Lagares D, García-Fernández RA, Jiménez CL, Magán-Marchal N, Busnadiego O, Lamas S, Rodríguez-Pascual F.2010. Endothelin 1 contributes to the effect of transforming growth factor beta1 on wound repair and skin fibrosis. Arthritis Rheum;62(3):878-89

Larsson J, Goumans MJ, Sjöstrand LJ, van Rooijen MA, Ward D, Levéen P, Xu X, ten Dijke P, Mummery CL, Karlsson S. 2001. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J. 20(7):1663-73.

Lazarus GS, Cooper DM, Knighton DR, Percoraro RE, Rodeheaver G, Robson MC. 1994. Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Repair Regen. 2(3):165-70.

Leal SM, Huang SS, Huang JS. 1999. Interactions of high affinity insulin-like growth factor-binding proteins with the type V transforming growth factor-beta receptor in mink lung epithelial cells. J Biol Chem 274:6711– 6717.

Leal SM, Liu Q, Huang SS, Huang JS. 1997. The type V transforming growth factor b receptor is the putative insulin-like growth factor-binding protein 3 receptor. J Biol Chem 272:20572–20576.

Leask A, Abraham DJ. 2004. TGF-beta signaling and the fibrotic response. FASEB Journal 18:816-827.

Leask A, Abraham DJ. 2006.All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci. 1;119(Pt 23):4803-10.

Leask A, Parapuram SK, Shi-Wen X, Abraham DJ. 2009. Connective tissue growth factor (CTGF, CCN2) gene regulation: a potent clinical bio-marker of fibroproliferative disease? J Cell Commun Signal 3: 89–94.

Leask A. 2006. Scar wars: is TGF beta the phantom menace in scleroderma?. Arthritis Research & Therapy 8:213.

Leask A. 2008. Targeting the TGF beta, endothelin-1 and CCN2 axis to combat fibrosis in scleroderma. Cell Signal 20:1409-1414.

Leask A. 2011. CCN2: a bona fide target for anti-fibrotic drug intervention. Journal of Cell Communication and Signaling 5:131-133.

Leask A. 2013a. CCN2: a novel, specific and valid target for anti-fibrotic drug intervention. Expert Opin Ther Targets; 17(9):1067-71

Leask A. 2013b. CCN2: a mechanosignaling sensor modulating integrin-dependent connective tissue remodeling in fibroblasts? J Cell Commun Signal; 7(3):203-5.

Leask A. Parapuram SK, Shi-wen X, Abraham DJ. 2009.Connective tissue growth factor (CTGF, CCN2) gene regulation: a potent clinical bio-marker of fibroproliferative disease? J. Cell Commun. Signal 3:89–94

Lin HK, Bergmann S, Pandolfi PP. 2004. Cytoplasmic PML function in TGF-beta signalling. Nature 431:205–211.

Lin X, Liang YY, Sun B, Liang M, Shi Y, Brunicardi FC, Shi Y, Feng XH. 2003. Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription. Mol Cell Biol. 23(24):9081-93.

Liu B, Dou CL, Prabhu L, Lai E. 1999. FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor beta signals. Mol Cell Biol.19(1):424-30.

Liu HH, Xie M, Schneider MD, Chen ZJ. 2006. Essential role of TAK1 in thymocyte development and activation. Proc Natl Acad Sci USA;103:11677–11682.

Liu Q, Huang SS, Huan g JS. 1997. Function of the type V transforming growth factor b receptor in transforming growth factor b-induced growth inhibition of mink lung epithelial cells. J Biol Chem 272:18891–18895.

Liu Q, Huang SS, Huang JS. 1994. Kinase activity of the type V transforming growth factor beta receptor. J Biol Chem 269:9221–9226.

Liu S, Kapoor M, Denton CP, Abraham DJ, Leask A. 2009. Loss of beta 1 Integrin in Mouse Fibroblasts Results in Resistance to Skin Scleroderma in a Mouse Model. Arthritis Rheum 60:2817-2821.

Liu S, Xu Shi-wen, Abraham DJ, Leask A. 2011. CCN2 Is Required for Bleomycin-Induced Skin Fibrosis in Mice. Arthritis Rheum 63:239-246.

Liu S, Xu Shi-wen, Kennedy L, Pala D, Chen Y, Eastwood M, Carter DE, Black CM, Abraham DJ, Leask A. 2007. FAK is required for TGF beta-induced JNK

phosphorylation in fibroblasts: Implications for acquisition of a matrix-remodeling phenotype. Mol Biol Cell 18:2169-2178.

Livak KJ and Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $^{\Delta\Delta Ct}$ Method. METHODS 25: 402–408

Lockman JW, Reeder MD, Robinson R, Ormonde PA, Cimbora DM, Williams BL, Willardsen JA.2011. Oxindole derivatives as inhibitors of TAK1 kinase. Bioorg Med Chem Lett;21(6):1724-7.

Lorimier S, Gillery P, Hornebeck W, Chastang F, LaurentMaquin D, Bouthors S, Droulle C, Potron G, Maquart FX. 1996. Tissue origin and extracellular matrix control neutral proteinase activity in human fibroblast three-dimensional cultures. J Cell Physiol 168

Lorimier S, Hornebeck W, Godeau G, Pellat B, Gillery P, Maquart FX, Laurent-Maquin D. 1998. Morphometric studies of collagen and fibrin lattices contracted by human gingival fibroblasts; Comparison with dermal fibroblasts. J Dent Res 77

Lovgren AK, Kovacs JJ, Xie T, Potts EN, Li Y, Foster M, Liang J, Meltzer EB, Jiang D, Lefkowitz RJ, Noble PW. 2011. beta-Arrestin Deficiency Protects Against Pulmonary Fibrosis in Mice and Prevents Fibroblast Invasion of Extracellular Matrix. Science Translational Medicine 3:74ra23.

Ma FY, Tesch GH, Ozols E et al. (2011) TGF-b1-activated kinase-1 regulates inflammation and fibrosis in the obstructed kidney. Am J Physiol Renal Physiol 300:F1410–21

Marshall RI, Bartold PM. 1999. A clinical review of drug-induced gingival overgrowth. Aust Dent J;44:219-232.

Massague J, Seoane J, Wotton D. 2005. Smad transcription factors. Genes Dev 19:2783-2810.

Massague J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753-791.

Mauviel A, Chen YQ, Dong W, Evans CH and Uitto J.1993. Transcriptional interactions of transforming growth-factor-beta with pro-inflammatory cytokines. Curr Biol 3(12):822-831.

Mauviel A, Chung KY, Agarwal A, Tamai K and Uitto J. 1996. Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-beta in fibroblasts and keratinocytes. J Biol Chem 271(18):10917-10923.

McWhirter A, Colosetti P, Rubin K, Miyazono K, Black C.1994. Collagen type I is not under autocrine control by transforming growth factor-beta 1 in normal and scleroderma fibroblasts. Lab Invest. 71(6):885-94.

Meckmongkol TT, Harmon R, McKeown-Longo P, VanDe Water L. 2007. The fibronectin synergy site modulates TGFbeta-dependent fibroblast contraction. Biochem Biophys Res Commun 360:709–14.

Mehra A, Wrana J. 2002. TGF-beta and the Smad signal transduction pathway. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 80:605-622.

Melisi D, Xia Q, Paradiso G, Ling J, Moccia T, Carbone C, Budillon A, Abbruzzese JL, Chiao PJ. 2011. Modulation of pancreatic cancer chemoresistance by inhibition of TAK1. J Natl Cancer Inst. 103(15):1190-204.

Meraw SJ and Sheridan PJ. Medically Induced Gingival Hyperplasia. 1998. Mayo Clin Proc 73:1196-1199

Michael KE, Dumbauld DW, Burns KL, Hanks SK, García AJ. 2009. Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. Mol Biol Cell.20(9):2508-19.

Mimura Y, Ihn H, Jinnin M, Asano Y, Yamane K, Tamaki K. 2005. Constitutive phosphorylation of focal adhesion kinase is involved in the myofibroblast differentiation of scleroderma fibroblasts. J Invest Dermatol 124

Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, Hanai J-I, Beppu H, Tsukazaki T, Wrana JL, Miyazono K, Sugamura K. 2000. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol Cell Biol 20:9346–9355.

Miyauchi, T., N. Suzuki, T. Yuhara, T. Akama, H. Suzuki, and H. Kashiwagi. 1992. Significance of plasma endothelin-1 levels in patients with systemic sclerosis. J. Rheumatol. 19:1566–1571.

Montagna W, Parakkal OF. 1974. The Structure and Function of Skin. Acad. Press

Moodley YP, Misso NLA, Scaffidi AK, Fogel-Petrovic M, McAnulty RJ, Laurent GJ, Thompson PJ, Knight DA. 2003. Inverse Effects of Interleukin-6 on Apoptosis of Fibroblasts from Pulmonary Fibrosis and Normal Lungs. Am J Respir Cell Mol Biol 29: 490–498.

Morelli S, Ferri C, Polettini E, Bellini C, Gualdi GF, Pittoni V, et al., 1995. Am. J. Med. 99: 255.

Mori T, Kawara S, Shinozaki M, Hayashi N, Kakinuma T, Igarashi A, Takigawa M, Nakanishi T, Takehara K. 1998. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: A mouse fibrosis model. J Cell Physiol.181(1):153-9.

Mortier J, Frederick R, Ganeff C, Remouchamps C, Talaga P, Pochet L, Wouters J, Piette J, Dejardin E, Masereel B. 2010. Pyrazolo[4,3-c]isoquinolines as potential inhibitors of NF-kappaB activation. Biochem Pharmacol. 79(10):1462-72.

Munger JS, Harpel JG, Gleizes PE, Mazzieri R, Nunes I, Rifkin DB. 1997. Latent transforming growth factorbeta: Structural features and mechanisms of activation. Kidney Int 51:1376–1382

Murray RN. 2009. Studies towards a fast and efficient total synthesis of LL-Z1640-2. PhD dissertation. University of Glasgow. Glasgow, United Kingdom

Myers BD, Newton L. Cyclosporine-induced chronic nephropathy: An obliterative microvascular renal injury. J Am Soc Nephrol 1991 Aug;2(2 Suppl. 1):S45-S52.

Nakamura T, Ebihara I, Fukui M, Tomino Y & Koide H .1995. Effect of a specific endothelin receptor A antagonist on mRNA levels for extracellular matrix components and growth factors in diabetic glomeruli. Diabetes 44, 895–899.

Nakib N, Ashrafi SS. 2011. Drug-Induced Gingival Overgrowth. Dis Mon 57:225-230

Nalini K, Andrabi KI, Ganguly NK, Wahi PL. 1990. Nifedipine administration impairs natural resistance of mice to Salmonella typhimurium. Mol Cell Biochem. 95(2):133-7.

Nanci A. 2013. Structure of the Oral Tissues. In "Ten Cate's Oral Histology. Development, Structure, and Function." Elsevier. 8th edition

Niewiaro.S, Regoeczi E, Mustard JF. 1972. Adhesion of Fibroblasts to Polymerizing Fibrin and Retraction of Fibrin Induced by Fibroblasts. Proceedings of the Society for Experimental Biology and Medicine 140

Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, Matsumoto K. 2003. A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. J Biol Chem 278:18485-18490.

O'Grady P, Kuo M-D, Baldassare JJ, Huang SS, Huang JS.1991. Purification of a new type high molecular weight receptor (Type V receptor) of transforming growth factor b (TGF-b) from bovine liver. J Biol Chem 266:8583–8589.

O'Grady P, Liu Q, Huang SS, Huang JS. 1992. Transforming growth factor beta (TGF-beta) type V receptor has a TGF-beta-stimulated serine/threonine-specific autophosphorylation activity. J Biol Chem 267:21033–21037.

Omori E, et al. 2006. TAK1 is a master regulator of epidermal homeostasis involving skin inflammation and apoptosis. J Biol Chem 281:19610–19617.

Palaiologou A, Yukna R, Moses R, Lallier T. 2001. Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors. J Periodontol 72:798-807.

Pannu J, Nakerakanti S, Smith E, et al. 2007. Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. J Biol Chem282:10405– 10413.

Papakonstantinou E, Aletras AJ, Roth M, Tamm M, Karakiulakis G. 2003. Hypoxia modulates the effects of transforming growth factor-beta isoforms on matrix-formation by primary human lung fibroblasts. Cytokine. 24: 25–35.

Parsons JT. 2003. Focal adhesion kinase: the first ten years. J Cell Sci 116

Perbal, B. 2004. CCN proteins: multifunctional signalling regulators. Lancet 363, 62-64.

Pilewski JM, Liu L, Henry AC, Knauer AV, Feghali-Bostwick CA. 2005. Insulin-Like Growth Factor Binding Proteins 3 and 5 are Overexpressed in Idiopathic Pulmonary Fibrosis and Contribute to Extracellular Matrix Deposition. Am J Pathol 166:399–407.

Pinzani M. 2008. Welcome to Fibrogenesis & Tissue Repair. Fibrogenesis Tissue Repair 1:1

Ponticos M, Holmes AM, Xu Shi-wen, Leoni P, Khan K, Rajkumar VS, Hoyles RK, Bou-Gharios G, Black CM, Denton CP, Abraham DJ, Leask A, Lindahl GE. 2009. Pivotal Role of Connective Tissue Growth Factor in Lung Fibrosis MAPK-Dependent Transcriptional Activation of Type I Collagen. Arthritis Rheum 60:2142-2155.

Rahimi RA, Leof EB. 2007. TGF-beta signaling: A tale of two responses. J Cell Biochem 102:593-608.

Rajkumar VS, Howell K, Csiszar K, Denton CP, Black CM, Abraham DJ. 2005. Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. Arthritis Research & Therapy 7

Rees TD, Levine RA. Systemic drugs as a risk factor for periodontal disease initiation and progression. CompendContin Educ Dent 1995;16:20-42.

Renzoni EA, Abraham DJ, Howat S, Shi-Wen X, Sestini P, Bou-Gharios G, Wells AU, Veeraraghavan S, Nicholson AG, Denton CP, Leask A, Pearson JD, Black CM, Welsh KI, and du Bois R. 2004. Gene expression profiling reveals novel TGFβ targets in adult lung fibroblasts. Respir Res. 5(1): 24.

Roberts A, Sporn M, Assoian R, Smith J, Roche N, Wakefield L, Heine U, Liotta L, Falanga V, Kehrl J, Fauci A. 1986. Transforming Growth-Factor Type-Beta - Rapid Induction of Fibrosis and Angiogenesis Invivo and Stimulation of Collagen Formation Invitro. Proc Natl Acad Sci USA 83:4167-4171.

Robson MC, Steed DL, Franz MG. 2001. Wound healing: biologic features and approaches to maximize healing trajectories. Curr Probl Surg 38

Rodríguez-Pascual F, Redondo-Horcajo M, Lamas S.2003. Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-beta-mediated induction of endothelin-1 expression. Circ Res;92(12):1288-95.

Sakurai H. 2012. Targeting of TAK1 in inflammatory disorders and cancer. Trends Pharmacol Sci.33(10):522-30

Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S. 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol. 6(11):1087-95.

Schor SL, Ellis I, Irwin CR, Banyard J, Seneviratne K, Dolman C, Gilbert AD, Chisholm DM. 1996. Subpopulations of fetal-like gingival fibroblasts: characterisation and potential significance for wound healing and the progression of periodontal disease. Oral Dis 2(2):155-66.

Sciubba JJ, Waterhouse JP, Meyer J. 1978. A fine structural comparison of the healing of incisional wounds of mucosa and skin. J Oral Pathol. 7(4):214-27.

Segarini PR, Nesbitt JE, Li D, Hays LG, Yates JR 3rd, Carmichael DF. 2001.The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. J Biol Chem.276(44):40659-67.

Seoane Leston JM, Aguado Santos A, Varela-Centelles PI, Vazquez Garcia J, Romero MA, Pias Villamor L. 2002. Oral mucosa: variations from normalcy, part I. Cutis.69(2):131-4.

Shephard P, Hinz B, Smola-Hess S, Meister JJ, Krieg T, Smola H. 2004. Dissecting the roles of endothelin, TGF-beta and GM-CSF on myofibroblast differentiation by keratinocytes. Thromb Haemost; 92(2):262-74.

Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K. 1996. TAB1: An activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science 272:1179-1182.

Shim JH, et al. 2009. TAK1 is an essential regulator of BMP signaling in cartilage. EMBO J. 28:2028–2041.

Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S. 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19:2668-2681.

Shi-Wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, Black CM, Abraham DJ.2001.Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1.J Invest Dermatol; 116(3):417-25.

Shi-wen X, Parapuram SK, Pala D, Chen Y, Carter DE, Eastwood M, Denton CP, Abraham DJ, Leask A. 2009. Requirement of Transforming Growth Factor beta-Activated Kinase 1 for Transforming Growth Factor beta-Induced alpha-Smooth Muscle Actin Expression and Extracellular Matrix Contraction in Fibroblasts. Arthritis Rheum 60:234-241.

Shi-Wen X, Rodríguez-Pascual F, Lamas S, Holmes A, Howat S, Pearson JD, Dashwood MR, du Bois RM, Denton CP, Black CM, Abraham DJ, Leask A. 2006. Constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. Mol Cell Biol;26(14):5518-27.

Shi-wen X, Stanton L, Kennedy L, et al. 2006. CCN2 is necessary for adhesive responses to TGF-beta1 in embryonic fibroblasts. J Biol Chem; 281:10715-26

Shi-wen X, Thompson K, Khan K, Liu S, Murphy-Marshman H, Baron M, Denton CP, Leask A, Abraham DJ. 2012. Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts. Rheumatology (Oxford); 51(12):2146-54

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 22;359(6397):693-9.

Singer A, Clark R. 1999. Mechanisms of disease - Cutaneous wound healing. N Engl J Med 341:738-746.

Sobral LM, Kellermann MG, Graner E, Martelli-Junior H, Coletta RD.2010.Cyclosporin A-induced gingival overgrowth is not associated with myofibroblast transdifferentiation. Braz Oral Res;24(2):182-8.

Somacarrera ML, Lucas M, Scully C, Barrios C. Effectiveness of periodontal treatments on cyclosporine induced gingival overgrowth in transplant patients. BrDent J 1997;183:89-94.

Sonnylal S, Shi-Wen X, Leoni P, Naff K, Van Pelt CS, Nakamura H, Leask A, Abraham D, Bou-Gharios G, de Crombrugghe B. 2010. Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. Arthritis Rheum. 62(5):1523-32

M.B. Sporn, Sonia B. Jakowlew. 2010. Transforming Growth Factor- ß in Cancer Therapy, Vol 1. Springer.

Squier CA and Kremer MJ. 2001. Biology of Oral Mucosa and Esophagus. JNCI Monographs Vol 2001(29):7-15.

Steinberg AD. 1981.Clinical management of phenytoin-induced gingival overgrowth in handicapped children. The American Academy of Pedodontics Vol. 3, Special Issue

Subramani T, Rathnavelu V, Alitheen NB. 2013.The possible potential therapeutic targets for drug induced gingival overgrowth. Mediators Inflamm;2013:639468

Tamilselvan S, Raju SN, Loganathan D, Kamatchiammal S, Abraham G, Suresh R.2007. Endothelin-1 and its receptors ET(A) and ET(B) in drug-induced gingival overgrowth. J Periodontol;78(2):290-5.

Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE. 2003. Myofibroblast differentiation by transforming growth factor-beta 1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J Biol Chem 278:12384-12389

Thompson K, Hamilton DW, Leask A. 2010. ALK5 Inhibition Blocks TGF beta-induced CCN2 Expression in Gingival Fibroblasts. J Dent Res 89:1450-1454.

Thomson P. 2012. Oral Precancer. Applied Anatomy of the Oral Cavity. In “Oral Precancer: Diagnosis and Management of Potentially Malignant Disorders.” Wiley-Blackwell.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nature Reviews Molecular Cell Biology 3

Toriseva MJ, Ala-aho R, Karvinen J, Baker AH, Marjomaki VS, Heino J, Kahari V. 2007. Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts. J Invest Dermatol 127:49-59.

Trojanowska M. 2008. Role of PDGF in fibrotic diseases and systemic sclerosis. Rheumatology 47: v2–v4.

Trojanowska M. 2009. Noncanonical transforming growth factor beta signaling in scleroderma fibrosis. Curr Opin Rheumatol 21:623-629.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. 1998. SARA, a FYVE domain protein that recruits smad2 to the TGFß receptor. Cell 95:779–791.

Uzel MI, Kantarci A, Hong HH, Uygur C, Sheff MC, et al. 2001.Connective tissue growth factor in drug-induced gingival overgrowth. J Periodontol 72: 921–931.

Vancheeswaran R, Azam A, Black C, Dashwood MR. 1994. Localization of Endothelin-1 and its Binding-Sites in Scleroderma Skin. J Rheumatol 21

Velnar T, Bailey T, Smrkoli V. 2009. The Wound Healing Process: an Overview of the Cellular and Molecular Mechanisms. J Int Med Res 37:1528-1542.

Verrecchia F, Mauviel A. 2007. Transforming growth factor-beta and fibrosis. World Journal of Gastroenterology 13:3056-3062.

Vi L. 2010. The Role of Integrin-Linked Kinase in TGF-β1 Induction of Dermal Myofibroblast Differentiation. Masters dissertation. University of Western Ontario, London, Ontario, Canada

Wang C, Deng L, Hong M, Akkaraju G, Inoue J, Chen Z. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346-351

Wang Q, Usinger W, Nichols B, Gray J, Xu L, Seeley TW, Brenner M, Guo G, Zhang W, Oliver N, Lin A, Yeowell D.2011.Cooperative interaction of CTGF and TGF-β in animal models of fibrotic disease. Fibrogenesis Tissue Repair. 4(1):4.

Wang W, Zhou G, Hu M, Yao Z, Tan T. 1997. Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. J Biol Chem 272:22771-22775.

Weber L, Kirsch E, Müller P, Krieg T. 1984. Collagen type distribution and macromolecular organization of connective tissue in different layers of human skin. J Invest Dermatol;82(2):156-60.

Werner S, Grose R. 2003. Regulation of wound healing by growth factors and cytokines. Physiol Rev 83

White BJ, Tarabishy S, Venna VR, Manwani B, Benashski S, McCullough LD, Li J. 2012. Protection from cerebral ischemia by inhibition of TGFβ-activated kinase. Exp Neurol;237(1):238-45.

Wikesjo UME, Nilveus RE, Selvig KA. 1992. Significance of Early Healing Events on Periodontal Repair - a Review. J Periodontol 63

Wilkes MC, Mitchell H, Gulati-Penheiter S, Dore´ JJ, Suzuki K, Edens M, Sharma DK, Pagano RE, Leof EB. 2005. Transforming growth factor-b activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. Cancer Res 65:10431–10440.

Wilkes MC, Murphy SJ, Garamszegi N, Leof EB. 2003. Cell-type-specific activation of PAK2 by transforming growth factor ß independent of Smad2 and Smad3. Mol Cell Biol 23:8878–8889.

Wolff K, Goldsmith L, Katz S, Gilchrest B, Paller A, Leffell D. 2007. Overview of Biology Development and Structure of Skin. In “Fitzpatrick's Dermatology in General Medicine.” McGraw-Hill Professional

Wu J, Powell F, Larsen NA, Lai Z, Byth KF, Read J, Gu RF, Roth M, Toader D, Saeh JC, Chen H. 2013.Mechanism and in vitro pharmacology of TAK1 inhibition by (5Z)-7-Oxozeaenol. ACS Chem Biol. 8(3):643-50

Wynn TA. 2008. Cellular and molecular mechanisms of fibrosis. J Pathol 214:199-210.

Xie M, Zhang D, Dyck JR et al. (2006) A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. Proc Natl Acad Sci USA 103(46):17378–83

Yamaguchi K, Shirakabe T, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. 1995. Identification of a Member of the Mapkkk Family as a Potential Mediator of Tgf-Beta Signal-Transduction. Science 270:2008-2011.

Yamane K, Miyauchi T, Suzuki N, Yuhara T, Akama T, Suzuki H, Kashiwagi H. 1992. Significance of plasma endothelin-1 levels in patients with systemic sclerosis.J. Rheumatol. 19(10):1566-71.

Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. 2008. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. Mol Cell 31:918-924.

Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. 1999.Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J. 18(5):1280-91.

Yasuda M. Miyachi Y, Ishikawa O, Takahashi K. 2006. Spatial expressions of fibronectin and integrins by human and rodent dermal fibroblasts. Cutaneous biology 155: 522–531.

Zeng G, McCue HM, Mastrangelo L, Millis AJ. 1996. Endogenous TGF-beta activity is modified during cellular aging:effects on metalloproteinase and TIMP-1 expression. Exp Cell Res. 228: 271–6.

Zhang D, Gaussin V, Taffet GE et al. 2000. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. Nat Med 6:556–63

Zhang D, Hu Y, Sun Q, Zhao J, Cong Z, Liu H, Zhou M, Li K, Hang C. 2013. Inhibition of transforming growth factor beta-activated kinase 1 confers neuroprotection after traumatic brain injury in rats. Neuroscience.238:209-17

Zhang YE. 2009. Non-Smad pathways in TGF-beta signaling. Cell Res 19:128-139.

Zheng B, Zhang Z, Black CM et al. 2002. Ligand-dependent genetic recombination in fibroblasts: a potentially powerful technique for investigating gene function in fibrosis. Am J Pathol 160:1609–17

Zhou S, Zawel L, Lengauer C, Kinzler KW, Vogelstein B.1998. Characterization of human FAST-1, a TGF beta and activin signal transducer. Mol Cell. 2(1):121-7.

Zhu Y, Richardson JA, Parada LF Graff JM, 1998. Smad3 Mutant Mice Develop Metastatic Colorectal Cancer. Cell 94(6):703-714.

Appendix

Supplemental Table 3.3

Supplemental Table 3.4

Permission to Reprint Figures

Curriculum Vitae

Supplemental Table 3.3. List of genes sensitive to TAK1 inhibition with over 1.7 fold induction in response to TGFβ-1 treatment (N=2).

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
7918857	GU971730	*TSPAN2*	12.5003
8067233	AJ308020	*PMEPA1*	9.40699
7950933	AB041035	*NOX4*	8.49418
8116921	AK291838	*EDN1*	6.7818
8109159	ENST00000384967	*MIR145*	4.99812
8156919	AK300099	*MURC*	4.93433
8112045	AK300634	*ESM1*	4.31011
8131600	BC033863	*TSPAN13*	4.15714
7970793	AK093932	*SLC46A3*	4.09995
8114572	BC033097	*HBEGF*	4.0607
7922162	AB209540	*SLC19A2*	4.05461
7972750	BC151220	*COL4A1*	3.80166
8175261	ENST00000385270	*MIR503*	3.63312
7962579	AY454159	*AMIGO2*	3.45511
7933075	AF086500	*FZD8*	3.37736
8095043	AY839725	*RASL11B*	3.35287
7954511	AB023182	*STK38L*	3.3435
8008885	ENST00000362134	*MIR21*	3.25119
8035517	AK074508	*COMP*	3.02008
8088550	AK127113	*PRICKLE2*	3.00856
8148304	AF205437	*TRIB1*	2.96456
8123936	AK292682	*NEDD9*	2.93624
7922328	ENST00000385289	*MIR199A2*	2.91144
8023220	AF010193	*SMAD7*	2.841
8077490	AF216709	*LMCD1*	2.81825
7966026	BC152462	*NUAK1*	2.68415
7987315	BC009978	*ACTC1*	2.64134
7958352	AK091276	*BTBD11*	2.63841
7962689	AF026260	*VDR*	2.55848
8160439	ENST00000362307	*MIR31*	2.53933
8113504	ENST00000446294	*NREP*	2.53329
8115490	AF134707	*ADAM19*	2.44597
7900510	AK130549	*CTPS1*	2.44545
8161701	AF137030	*TMEM2*	2.43863
8023995	AK291958	*FSTL3*	2.43461
8056184	AK290300	*ITGB6*	2.41065
8147012	BC022265	*PKIA*	2.41012
8024485	AF078077	*GADD45B*	2.39958
7990873	AB095929	*MEX3B*	2.38217
7974335	ENST00000515947	*RN5S385*	2.38034

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
8159900	BC033899	*GLIS3*	2.37678
8160435	AY358570	*IFNE*	2.3663
8143154	AK091081	*DGKI*	2.34734
8109157	NR_029684	*MIR143*	2.33962
7958202	AB042326	*CHST11*	2.28914
8169240	AK297968	*PRPS1*	2.2807
8136341	BC017050	*BPGM*	2.27611
8151334	BC006313	*MSC*	2.26058
8121257	AF084199	*PRDM1*	2.25456
7950391	AB019210	*PGM2L1*	2.25386
7926545	AF378757	*PLXDC2*	2.23225
7933872	AF139463	*EGR2*	2.22617
7934434	AF240633	*MYOZ1*	2.2035
8135069	AK293248	*SERPINE1*	2.17387
7993588	AK094664	*TMC7*	2.17183
8112803	BC136335	*LHFPL2*	2.14818
7970033	ENST00000400163	*COL4A2*	2.14259
8057990	AK301209	*ANKRD44*	2.13067
7997642	AK027395	*CRISPLD2*	2.12702
7959361	BC151841	*MLXIP*	2.12286
8160637	AK297541	*B4GALT1*	2.12002
7972557	BC020752	*GPR183*	2.10273
8023415	AK299169	*TCF4*	2.10147
7923173	ENST00000385240	*MIR181B1*	2.09207
8137670	AK292217	*PDGFA*	2.07886
8041206	AF110224	*LBH*	2.07127
8072678	BC001491	*HMOX1*	2.05866
8109426	ENST00000524264	*FLJ38109*	2.05775
8022674	BC036470	*CDH2*	2.04821
8139207	AK290584	*INHBA*	2.04306
8114970	AY358656	*C5orf46*	2.04245
7950374	AK300598	*P4HA3*	1.99257
7905428	AK299925	*TUFT1*	1.98978
8116418	AB016789	*GFPT2*	1.98967
7906995	BT006860	*UCK2*	1.98699
8111430	EF560721	*AMACR*	1.97704
8123104	BC146783	*FNDC1*	1.9678
8156571	ENST00000385129	*MIR27B*	1.96393
7936322	AL833061	*GPAM*	1.95685
7979710	AF228603	*PLEK2*	1.95078
7922773	AF527950	*NCF2*	1.94535
8162940	AB055982	*ABCA1*	1.94403
7965094	BC017481	*E2F7*	1.93429

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
7991080	AK302992	*BNC1*	1.93384
7972239	BC101070	*SLITRK6*	1.93138
7922976	AK292167	*PTGS2*	1.92186
8063634	BC002831	*MGC4294*	1.91941
8021707	BC020082	*SOCS6*	1.91887
8094751	BC113549	*CHRNA9*	1.91164
8156573	AF480560	*MIR24-1*	1.91017
8102232	AF198532	*LEF1*	1.90975
7943803	AK055899	*DIXDC1*	1.90939
8035896	AK293267	*TSHZ3*	1.9092
7953981	AK289441	*ETV6*	1.90186
7934997	AK299392	*PPP1R3C*	1.89232
8139488	AK298143	*IGFBP3*	1.88748
7986789	BC038712	*ATP10A*	1.88652
8040473	AF498971	*RHOB*	1.88318
7944302	AB094090	*PHLDB1*	1.87993
8026047	BC004250	*JUNB*	1.87672
7974316	BC020521	*FRMD6*	1.8761
8154962	AF088982	*DNAJB5*	1.87319
7922889	ENST00000367498	*IVNS1ABP*	1.87315
7950555	BC070079	*LRRC32*	1.86387
8167013	ENST00000424392	*PHF16*	1.86357
8088776	AF146696	*FOXP1*	1.86169
8161945	ENST00000376447	*RASEF*	1.85336
8121685	AJ420528	*DCBLD1*	1.85226
8039484	AK290572	*IL11*	1.84543
8005661	AK304186	*SPECC1*	1.84376
8109407	AB078145	*GALNT10*	1.82627
8060850	ENST00000378827	*BMP2*	1.81767
8020455	U66075	*GATA6*	1.81052
7909494	BC130323	*SYT14*	1.80486
8103695	AK290041	*MFAP3L*	1.79877
8095767	AK074237	*C4orf26*	1.79371
8026198	ENST00000440752	*ZSWIM4*	1.79067
7965112	ENST00000550006	*PAWR*	1.78964
7922326	ENST00000385214	*MIR3120*	1.78538
8092836	BC031569	*CPN2*	1.77686
8054997	AK074577	*MAP3K2*	1.77627
8056257	U76833	*FAP*	1.77509
7999909	AF181862	*GPRC5B*	1.76782
7944722	AF425252	*UBASH3B*	1.75863
8157253	ENST00000374232	*SNX30*	1.75667
8072705	AF279143	*RASD2*	1.75216

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
8157802	ENST00000385004	*MIR181B2*	1.74598
8142415	AK299901	*C7orf60*	1.74074
8058477	AK297312	*KLF7*	1.73553
8047738	AL833606	*NRP2*	1.73487
8010061	AF200328	*SPHK1*	1.72518
8025402	AB056477	*ANGPTL4*	1.72498
8151816	BC022010	*GEM*	1.72429
7956271	AF016509	*HSD17B6*	1.7231
8104035	AK056628	*SORBS2*	1.72124
8155192	AK091288	*GLIPR2*	1.71552
7943998	BC000234	*NNMT*	1.711
7901993	AK299361	*CACHD1*	1.70334
8141241	ENST00000361125	*SMURF1*	1.70312

Supplemental Table 3.4. List of genes with over 1.7 fold induction in response to TGFβ-1 treatment (N=2).

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
7918857	GU971730	*TSPAN2*	12.5003
8067233	AJ308020	*PMEPA1*	9.40699
7950933	AB041035	*NOX4*	8.49418
8116921	AK291838	*EDN1*	6.7818
7974689	AF251079	*DACT1*	5.38907
8109159	ENST00000384967	*MIR145*	4.99812
8156919	AK300099	*MURC*	4.93433
8112045	AK300634	*ESM1*	4.31011
8131600	BC033863	*TSPAN13*	4.15714
7970793	AK093932	*SLC46A3*	4.09995
8114572	BC033097	*HBEGF*	4.0607
7922162	AB209540	*SLC19A2*	4.05461
7972750	BC151220	*COL4A1*	3.80166
8175261	ENST00000385270	*MIR503*	3.63312
7962579	AY454159	*AMIGO2*	3.45511
7933075	AF086500	*FZD8*	3.37736
8095043	AY839725	*RASL11B*	3.35287
7954511	AB023182	*STK38L*	3.3435
8008885	ENST00000362134	*MIR21*	3.25119
8035517	AK074508	*COMP*	3.02008
8088550	AK127113	*PRICKLE2*	3.00856
8083876	AK300053	*SKIL*	2.96522
8148304	AF205437	*TRIB1*	2.96456
8123936	AK292682	*NEDD9*	2.93624
7922328	ENST00000385289	*MIR199A2*	2.91144
8023220	AF010193	*SMAD7*	2.841
8077490	AF216709	*LMCD1*	2.81825
7966026	BC152462	*NUAK1*	2.68415
7987315	BC009978	*ACTC1*	2.64134
7958352	AK091276	*BTBD11*	2.63841
8178435	AF083421	*IER3*	2.57544
8129562	AK290884	*CTGF*	2.55987
7962689	AF026260	*VDR*	2.55848
8160439	ENST00000362307	*MIR31*	2.53933
8077441	AB004066	*BHLHE40*	2.5374
8113504	ENST00000446294	*NREP*	2.53329
8124848	BC005080	*IER3*	2.51526
8179704	AF083421	*IER3*	2.51526

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
8115490	AF134707	*ADAM19*	2.44597
7900510	AK130549	*CTPS1*	2.44545
8161701	AF137030	*TMEM2*	2.43863
8023995	AK291958	*FSTL3*	2.43461
8056184	AK290300	*ITGB6*	2.41065
8147012	BC022265	*PKIA*	2.41012
8024485	AF078077	*GADD45B*	2.39958
7990873	AB095929	*MEX3B*	2.38217
7974335	ENST00000515947	*RN5S385*	2.38034
8159900	BC033899	*GLIS3*	2.37678
8160435	AY358570	*IFNE*	2.3663
8143154	AK091081	*DGKI*	2.34734
8109157	NR_029684	*MIR143*	2.33962
7958202	AB042326	*CHST11*	2.28914
8169240	AK297968	*PRPS1*	2.2807
8136341	BC017050	*BPGM*	2.27611
8151334	BC006313	*MSC*	2.26058
8121257	AF084199	*PRDM1*	2.25456
7950391	AB019210	*PGM2L1*	2.25386
7926545	AF378757	*PLXDC2*	2.23225
7933872	AF139463	*EGR2*	2.22617
7934434	AF240633	*MYOZ1*	2.2035
8152215	BT006634	*KLF10*	2.18971
8135069	AK293248	*SERPINE1*	2.17387
7993588	AK094664	*TMC7*	2.17183
8112803	BC136335	*LHFPL2*	2.14818
7970033	ENST00000400163	*COL4A2*	2.14259
8057990	AK301209	*ANKRD44*	2.13067
7997642	AK027395	*CRISPLD2*	2.12702
7959361	BC151841	*MLXIP*	2.12286
8160637	AK297541	*B4GALT1*	2.12002
7972557	BC020752	*GPR183*	2.10273
8023415	AK299169	*TCF4*	2.10147
7923173	ENST00000385240	*MIR181B1*	2.09207
8137670	AK292217	*PDGFA*	2.07886
8041206	AF110224	*LBH*	2.07127
8072678	BC001491	*HMOX1*	2.05866
8109426	ENST00000524264	*FLJ38109*	2.05775
8022674	BC036470	*CDH2*	2.04821
8139207	AK290584	*INHBA*	2.04306
8114970	AY358656	*C5orf46*	2.04245
7950374	AK300598	*P4HA3*	1.99257
7905428	AK299925	*TUFT1*	1.98978

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
8116418	AB016789	*GFPT2*	1.98967
7906995	BT006860	*UCK2*	1.98699
8111430	EF560721	*AMACR*	1.97704
8123104	BC146783	*FNDC1*	1.9678
8156571	ENST00000385129	*MIR27B*	1.96393
7936322	AL833061	*GPAM*	1.95685
7979710	AF228603	*PLEK2*	1.95078
7922773	AF527950	*NCF2*	1.94535
8162940	AB055982	*ABCA1*	1.94403
7965094	BC017481	*E2F7*	1.93429
7991080	AK302992	*BNC1*	1.93384
7972239	BC101070	*SLITRK6*	1.93138
7922976	AK292167	*PTGS2*	1.92186
8063634	BC002831	*MGC4294*	1.91941
8021707	BC020082	*SOCS6*	1.91887
8094751	BC113549	*CHRNA9*	1.91164
8156573	AF480560	*MIR24-1*	1.91017
8102232	AF198532	*LEF1*	1.90975
7943803	AK055899	*DIXDC1*	1.90939
8035896	AK293267	*TSHZ3*	1.9092
7953981	AK289441	*ETV6*	1.90186
7934997	AK299392	*PPP1R3C*	1.89232
8139488	AK298143	*IGFBP3*	1.88748
7986789	BC038712	*ATP10A*	1.88652
8040473	AF498971	*RHOB*	1.88318
7944302	AB094090	*PHLDB1*	1.87993
8026047	BC004250	*JUNB*	1.87672
7974316	BC020521	*FRMD6*	1.8761
8154962	AF088982	*DNAJB5*	1.87319
7922889	ENST00000367498	*IVNS1ABP*	1.87315
7950555	BC070079	*LRRC32*	1.86387
8167013	ENST00000424392	*PHF16*	1.86357
8088776	AF146696	*FOXP1*	1.86169
8161945	ENST00000376447	*RASEF*	1.85336
8121685	AJ420528	*DCBLD1*	1.85226
8039484	AK290572	*IL11*	1.84543
8005661	AK304186	*SPECC1*	1.84376
8109407	AB078145	*GALNT10*	1.82627
8060850	ENST00000378827	*BMP2*	1.81767
8020455	U66075	*GATA6*	1.81052
7909494	BC130323	*SYT14*	1.80486
8103695	AK290041	*MFAP3L*	1.79877

Affymetrix ID	Genbank Accession	Gene Name	Fold Increase
8095767	AK074237	*C4orf26*	1.79371
8026198	ENST00000440752	*ZSWIM4*	1.79067
7965112	ENST00000550006	*PAWR*	1.78964
7922326	ENST00000385214	*MIR3120*	1.78538
8092836	BC031569	*CPN2*	1.77686
8054997	AK074577	*MAP3K2*	1.77627
8056257	U76833	*FAP*	1.77509
7999909	AF181862	*GPRC5B*	1.76782
7944722	AF425252	*UBASH3B*	1.75863
8157253	ENST00000374232	*SNX30*	1.75667
8072705	AF279143	*RASD2*	1.75216
8157802	ENST00000385004	*MIR181B2*	1.74598
8142415	AK299901	*C7orf60*	1.74074
8058477	AK297312	*KLF7*	1.73553
8047738	AL833606	*NRP2*	1.73487
8010061	AF200328	*SPHK1*	1.72518
8025402	AB056477	*ANGPTL4*	1.72498
8151816	BC022010	*GEM*	1.72429
7956271	AF016509	*HSD17B6*	1.7231
8104035	AK056628	*SORBS2*	1.72124
8155192	AK091288	*GLIPR2*	1.71552
7943998	BC000234	*NNMT*	1.711
7901993	AK299361	*CACHD1*	1.70334
8141241	ENST00000361125	*SMURF1*	1.70312

PERMISSION TO REPRINT FIGURES

ELSEVIER LICENSE
TERMS AND CONDITIONS
Feb 14, 2014

This is a License Agreement between Hanna Kuk ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Registered Company Number	1982084
Customer name	Hanna Kuk

License number	3327130268498
License date	Feb 13, 2014
Licensed content publisher	Elsevier
Licensed content publication	Progress in Lipid Research
Licensed content title	Bioactive lipid mediators in skin inflammation and immunity
Licensed content author	Alexandra C. Kendall, Anna Nicolaou
Licensed content date	January 2013
Licensed content volume number	52
Licensed content issue number	1
Number of pages	24
Start Page	141
End Page	164
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	TAK1 mediates TGFb1 responses in gingival fibroblasts

Expected completion date	May 2014
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis
Date: Sat, 15 Feb 2014

Dear Hanna - all such requests should be addressed to the publishers.
Kind regards
A.Nicolaou

Subject: Re: Permission to Use Copyrighted Material in a Master's Thesis
Date: February 14, 2014

Dear Dr. Nicolaou,

I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.

I would like permission to allow inclusion of the following material in my thesis:
Alexandra C. Kendall, Anna Nicolaou.Bioactive lipid mediators in skin inflammation and immunity.Progress in Lipid Research 52 (2013) 141–164.
http://dx.doi.org/10.1016/j.plipres.2012.10.003
The material will be attributed through a citation.
Please confirm in writing that these arrangements meet with your approval.
Sincerely,
Hanna Kuk

ELSEVIER LICENSE TERMS AND CONDITIONS
Feb 06, 2014

This is a License Agreement between Hanna Kuk ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Registered Company Number	1982084
Customer name	Hanna Kuk

License number	3323100829055
License date	Feb 06, 2014
Licensed content publisher	Elsevier
Licensed content publication	The American Journal of Pathology
Licensed content title	The Myofibroblast: One Function, Multiple Origins
Licensed content author	Boris Hinz,Sem H. Phan,Victor J. Thannickal,Andrea Galli,Marie-Luce Bochaton-Piallat,Giulio Gabbiani
Licensed content date	June 2007
Licensed content volume number	170
Licensed content issue number	6
Number of pages	10
Start Page	1807
End Page	1816
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	TAK1 mediates TGFb1 responses in gingival fibroblasts
Expected completion date	May 2014

Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Date: Wed, 5 Feb 2014
Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis

Hi Hanna,
From my perspective, you are invited to use the requested material for your Master thesis. However, you will have to obtain copyright clearance from the publisher (AJP).
This is usually free of charge for scientific purposes and can be done through a web provider such as www.copyright.com<http://www.copyright.com>.
Best regards
Boris Hinz

Date: February 5, 2014
Subject: Permission to Use Copyrighted Material in a Master's Thesis

To whom it may concern,
I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.
I would like permission to allow inclusion of the following material in my thesis:

Figure 2. One cell, multiple origins, *in* "The myofibroblast: one function, multiple origins. Am J Pathol. 2007 Jun;170(6):1807-16. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G."

The material will be attributed through a citation.
Please confirm in writing or by email that these arrangements meet with your approval.
Sincerely,
Hanna Kuk
Date: Mon, 10 Feb 2014

Subject: 00510010

Dear Hanna

Thank you for contacting BioMed Central.

Reproduction of figures or tables is permitted free of charge and without formal written permission from the publisher or the copyright holder, provided that the figure/table is original, BioMed Central is duly identified as the original publisher, and that proper attribution of authorship and the correct citation details are given as acknowledgement.

If you have any questions please do not hesitate to contact me.

Best wishes
Eleanor Lee
Customer Services

Subject: Permission to Use Copyrighted Material in a Master's Thesis
Date: February 8, 2014

To whom it may concern
I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.

I would like permission to allow inclusion of the following material in my thesis:
Figure 2. Schematic diagram of general and gene-specific transforming growth factor (TGF) signaling in fibroblasts. in "Scar wars: is TGFβ the phantom menace in scleroderma?"Andrew Leask. Arthritis Res Ther. 2006; 8(4): 213. doi: 10.1186/ar1976

The material will be attributed through a citation.
Please confirm in writing that these arrangements meet with your approval.
Sincerely,
Hanna Kuk

OXFORD UNIVERSITY PRESS LICENSE
TERMS AND CONDITIONS
Feb 06, 2014

This is a License Agreement between Hanna Kuk ("You") and Oxford University Press ("Oxford University Press") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Oxford University Press, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3323110071613
License date	Feb 06, 2014
Licensed content publisher	Oxford University Press
Licensed content publication	Cardiovascular Research
Licensed content title	The role of TGF-β signaling in myocardial infarction and cardiac remodeling:
Licensed content author	Marcin Bujak, Nikolaos G. Frangogiannis
Licensed content date	05/01/2007
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	TAK1 mediates TGFb1 responses in gingival fibroblasts
Publisher of your work	n/a
Expected publication date	May 2014
Permissions cost	0.00 USD
Value added tax	0.00 USD
Total	0.00 USD
Total	0.00 USD

Date: Wed, 5 Feb 2014
Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis

Dear Ms Kuk,
please feel free to use material from this manuscript.
with best regards

Nikolaos Frangogiannis, MD
Albert Einstein College of Medicine

Subject: Permission to Use Copyrighted Material in a Master's Thesis
Date: February 5, 2014

Dear Dr. Frangogiannis,

I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.

I would like permission to allow inclusion of the following material in my thesis:
Figure 2. The diverse, multifunctional, and pleiotropic effects of TGF-β on cell types involved in infarct healing, *in* "The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res. 2007 May 1;74(2):184-95. Epub 2006 Oct 7. Bujak M, Frangogiannis NG."

The material will be attributed through a citation.

Please confirm in writing or by email that these arrangements meet with your approval.

Sincerely,
Hanna Kuk

Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis
Date: Fri, 7 Feb 2014
Dear Hanna Kuk,

Permission is granted with no charge.

The acknowledgement should state "reproduced / adapted with permission" and give the source journal name - the acknowledgement should either provide full citation details or refer to the relevant citation in the article reference list - the full citation details should include authors, journal, year, volume, issue and page citation.
Where appearing online or in other electronic media, a link should be provided to the original article (e.g. via DOI).

Development: dev.biologists.org
Disease Models & Mechanisms: dmm.biologists.org
Journal of Cell Science: jcs.biologists.org
The Journal of Experimental Biology: jeb.biologists.org

Best wishes,
Kasia Ladds

Subject**:** Permission to Use Copyrighted Material in a Master's Thesis
Date: February 6, 2014

To whom it may concern
I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.
I would like permission to allow inclusion of the following material in my thesis:
Figure 2. Signaling by CCN family members, *in* "All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci. 2006 Dec 1;119 (Pt 23):4803-10. Leask A, Abraham DJ."

The material will be attributed through a citation. Please confirm in writing that these arrangements meet with your approval.

Maney Publishing
Research • Knowledge • Innovation

www.maneypublishing.com

Hanna Kuk

Friday 7th March 2014

Dear Ms. Kuk

REF: Clocheret K1, Dekeyser C, Carels C, Willems G., 'Idiopathic gingival hyperplasia and orthodontic treatment: a case report.', Journal of Orthodontics, 2003 Mar;30(1):13-9., Figure 1b

I am writing to you in response to your request for permission to use the above figure to republish it in your Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts" at the University of Western Ontario. Permission is given subject to the proper acknowledgement of the publisher and copyright holder. Permission is also subject to acknowledgement of the original place of publication of the material listed above, in your forthcoming publication.

Please also list the URL for the online version of this journal:

http://jorthod.maneyjournals.org/

Please note that any elements of the article, whose copyright is owned by those other than the journal's copyright holder, will require further permissions prior to reuse. Please check all items carefully.

Please do contact me here with any further requirements or requests that you may have with regard to this matter.

Yours sincerely,

Rose Worrell
Editorial Assistant

Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis
Date: Thu, 6 Feb 2014

Dear,
You have my permission on the condition that you mail me a pdf version for personal use of your thesis. In addition you should also ask J Orthod for permission!
---
prof. dr. Guy Willems
orthodontie - forensische tandheelkunde
afdelingshoofd
orthodontics - forensic odontology
program director

Subject: Re: Permission to Use Copyrighted Material in a Master's Thesis
Date: February 5, 2014
Re: **Permission to Use Copyrighted Material in a Master's Thesis**

Dear Prof. Dr. Willems,
I am a University of Western Ontario graduate student completing my Master's thesis entitled "TAK1 Mediates TGFb1 Responses in Gingival Fibroblasts". My thesis will be available in full-text on the internet for reference, study and / or copy. Except in situations where a thesis is under embargo or restriction, the electronic version will be accessible through the Western Libraries web pages, the Library's web catalogue, and also through web search engines. I will also be granting Library and Archives Canada and ProQuest/UMI a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you.
I would like permission to allow inclusion of the following material in my thesis:
Figure 1(b) in Idiopathic gingival hyperplasia and orthodontic treatment: a case report. Clocheret K, Dekeyser C, Carels C, Willems G.J Orthod. 2003 Mar;30(1):13-9
The material will be attributed through a citation.
Please confirm in writing or by email that these arrangements meet with your approval.
Sincerely,
Hanna Kuk

CURRICULUM VITAE

Name: Hanna Kuk

Post-secondary Education and Degrees:
University of Western Ontario
London, Ontario, Canada
2008-2012 BMSc

The University of Western Ontario
London, Ontario, Canada
2012-2014 MSc

Honours and Awards:
Western Graduate Research Stipend – Physiology and Pharmacology
2012-2014

Dean's Honour List
2008-2009, 2010-2011, 2011-2012

Related Work Experience:
Teaching Assistant
The University of Western Ontario
2013-2014

Abstracts and Poster Presentations:
2013 Charles W. Gowdey Distinguished Lecture & Research Day
November 4, 2013.
The University of Western Ontairo

London Health Research Day
March 18, 2014
London, Ontario

Publications:
Kuk H. Carter D. Leask A.2014. 5Z-7-Oxozeanol inhibits the effects of TGFβ1 on human gingival fibroblasts. Wound Repair Regen. *Under Review*...

Relevant Technical Experience:

- Cell culture maintenance, amplification and cryo-preservation (Human Gingival Fibroblast; Human Foreskin Fibroblast cells)
- Isolation of total RNA from cell monolayers; phenol/chloroform extraction method
- TAQMan One-Step RT-qPCR
- Indirect Immunofluorescence procedure
- Western blot procedure
- Regular mice handling and breeding practices (C57BL/6J background)
- Genotyping (ear clip samples; Polymerase Chain Reaction)
- Intraperitoneal injections
- Euthanasia
- Harvest and fixation of lungs
- Tissue slides preparation for histological analysis