Elucidating the regulatory mechanisms of pterygium

Chunlai Fang

School of Biological Sciences,

University of East Anglia, Norwich, UK

June 2013

Acknowledgements

My most sincere thanks to my supervisor Dr. Michael Wormstone for his valuable ideas, advice and discussions over the past few years. I will never forget your help and guidance during my PhD. I would also like to thank all members of the Norwich Eye Group. Thanks for your generous help and friendship during our studies and non-academic lives. I am very proud to be a member of this group. Special thanks go to Dr Julie Eldred for her patiently technical support. In addition, I would like to thank the staff at the Norwich Eye Bank and Harbin Ophthalmology Hospital, especially Dr. Christopher D. Illingworth and Dr Limin Qian for providing donor research materials. I would like to thank the Humane Research Trust for their contribution to my studies. Finally, I offer endless thanks to my parents and my wife Ning Ma for their unconditional love, encouragement and support.

Abstract

Purpose: Pterygium is characterised as invasive, proliferative fibrovascular altered conjunctival tissue. The extensive vascular network is likely to significantly contribute to the progression of the disease. In the present study we investigated the effects of reduced serum (to mimic a suppressed blood supply) and treatment with transforming growth factor β on cell signalling and function of pterygial derived fibroblasts.

Methods: Pure fibroblast cultures were established from cell outgrowths of pterygial tissue. Immunochemistry techniques were used to identify cell phenotype, tissue features and cell signalling. Growth and migration of pterygial-derived fibroblast were evaluated using a patch growth assay, MTS assay and a scratch wound assay. Intracellular calcium levels were determined using Fura-2 detection in response to ligand stimulation using a 96-well plate format. RT-PCR and Western blot were utilized to detect cell transdifferentiation. Human angiogenesis protein array was used for investigating angiogenic activities in pterygial fibroblasts. Gene microarray was employed to provide a global profile of gene expression in unstimulated and treated (1ng/ml TGFβ2 and 10% serum) conditions.

Results: A progressive increase in serum level resulted in promotion of pterygial cell growth. A significant increase in intracellular calcium level was observed in response to histamine, ATP, acetylcholine and epidermal growth factor in serum-maintained cells. However, no significant changes were observed when cells were maintained in serum-free medium. 1μM thapsigargin induced a significantly greater increase in intracellular calcium level in the serum maintained group relative to serum starved cells. Pre-incubation of cells with 1μM thapsigargin ablated ligand-induced calcium responses. Disruption of calcium signalling through thapsigargin treatment significantly perturbed cell growth and migration. Smad2/Smad3 translocated to the nucleus in response to TGFβ in pterygial fibroblasts. TGFβ2 stimulated transdifferentiation of pterygial fibroblasts to myofibroblasts. Proteome Profiler™ Array data revealed that pterygial fibroblasts release angiogenic factors including IL-8 and VEGF following treatment with 10% serum and TGFβ2. Gene microarray demonstrated that a total of 103 genes were up-regulated and 53 genes downregulated by more than 2 fold in pterygial fibroblast treated with 10% serum. A total of 198 genes were up-regulated and 197 genes were down-regulated by more than 2 fold in pterygial fibroblast exposed to TGFβ2.

Conclusions: Calcium signalling was suppressed in pterygial-derived fibroblasts in response to serum-deprivation. The store plays a key role in cell growth and migration of pterygial derived fibroblasts. TGFβ induce the Smad signalling pathway and transdifferentiation in pterygial derived fibroblasts. Pterygial fibroblasts release a number of angiogenic factors and up-regulate transdifferentiation genes in response to serum or TGFβ2. Serum level/blood supply and TGFβ2 are likely to play a key role in the pathogenesis of pterygium.

TABLE OF CONTENT

Abbreviations

AAH	**Artificial aqueous humour**
ACh	**Acetylcholine**
ADP	**Adenosine Diphosphate**
ATP	**Adenosine Triphosphate**
AM	**Acetoxymethylester**
DAG	**Diacylglycerol**
DAPI	**4, 6-diamidino-2-phenylindole**
BCA	**Bicinchoninic acid assay**
DMSO	**Dimethyl sulfoxide**
DPBS	**Dulbecco's Phosphate Buffered Saline**
ECM	**Extracellular matrix**
EDTA	**Ethlenediaminetet raacetic acid**
EMEM	**Eagle's minimum essential medium**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
ER	**Endoplasmic reticulum**
ERK	**Extracellular signal-regulated kinase**

FCS	**foetal calf serum**
GAPDH	**Glyceraldehyde-3-phosphate**
GPCR	**G-protein coupled receptor**
HEPES	**hydroxyethyl piperazine ethanesulfonic acid**
IgG	**Immunoglobulin gamma**
IP_3	**Inositol (1, 4, 5) trisphosphate**
IL	**Interluekin**
MAPK	**Mitogen-activated protein kinase**
M-PER	**mammalian protein extraction reagent**
MMP	**Matrx metalloproteinase**
PBS	**Phosphate buffered saline**
PI3	**Phosphatidylinositol 3**
PIP2	**Phosphatidyl inositol 4, 5-bisphosphate**
PLC	**Phospholipase C**
PVDF	**polyvinylidene difluoride**
RT	**Room temperature**
RT-PCR	**Reverse transcriptase-polymerase chain reaction**
SEM	**Standard error mean**
SF	**Serum free**
ASMA	**Alpha smooth muscle actin**
SMAD	**Small body size mothers against DDP**

Tg	**Thapsigargin**
TGF	**Transforming growth factor**

CHAPTER 1

General introduction

1.1 Pterygium

Pterygium is characterized as invasive, proliferative fibrovascular altered conjunctival tissue and fleshy outgrow over the cornea. It is triangular shaped fibrovascular tissue on the epibulbar conjunctiva surface (figure 1.1). Pterygium grows most commonly from the nasal aspect of the sclera, proliferating on the naso-temporal. It is linked and thought to be caused by ultraviolet radiation (UVR), low humidity, and dust (Coroneo 1993). It is also related to other long-term chronic stimulus. In terms of its shape, pterygium is divided into three component regions. The first region is known as the head and is the portion that is seen growing on the cornea. The second component is termed the neck, which is appears as a small bulge located in the cornea-scleral limbus region. The final part is the main body, which extends across the sclera surface. Pterygium is characterized as a highly vascular tissue. Reports suggest vascular endothelial growth factor (VEGF) is highly expressed and more von Willebrand factor (vWF) is stained in new vessels in pterygium tissue compared with

normal conjunctiva (Marcovich, Morad *et al*. 2002). Therefore, angiogenesis is likely to play a role in pterygium (Marcovich, Morad *et al*. 2002; Aspiotis, Tsanou *et al*. 2007). The symptoms of pterygium include visual impairment when it invades the cornea, histogenesis degeneration, hypertrophy, hyperplasia, persistent redness, inflammation, foreign body sensation, dry eyes.

Pterygium can cause inflammation, irritation and may affect vision by inducing astigmatism or involvement of the visual axis. Pterygium has some tumour-like features like invading normal tissue and high recurrence rates after surgical removal. Although the pathogenesis of pterygium is unclear, the effect of ultraviolet (UV) exposure leading to limbal epithelial cell damage is commonly accepted as involved (Coroneo 1993; Coroneo, Di Girolamo *et al*. 1999). Pterygium is also associated with hypersensitive reactions, accelerated fibroblastic proliferation (Hill and Maske 1989) and it is suggested that pterygium is the result of subconjunctival invasion of fibroblasts that enter the cornea along natural tissue planes surrounding Bowman's layer (Cameron 1983). Furthermore, inflammatory cell infiltration and overexpression of extracellular matrix with alteration of the collagen and elastic fibres are also contributing factors (Cameron 1983; Karukonda, Thompson *et al*. 1995; Coroneo, Di Girolamo *et al*. 1999). In addition, heredity is also reported to play a role in pterygium (Hilgers 1960).

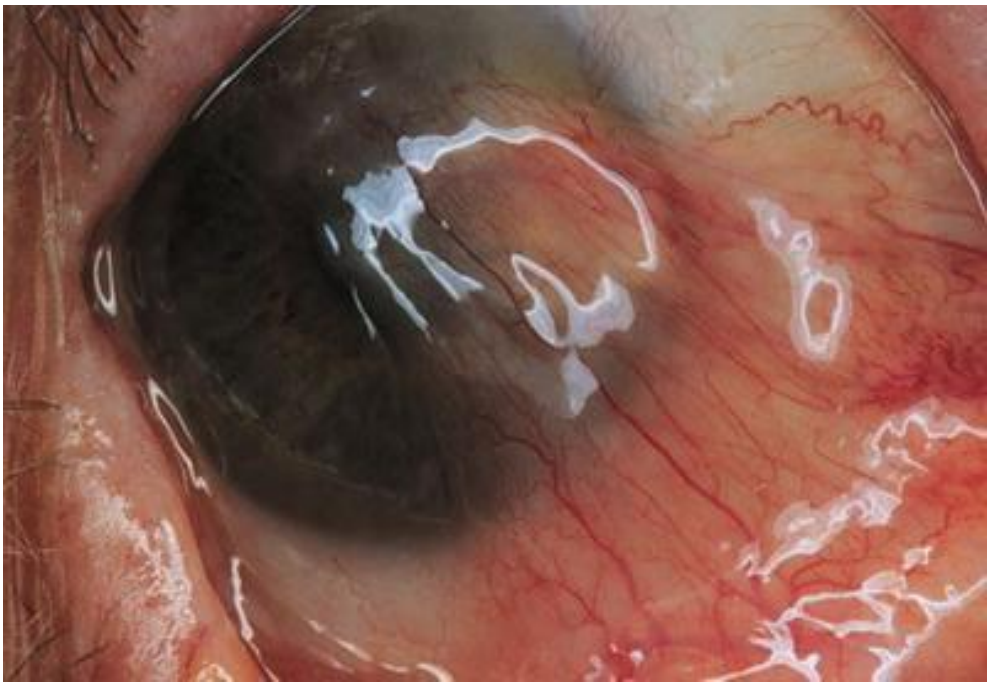


Figure 1.1. Pterygium formation in a patient. Note the triangular shape and extensive blood supply. Adapted from doctor.eye999.com/ 20080911/10280313.shtml

Today the choice of treatment for pterygium is controversial. A variety of options are available for the management of pterygium. At present, static type pterygium which has not violated the cornea and affected vision do not require surgery. If complications arise such as trachoma or chronic conjunctivitis, antibiotics or steroid eye drops can be used. If the pterygium covers the pupil, obstructs vision or presents with acute symptoms, current therapeutic approaches often employ conjunctival autografting, sliding conjunctival flaps, excimer laser treatment, and amniotic membrane transplants (Hirst 2003). In addition, due to the high recurrence rate, adjunctive therapies like radiation therapy (Nishimura, Nakai *et al*. 2000), intraoperative and postoperative mitomycin (Fruchtpery and Ilsar 1994; Lam, Wong *et al*. 1998) have been employed for preventing recurrence of pterygium.

1.2 Ultraviolet radiation and pterygium

It has been reported Ultraviolet radiation leads to limbal epithelial cell damage and induces the pterygium progression (Coroneo 1993; Coroneo, Di Girolamo *et al*. 1999). It showed dose dependent association between the ocular solar radiation and pterygium (Threlfall and English 1999). There are a number of epidemiological studies support the link between sun exposure and pterygium (McCarty, Fu *et al*. 2000; Paula, Thorn *et al*. 2006; Rim, Nam *et al*. 2013). Therefore, pterygium occurs frequently in tropical and equatorial climates. Many investigations confirmed a large number of

cases, outdoor workers (such as farmers, fishermen, pastoralists, etc.) who experience long-term radiation of sunlight, dust, snow and other stimulus result in the development of pterygium (Khoo, Saw *et al*. 1998; Wong, Foster *et al*. 2001; Al-Bdour and Al-Latayfeh 2004). Interestingly, the shadow of the nose partly reduces the intensity of sunlight focused on the lateral temporal limbus, thus pterygium normally occurs in nasal side (Coroneo 1993). The focal limbal irradiation may activate stem cells in the limbus (Di Girolamo, Chui *et al*. 2004) and UVB may induce the matrix metalloproteinases (MMPs) expression which result in extracellular matrix remodelling in development of pterygium through the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) intracellular pathway (Di Girolamo, Coroneo *et al*. 2003). It has been reported UVB increases various chemokines, cytokines and growth factors like IL-6, IL-8, and VEGF production in pterygium via MAPK pathway (Di Girolamo, Wakefield *et al*. 2006).

1.3 Physiological features of pterygium

1.3.1 Inflammation

Inflammation is a process of self-protection in human body to remove irritation. It happens in both normal physiological process like wound healing and pathology of disease like cancer. Chronic inflammation is from long-term irritation and consistently interferes with healthy tissues. It leads to increased blood flow, capillary permeability

elevation and induces white blood cell recruitment, which in turn release pro-inflammatory chemicals to results in angiogenesis (Leibovich, Polverini *et al*. 1987; Mantovani, Allavena *et al*. 2008). The chronic inflammatory process is an important characteristic in pterygium progression (Coroneo, Di Girolamo *et al*. 1999). UV light exposure promotes this inflammatory response by release of pro-inflammatory cytokines (Kennedy, Kim *et al*. 1997). Pterygial fibroblasts show increasing pro-inflammatory factors and their receptors (Chen, Tsai *et al*. 1994; Kria, Ohira *et al*. 1998). EGFR phosphorylation, NF-kappaB activation and increasing production of IL-1, IL-6, IL-8, and TNF alpha were detected in UV-mediated corneal cells (Kennedy, Kim *et al*. 1997; Song, Abraham *et al*. 2002). It was reported that pterygial cell responses with certain growth factors like EGF, TGF-alpha, IL-1 beta, bFGF, TGF-beta 1, TNF-alpha, or IL-6 to induce secretion of proinflammatory cytokines expression of MMPs. MMPs subsequently activate ECM remodelling, angiogenesis and fibroblast proliferation in pterygium (Soloman, Li *et al*. 2000). The end result of chronic inflammation is tissue fibrosis which is defined as cell proliferation and excessive extracellular matrix deposition (Wynn 2008).

1.3.2 Cell proliferation and migration

In normal conditions, cell growth refers to cell development and cell division. There are four phases of the cell cycle, which regulates cell division. These phases are G1, S,

G2 and M phase (mitosis). In division, one cell will produce two cells (Schafer 1998). Deregulation of cell proliferation is the hallmark in many cancers with cell overgrowth and increased cell division the result (Sherr 1996; Levine 1997). However, ultraviolet radiation exposure or other stimuli can induce conjunctival epithelial to undergo abnormal growth (proliferation) to produce excessive cells along the nasal limbus in the progression of pterygium (Kwok and Coroneo 1994). Cell migration is defined as the movement of cells to specific positions and is critical in tissue formation. Migration also plays an important role during wound healing responses (Martin 1997). Altered basal limbal epithelial cell migration is described as a key feature of pterygium formation (Dushku, John *et al*. 2001).

1.3.3 Angiogenesis

Angiogenesis is an important physiological process occurring in the human body with the growth of new blood vessels from pre-existing vessels in response to tissue demands (Risau 1997). It is a normal process in growth and development such as wound healing and dysregulation of angiogenesis also impacts on human health (Carmeliet 2003). Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently. Excessive angiogenesis occurs when diseased cells produce overwhelming angiogenic growth factors. It results in diseases such as cancer, infectious disease and age-related macular degeneration (Meyer, Clauss *et al*.

1999; Coussens and Werb 2002). In the case of cancer, the tumour recruits new blood vessels to support cell growth, surround the new capillary sprouts and metastases (Folkman 1995) and insufficient angiogenesis occurs in diseases such as stroke, heart and brain ischemia and hypertension (Boudier 1999; Kalimo, Ruchoux *et al*. 2002) when blood vessel growth is inadequate and circulation is not properly restored due to insufficient angiogenic growth factor production.

Angiogenesis is initiated when pre-existing vasculature becomes permeable due to diffusion of angiogenic growth factors. Once growth factors bind to their receptors, the endothelial cells (EC) are activated to send signals from the cell's surface to the nucleus. This results in degradation of extracellular matrix, cell proliferation and migration that ultimately form new blood vessels (Risau 1997). In the pathogenesis of pterygium, it has been reported that the abundance of the pterygium angiogenesis factors may irritate the limbal basal cells and produce vessel ingrowths to facilitate the formation of pterygium (Coroneo 1993). In addition, angiogenesis is controlled by the net balance of various pro-angiogenic and anti-angiogenic factors that regulatory angiogenic activity (Pepper 1997). This concept is known as ‘‘angiogenic switch,’’ the main "on" switches are known as angiogenesis-stimulating growth factors and angiogenesis inhibitors are the main "off switches". When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is inclined to blood

vessel growth while angiogenesis is stopped when the levels of inhibitors exceed the level of angiogenic stimulators. Numerous inducers of angiogenesis have been identified. The first angiogenic factor was isolated from tumours in the early 1970s and more factors were discovered in following years (Folkman and Klagsbrun 1987; Folkman and Shing 1992; Folkman 1995).

1.3.4 Transdifferentiation

Transdifferentiation is a process that permits a cell with a defined phenotype to transiently adopt the phenotype of another cell, which is typically a myofibroblast. This process can play a positive role in tissue reparation or occurs during pathological processes (Sisakhtnezhad and Matin 2012). Granulation tissue fibroblast (Myofibroblast) is a cell in differentiation between a fibroblast and a smooth muscle cell. Myofibroblasts synthesize extracellular matrix components such as collagen types I and III (Gabbiani 2003) and contract by using smooth muscle type actin-myosin complex that show abundant expression in the form of alpha-smooth muscle actin during normal wound healing or fibrocontractive diseases (Desmouliere, Geinoz *et al*. 1993). After normal wound healing is complete, myofibroblasts disappear by apoptosis but in some cases growth of myofibroblasts can persist. Continued myofibroblast proliferation and contraction can give rise to fibrotic disorders and cancer (Gabbiani 2003; Kellermann, Sobral *et al*. 2008).

Transdifferentiation is considered an important pathological process in pterygium; extracellular matrix deposition and connective tissue remodelling are major contributing factors (Cameron 1983; Karukonda, Thompson *et al*. 1995; Coroneo, Di Girolamo *et al*. 1999). Contractile myofibroblasts have previously been characterised in pterygium (Kellermann, Sobral *et al*. 2008).

1.3.5 Extracellular matrix remodelling

Extracellular matrix (ECM) is defined as connective tissue in extracellular protein network. ECM supports tissue structure and can mediate intercellular signal transduction (Giancotti and Ruoslahti 1999). Extracellular matrix plays an important role in cell growth and differentiation (Lin and Bissell 1993; Meredith, Fazeli *et al*. 1993). Collagens are the primary abundant proteins in the ECM that initiate fibrillogenesis (Kadler, Hill *et al*. 2008). When the ECM is deregulated, a variety of human diseases can occur. A number of studies demonstrated that fibrogenic cytokines like TGFβ and VEGF induce the expression of extracellular matrix proteins, mainly collagen deposition (Park, Keller *et al*. 1993; Leask and Abraham 2004; Bataller and Brenner 2005). In addition, MMPs are capable of degrading extracellular matrix (ECM) proteins and contribute pathological processes like tissue remodelling, angiogenesis and metastasis (Mott and Werb 2004). Extracellular matrix remodelling can promote inflammation and restructure the tissue (Stamenkovic 2003). It has

been suggested that extracellular matrix remodelling through the actions of MMPs and TIMPs are involved the pathogenesis of pterygium (Di Girolamo, Chui *et al*. 2004). In wound healing and fibrotic disease, contracting granulation tissue (myofibroblasts) activates the tissue dynamics and synthesizes ECM for rapid wound closure (Guidry 1992; Desmouliere, Geinoz *et al*. 1993; Gabbiani 2003).

1.4 Calcium signalling

Calcium signalling is widely known to play a number of important roles in the maintenance and physiological function of cells throughout the body. Our understanding of calcium signalling in pterygium is limited and it is of importance to address this key gap in our knowledge of this disease.

1.4.1 Intracellular Calcium

The primary signals received at the cell surface mediate this communication and then affect intracellular behaviour via second messenger relay signals from surface receptors (Parekh and Penner 1997). Calcium regulates the key cellular physiology processes like gene transcription, cell proliferation and metabolism (Berridge 1993). Unregulated cellular proliferation, transdifferentiation and angiogenesis are linked to Ca^{2+} signalling activities (Coroneo, Di Girolamo *et al*. 1999). The concentration of Ca^{2+} in the cytoplasm is typically kept in the range of 10–100nM. The binding of specific

receptor proteins on the plasma membrane (PM) results in a rapid increase to the 500-1000nM range from opening of calcium channels. The endoplasmic reticulum (ER) is the main intracellular Ca^{2+} store/release organelle. The cells can either elevate cytosolic Ca^{2+} by releasing Ca^{2+} from intracellular store or uptake Ca^{2+} into the cell from extracellular solution by store-operated calcium entry (SOCE) (Parekh and Penner 1997). The main signalling mechanism regulating calcium release from the endoplasmic reticulum is through phospholipase C pathway (PLC) which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the intracellular messengers inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (Bootman, Collins *et al*. 2001). Then InsP3 diffuses into the cell where it binds its receptors (InsP3Rs) on the ER/SR to release the calcium.

1.4.2 Receptor mediated pathway

Although IP_3 is the key in opening InsP3Rs, their activation is regulated by the calcium concentration at their cytosolic surface (Bootman, Collins *et al*. 2001). Phospholipase C enzymes are activated directly and indirectly by different cell surface receptors including growth factors, cytokines and G-protein coupled receptors (GPCR). The molecules that activate receptors are ligands. Cells could produce a response following stimulation by the extracellular ligand via second messengers. Therefore, the ligand-receptor interactions play a fundamental role on cell signalling. Two major

receptor-mediated pathways are involved in the formation of Inositol trisphosphate (IP_3) to release Ca^{2+} from ER. G-protein coupled receptors including histaminergic, purinergic and muscarinic receptors activate a common pathway by stimulation of PLCβ. Alternatively, receptor activation can activate PLCgamma to elevate IP_3 and mobilize the ER calcium store (Berridge 1993); this is typically associated with growth factors such as epidermal growth factor.

G protein coupled receptors (GPCRs) are one of largest superfamily of transmembrane receptor proteins in humans and affect a wide variety of cell physiological processes and biological functions. G protein coupled receptors form a modular system that detects extracellular molecules and transmits the signals over the plasma membrane into the intracellular domain and ultimately activates cellular responses. GPCRs are also called seven-transmembrane (7TM) domain receptors because they pass through the cell membrane seven times. Common structural features of GPCRs include a bundle of seven transmembrane alpha helices connected by six loops of varying lengths (Palczewski, Kumasaka *et al*. 2000). 7TM receptors play an important role in physiological processes in human which are the key targets for many pharmaceuticals drugs. Activation of PLC hydrolyzes the lipid precursor phosphatidylinositol 4, 5-bisphosphate (PIP2) to release DAG and IP_3. Then IP_3 binds to its receptor IP_3R to mobilize the intracellular calcium (Berridge 1993)(Figure 1.2).

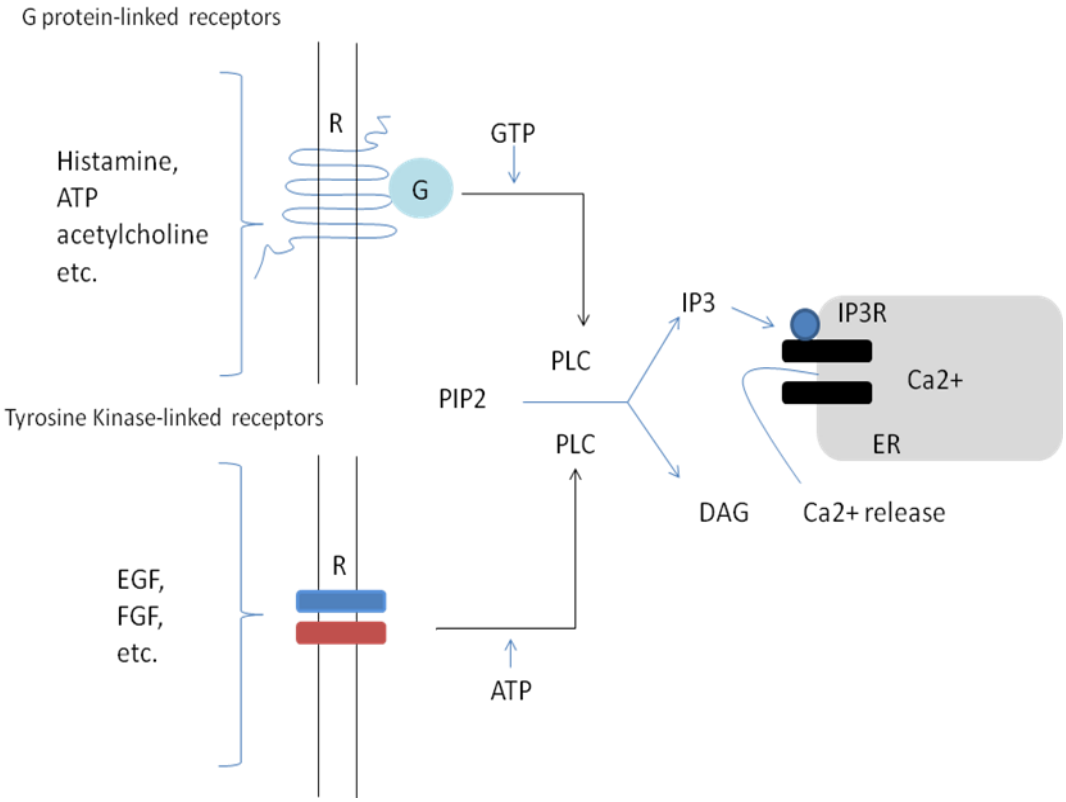


Figure 1.2. Two major receptor-mediated pathways involved in the formation of Inositol trisphosphate (IP_3) to release Ca^{2+} from ER. Modified from Calcium signalling, Martin D. Bootman 2001.

Receptor tyrosine kinases (RTK) are a diverse group of transmembrane proteins that act as high-affinity cell surface receptors for many polypeptide growth factors, cytokines, hormones and other signalling molecules. Receptor tyrosine kinases are involved in a variety of cellular processes, including growth, differentiation and angiogenesis. All tyrosine kinase receptors have a similar structure. They each have a tyrosine kinase domain that phosphorylates proteins on tyrosine residues, a hormone binding domain and a carboxyl terminal segment with multiple tyrosines for autophosphorylation. In calcium signalling, RTK stimulates PLCγ which generates IP_3 to activate the calcium store (Berridge 1993).

1.5 Transforming growth factor beta signalling

The pro-fibrotic factor transforming growth factor beta involves in many biological processes including cell growth, differentiation, apoptosis, angiogenesis and fibrosis (Roberts, Sporn *et al*. 1986; Massague 1990; Padgett and Patterson 2001). It is critical in tissue repair in human but excessive action of TGFβ also contributes to fibrosis disorder (Border and Ruoslahti 1992). TGFβ can induce angiogenesis and stimulates fibroblasts to produce collagens (Roberts, Sporn *et al*. 1986; Connor, Roberts *et al*. 1989) and it induces deposition of extracellular matrix and stimulates extracellular matrix component including collagens synthesis (Roberts, Sporn *et al*. 1986; Kagami,

Border *et al*. 1994). Pterygium is regarded as a fibrotic disease and TGFβ is commonly implicated in such conditions (Coroneo, Di Girolamo *et al*. 1999; Verrecchia and Mauviel 2007), yet it has been poorly studied in pterygium formation. Pterygium demonstrates the accelerated fibroblastic proliferation (Cameron 1983). Pterygial fibroblasts indicate a greater growth response as well as more release of growth factors compared with normal conjunctival fibroblasts in same conditions (Chen, Tsai *et al*. 1994; Kria, Ohira *et al*. 1998). It is reported that the fibrogenic stimuli may induce the transdifferentiation from fibroblasts to myofibroblasts which were also found in fibrovascular tissues of primary and recurrent pterygium (Dushku and Reid 1994; Kalluri and Neilson 2003). Transforming growth factorβ is a multifunctional protein, which belongs to TGFβ super family that is known to regulate cell growth, differentiation, apoptosis and immune functions (Border and Ruoslahti 1992). The TGFβ superfamily is comprised of a large group of proteins, including inhibitin, activin, anti-mullerian hormone, bone morphogenetic protein (BMPs) and growth differentiation factors (GDFs). TGFβ receptors are serine/threonine-protein kinase receptors and there are three different types of TGFβ receptors, TGFβ receptor 1, TGFβ receptor 2 and TGFβ receptor 3; these can be distinguished by their structural and functional properties (Cohen 1997; Massague 2000). Three isoforms namely TGFβ1, TGFβ2 and TGFβ3, they functionally interact with each other (Border and Ruoslahti 1992). Recent study has found that antagonists can block TGFβ signalling, which can inhibit the effects of fibrosis (Liu, Hu *et al*. 2006). The TGFβ response is cell

type specific and subject to activation of various signal transduction pathways (Figure 1.3). The Smad signalling pathway is the most studied mode of signal transduction in response to TGF-β (Derynck, Zhang *et al*. 1998). Smad proteins are distinguished by three functional classes: receptor-regulated Smad (Smads 2 and 3); co-Smad (Smad 4) and inhibitor Smad (Smad 7). Transforming growth factorβ ligands can specifically bind to and activate serine/threonine kinase cell-surface receptor (type II receptor) and activate various responses. Type II receptor is able to recruit and phosphorylates a type I receptor. Type I receptor phosphorylates receptor-regulated Smad2/3, which engages with Smad4 to form a complex to translocate to the nucleus to stimulate transcriptional regulation (Wu, Hu *et al*. 2001). Nuclear cofactors that associate with the Smad complex can regulate the transcription of target genes (Shi, Wang *et al*. 1998). Smad7 can negatively regulate TGFβ signalling by competing with R-Smads and consequently regulate gene transcription (Landström, Heldin *et al*. 2000).

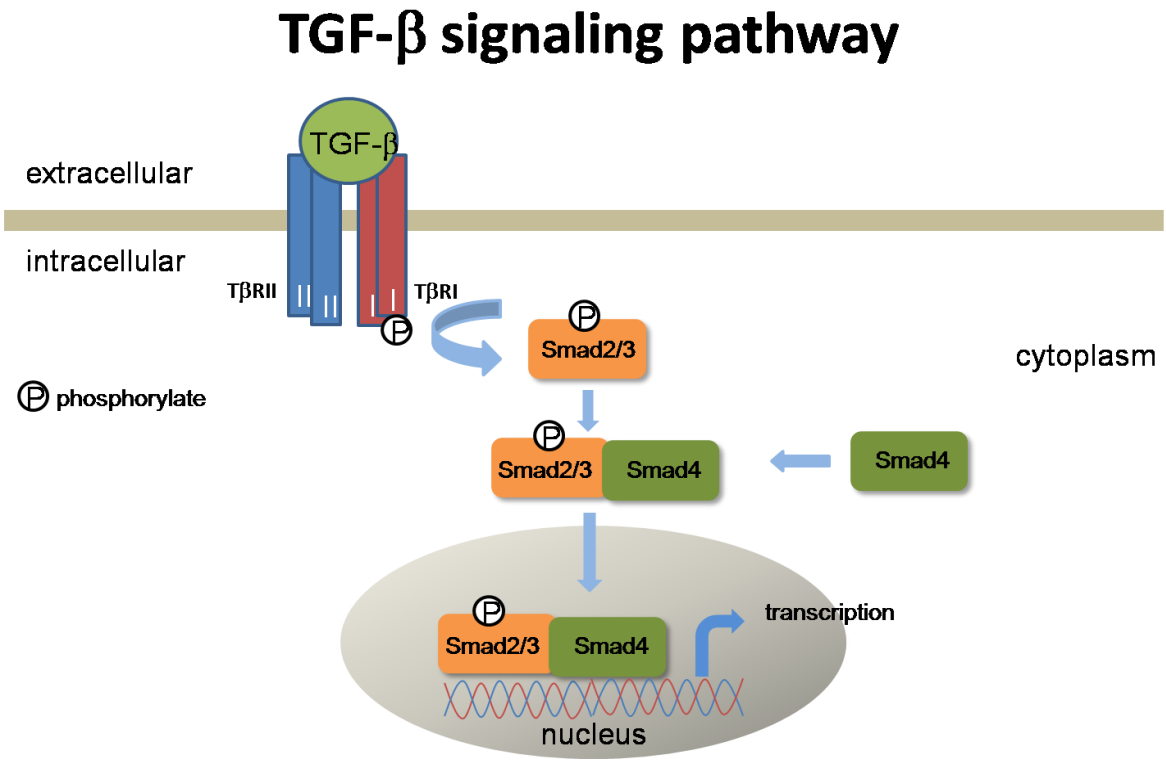


Figure 1.3. TGFβ signalling/Smad pathway. The current model of induction of signalling responses by TGF-β-related factors is a linear signalling pathway from the type II to the type I receptor kinase to Smad activation, resulting in ligand-induced transcription.

1.6 Genetic factors, viral infections and pterygium

UV irradiation leads to DNA damage which follows with gene mutation and genomic instability. Although the relation between pterygium and genetic factors are unclear but a family history is frequently reported. Elevation of P53 expression alteration, loss of heterozygosity (LOH) and genetic polymorphism of hOGG1 may be the co-factors to pterygium (Detorakis, Drakonaki *et al*. 2000; Kau, Tsai *et al*. 2004; Rodrigues, Arruda *et al*. 2008). The Ku70 promoter T-991C polymorphism is also a potential genetic marker for pterygium susceptibility (Tsai, Bau *et al*. 2007). It was reported that VEGF-460C polymorphism in female patients increases the risk of pterygium (Tsai, Chiang *et al*. 2008).

In addition, a number of studies identified oncogenic viruses in pterygia. Human papilloma virus (HPV) and herpes simplex virus (HSV) were detected in pterygium in several studies (Detorakis, Drakonaki *et al*. 2000). It has been reported HPV 16/18 was detected in 24% of the pterygium tissues but not in normal conjunctival tissues and 48.3% of HPV 16/18 DNA-positive pterygium tissues expressed HPV 16/18 E6 oncoprotein (Tsai, Chang *et al*. 2009). Epstein-Barr virus (EBV) was also detected in 10% of primary pterygial patients (Otlu, Emre *et al*. 2009).Therefore, viral infections may also contribute to the development of pterygium.

1.7 Aim

Pterygium is a highly vascular fibrotic disease. It is therefore important to understand the role the vascular system plays in the physiological behaviour of cell populations within pterygia and the factors that can promote fibrotic change. The current project involved studies aimed to address these issues. The main aims of the project were:

- To determine receptor mediated calcium signalling responses in the presence and absence of serum, which mimic a rich and poor blood supply.
- To determine the functional importance of calcium signalling in pterygial-derived fibroblasts
- To determine the ability of TGFbeta to induce fibrotic responses in pterygial-derived fibroblasts

CHAPTER 2

Materials and methods

All reagents were purchased from Sigma (Sigma, Poole, Dorset) unless otherwise stated.

2.1 Tissue culture

2.1.1 Isolation and culture of pterygial-derived fibroblasts

The pterygial tissues were obtained from patients at the Norfolk and Norwich University Hospital and Harbin Ophthalmological Hospital in China after surgery. The conjunctiva tissues were kindly provided by Norfolk and Norwich University Hospital. The use of human tissue in this study was in accordance of the Declaration of Helsinki. For experimental purposes cells were employed between passage 3 and 9. In some cases conjunctiva and pterygium were fixed and analysed using immunohistochemistry. In the majority of cases, pterygial samples were placed into an Eppendorf tube which contained Eagle's minimum essential medium (EMEM; Sigma, Poole, Dorset, U.K.) supplemented with 10% foetal calf serum (FCS; Gibco, Paisley) and 50 mg/l gentamicin. The tissue was dissected into ~5mm^2 pieces and transferred from the tube to a 35mm round tissue culture dish with 1.5ml fresh medium. Under the microscope the tissue was secured to the tissue culture dish

using entomological pins (D1, Watkins and Doncaster Ltd, Cranbook Kent, U.K)(figure 2.1A). The tissues were cultured in an incubator (maintained at 35^0 C in a 5% CO_2 atmosphere). Medium was replaced every 3-4 days. Viable preparations demonstrated pterygial cell outgrowth from the tissue onto culture dishes, these primary cultures demonstrated a mixed population of epithelial cells and fibroblasts (Figure 2.1B). Once a sufficient number of cells had grown onto the culture dish, the tissue was transferred to a new dish and the medium was removed from cells. Then the cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and then the pterygial cultures were trypsined with 0.02% (w/v) ethlenediaminetetraacetic acid (EDTA) in Hank's balanced saline solution for 2 to 5 minutes in incubator to detach the cells from the bottom but the remaining cells were released by tapping the base of the dish. The cell number was counted under the microscope with a haemocytometer. Afterwards, the cell suspension was centrifuged for 5 minutes at 1000rpm and the supernatant was removed. Cells were re-suspended in the new culture medium and plated either to culture dishes, flasks or coverslips and maintained at 35^0 C in 5% CO_2 atmosphere. Medium was changed every 2-3 days and were passaged to the new dishes. Finally, cultures contained pure population of fibroblasts (Figure 2.1D) that were identified immunocytochemically. The pterygial fibroblasts were seeded in the 96 well plates for MTS, calcium imaging, tissue culture dishes for immunocytochemistry, RT-PCR, Western blot, microarray, patch assay and scratch assay. Part of pterygial tissues were kept in 4% Formaldehyde and stored

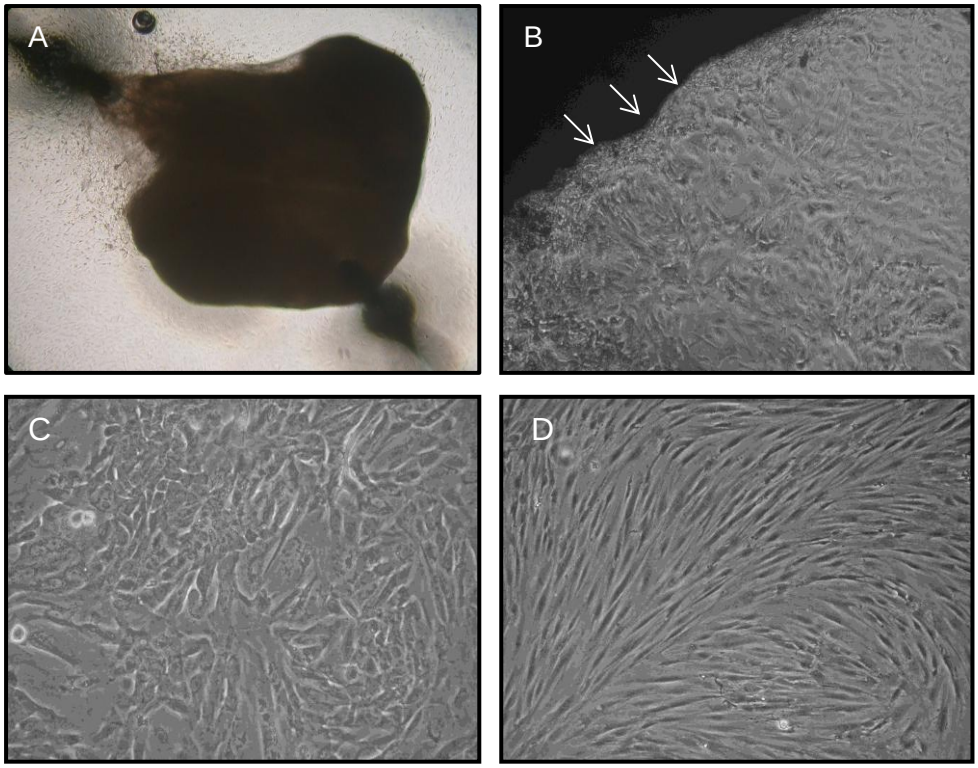


Figure 2.1: Morphologic characterization of pterygial cell outgrowth. (A) Native pterygial tissue pinned out onto a 35mm culture dish; (B) phase micrograph shows the cells outgrow from edge of the pterygial tissue (arrowed). (C) mixed populations of pterygial-derived (epithelial cells and fibroblasts) cells; (D) Cell cultures of passaged cells exhibiting a consistent fibroblast morphology. The field of view represents 4.2 x 3 mm (A), 2.1 x 1.5 mm (B) and 800 x 571 μm (C&D).

at 4°C for immunohistochemistry experiments.

2.1.2 Storing and recovering the cells

Cells were stored for future studies in case of cell contamination. Therefore cryopreservation was employed to store the cell lines. Following trypsinisation cells were resuspended in freezing medium (1ml 5-10% (v/v) DMSO) (Sigma, Poole, UK) to create a cell suspension of $1x10^6$ cells/ml and 1ml cell suspension was transferred to cryogenic vials (Corning, NY, USA). Vials were placed immediately to -20°C for one hour, followed by -80°C overnight before long term storage in liquid nitrogen (-197°C). When recovering the cells, medium was pre-warmed and cryovials were removed from liquid nitrogen and immediately placed in a 37°C water bath to quickly melt the freezing medium. The suspension was pipetted out into a flask with 10% FCS EMEM and placed in incubator at 35°C in a 5% CO_2 atmosphere. The medium was changed every 2-3 days until sufficient populations were established to permit experiments to take place.

2.1.4 Photomicroscopy

Phase contrast images of native and cultured pterygial cells were taken using a Nikon TE200 Eclipse Microscope (Nikon industries, Tokyo Japan) fitted with a Nikon coolpix 950 digital camera and MDC lens (Nikon industries, Tokyo Japan).

2.2 Histology

2.2.1 Paraffin embedding and slicing the sample

Fresh human conjunctival and pterygial tissue (typically thickness less than 0.5 cm) were stored in fixative solution (4% formaldehyde) to maintain the original morphology of cells. The tissue was dehydrated through transfer from low concentration to high concentrations of ethanol (30%, 50%, 70%, 90%, and 100% for 30 minutes respectively). Tissues were then placed in a half ethanol/half xylene solution for 30 minutes then another half an hour in solely xylene to make the tissue transparent. Subsequently, specimens were placed in 50% xylene and 50% paraffin solution at 60℃ for one hour and then heated in pure paraffin for another hour. The samples were cooled in the sink with water and stored in the fridge. Using a microtome, the fixed paraffin embedded specimens were sliced into 6μM sections and placed on glass slides.

2.2.2 Hematoxylin and eosin stain (H&E stain)

The specimen sections were placed in xylene for 5 minutes twice and then they were hydrated through transfer from 95% alcohol to 30% every 3 minutes then placed into distilled water for 1 minute. The nuclei were stained with the alum haematoxylin for 10 minutes followed by rinsing under running tap water for several seconds to remove the over-staining. Subsequently, they were differentiated with 1% acid alcohol and rinsed in

running tap water for a further 10 seconds. The specimens were counterstained with xanthene dye eosin for 3 minutes and rinsed in running tap water to remove the over-staining. In this process, cytoplasm, collagen and keratin were stained red. Afterwards, the samples were dehydrated with 30% to 90% alcohol 1 minute respectively and 2 minutes in 95%, twice 5 minutes in xylene. Then coverslips were placed above the samples using hydromount solution (National Diagnostic, Hessle Hull, U.K.).

2.2.3 Immunohistochemistry

The tissues were sectioned into 6µm thick sections and put onto slides overnight. The samples were hydrated and the glass slides were placed in plastic square dishes with PBS poured into the corner. PBS was slowly poured into dishes to avoid washing the samples away from the slides. Then the square dish was placed on a shaker for 10 minutes and the washing process was repeated three more times. The slides were picked from PBS and dried carefully with tissue. 50µl of blocking solution (5% Goat serum, 0.2% Triton X-100 in PBS) was then added to each sample at room temperature for 90 minutes; a plastic coverslip was placed over each sample to ensure even distribution of solution and prevent evaporation. After that, plastic coverslips were removed with forceps. 50µl of primary antibody (anti-vimentin mouse monoclonal antibody; anti-cytokeratin mouse monoclonal antibody; anti-aSMA mouse monoclonal antibody) diluted 1:100 in blocking solution was then

added to each specimen and plastic slips were placed back. The samples were incubated with first antibody overnight at 4°C.

Samples were then washed for 5 minutes with 0.02% BSA-0.05% IGEPAL in PBS. The samples were then incubated in ALEXA 488-conjugated secondary antibodies (Molecular Probes, Leiden, The Netherlands) for 2 hours at room temperature. After washing with PBS, 1:100 DAPI-phalloidin (Invitrogen, Eugene, Oregon, USA) diluted in PBS was added to tissues for 10 minutes in the dark. DAPI was used to dye the cell nuclei and the actin cytoskeleton was stained with phalloidin conjugated to Texas Red. After final washes, the specimens were mounted on a glass microscope slide by inverting the coverslip onto a drop of hydromount (National Diagnostis, Hessle Hull, U.K.). Slides were left to dry for 1 hour at room temperature and stored in the fridge in the dark until imaged. The samples were viewed with a Zeiss Axiovert fluorescent microscope (Melville, NY, U.S.A) and Zeiss Axiovision software.

2.2.4 Immunocytochemistry

Pterygial-derived fibroblasts were seeded at a density of 5000 cells/100μl on a square sterile coverslip housed in a tissue culture dish before the day of the experiment. After the cells adhered to the coverslip, 2ml of EMEM supplemented with 10 % FCS was added to each dish and culture dishes maintained at 35°C in a 5% CO_2 atmosphere. The medium was changed to serum-free for 24 hours later. For

phenotypic characterisation cells were fixed at this time point using 4% formaldehyde in phosphate buffered solution (PBS) at RT for 30 minutes. In some cases, TGFβ was added and cultures were maintained up to 4 hours in experimental conditions before fixation. Preparations were then washed twice for 5 minutes with PBS. Cells were then treated with 0.5% Triton X-100 in PBS for 30 minutes followed by three washes with 0.02% BSA-0.05% IGEPAL in PBS. 40µl normal goat serum (NGS) made up in 1% BSA PBS at 1:50 dilutions was added to bind non specific sites. This was followed by addition of 40µl primary antibody (anti-vimentin mouse monoclonal antibody; anti-cytokeratin mouse monoclonal antibody; anti-aSMA mouse monoclonal antibody; anti-smad2/3 mouse monoclonal antibody), which were diluted 1:100 in 1% BSA PBS and applied overnight at 4°C or incubated at 37°C for 1 hour. Preparations were then washed three times with 0.02% BSA-0.05% octylphenoxy polyethoxyethanol IGEPAL in PBS. Following washes, the cells were incubated in appropriate ALEXA 488-conjugated secondary antibodies (Molecular Probes, Leiden, The Netherlands) for 1 hour at room temperature in the dark. After washing three times with PBS alone, 1:100 diluted DAPI-phalloidin (invitrogen, Eugene, Oregon, USA) in PBS was added to tissues for 10 minutes in the dark. DAPI was used to stain the cell nuclei and the actin cytoskeleton was staining by phalloidin conjugated to Texas Red. After final 3 washes, the specimens were mounted on microscope slides using Hydromount solution (National diagnostis, Hessle Hull, U.K.). The samples were viewed with a Zeiss Axiovert fluorescence microscope (Melville, NY, U.S.A) and Zeiss axiovision

software. ImageJ software was applied to analyse the captured images when appropriate.

2.2.4.1 Quantification of Smad2/3 nuclear accumulation

Smad2/3 nuclear accumulation was quantified using ImageJ software. Triple or dual colour immunocytochemistry images were analyzed using Image J analysis software. DAPI staining defined the nucleus and the nuclear boundary for each cell was then drawn and a pre-defined area in the cytoplasm surrounding the nucleus was identified. Thus a nuclear and cytoplasmic compartment was identified. The fluorescent SMAD intensity was averaged over the measured area for each cell analysed and the difference in values was calculated.

2.3 MTS assay

Pterygial fibroblasts in 200ul (EMEM supplemented with 10% FCS) medium were seeded in each well of a 96-well tissue culture plate and maintained at 35°C in 5% CO_2 incubator. Then the medium was replaced with non-supplemented EMEM and cultured overnight. The cells were then placed in experimental conditions for 24 or 48 hours. Pterygial fibroblast number was determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Southampton, UK). Following the experimental duration, medium is removed from each well and replaced with, 100µl

MTS solution and incubated at 35°C in a 5% CO_2 atmosphere for one hour. The amount of coloured product was directly proportional to the number of cells. The plate was read at 490 nm using a BMG labtech FluoStar Plate reader (Aylesbury Bucks, USA).

2.4 Scratch wound assay

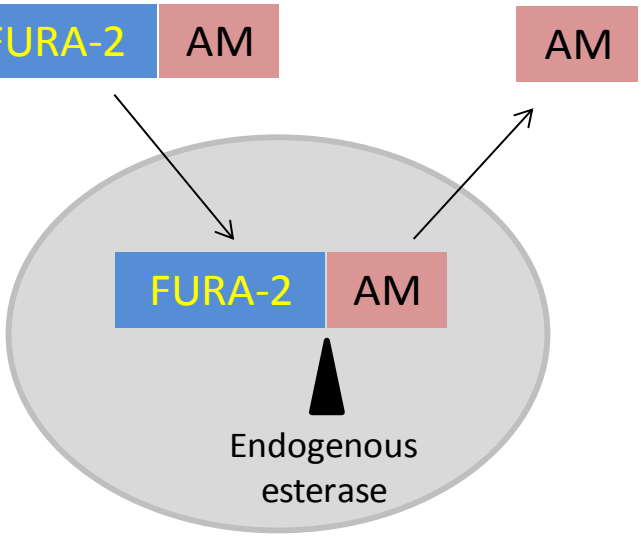
Pterygial fibroblasts were seeded on a 35mm tissue culture dish at a density of 5000 /200 µl of 5% FCS-EMEM and allowed to establish over a 48 hours period, such that a distinct patch of cells (~1cm in diameter) was observed. The medium was then replaced with non-supplemented EMEM and cultured for a further 24 hours. At this time-point a scratch was made through the middle of the confluent sheet using a plastic pipette tip. Indentations within the wound area were made to establish points of reference. The cells were then exposed to experimental conditions and maintained for 24 hours with the medium being changed every 2 days. Images were captured and the movement determined using image analysis software (Image J).

2.5 Calcium signalling

Pterygial fibroblasts were seeded in each well of a transparent 96-well microtitre plate at a density of 5000 cells/200µl of 10% FCS-EMEM and allowed to establish. Medium was removed and replaced with serum free EMEM or EMEM supplemented

with 10% serum for a 48 hour period. The cells were then loaded with 5µM Fura 2-acetoxymethylester (Fura 2-AM) in EMEM for 40 minutes (Figure 2.2A). Following this period, the medium in each well was changed to Standard Ringer solution (AAH; 5mM KCl, 5mM $NaHCO_3$, 5mM glucose, 20mM HEPES, 130mM NaCl, 0.5mM $MgCl_2$, 1mM $CaCl_2$, pH 7.25) or calcium free Ringer solution (5mM KCl, 5mM $NaHCO_3$, 5mM glucose, 20mM HEPES, 130mM NaCl, 0.5mM $MgCl_2$, 1mM EGTA, pH 7.25) (Rhodes, Russell *et al*. 2009). The plate was placed in a FLUOstar Omega multidetection microplate reader (BMG LABTECH Ltd, Offenburg, Germany), which was maintained at 35°C (Figure 2.2B). Fura 2 was excited alternately with 340 and 380nm wavelengths with emission detected at 520nm. Calcium levels were initially determined by the ratio of FURA-2 signal at 340nm/signal at 380nm. The ratio value at the onset of the experiment (t=0) served as a reference signal (F0); all subsequent readings were established as an F1/F0 ratio. Peak responses following stimulation were determined using the MAX feature on Excel (Microsoft).

A



B

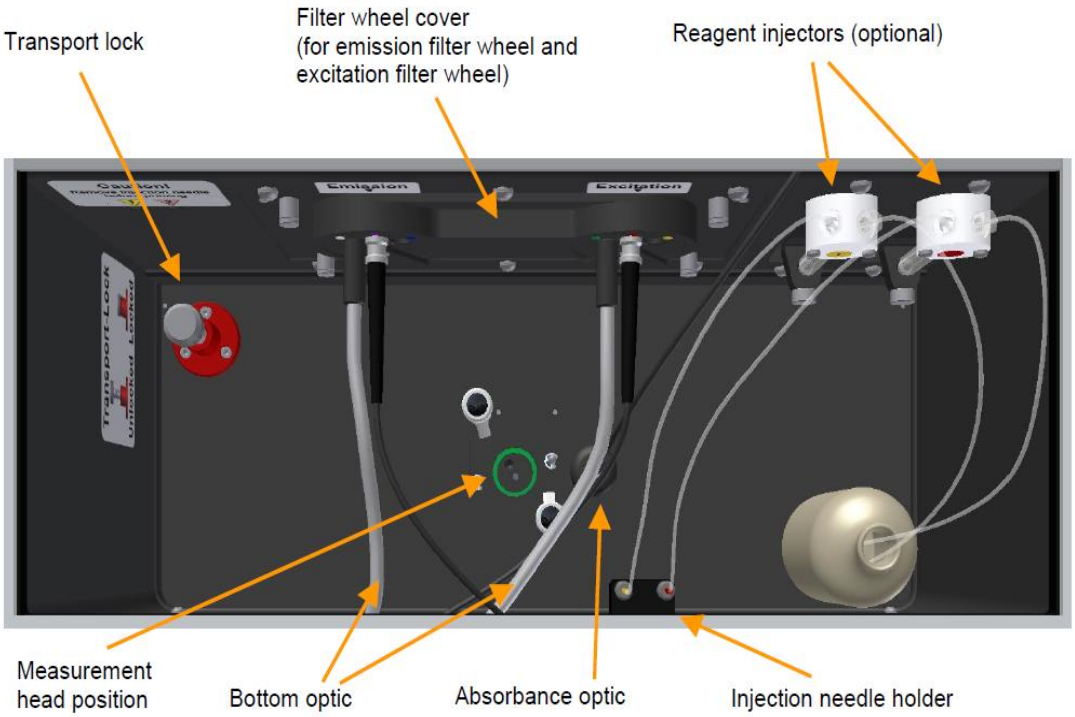


Figure2.2. A: The principle of FURA2 entry and entrapment within cells. B: Top view of reagent box in BMG labtech FluoStar Plate reader.

2.6 Patch growth assay

The patch assay technique was employed on pterygial fibroblasts to investigate the cell ability in migration and proliferation (Figure 2.3). Pterygial fibroblasts were seeded on a 35mm tissue culture dish at 5000 cells in 200 µl of 10% FCS-EMEM and allowed to establish over a 48 hour period, such that a distinct patch of cells (~1cm in diameter) was observed. Pterygial fibroblasts were placed in experimental conditions for 48 hours; conditions were 10% FCS EMEM ±1µM thapsigargin. At end-point, cells were washed with PBS and stained with Coomassie blue (Merck, Germany) for 1 hour to enable the patches to be visualized and measured. The dye was removed and the cells were washed with PBS several times to remove excess dye. Images of individual patches were captured on a CCD camera using Genesnap software (Synoptics, Cambridge, UK). Cell coverage was determined using Image J software. To provide an estimate of total cell number, the Coomassie blue dye within the cells was extracted by removal of PBS and addition of 1ml 70% ethanol. 100µl samples of each dish were transferred to a 96 well plate. The level of dye content was assessed by measuring absorbance at 550nm using a BMG labtech FluoStar Plate reader (Aylesbury Bucks, USA).

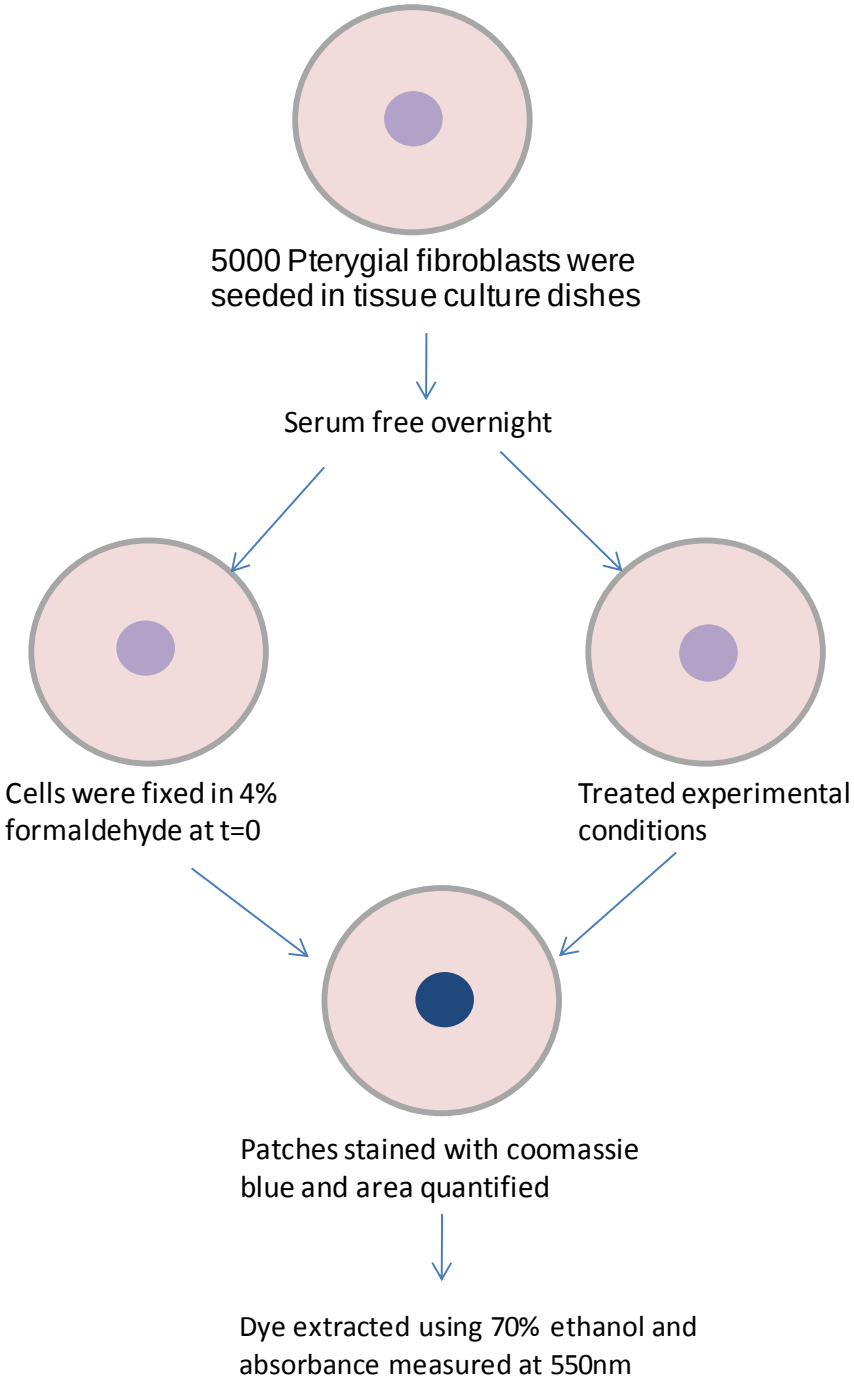


Figure 2.3. Diagram of patch assay.

2.7 RT-PCR

2.7.1 RNA Extraction

Total RNA was isolated from cultured pterygial fibroblasts using RNeasy Mini Kit column based method according to the manufacturer's instructions (Qiagen Ltd, Crawley, UK). Cell-culture medium was aspirated and 350µl of buffer RLT was added to the cell-culture dish. Cells were scraped off the dish using a cell scraper. Cell lysate was collected into an Eppendorf tube. 350µl of 70% ethanol was added to the homogenized lysate and mixed by pipetting with a gauge needle to a RNase free syringe without centrifuge. The samples were spun at 13000 rpm for 3 minutes and supernatant was collected. And then sample was put into an RNeasy mini column and placed in a 2 ml collection tube; Centrifuged for 30s >=8000xg. Discarded the flow-through and 700µl buffer RW1 was added to the RNeasy column, centrifuged for 60s and discarded the flow through. The Rneasy column was transferred into a new collection tube and 500 µl Buffer RPE containing ethanol was added. Another centrifuge for 15s and the flow-through was discarded. 500µl Buffer RPE was added to the Rneasy column and then centrifuged it for 2 minutes to dry the Rneasy silica-gel membrane. Then transferred the Rneasy column in new 1.5ml collection tube and 50µl Rneasy- free water was added onto the Rneasy silica-gel membrane. It was either used immediately for cDNA synthesis or stored short term at -80℃. The concentration of RNA was measured by using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

2.7.2 Synthesis of cDNA

1000ng of total RNA was used as a template for first strand cDNA synthesis. 1µl random primers and 1µl 10mM dNTP mix were added into each tube. Then 1000ng of total RNA and double diluted water in 12µl total amount were added to each tube, and then it was spun down at maximum 13000 rpm for 15s. The thermocycler DNA engine (MJ Research, Inc, USA) was applied to heat samples to 65°C for 5 minutes and followed by quick chill on ice. The contents of the tube was collected by briefly centrifuging and then added 4µl 5x First-Strand Buffer, 2µl 0.1 M DTT and 1µl RNaseOUT Recombinant Ribonuclease Inhibitor (40 units/µl) to each tube. The tube was incubated at 25°C for 10 minutes and 42°C for 2 minutes. Subsequently, 1µl superscript II was quickly added into each tube. This was followed by incubating the tube at 42°C for 15 minutes and 70°C for 15 minutes to stop the reaction. The cDNA product was either stored at -20°C or used straight away for PCR.

2.7.3 QRT-Polymerase chain reaction

Primer oligonucleotide sequences specific for the genes examined are shown in (Table 2.1). The level of product was determined by SYBR green (Finnzymes, Espoo, Finland), it binds exclusively to double-stranded DNA which leads to a fluorescence emission. Therefore, the product is proportional to fluorescence. Subsequently, SYBR green/ primer mix was made up as follows: SYBR green

equate to half the total mix; forward primer equate to 1/10 of SYBR green; reverse primer equate to 1/10 of SYBR green; the remainder of the mix volume was made with RNase free water. 45µl mix and 5 µl appropriate sample cDNA/calibration were added to 0.5ml Eppendorf tubes. Serial dilutions of cDNA known to express the gene of interest were prepared to permit relative levels between test samples to be determined. QRT-PCR was performed with the following program: step 1, initial denaturation for 94°C for 4 minutes; step 2, denaturation for 94°C for 20 seconds; step 3, annealing at 55°C for 30 seconds; step 4, extension at 72°C 20 seconds; step 5, cutoff for 10 seconds at 80°C to denature potential primer dimers, followed by fluorescent dye measurement. Steps 2 to 5 were repeated for 35 cycles. In addition, melting curve analysis was performed to determine the quality of the product.

Table 2.1. Primers employed for QRT-PCR

Gene of Interest	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
Alpha SMA	CCCAGCCAAGCACTGTCA	TCCAGAGTCCAGCACGATG	Lee and Joo, 1999
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	Yang *et al*., 1996

2.8 Western blot analysis

2.8.1 Protein extraction

Cells were incubated in serum-free medium overnight and then changed to the experimental conditions. Mammalian protein extraction reagent (M-PER; Thermoscientific, Rockford, USA) was supplemented with 10µl/ml protease inhibitors from the Halt ^{TM} phosphatase inhibitor kit (Thermo Scientific, Rockford, USA). Additionally, 10µl/ml of 0.5M ethylenediaminetetraacetic acid (EDTA) was added to dishes to act as a metalloproteinase inhibitor. Cells were rinsed with 500µl ice-cold PBS. Afterward, 350µl of M-PER and inhibitors were added to each sample dish. The dish was placed on a shaker for 5 minutes and then the cells were scraped from the base of the dishes using a pre-cleaned cell scraper. Lysates were pre-cleared by centrifuging at 14000 rpm to pellet the cell debris. The supernatant was transferred to 0.5ml Eppendorf tubes and stored at -20 or -80°C prior to use.

2.8.2 BCA protein assay

The protein content was assayed by Bicinchoninic acid assay (BCA) so that equal amounts of protein per sample were loaded onto SDS-Page gels for electrophoresis and transfer to PVDF (polyvinylidene difluoride) membrane. The BCA assay combines two reactions. Primarily, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+}. The amount of Cu^{2+} reduced is proportional to the amount of

protein in the alkaline medium. Secondly, bicinchoninic acid chelates with each Cu^{+} ion and a purple-colored reaction mixture was formed that strongly absorbs light at 562 nm. Protein standards were prepared by diluting 2mg/ml bovine serum albumin (BSA) over a working range of 25-1000µg/ml in lysis buffer stock solution. 40 µl of ddH_2O was added to each well of a 96 well plate. 10µl of samples, standards or blanks were pipetted into separate wells in duplicates. The BCA working reagents (WR) contained 50 parts of the reagent A for 1 part of reagent B and 200µl was added to each well. The plate was mixed well on the plate shaker for 30 seconds then covered it and incubated at 37°C for 1 hr. Plates were cooled to room temperature and the absorbance was read at 550nm on a multi-well plate reader (Fluostar Omega, BMA Labtech). The results were analyzed in Microsoft Excel.

2.8.3 SDS-PAGE gel electrophoresis and protein transfer

Each sample required 7µl of loading buffer containing 12.5% beta-Mercaptoethanol. Each tested protein sample consists of 28µl protein and 7µl loading buffer in a 0.5ml Eppendorf tube. The samples were centrifuged at 13000 rpm at 4°C for 1-2 minutes and heated for 5 minutes at 100°C using the MJ research gene engine (Alpha Unit™ Block Assembly for PTC DNA Engine™ System) (MJ Research, Inc. Watertown, USA). Then the samples and markers were loaded with long-nosed pipette tips to the SDS gels. The gels were run at 0.06 AMPs 60 minutes. Polyvinylidene fluoride (PVDF) membrane was activated in methanol for 30s. And then the gels, blotting paper and

PVDF membrane were equilibrated for 25-30 minutes at RT in transfer buffer. Afterwards, the proteins were transferred from the gel to the PVDF membrane in the Bio-Rad power Pac setting the volts at 15 V and AMPs at 0.3A per gel for 35 minutes.

2.8.4 Immunoblotting and development

After transfer, PBS-T blocking solution was made up in 0.5% Tween-20 (Fisher Scientific, New Jersey, USA) in PBS with 5% fat-reduced milk powder (Marvel). The membrane was placed in a square petri-dish filled with PBS-T and positioned on a shaker for one hour at RT to block the non-specific sites on the membrane. Subsequently, the membrane was incubated with the primary antibody (Abcam, Cambridge, U.K.) diluted 1:1000 in Marvel PBS-T overnight at 4°C. Secondary antibody used a Horse Radish Peroxidase (HRP) (Cell Signalling Technology) at a concentration of 1:1000 in Marvel PBS-T and additionally 0.5µl other secondary component for the Dual Vue makers (CPK 1070) was added to the solution. They were applied for 1 hour and bands were detected using ECL plus Western blotting detection reagents (GE Healthcare USA, Piscataway, NJ) according to manufacturer's instructions. The photographic film was developed in the dark room then it can be scanned and measured using imagineJ analysis software.

2.9 Proteome Profiler™ Array

Pterygial fibroblasts were maintained in experimental conditions for 48 hours. The bathing medium was sampled and analysed using a commercially available proteome profiler array. This permits evaluation of a number of targets established through capture antibodies spotted in duplicate on nitrocellulose membranes. A layout of the membrane and a list of angiogenic factors assessed are provided in (Table 2.2). The blocking buffer was added to each well of a 4-Well Multi-dish containing a proteome profiler membrane. Samples are diluted and mixed with a cocktail of biotinylated detection antibodies then incubated with the Human Angiogenesis Array overnight at 2-8 °C on a rocking platform shaker. Any protein/detection antibody complex present was bound by its cognate immobilized capture antibody on the membrane. Each membrane was washed three times with 1X Wash Buffer for 3 X 10 minutes on a rocking platform shaker to remove unbound material. The membrane was incubated with diluted streptavidin-HRP in the 4-Well Multi-dish for 30 minutes at room temperature on a rocking platform shaker. After another three washes, 1 ml chemi Reagent Mix was added onto each membrane. Finally, ECL plus reagents (GE Healthcare USA, Piscataway, NJ) was used according to manufacturer's instructions. The photographic film was developed in the dark room. Light was produced at each spot in proportion to the amount of analyte bound.

Table 2.2. Coordinate reference for analyte identification.

Coordinate	Target/Control	Alternate Nomenclature	Coordinate	Target/Control	Alternate Nomenclature
A1,A2	Positive Control	Control (+)	C15,C16	IL-1β	Interleukin-1 beta
A5,A6	Activin A	ACVR1	C17,C18	IL-8	Interleukin-8
A7,A8	ADAMTS-1		C19,C20	LAP(TGF β1)	
A9,A10	Angiogenin	ANG	C21,C22	Leptin	LEP
A11,A12	Angiopoietin-1	ANG-1	C23,C24	MCP-1	CCL2
A13,A14	Angiopoietin-2	ANG-2	D1,D2	MIP-1α	CCL3
A15,A16	Angiostatin	PLG	D3,D4	MMP-8	
A17,A18	Amphiregulin	AREG	D5,D6	MMP-9	
A19,A20	Artemin	ARTN	D7,D8	NRG1- β1	HRG1- β1
A23,A24	Positive Control	Control (+)	D9,D10	Pentraxin 3 (PTX3)	TSG-14
B1,B2	Coagulation Factor III	TF	D11,D12	PD-ECGF	TYMP
B3,B4	CXCL16	Chemokine ligand 16	D13,D14	PDGF-AA	
B5,B6	DPPIV	DPP4	D15,D16	PDGF-AB/BB	
B7,B8	EGF		D17,D18	Persephin	PSPN
B9,B10	EG-VEGF		D19,D20	PIGF	
B11,B12	Endoglin	ENG/CD105	D21,D22	Prolactin	PRL
B13,B14	Endostatin	COL18A1	E1,E2	Serpin B5	Maspin
B15,B16	Endothelin-1	ET-1/EDN1	E3,E4	Serpin E1	PAI-1
B17,B18	FGF acidic	FGF-1	E5,E6	Serpin F1	PEDF
B19,B20	FGF basic	FGF-2	E7,E8	TIMP-1	
B21,B22	FGF-4		E9,E10	TIMP-4	
B23,B24	FGF-7		E11,E12	Thrombospondin-1	TSP-1
C1,C2	GDNF		E13,E14	Thrombospondin-2	TSP-2
C3,C4	GM-CSF		E15,E16	uPA	PLAU
C5,C6	HB-EGF	Heparin bing EGF	E17,E18	Vasohibin	VASH
C7,C8	HGF		E19,E20	VEGF	
C9,C10	IGFBP-1		E21,E22	VEGF-C	
C11,C12	IGFBP-2		F1,F2	Positive Control	Control (+)
C13,C14	IGFBP-3		F23,F24	Negative Control	Control (-)

2.9.1 Data analysis

The positive signals on the developed film were identified. A transparent overlay was placed on the array images and aligned with the three pairs of positive control spots in the corners of each array. Profiles were created by quantifying the mean spot pixel densities. Array signals from scanned X-ray film images were analyzed by Image J analysis software.

2.10 Illumina Gene Array

Routinely cultured pterygial fibroblasts maintained in EMEM were treated with either TGFβ2 at 1ng/ml or 10% serum in EMEM for 24 hour. Then total RNA was isolated from cultured pterygial fibroblasts using RNeasy Mini Kit column based method according to manufacturer's instructions. The Illumina microarray platform was selected to provide a comprehensive view of multiplex gene expression and genotyping under the different conditions. A Human-HT12 Expression BeadChip (Illumina, San Diego, CA. BD-25-113) array platform was employed. RNA samples were prepared as described section 2.7 and shipped to a commercial microarray facility and quality assessed using Nanodrop and an Agilent RNA pico labchip; only samples with an RNA integrity number (RIN) ≥ 9 were used in the study. Each Illumina BeadChip allows simultaneous analysis of 12 arrays, each with > 48,000 probes derived from human genes in the National Centre for Biotechnology Information

(NCBI). Oligonucleotide spots were synthesized onto the silica beads. The Bead chips were imaged using Illumina's Bead BeadStation scanner. The Bioconductor package (http://www.bioconductor.org) in R (http://www.r-project.org) was used to analyze the Illumina microarray (BeadArray) unnormalised probe profile data (Lim, Chung *et al*. 2008). Firstly, the data from different chips were loaded into R to be background corrected, quantile normalised and variance stabilised. Gross changes in gene expression were computed by a log fold change (lfc). For finding the meaningful biologically role of the genes, a p value threshold criteria was utilized for each gene using an extension of the empirical Bayes moderated t-statistic known as TREAT (McCarthy and Smyth 2009). Differentially expressed genes that had a fold change threshold of 2.0 (1 log fold change) and a p value of <0.05 were identified as significant.

CHAPTER 3

Serum deprivation can suppress calcium cell signalling in pterygial derived fibroblasts

3.1 Introduction

Pterygium show higher vasculature and a greater growth response relative to normal conjunctiva (Chen, Tsai *et al*. 1994; Kria, Ohira *et al*. 1998)(Aspiotis, Tsanou *et al*. 2007). Abundance of serum is possible to support the pterygial fibroblasts by supplying the tissue with oxygen and growth factors to the cells survival and proliferation. An investigation of serum supplement was used in pterygial derived cells and showed that cells cultured in the presence of serum are more irregular and grew faster than cells cultured in serum free medium (Di Girolamo, Tedla *et al*. 1999). A rich blood supply can therefore promote the rate of pterygium formation and understanding how this source of nutrients, survival factors and oxygen facilitates these changes is an important aspect of pterygia biology that requires further study. To mimic a rich and poor blood supply cells were maintained in either serum rich or serum depleted medium and comparisons made. In particular differences in calcium signalling, gene expression patterns and the regulation of angiogenic factors were assessed.

Calcium is an essential element in maintaining normal physiological function. It is considered a versatile second messenger that is able to regulate many cellular physiology processes (Berridge 1993). Many of the physiological features of pterygium such as persistent cellular proliferation, transdifferentiation and angiogenesis could be attributed to Ca^{2+} signalling activities (Coroneo, Di Girolamo *et al*. 1999). However, the fluctuation of the calcium will impact physiological function directly or even leading to many kinds of disease. Therefore, calcium has long been researched because of its key importance in human disease. Pterygial tissue is characterized by extensive cellular proliferation, transdifferentiation and angiogenesis which are linked to Ca^{2+} signalling activities (Coroneo, Di Girolamo *et al*. 1999). G-protein coupled receptors including histaminergic, purinergic and muscarinic receptors release calcium from intracellular stores through activation of PLCβ. Alternatively, epidermal growth factor (EGF) is a tyrosine kinase-linked receptor that can utilise PLCgamma to elevate IP_3 and mobilise the ER calcium store (Berridge 1993). The regulation of calcium signalling through recruitment of the endoplasmic reticulum store has not been well studied in pterygial cells. The present study therefore aimed to understand the effects of serum deprivation on calcium cell signalling events and the functional role of calcium signalling per se in pterygial-derived fibroblasts.

3.2 Results

3.2.1 Clinical features of conjunctival and pterygial tissues

In order to observe the histological structure of pterygium and conjunctiva tissue, hematoxylin-eosin (H&E) staining was carried out. It is notable that the structure of the two tissues differ as pterygium demonstrates more compact organization of fibroblasts showing intense eosin cytoplasmic staining (pink/red) (Figure 3.1A), while the conjunctiva tissue has a less dense arrangement of fibroblasts (Figure 3.1B). Conjunctival and pterygial tissue sections were also stained for alpha smooth muscle actin (αSMA), which binds smooth muscle within the wall of blood vessels and serves as a marker of fibroblast to myofibroblast transdifferentiation. While the distinct epithelial and stromal layers are evident, it is also apparent that the two tissues are different. The first difference is the frequency of blood vessels, which are defined by the bright circular appearance of (αSMA); in pterygium these are far more abundant (Figure 3.1C and D). The second difference is the increased level of αSMA staining in the fibroblast cell population of pterygium, indicating transdifferentiation has occurred (Figure 3.1C and D).

3.2.2 Concentration-dependent effects of serum on cell growth

A concentration dependent effect of serum over a 48 hour culture period was observed with pterygial fibroblasts (Figure 3.2). Concentrations of 4%v/v FCS were required to produce a significant increase in growth compared with SF EMEM cultures. A peak

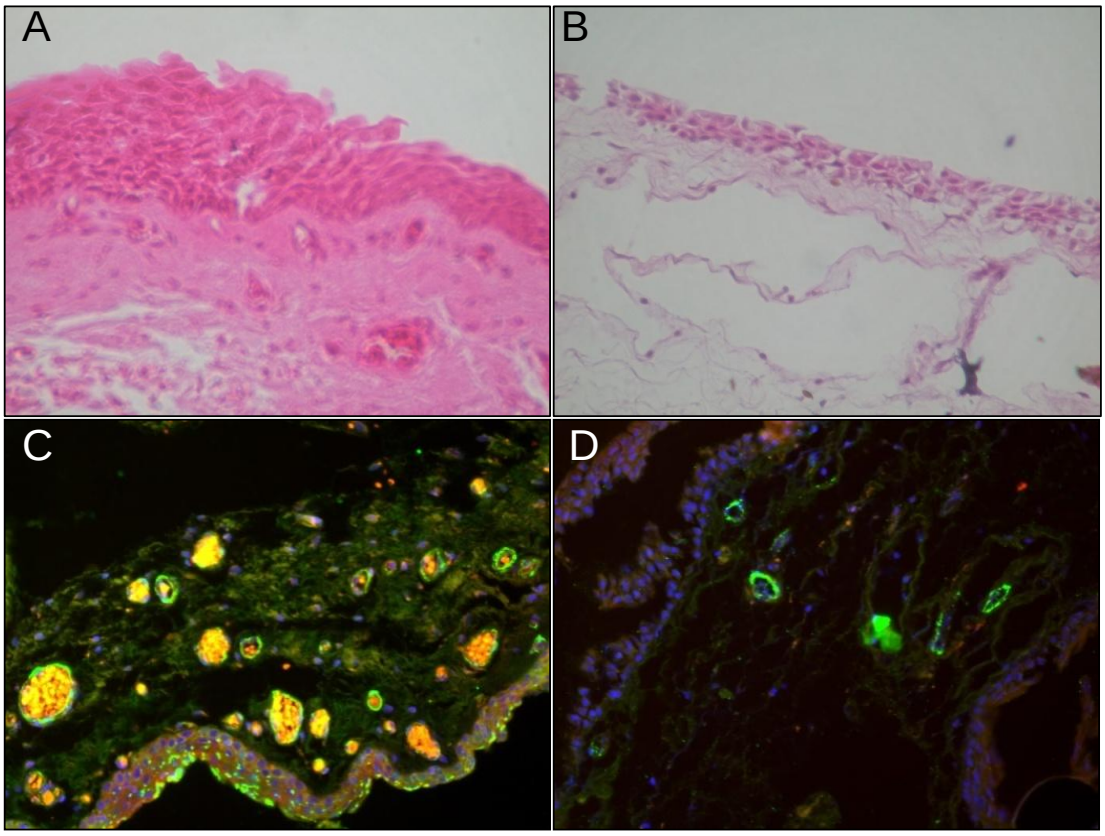


Figure 3.1. A comparison of cell organisation and tissue structure in pterygium (A & C) and normal conjunctival (B & D) tissue. (A & B) Haemotoxylin and eosin staining of sections derived from (A) apterygium and (B) conjuctiva. (C & D) Fluorescence micrographs showing aSMA expression (green) in (A) pterygium and (B) conjunctival tissue; sections were counterstained to visualise chromatin (blue) and the F-actin cytoskeleton (red). The field of view represents 800 X 571μm (A&B) and 448 x 342 μm (C&D).

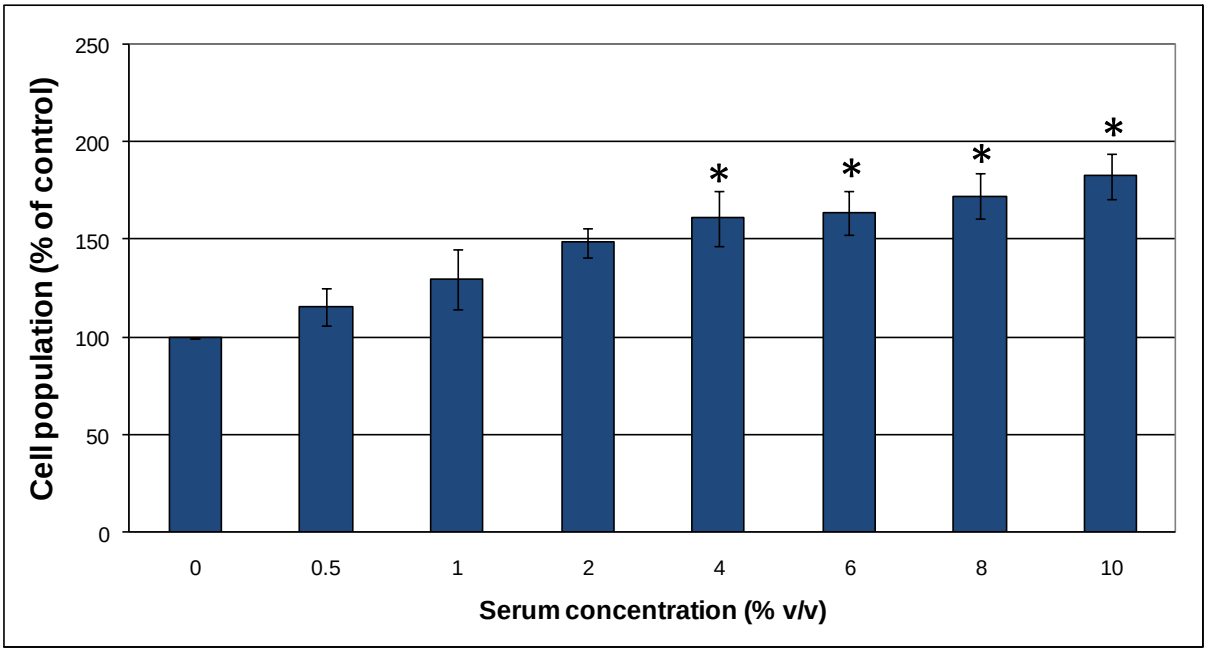


Figure 3.2. Concentration-dependent effects of serum on cell growth of pterygial-derived fibroblasts detected using the MTS assay. The data represent means ± S.E.M. from experiments performed on pterygial derived fibroblasts from three different donors. Cells were exposed to experimental conditions for a 48 hour culture period. * indicates a significant difference between serum free maintained and serum treated group ($p \leq 0.05$; ANOVA with Dunnett's post hoc test).

response was obtained with 10% FCS addition, such that the cell population was 183 ± 11.67% relative to non-stimulated Serum-free control.

3.2.3 Intracellular calcium signalling

To assess putative changes in calcium signalling in serum-maintained and serum-free conditions, we employed several agonists that are known to mobilise intracellular calcium. These ligands were histamine, ATP, ACh and EGF. Moreover, these ligands were selected because they have been associated with pterygium or have the potential to influence this condition. For example, active histamine and epidermal growth factor receptors have been determined in human pterygium (Maini, Collison *et al*. 2002). Extracellular ATP levels are also known to rise in stressed or pathological states (Eldred, Sanderson *et al*. 2003) and ACh is believe to influence cell function as a consequence of innervation, but can also transmit signals independent of innervation (Duncan and Collison 2003).

In serum-maintained cells, histamine, ATP, ACh and EGF were shown to increase intracellular calcium levels through mobilisation of the ER calcium store in a concentration dependent manner. Significant elevation of intracellular calcium was observed with concentrations ≥ 10µM of histamine, ATP and ACh and 10ng/ml in the case of EGF. In contrast, cells maintained in serum-free medium and exposed to the four

ligands did not show any significant increase in intracellular calcium levels at any of the concentrations tested suggesting that serum deprivation can reduce the sensitivities of cell in response to these ligands. Responses obtained with histamine, ATP, ACh at 100µM and EGF at 10ng/ml differed significantly between the serum maintained and serum starved cells (Figure 3.3-3.6).

Moreover, thapsigargin which is an intracellular Ca^{2+} pump inhibitor was applied to deplete the calcium store in pterygial fibroblasts in the presence and absence of serum and thus reveal the level of calcium stored within the ER. 1µM thapsigargin induced a significant increase in intracellular calcium level of cells maintained in serum and serum starved conditions (Figure 3.7). However, a significantly greater increase in calcium was observed in the serum maintained cells; the peak intracellular calcium level in serum free maintained cells group was 47% of that obtained in serum maintained cells (Figure 3.7). To assess the role of calcium influx, through store operated calcium entry, in the thapsigargin elevation of intracellular calcium experiments were performed in calcium-free extracellular medium (Figure 3.7). These experiments demonstrated an overall reduction in intracellular calcium levels in response to 1µM thapsigargin, but peak responses were not significantly different from cells treated in the presence of calcium in the extracellular medium. Therefore, ER store play a dominant role in mediating intracellular calcium mobilization.

In order to confirm that the ligand induced calcium responses involved the ER calcium store we pre-treated cells with thapsigargin for >1 hours to ensure the calcium store was drained. Control cells maintained in serum-free or serum, that exhibit a normal ER store, increased intracellular calcium in response to all ligands with a greater response observed in serum-maintained cells (Figure 3.8-3.11). In all cases, cells pre-treated with thapsigargin did not elicit an elevation in calcium in response to any of the four ligands tested.

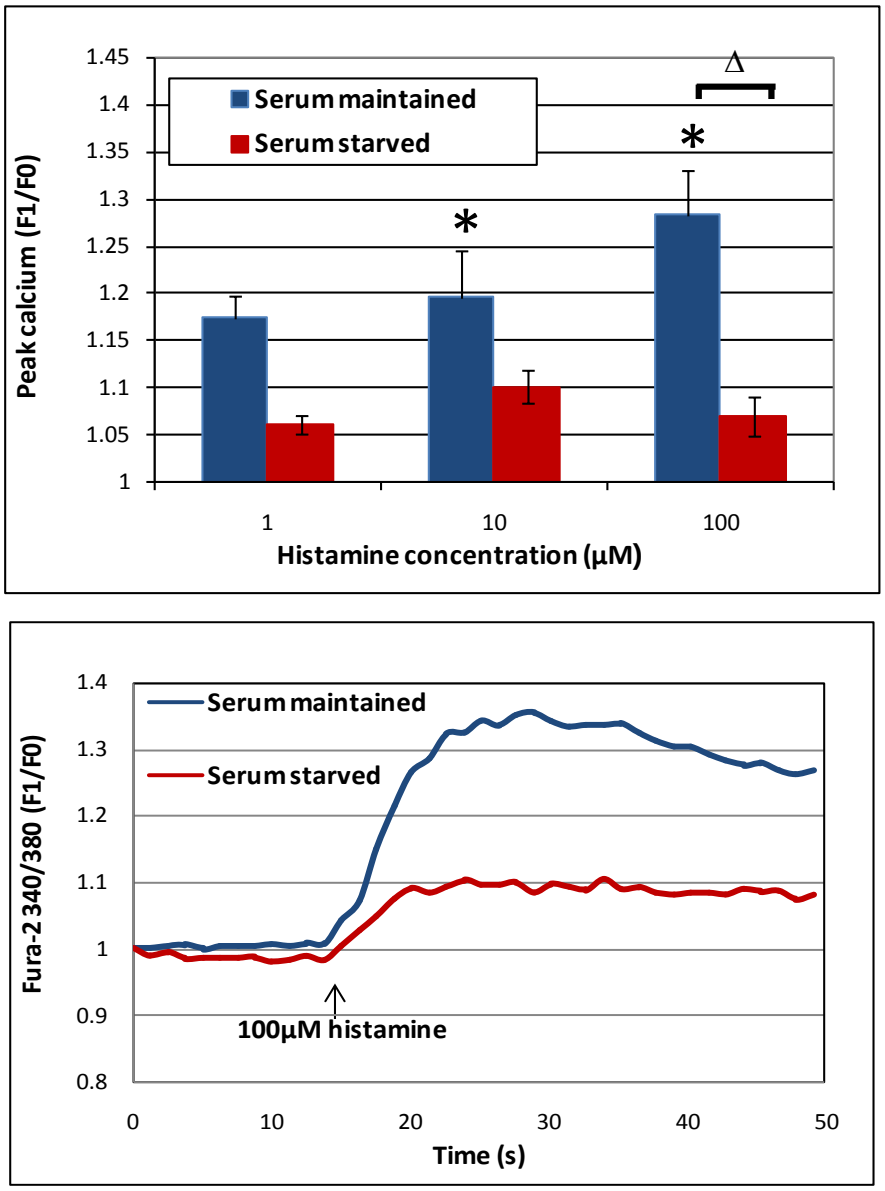


Figure 3.3. Characterization of calcium mobilization in response to histamine in pterygial derived fibroblasts in the presence and absence of serum. Baseline calcium levels were established prior to injection of histamine after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.E.M. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ Significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

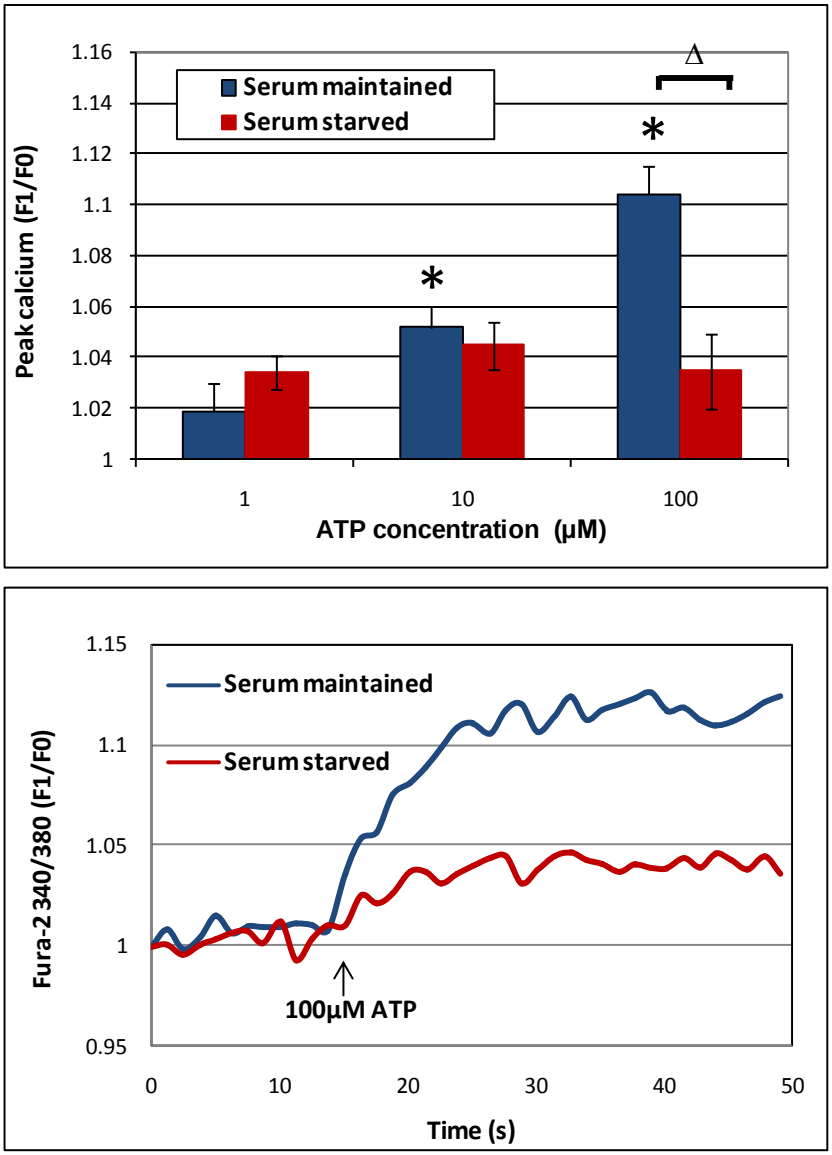


Figure 3.4. Characterization of calcium mobilization in response to ATP in pterygial derived fibroblasts in the presence and absence of serum. Baseline calcium levels were established prior to injection of ATP after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.E.M. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ Significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

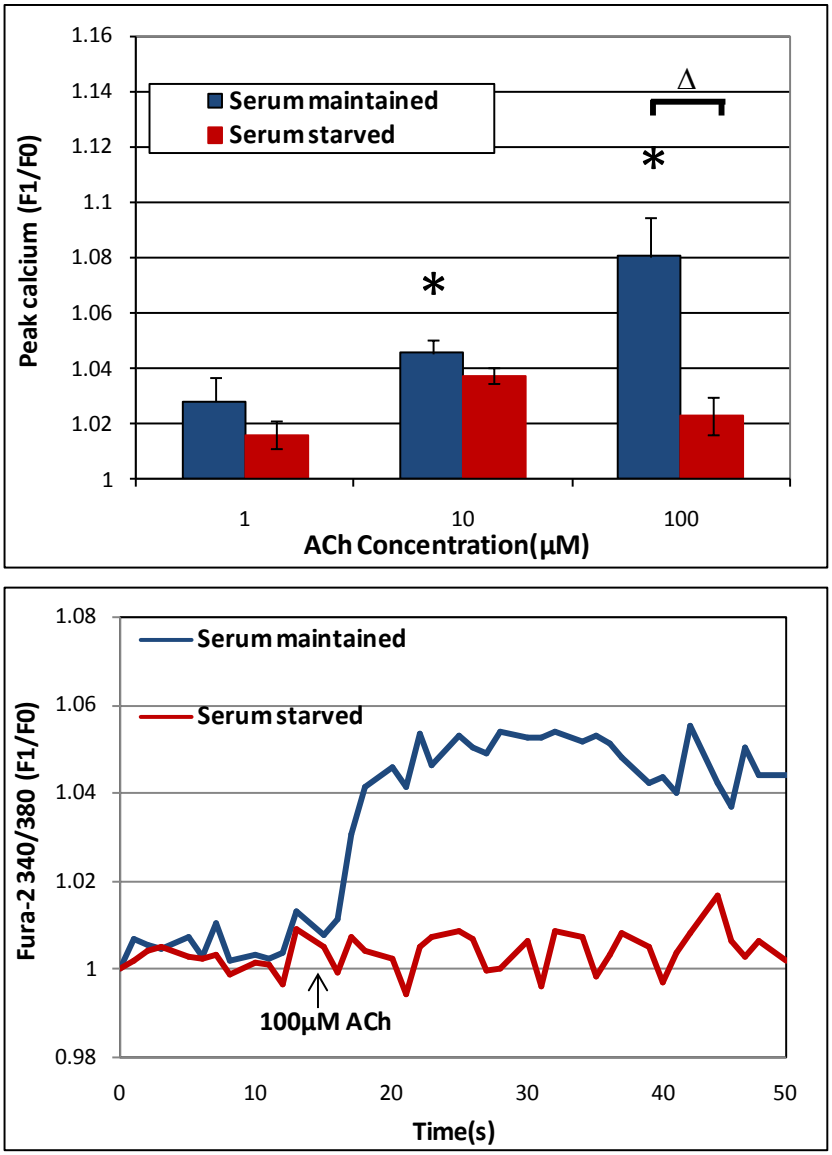


Figure 3.5. Characterization of calcium mobilization in response to ACh in pterygial derived fibroblasts in the presence and absence of serum. Baseline calcium levels were established prior to injection of acetylcholine after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.E.M. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ Significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

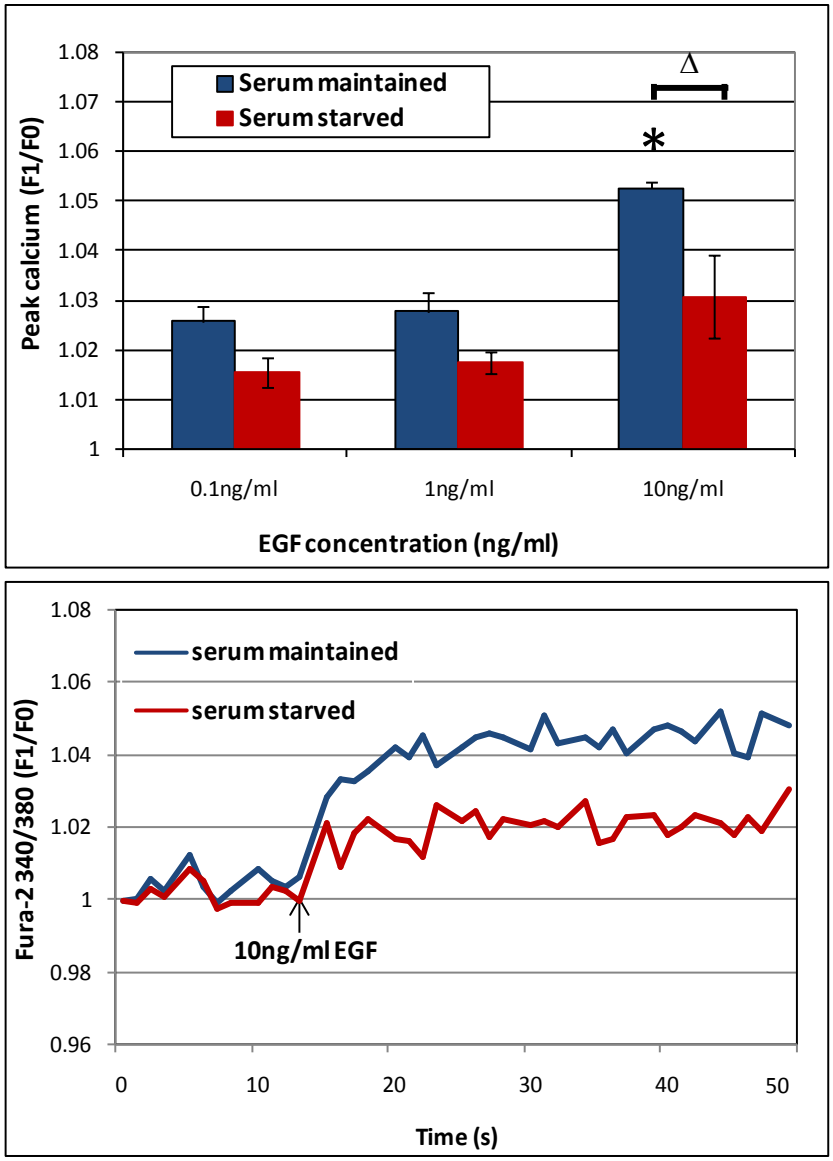


Figure 3.6. Characterization of calcium mobilization in response to EGF in pterygial derived fibroblasts in the presence and absence of serum. Baseline calcium levels were established prior to injection of EGF after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.E.M. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ Significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

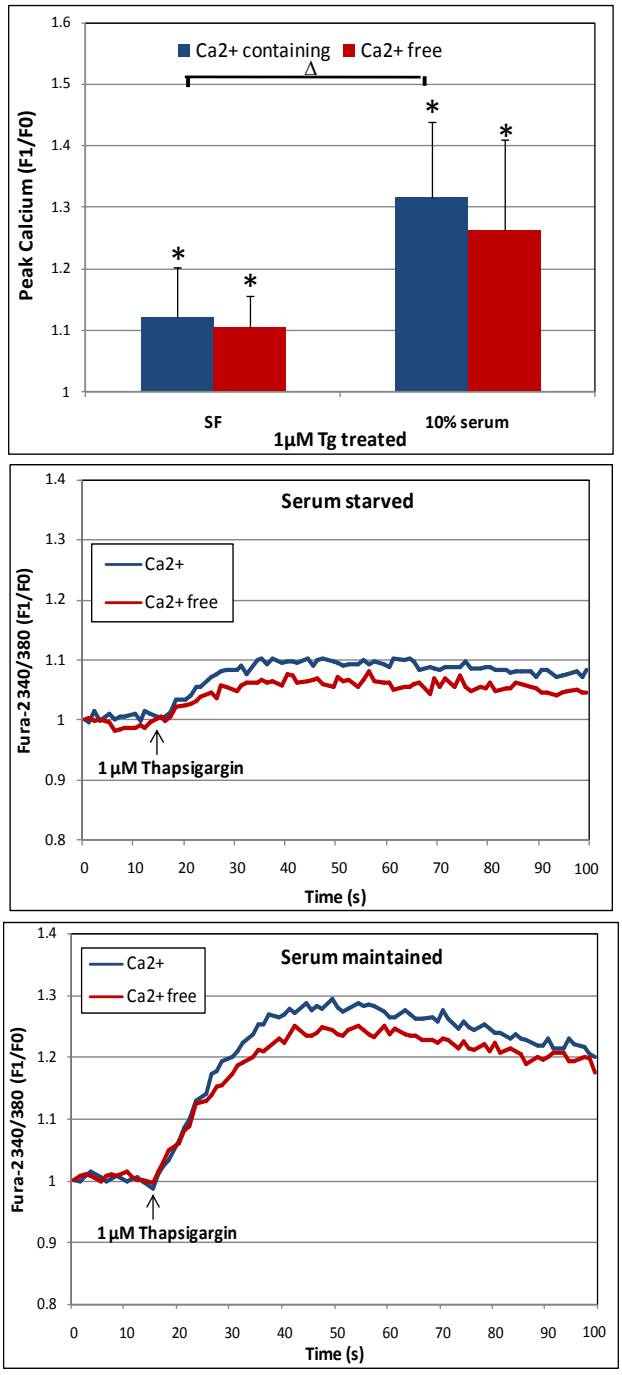


Figure 3.7. The effect of serum starvation on ER calcium store depletion in response to the CaATPase inhibitor thapsigargin in the presence and absence of extracellular calcium. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from four different donors.* Indicates a significant difference from baseline; Δ Significant difference between serum maintained and serum starved groups (Student's t test, $p \leq 0.05$).

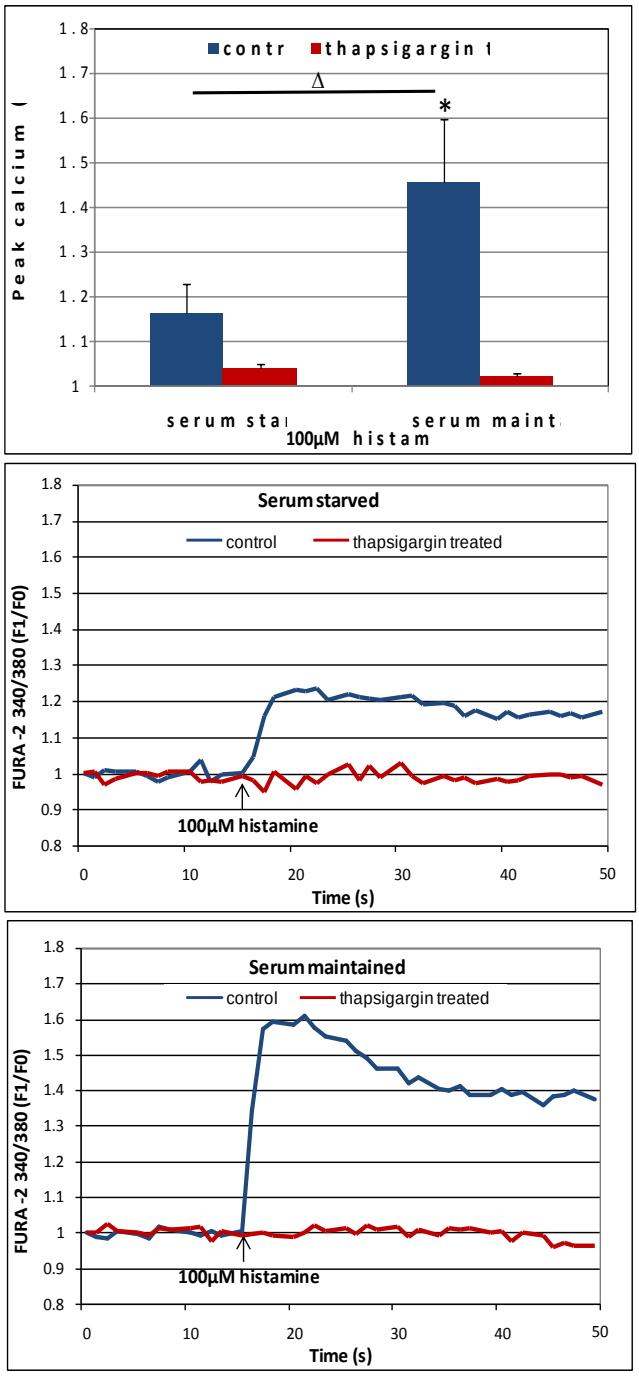


Figure 3.8. The effect of ER calcium store depletion by thapsigargin on histamine mediated calcium responses. Cells were maintained in control medium or exposed to 1µM thapsigargin for the final hour of culture prior to FURA-2 loading. Baseline calcium levels were established prior to injection of histamine after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

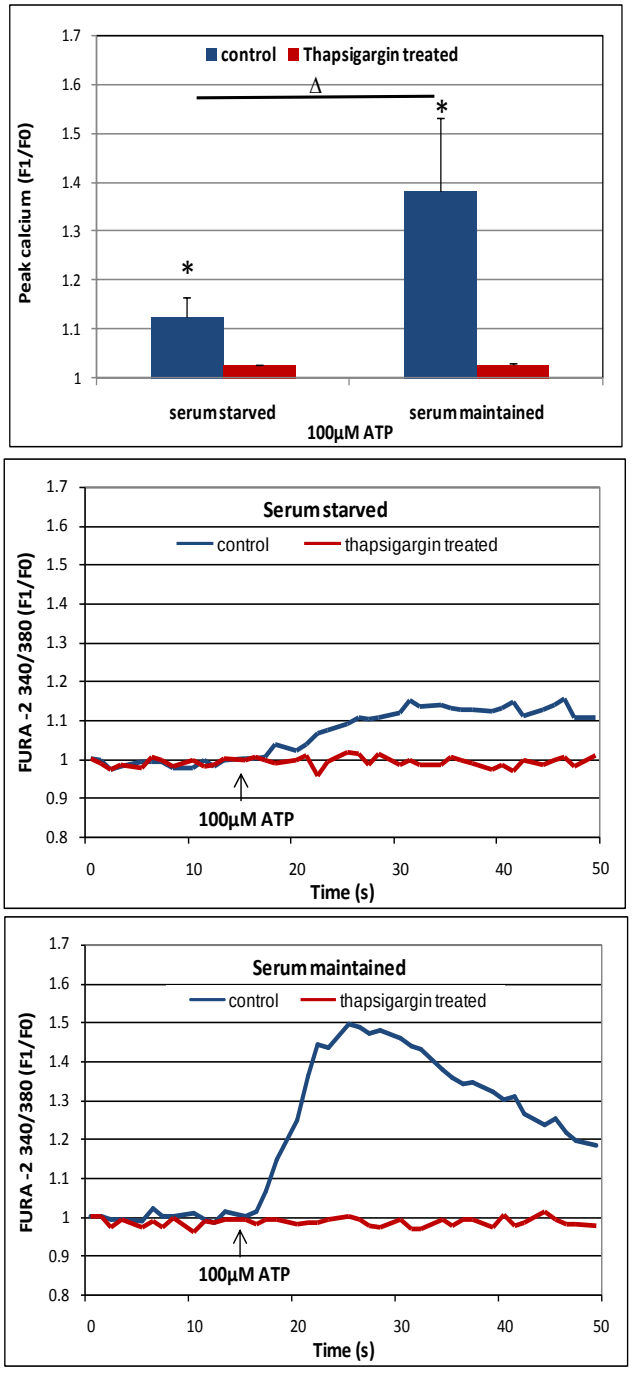


Figure 3.9. The effect of ER calcium store depletion by thapsigargin on ATP mediated calcium responses. Cells were maintained in control medium or exposed to 1µM thapsigargin for the final hour of culture prior to FURA-2 loading. Baseline calcium levels were established prior to injection of ATP after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

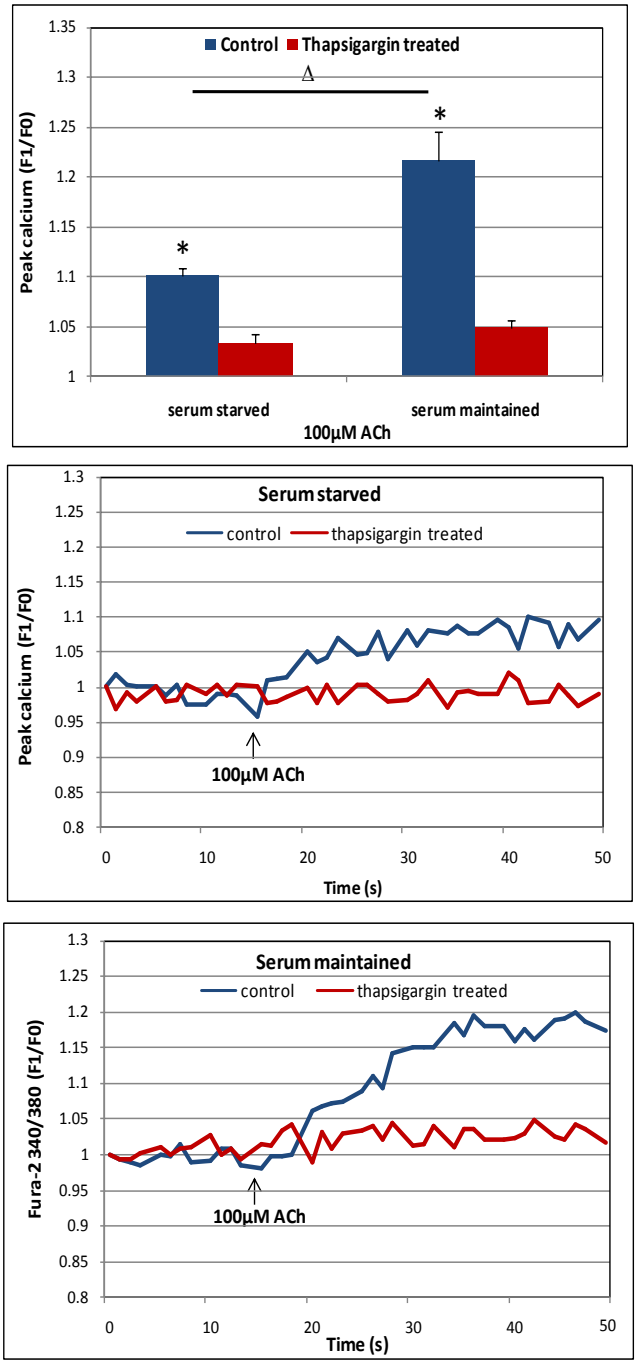


Figure 3.10. The effect of ER calcium store depletion by thapsigargin on ACh mediated calcium responses. Cells were maintained in control medium or exposed to 1µM thapsigargin for the final hour of culture prior to FURA-2 loading. Baseline calcium levels were established prior to injection of ACh after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

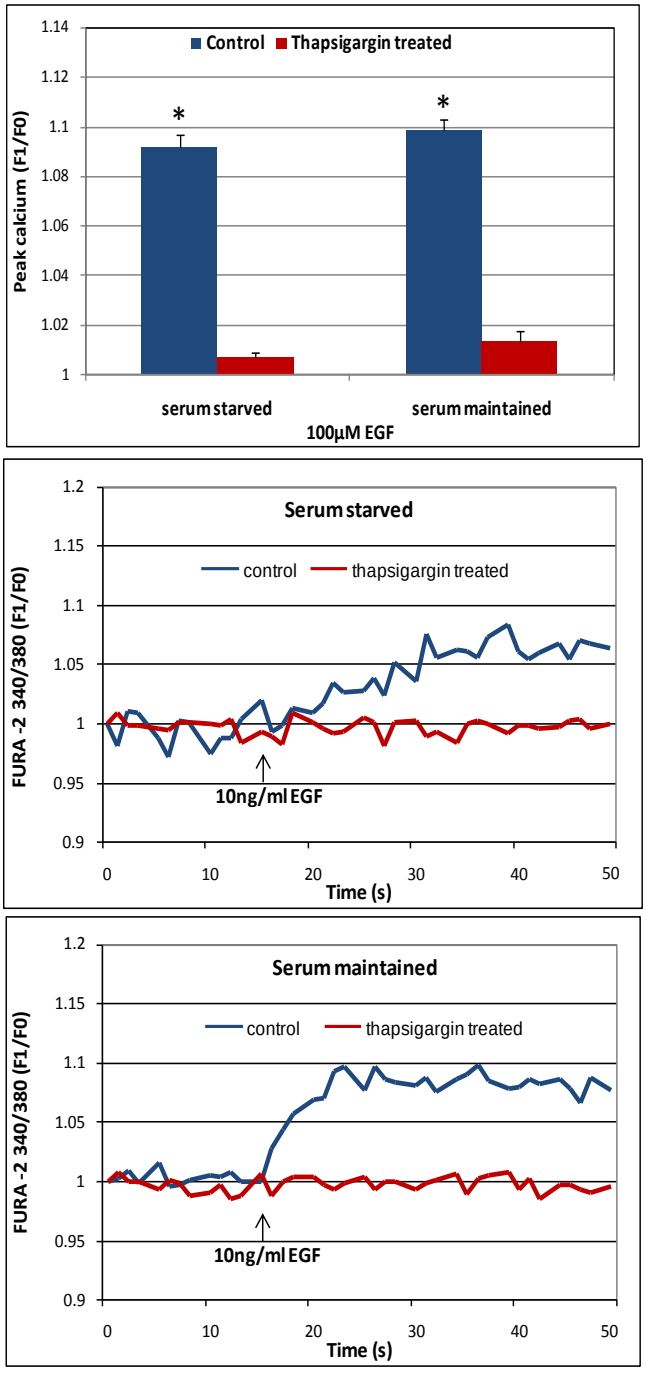


Figure 3.11. The effect of ER calcium store depletion by thapsigargin on EGF mediated calcium responses. Cells were maintained in control medium or exposed to 1µM thapsigargin for the final hour of culture prior to FURA-2 loading. Baseline calcium levels were established prior to injection of EGF after 15s as indicated; the ligand was retained in the bathing medium for the remainder of the experiment. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * Indicates a significant difference from baseline; Δ significant difference between serum maintained and serum starved groups (ANOVA with Turkey's post hoc test, $p \leq 0.05$).

3.2.4 The effects of calcium signalling disruption on cell growth and migration

To determine the importance of calcium signalling on pterygial cell function, we employed several assays to evaluate growth and migration. A patch assay was initially used to assess coverage of cells on a tissue culture dish. 1μM thapsigargin was used to disrupt calcium signalling. Thapsigargin treated cells covered a significantly smaller area, such that area covered was 65.3 ± 9.34% that of the control group (Figure 3.12C). Cell population numbers also differed significantly; in this case thapsigargin treated cells were 86.3 ± 2.73% compared with controls (Figure 3.12D). These data suggested an effect on both migration and cell growth. We then expanded on these findings and employed an MTS assay to further evaluate effects on cell populations and a scratch assay to study effects on migration. The MTS assay showed a similar outcome to the patch assay, such that populations in the thapsigargin treated group were 81.7 ± 5.3% of the non-stimulated group (Figure 3.13). The scratch wound assay clearly demonstrated the ability of thapsigargin to inhibit coverage of the wounded area (Figure 3.14). Within 24 hours, control cells have rapidly covered the cell-free area, whereas progress is significantly retarded in the thapsigargin treated group.

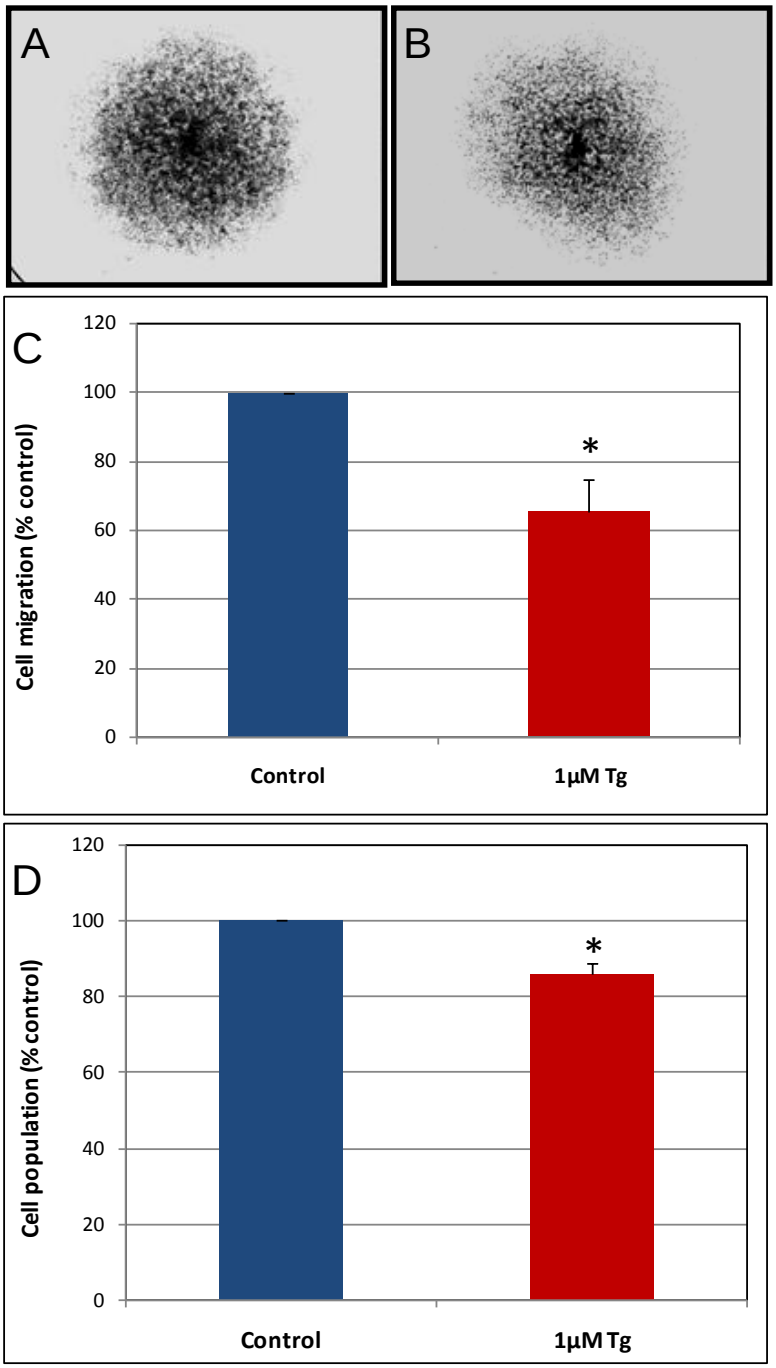


Figure 3.12. The effect of thapsigargin on pterygial-derived fibroblast growth detected using the patch assay. Pterygial fibroblasts were cultured for 48 hours in EMEM supplemented with 10% FCS only (A) or treated with 1µM thapsigargin (B); the coverage (C) and number (D) of cells were assessed. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * indicates a significant difference between non-stimulated control and treated group ($p \leq 0.05$; Student's t test).

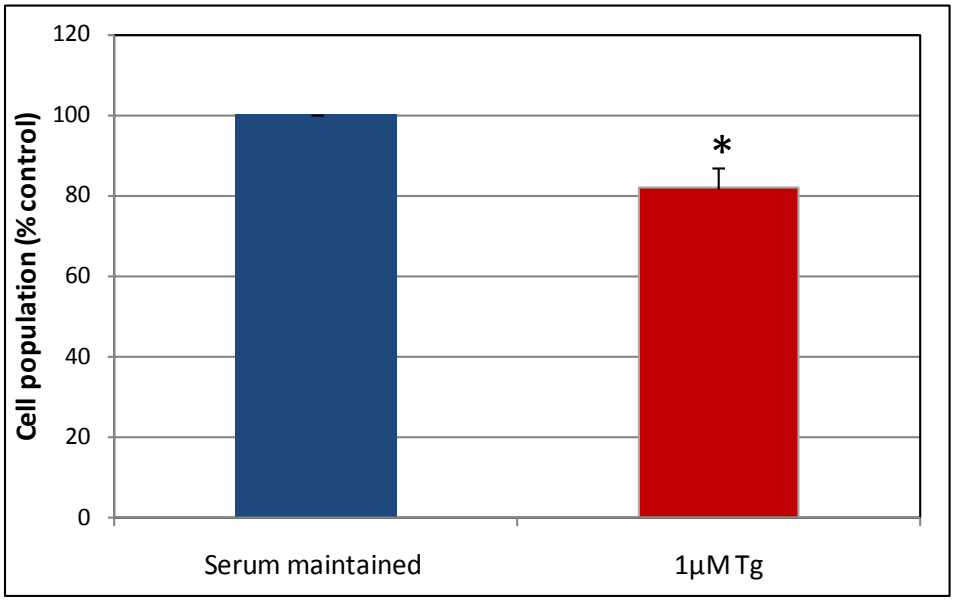


Figure 3.13. Effects of thapsigargin on cell growth of pterygial-derived fibroblasts detected using the MTS assay. Pterygial fibroblasts were cultured for 24 hours in EMEM supplemented with 10% FCS ± 1µM thapsigargin. The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * indicates a significant difference between non-stimulated control and treated group ($p \leq 0.05$; Student's t test).

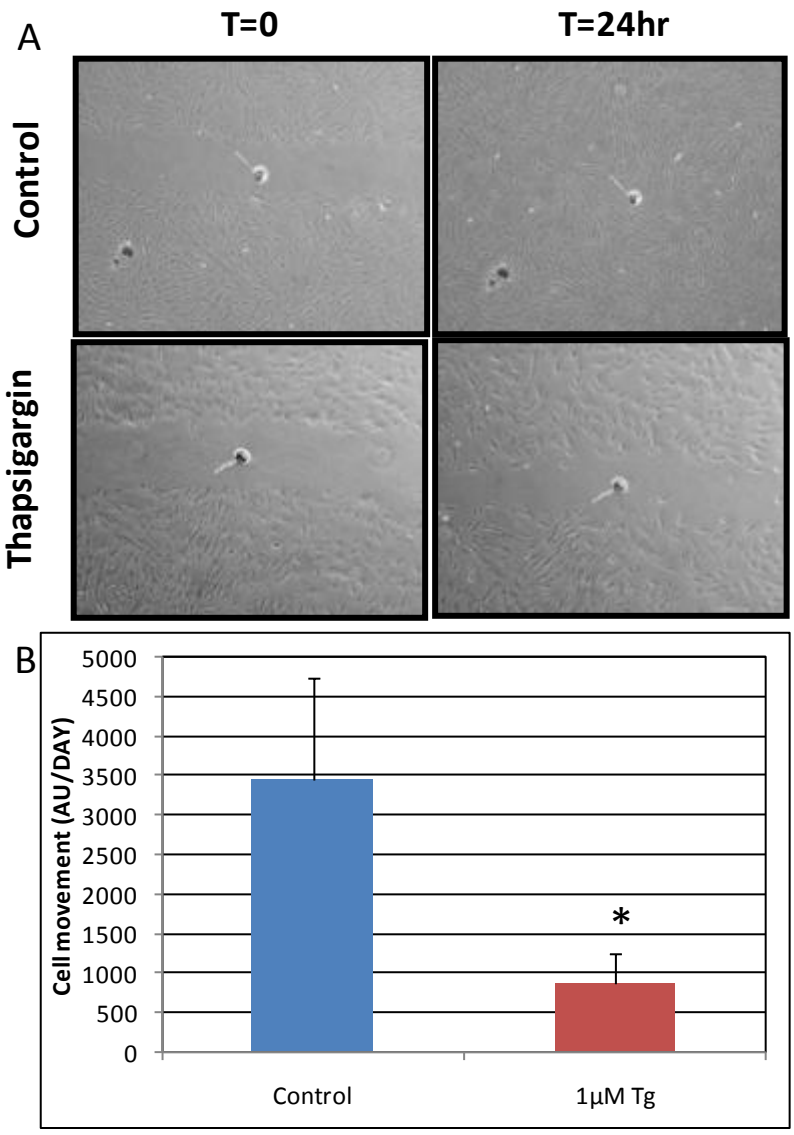


Figure 3.14. Effects of thapsigargin on cell migration of pterygial-derived fibroblasts detected using the scratch assay. Pterygial fibroblasts were cultured for 24 hours in EMEM supplemented with 10% FCS ± 1µM thapsigargin (A). The cell movement is significantly retarded in the thapsigargin treated group (B). The data represent means ± S.D. from experiments performed on pterygial derived fibroblasts from three different donors. * indicates a significant difference between non-stimulated control and treated group ($p \leq 0.05$; Student's t test).

3.2.5 Genes up-regulated and down-regulated by 10% serum in pterygial derived fibroblasts

A total of 104 genes were up-regulated by more than 2 fold in pterygial fibroblast in response to 10% serum. Many of these up-regulated genes have biological functions of relevance to pterygium, such as cell cycle division, growth, proliferation, differentiation and contraction. A total of 53 genes were down-regulated by more than 2 fold in pterygial fibroblast following the treatment with 10% serum. Many of these down-regulated genes have biological functions associated with regulation of cholesterol biosynthesis, anti-angiogenesis, and negative regulation of cell growth, differentiation and transcriptional activities. The 50 genes showing the greatest level of increase and decrease in response to serum are shown in Appendix I.

3.2.6 Gene expression profile of histaminergic, purinergic, cholinergic and EGF receptors in pterygial fibroblasts

Four histamine receptor HRH1, 2, 3 and 4 were detected in pterygial fibroblast giving a low baseline signal value (Figure 3.15). Gene expression of these receptors was unchanged following treatment with 10% serum (Figure 3.15). Of the 18 cholinergic receptors, both muscarinic and nicotinic receptor subtypes were detected in pterygial fibroblasts, the majority of these genes were associated with a relatively low signal. Nicotinic α5 and β1 had a relatively higher baseline signal (Figure 3.15). The gene

expression of all cholinergic receptors remained unchanged following addition of 10% serum (Figure 3.15). Epidermal growth factor (EGF) receptor was detected and again expression was unaffected by 10% serum exposure. Of the 20 purinergic receptors (P2X and P2Y subtypes) all were detected in pterygial fibroblasts, these receptors show varying baseline signal values with P2RX4 and P2RY5 having relatively higher signal values (Figure 3.15). However, only P2RY5 was down-regulated 1.3 fold and other purinergic receptors remained unchanged following treatment with 10% serum (Figure 3.15). All three IP_3 receptors are present in pterygial fibroblasts. Type1 and 3 were detected with a high baseline signal relative to IP_3R1 (Figure 3.16). IP_3R1 was down-regulated 1.3 fold, following treatment with 10% serum, while expression of the other two IP_3 receptor gene expressions were unchanged (Figure 3.16). Three SERCA Ca^{2+}-ATPases were detected in pterygial fibroblasts. ATP2A2 had the highest baseline signal value; serum treatment had no effect on expression. 19 phospholipase C family members were detected with varying baseline signal values; PLCD1, D3, G1, L2 and XD1 had relatively high signal levels. PLCL2 was down-regulated 1.3 fold and PLCXD3 was up-regulated 1.3 fold while gene expressions of all other members remained unchanged with exposure to serum. The guanine nucleotide binding protein (GNAQ) is expressed in pterygial cells and its gene expression pattern was unchanged by 10% serum.

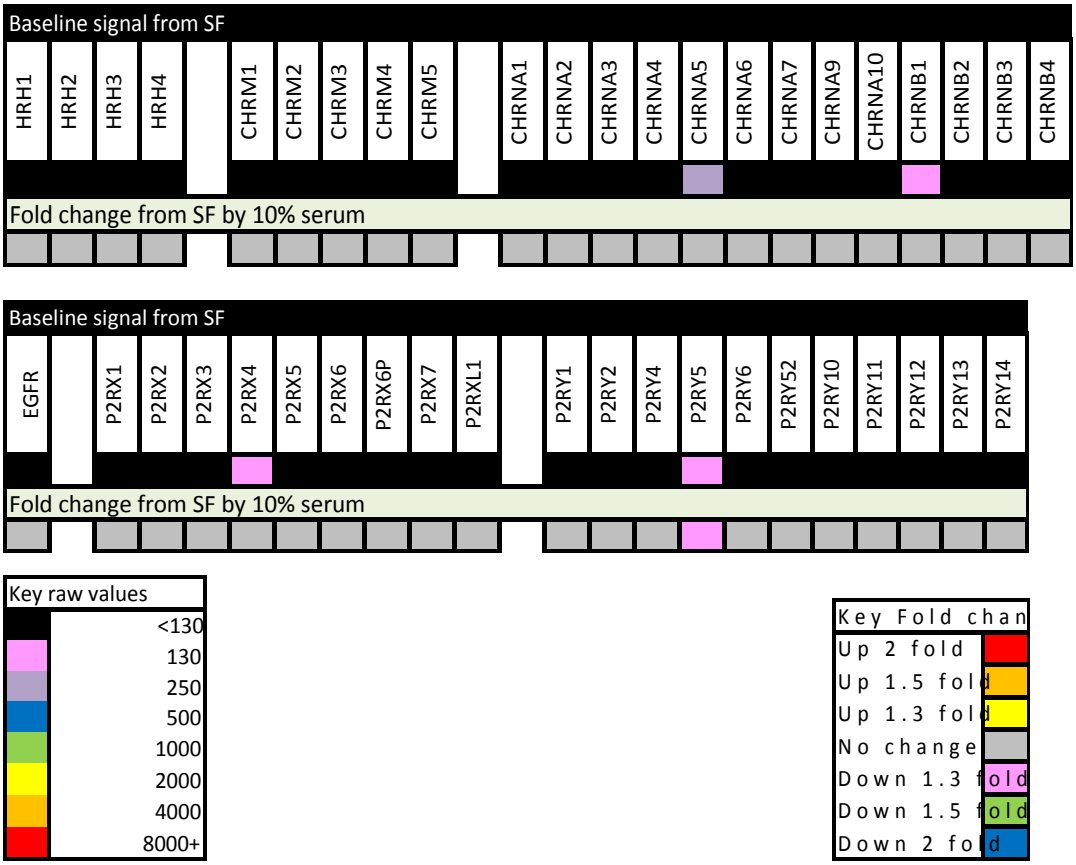


Figure 3.15. A gene expression profile of histaminergic, purinergic, cholinergic, EGF receptors detected in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 10% serum relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

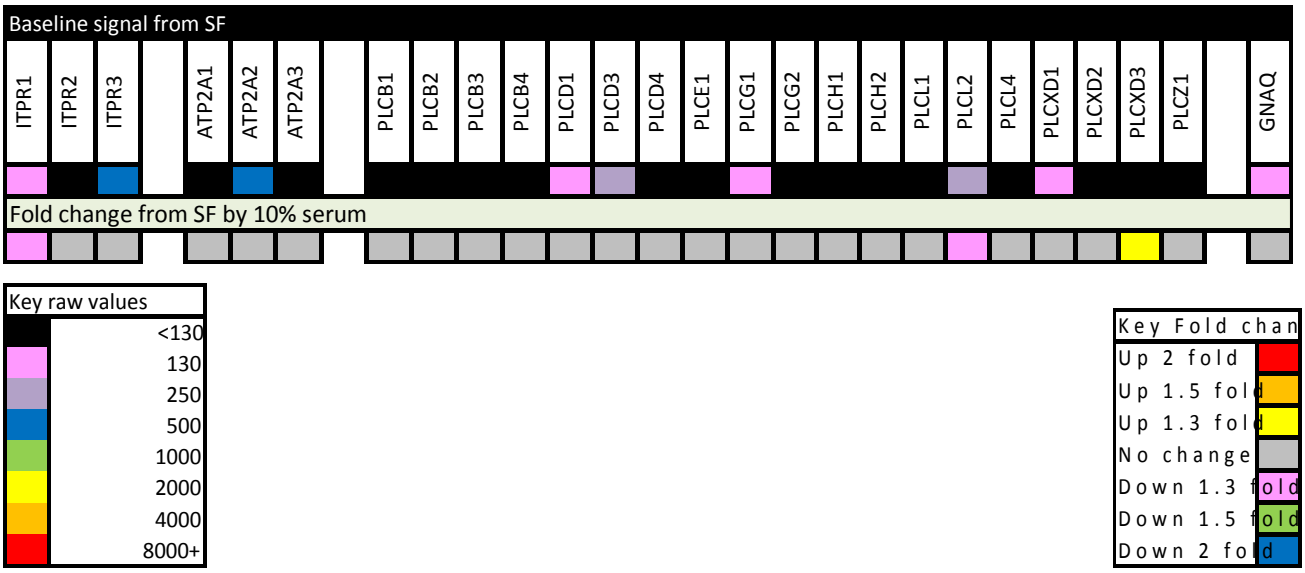


Figure 3.16. A gene expression profile of calcium signalling components detected in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 10% serum relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

3.2.7 Angiogenesis factors expression

Proteome Profiler™ Array data revealed that pterygial-derived fibroblasts release angiogenic factors into the bathing medium (Figure 3.17). 24 proteins were detectable using image analysis software (Image J). Of these, 19 have been reported to be pro-angiogenic factors and five anti-angiogenic factors (Figure 3.18). Serum exposure resulted in a significant increase IL-8 protein level to 181.48% ± 39.24%, MMP9 to 122.77% ± 7.48%, VEGF to 186.62% ± 53.77% and MCP-1 115.67% ± 7.29%, but significantly reduced the levels of Insulin-like growth factor-binding protein 3 (IGFBP-3) to 75.94 ± 10.56%, FGF-4 to 60.58 ± 17.05%, EG-VEGF to 56.14 ± 11.25%, IGFBP-1 to 33.33 ± 33.85%, artemin to 48.28 ± 13.04%, FGF-1 to 39.18 ± 33.7%, angiogenin-1 to 38.06 ± 21.9%, platelet derived growth factor-AA (PDGF-AA) to 36.67 ± 5.04%, IL-1β to 22.29 ± 19.4% and fibroblast growth factor 2 (FGF-2) to 15.16 ± 22.6% when compared with untreated control SF EMEM control. Serum maintenance also affected the anti-angiogenic proteins, such that it significantly decrease angiostatin protein expression to 17.8 ± 8.2%, SerpinE1 to 66.75 ± 20.4%, metallopeptidase inhibitor 1 (TIMP-1) to 65.11 ± 20.2% and thrombospondin-1 to 42.62 ± 12.5% of SF EMEM control.

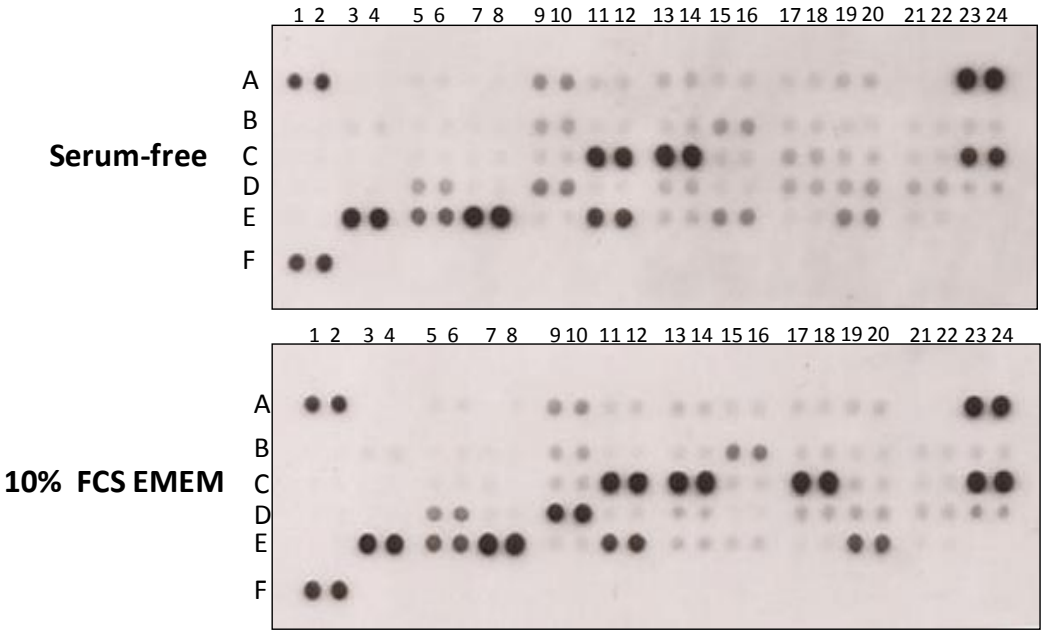
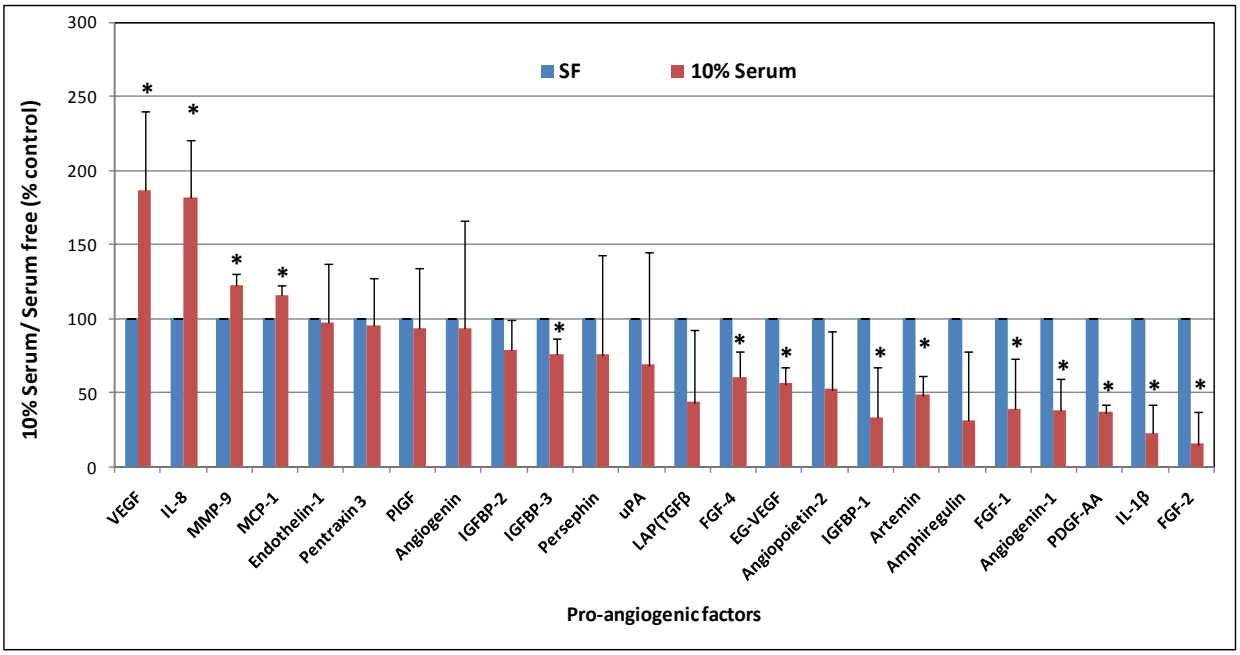


Figure 3.17. The human angiogenesis array detects multiple analytes in serum free and 10% serum pterygial-derived fibroblasts culture supernatants. Array images were exposure in the X-RAY film and the detectable protein spots were labelled on the film. The coordinate reference for analyte identification is listed in the Methods section, Table 2.2.



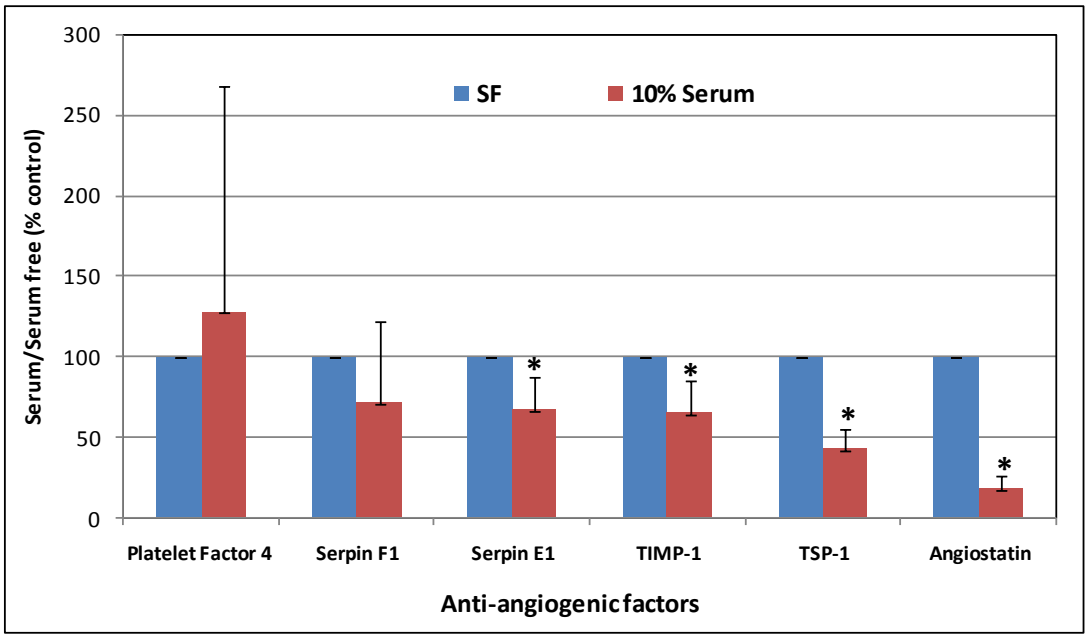


Figure 3.18. Proteome profile of angiogenic factors released from pterygial-derived fibroblasts maintained in the presence or absence of serum for a 24 hour period. The data represent means ± S.D. compiled from three separate experiments. * Indicates a significant difference between serum maintained and serum starved groups (Student's ttest, $p \leq 0.05$).

3.3 Discussion

Pterygium exhibits a high degree of vasculature relative to normal conjunctiva. The pro-angiogenic VEGF has been identified in pterygial specimens and its presence links in well with the abundance of blood vessels (Liang, Jiang *et al*. 2012). Therapeutic approaches using anti-VEGF have been used and some benefit has been reported (Leippi, Grehn *et al*. 2009). It was therefore of interest to determine the importance of this rich vasculature and determine the impact of restricting nutrient supplies to pterygial cells. A simple approach was used, which involved serum maintenance or serum starvation; the intention of this strategy was to mimic an environment that has a rich or diminished blood supply and one that is poor. As predicted a progressive decrease in cell growth was observed as serum levels declined. Pterygial cells are therefore reliant on a supply of growth and survival factors. Having established that serum starvation reduces cell growth, analysis of calcium signalling was performed.

Pterygial tissue is characterized by extensive cellular proliferation, transdifferentiation and angiogenesis which is linked to Ca^{2+} signalling activities (Coroneo, Di Girolamo *et al*. 1999). The endoplasmic reticulum (ER) is the main intracellular Ca^{2+} store/release organelle. The cells can either elevate cytosolic Ca^{2+} by releasing Ca^{2+} from the intracellular store or uptake Ca^{2+} into the cell from extracellular solution. Store-operated Calcium entry (SOCE) is the dominant Ca^{2+} entry pathway (Parekh and Penner 1997). In

my study, Histamine, ATP, Acetylcholine and epidermal growth factors were evaluated for their effect on intracellular calcium levels of pterygial fibroblasts in the presence and absence of serum. All ligands demonstrated a reduced ability to raise intracellular calcium concentration following serum starvation. It demonstrates that receptors are available on the surface of pterygial cells. The active histamine and epidermal growth factor receptors have been determined in human pterygium (Maini, Collison *et al*. 2002). Calcium signalling is impaired and may be the result of differences in receptor expression or modification of signalling molecules common to these ligand/receptor systems. In gene microarray analysis, four histamine receptors H1, 2, 3 and 4 were detected in pterygial fibroblast but their receptors gene expressions were unchanged following treatment with 10% serum. HRH1 is the dominant receptor and is known to elevate intracellular Ca^{2+} level (Francis, Glaser *et al*. 2008). Mast cells that can produce histamine and in pterygium these cells are nearly twice as abundant as observed in normal conjunctiva (Nakagami, Murakami *et al*. 1999). Therefore, a rich blood supply is likely to maintain the stimulation of pterygial cells by the enhanced levels of histamine available. Moreover, due to the inflammatory nature of pterygium the histamine stored in mast cells is likely to be released. Muscarinic and nicotinic acetylcholine receptors were detected in pterygial fibroblasts. Cholinergic nicotinic receptor α5 and β1 had the relatively high baseline signal. They belong to nicotinic acetylcholine receptor superfamily and are triggered by the binding of the neurotransmitter acetylcholine (ACh) to form ligand-gated ion channels that mediate signal transmission across plasma membrane

(Brejc, van Dijk *et al*. 2001). It is reported that nACh can modulate endothelial cell survival, proliferation, and migration to result in pathological angiogenesis (Heeschen, Jang *et al*. 2001; Cooke and Ghebremariam 2008). The receptors may be activated by acetylcholine or nicotine to induce endothelial-cell growth and tube formation and accelerate fibrovascular growth in pathophysiological disorder (Heeschen, Jang *et al*. 2001; Zhu, Heeschen *et al*. 2003). Muscarinic receptors M1, M3 and M5 were suggested to play a role in intracellular Ca^{2+} by coupling to G proteins to activate phospholipase C (Caulfield 1993). The gene expression of all acetylcholine receptors remained unchanged following addition of 10% serum. Epidermal growth factor (EGF) receptor was expressed but showed no change in expression in 10% serum relative to serum-free medium. ATP purinergic receptors (P2X and P2Y) were detected in pterygial fibroblasts. The purinergic receptor P2Y, G-protein coupled 4 and 5 were abundant under non-stimulated conditions. However, P2RY5 was down-regulated following treatment with 10% serum. P2Y G protein-coupled receptor functions as a receptor for extracellular ATP and ADP and mobilizes intracellular calcium ions via activation of phospholipase C (Abbracchio, Burnstock *et al*. 2006). Recent studies have suggested that both ATP-gated P2X (ion channel) and P2Y (G protein-coupled) signalling may contribute to platelet aggregation, cell proliferation, differentiation and migration in human vascular diseases (Gachet 2006; Erlinge and Burnstock 2008). However, these ligands can stimulate a number of pathways in cells. Scrutiny of calcium signalling components within the gene array data provided assessment of changes at the message level. Many studies have investigated inositol

trisphosphate in calcium signalling, inositol 1, 4, 5-trisphosphate (IP_3) is a secondary messenger molecule used in signal transduction in cells. It is produced by phospholipase C pathway (PLC) which catalyses the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) (Berridge 1993; Clapham 1995; Bootman, Collins *et al*. 2001). With regard to IP_3 receptors present on the microarray, type1 and 3 were detected with high baseline signals and IP_3R1 was down-regulated by 10% serum in pterygial fibroblasts. Of the 19 phospholipase C family members, PLCD1, D3, G1, L2 and XD1 had relatively high signal levels. PLCL2 was down-regulated and PLCXD3 was up-regulated with abundance of serum. A study suggested that PLC like-2 protein is involved in IP_3 regulation around endoplasmic reticulum (ER) (Otsuki, Fukami *et al*. 1999). PLCX-domain containing protein 3 was identified in cytoplasm and suggested different functions in animal models in a recent study (Gellatly, Kalujnaia *et al*. 2010). Above all, explicitly linking calcium mobilization to a specific function is not straightforward. To demonstrate a functional consequence of calcium signalling we have determined the effects of calcium signalling ablation, using intracellular Ca^{2+} pump inhibitor thapsigargin to investigate the effects on pterygial fibroblast proliferation and migration. However, all ligands show a similar pattern of response which suggests that a common factor is changing. To address this issue we concentrated our efforts on the calcium store itself. The Ca^{2+}ATPase inhibitor thapsigargin can be used to establish store content. It depletes the ER store slowly and evokes the cytosolic free Ca^{2+} without elevation in inositol polyphosphates (Parekh and Penner 1997). Application of thapsigargin to cells maintained in serum free medium

demonstrated that the calcium level within the endoplasmic reticulum store was depleted relative to serum maintained cells. Therefore, these data illustrate that intracellular calcium stores are sensitive to serum deprivation: this could have a marked effect on cell behaviour. The ER calcium store is regulated by a number of components. The receptor channels inositol-1, 4, 5-phosphtate- (IP_3R) and ryanodine-receptors (RYR) release Ca^{2+} from intracellular stores (Bergner and Huber 2008). Therefore reduction in these channels would impair release from the store. In addition, sarcoplasmic/endoplasmic reticulum-CaATPASE (SERCA), is an ER trans-membrane protein that pumps calcium back into the ER; without this pump calcium recruitment in the ER cannot occur. The gene expressions of SERCA Ca^{2+}-ATPases were detected in pterygial fibroblasts and ATP2A2 had correspondingly high baseline signal values. ATP2A2 encoded SERCA Ca^{2+}-ATPases are intracellular pumps located in the sarcoplasmic or endoplasmic reticula of muscle cells, it is involved in regulation of the contraction and relaxation cycle (Sakuntabhai, Ruiz-Perez *et al*. 1999). Further regulation can occur within the ER: Calreticulin (CRT) and calsequestrin (CASQ) are two major Ca^{2+} -binding proteins inside the ER membranes that act as Ca^{2+} buffers (Milner, Famulski *et al*. 1992; Mery, Mesaeli *et al*. 1996; Bergner and Huber 2008). Both calreticulin and calsequestrin play a critical role in Ca^{2+} homeostasis in the lumen of the ER (Michalak, Corbett *et al*. 1999; Beard, Laver *et al*. 2004). Calsequestrin is a regulator of RyR activity and many studies show that it regulates protein synthesis (Helenius, Trombetta *et al*. 1997; Saito, Ihara *et al*. 1999) while calreticulin is a versatile lectin-like chaperone and also has been

implicated in a variety of cellular functions (Michalak, Corbett *et al*. 1999). The major function of these Ca^{2+}-binding chaperones is to increase the Ca^{2+} storage capacity of the ER lumen. Therefore, expression of such proteins is vital for active calcium signalling. Scrutiny of these expression patterns in serum-maintained and serum-deprived cells will be a worthwhile topic of study in the future.

Having demonstrated that the ER store can be modulated by serum deprivation it was important to determine a functional role for calcium signalling in pterygium. The agonists used to assess changes in calcium signalling can stimulate a number of pathways; therefore explicitly linking calcium mobilization to a specific function by a ligand isn't straightforward. Further investigation of the signalling pathways activated by these ligands and their functional roles in pterygium will be a valuable topic of investigation in the future. However, to demonstrate a functional consequence of calcium signalling it was necessary to utilise thapsigargin to deplete the store and disrupt signalling. Using this approach we have shown that disruption of the ER store and thus calcium signalling could affect cell growth and migration of pterygial-derived fibroblast. This suggests that regulation of the ER store is an important consideration in the management of pterygium. These data tie in well with studies in other cells and tissues that have demonstrated that the ER store is required for cell division (Wang, Wormstone *et al*. 2005) and migration.(Duncan, Wormstone *et al*. 1997; Nicola, Timoshenko *et al*. 2005) Persistent depletion of the store can result in reduced protein synthesis, ER stress and

apoptosis.(Shi, Wang *et al*. ; Nicola, Timoshenko *et al*. 2005; Wang, Wormstone *et al*. 2005; Zhang, Duncan *et al*. 2007) It is feasible that serum starvation over prolonged periods of time could lead to an increasingly diminished store. This in turn could affect basic functions associated with the progression of pterygium. In the first instance a reduction in migration and proliferation would be predicted followed by ER stress lead processes such as reduced protein synthesis and potentially cell death by apoptosis.(Shi, Wang *et al*. ; Nicola, Timoshenko *et al*. 2005; Wang, Wormstone *et al*. 2005; Zhang, Duncan *et al*. 2007) Applying this principle to the clinic, strategies that reduce the blood supply to the pterygium, such as anti-VEGF (Leippi, Grehn *et al*. 2009), could facilitate a reduction of calcium in the ER store; when this reaches a threshold, cell functions will be impaired leading to reduced growth and potentially cell death. Future work into calcium signalling and the store per se should examine the effects of long-term depletion of the ER store and ER stress related events in relation to cell growth, migration and survival of pterygial fibroblasts when considering putative therapies.

It is evident that a rich blood supply is required to facilitate growth and migration of pterygial cells and that modification to this nutrient supply affects signalling potential, which provides a rational reduced cell growth. It is therefore of interest to assess the effect of serum on the promotion of angiogenenic factor release by fibroblasts and their role in the maintenance of the high level of vasculature in pterygia.

Using a Proteome Profiler™ Array, it was found that serum was capable of promoting release of pro-angiogenic factors and suppressing the anti-angiogenic factors. This suggests that if a rich blood supply exists it is likely to fuel continued production of new blood vessels that will further enrich the pterygial cell environment and progress the disease at a more rapid rate.

Interleukin 8 is a major mediator of the inflammatory response and potent angiogenic factor in many human diseases (Huang, Mills *et al*. 2002; De Paepe, Creus *et al*. 2012; Welling, Fu *et al*. 2012). IL8 was up-regulated in response to 10% serum and is reported to be involved in ultraviolet radiation initiated blood vessel formation, inflammation and cellular proliferation in pterygium (Di Girolamo, Kumar *et al*. 2002). As UV is an initiating factor in pterygia then IL8 is an interesting topic for future investigations. MMP9 was also found to increase in the medium of serum maintained cells. A recent study suggested that pterygium cells may produce the MMPs to stimulate stromal fibroblasts growth by dissolving Bowman's layer (Reid and Dushku 2010). MMP-9 was found highly expressed in advanced stage pterygial tissues and fibroblasts which propose to play a role in progression of pterygium (Naib-Majani, Eltohami *et al*. 2004; Yang, Lin *et al*. 2009). Monocyte chemoattractant protein-1 (MCP-1) was elevated in the presence of serum and has previously been identified as a TGFβ target gene in endothelial cells (ECs) (Ma, Wang *et al*. 2007). It reported that MCP-1 mediates TGFβ–stimulated angiogenesis by increasing cell migration and promoting the formation of blood vessels in vivo, it plays a

direct role in wound healing, angiogenesis and tumour progression (Salcedo, Ponce *et al*. 2000; Low, Drugea *et al*. 2001; Ma, Wang *et al*. 2007). Most interestingly, vascular endothelial growth factor (VEGF) was up-regulated significantly by 10% serum. As a vascular permeability factor, it is potent mitogen acting on the endothelium. In the presence of this growth factor, plated endothelial cells will proliferate, migrate and recruit new capillaries. It plays important roles in angiogenic diseases (Ferrara and DavisSmyth 1997). The current data confirms that in abundant serum environment pterygial fibroblasts are more like to synthesise and release VEGF to promote angiogenesis and the cells still release VEGF to maintain this rich environment.

With regard to anti-angiogenic factor, 10% serum significantly reduced the protein production of anti-angiogenic factors angiostatin, SerpinE1, TIMP-1 and Thrombospondin-1. Angiostatin was reported an angiogenesis inhibitor that suppress neovascularisation and growth of metastases in mouse tumour (Oreilly, Holmgren *et al*. 1994). As protease inhibitors, serpins locate in blood plasma and are involved in angiogenic processes like regulating blood clotting, the complement pathway and extracellular matrix remodelling (Potempa, Korzus *et al*. 1994). SerpinE1 inhibits the activity of matrix metalloproteinases to regulate the progression to fibrosis (Irigoyen, Munoz-Canoves *et al*. 1999). Thrombospondin-1 is a potent inhibitor of angiogenesis; it interacted with ECs surface receptor CD36 to inhibit the proliferation and migration of endothelial cells (Reiher, Volpert *et al*. 2002). It was reported p53 can positively activate

endogenous TSP-1 gene expression to inhibit angiogenesis in fibroblasts (Dameron, Volpert *et al*. 1994). Tissue inhibitors of metalloproteinases function as inhibitors of angiogenic factors, matrix metalloproteinases and induction of apoptosis (Brew, Dinakarpandian *et al*. 2000), it was demonstrated that TIMP-1 can potentially inhibit pterygium invasion (Tsai, Chiang *et al*. 2010).

It seems evident that serum can promote the release of pro-angiogenic factors and suppress the release of anti-angiogenic factors. It should however be noted that angiogenic factors are still released from cells maintained in serum-free conditions. While the net potential of these cells to promote angiogenesis is less than their serum cultured counterparts they still feasible could promote angiogenesis to some degree. The reason of this phenomenon may result from that pterygial fibroblast in serum free medium releasing pro-angiogenic signals in order to promote new blood vessel formation, which could sustain their survival through nutrient enrichment. It has been reported that serum deprivation could induce low oxygen pressure and nutrition depletion (Baek, Jang *et al*. 2000). Hypoxia can activate hypoxia-inducible factor (HIF) to increase VEGF to benefit angiogenesis (Risau 1997; Pousa and Gisbert 2006). The current data support the previous studies that hypoxia mediates angiogenesis by up-regulation of VEGF and various angiogenic factors like FGF-2, IGFBP-1 and angiogenin (Tazuke, Mazure *et al*. 1998; Hartmann, Kunz *et al*. 1999; Kroon, Koolwijk *et al*. 2001). Therefore, both serum starved and maintained conditions could induce the pro-angiogenic factor release from pterygial

derived fibroblast from different mechanisms. However, except IGFBPs all up-regulated angiogenic factors by SF EMEM show very weak protein signal level compared with serum maintained condition.

A rich blood supply can fuel cell growth and migration retain efficient calcium signalling and has the potential to further enrich the vasculature within pterygial tissue. The data therefore support the notion that restriction of the blood supply in pterygium will be of therapeutic benefit to patients.

CHAPTER 4

TGFβ signalling in pterygial fibroblasts

4.1 Introduction

Transforming growth factor beta (TGFβ) is a typical mediator and master switch of fibrosis (Sime and O'Reilly 2001). It involves in the regulation of cell behaviour in ocular tissues. A study suggested TGFβ can induce eye inflammatory responses as well as tissue fibrosis (Connor, Roberts *et al*. 1989). It is expressed more in epithelial and stromal layers of pterygium compared to similar regions in normal conjunctiva (Bianchi, Scarinci *et al*. 2012). TGFβ is believed to play a critical role in inducing transdifferentiation and regulation of the extracellular matrix (Desmouliere, Geinoz *et al*. 1993; Miettinen, Ebner *et al*. 1994). Both TGFβ1 and TGFβ2 are reported to stimulate myofibroblast formation and expression of alpha SMA in different human ocular cells (Lee, Kwon *et al*. 2001; Wormstone, Tamiya *et al*. 2002). In the anterior segment of the human eye, TGFβ2 was found to be predominant in superficial limbal epithelial cells, conjunctival stroma and human aqueous humour (Jampel, Roche *et al*. 1990; Pasquale, Dormanpease *et al*. 1993). TGFβ2 could increase the expression of cell adhesion receptors termed integrins on the surface of cells to control cell proliferation and differentiation (Ignotz and Massague 1987). Furthermore, over-expression of TGFβ may play an important role in the

pathogenesis of pterygium by increasing angiogenesis and lead to the formation of new blood vessels from the pre-existing vasculature (Kria, Ohira *et al*. 1996; Sun and Ke 2007; Bianchi, Scarinci *et al*. 2012). However, the pathogenesis of fibrotic activity in pterygium still needs further investigation. The present study aimed to understand the effects of the inflammatory cytokine TGFβ signalling events on pterygial-derived fibroblasts and contribution with respect to Smad2/3 signalling, transdifferentiation and angiogenesis which could contribute to the process of this disease.

4.2 Results

4.2.1 TGFβ/Smad signalling in pterygial fibroblasts

5000 cells in 100μl were seeded on a square sterile coverslip housed in a tissue culture dish. Cells were allowed to establish and then placed in serum-free medium for 24 hours prior to addition of TGFβ. Over 20 cell nuclei on each image were quantified. Immunocytochemistry revealed that Smad2/Smad3 was translocated to the nucleus in response to TGFβ1 and 2 in pterygial fibroblasts (Figure 4.1-4.2). Non-stimulated (control) cells did not demonstrate significant levels of nuclear Smad2/3 over the experimental duration. Stimulation with 0.001ng/ml TGFβ1 or 2 did not induce a significant mobilization of Smad2/3 to the nucleus relative to control cells at any time point studied (Figure 4.3-4.4). Application of 0.01ng/ml TGFβ2 again did not show a significant accumulation of Smad2/3 against control (Figure 4.4), but stimulation was observed with

application of 0.01ng/ml TGFβ1 at the 120min time-point (Figure 4.3). Addition of TGFβ1 and TGFβ2 at 0.1ng/ml leads to a marked translocation of Smad2/3 to the nucleus (Figure 4.3-4.4). TGFβ1 invoked a significant accumulation of Smad2/3 that was first observed following a 30 min exposure period, whereas this change was first observed with TGFβ2 at the 1 hour time point. Stimulation with 1ng/ml TGFβ1 or TGFβ2 gave the greatest response of all concentrations tested. Significant changes in nuclear Smad2/3 were seen following 30 minutes and remained elevated for the experimental duration. With both isoforms peak Smad2/3 accumulation was observed at the 120 min time point.

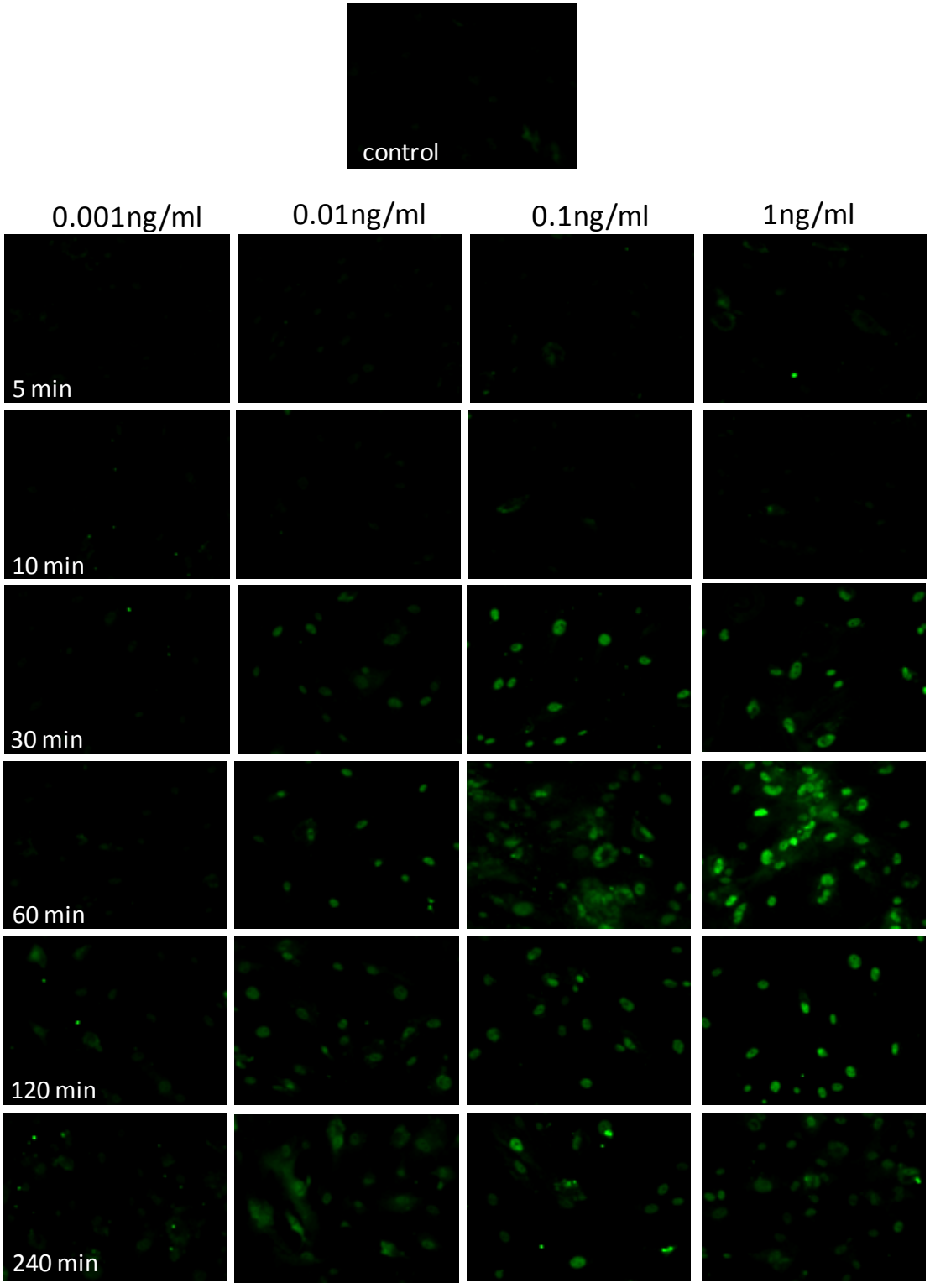


Figure 4.1. Fluorescent micrographs showing Smad 2/3 distribution in pterygial-derived fibroblasts following TGFβ1 stimulation. Smad 2/3 is depicted as green. The field of view in each case represents 448 x 342 μm.

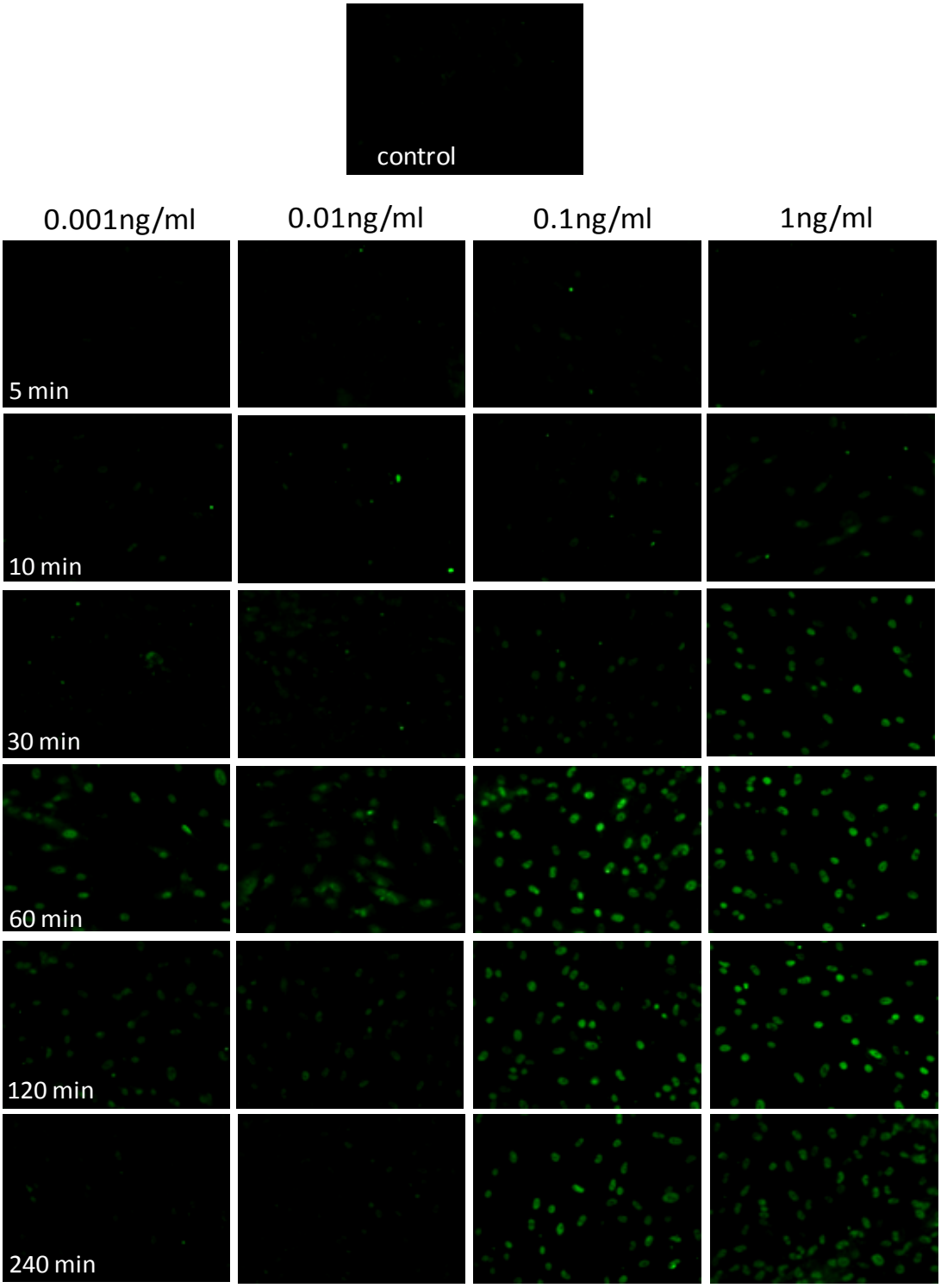


Figure 4.2. Fluorescent micrographs showing Smad 2/3 distribution in pterygial-derived fibroblasts following TGFβ2 stimulation. Smad 2/3 is depicted as green. The field of view in each case represents 448 x 342 μm.

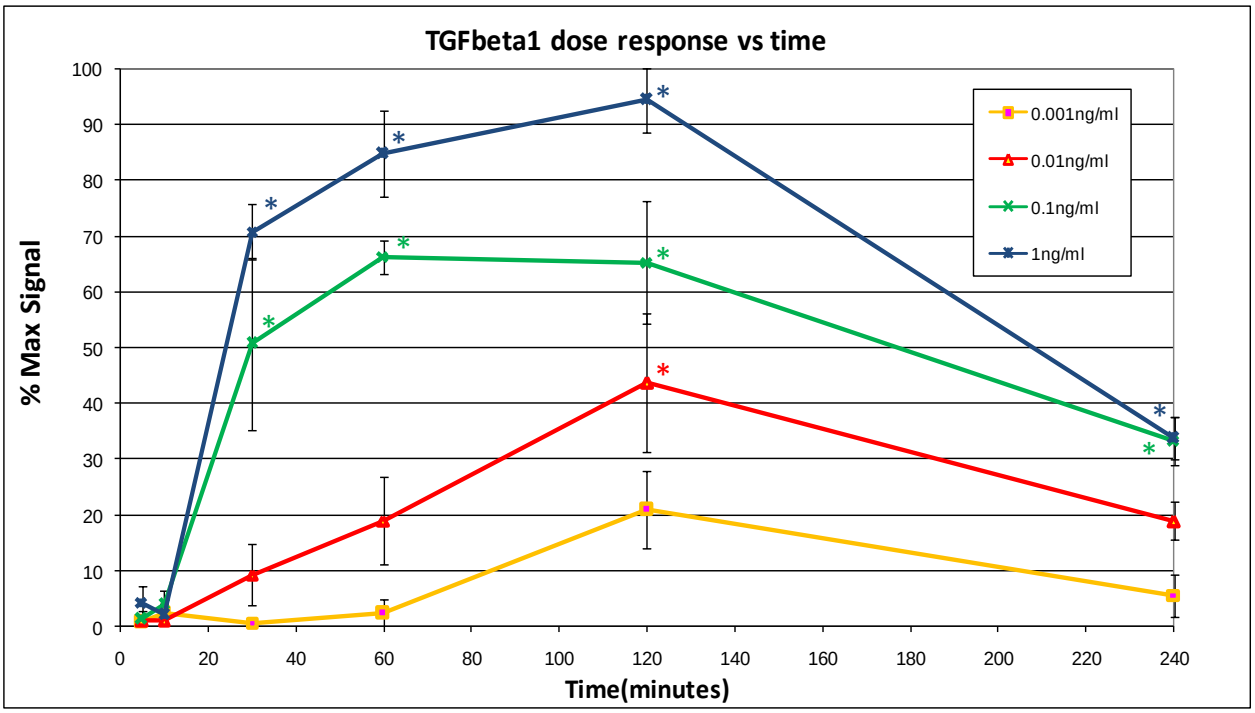


Figure 4.3. TGFβ1 induced Smad 2/3 translocation into the nuclei of pterygial fibroblasts as a function of dose and time. The data represent means ± S.E.M. compiled from three separate experiments. * indicates a significant difference between non-stimulated control and treated group ($p \leq 0.05$; ANOVA with Dunnett's post hoc test).

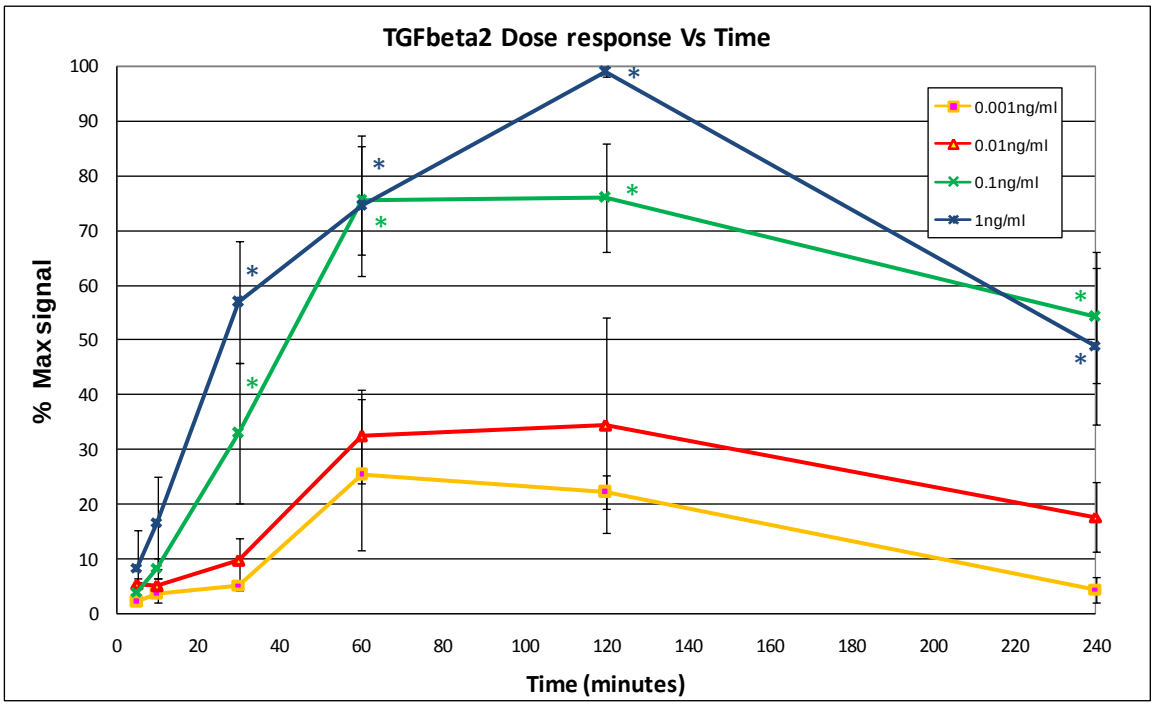


Figure 4.4. TGFβ2 induced Smad 2/3 translocation into the nuclei of pterygial fibroblasts as a function of dose and time. The data represent means ± S.E.M compiled from three separate experiments. * indicates a significant difference between non-stimulated control and treated group ($p \leq 0.05$; ANOVA with Dunnett's post hoc test).

4.2.2 Transdifferentiation of pterygial fibroblasts

In order to determine the effect of serum, TGFβ1 and TGFβ2 on pterygial fibroblast transdifferentiation to a myofibroblast, expression of αSMA was used as a marker. This evaluation was carried out on cultured pterygial fibroblasts derived from three different donors. Real-time PCR was performed to assess αSMA gene expression. 10% serum was found to promote αSMA expression to levels of 255.33% ± 262.1% compared to non-stimulated controls (Figure 4.5). Treatment of pterygial fibroblasts with 1ng/ml TGFβ1 caused a 488.06 ± 390.1% increase in αSMA expression when compared to control. TGFβ2 (1ng/mL) stimulation resulted in a significant increase in gene expression to 491.33 ± 237.3% in comparison with control (Figure 4.5).

Similarly, Western blot analysis reveals that αSMA was expressed in pterygial fibroblasts. There were weak but detectable bands in serum free controls, but αSMA protein was up-regulated following 24 hours stimulation with serum, such that expression levels rose to 154.85% ± 36.93% compared with non-stimulated control cells. In addition, the αSMA protein was also up-regulated with TGFβ2. in this case protein levels increased to 365.37 ± 326.4% compared to the control group (Figure 4.6).

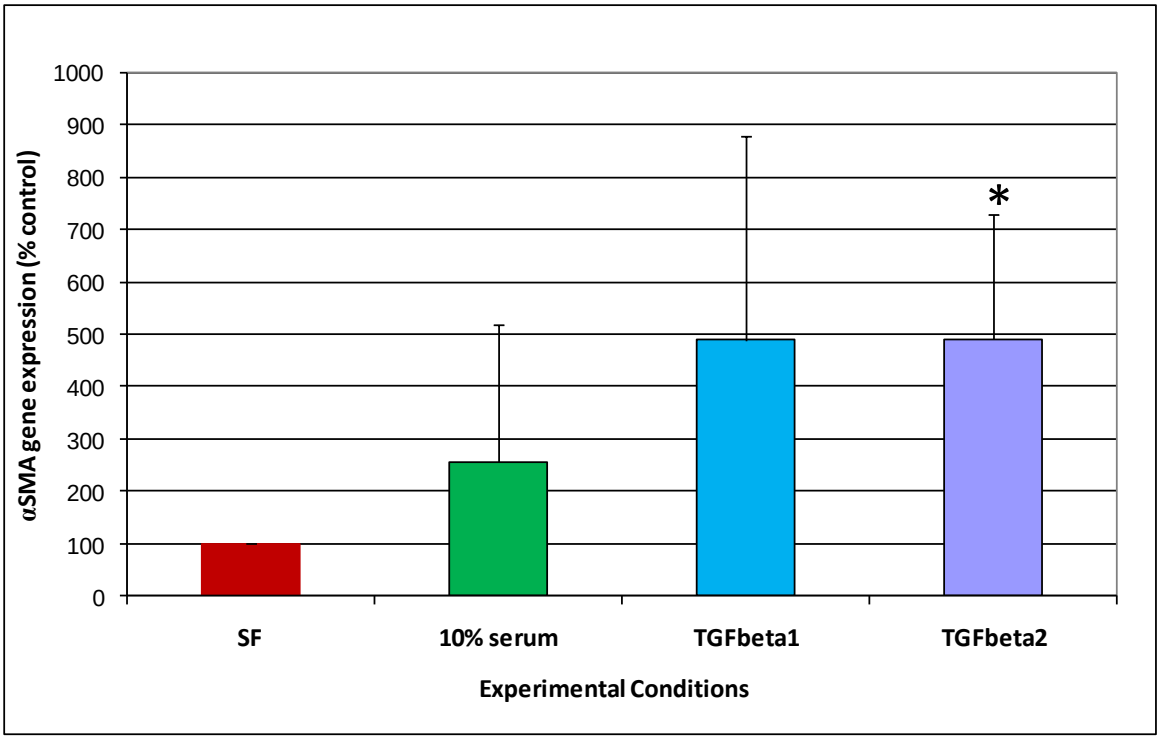


Figure 4.5. αSMA gene expression in pterygial fibroblasts detected using QRT-PCR. The data represent means ± S.D. compiled from three separate experiments. TGFβ concentration applied was 1ng/ml. * Indicates a significant difference between treated and control groups (Student's ttest, $p \leq 0.05$).

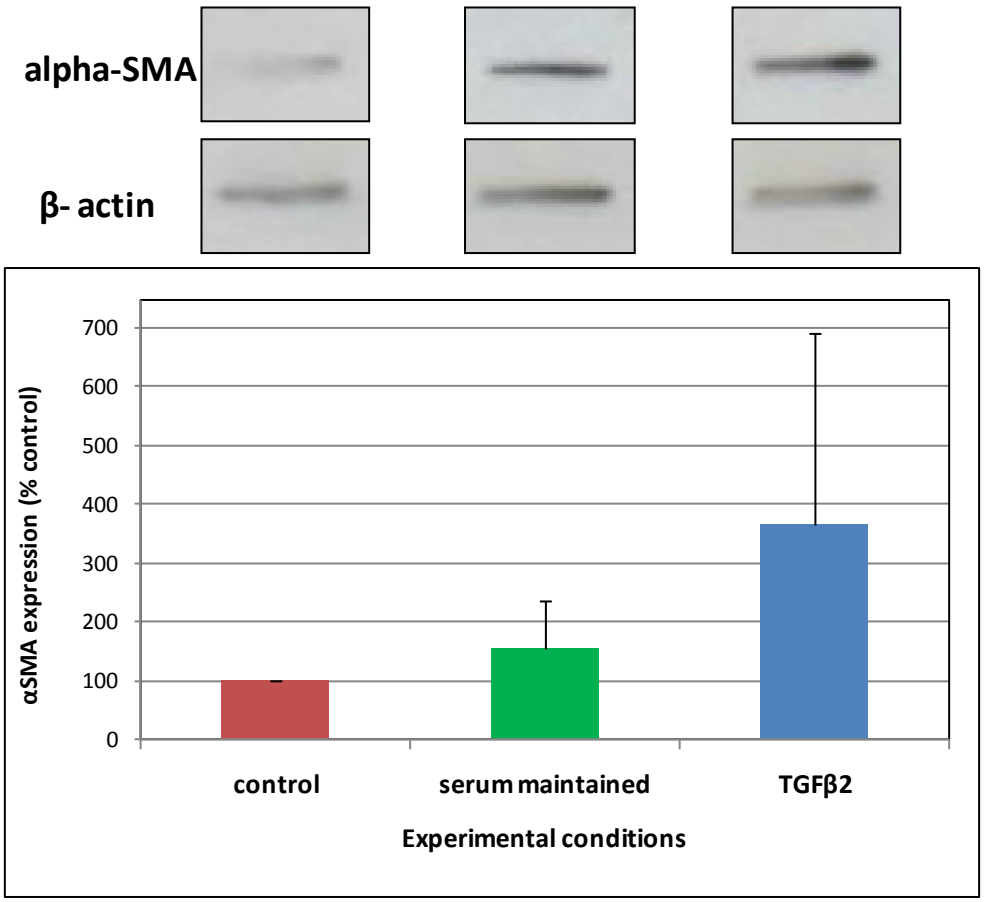


Figure 4.6. Protein level of αSMA was detected in pterygial fibroblasts using the Western blot method. Pterygial-derived fibroblasts maintained in the presence or absence of TGFβ2 for a 24 hour period. The data represent means ± S.D. compiled from four separate experiments.

4.2.3 Angiogenic factors expression

Proteome Profiler™ Array data revealed that pterygial-derived fibroblasts release angiogenic factors into the bathing medium. 31 proteins were detectable using image analysis software (Image J) from three separate experiments (Figure 4.7). 24 of these factors are reported to be pro-angiogenic and 7 are anti-angiogenic factors. Measurement of the cell culture supernatant revealed the protein profile associated with each treatment (Figure 4.7). The data reveals that the release of angiogenic factors, namely fibroblast growth factor 1, 2, 4, 7, amphiregulin, IL-1β, persephin, LAP(TGFβ), placental growth factor (PlGF), PDGF-AA, Artemin, Prolactin, Angiopoietin-1 and Angiogenin were up-regulated significantly in response to 1ng/ml TGFβ2 in pterygial fibroblast. Importantly, TGFβ2 exposure resulted in a significant increase in production of IL-8 to 542.3 ± 107.97%, VEGF to 183.93 ± 23.64%, EG-VEGF to 166.83 ± 17.65%, endothelin-1 to 167.3 ± 20.33%, , MMP9 to 160.08% ± 8% and angiogenin 166.83 ± 17.65% when compared with untreated control medium (Figure 4.8). TGFβ2 stimulation also affected the release of anti-angiogenic proteins; it significantly activates production of angiostatin, Thrombospondin-2 and Platelet Factor 4 (Figure 4.8).

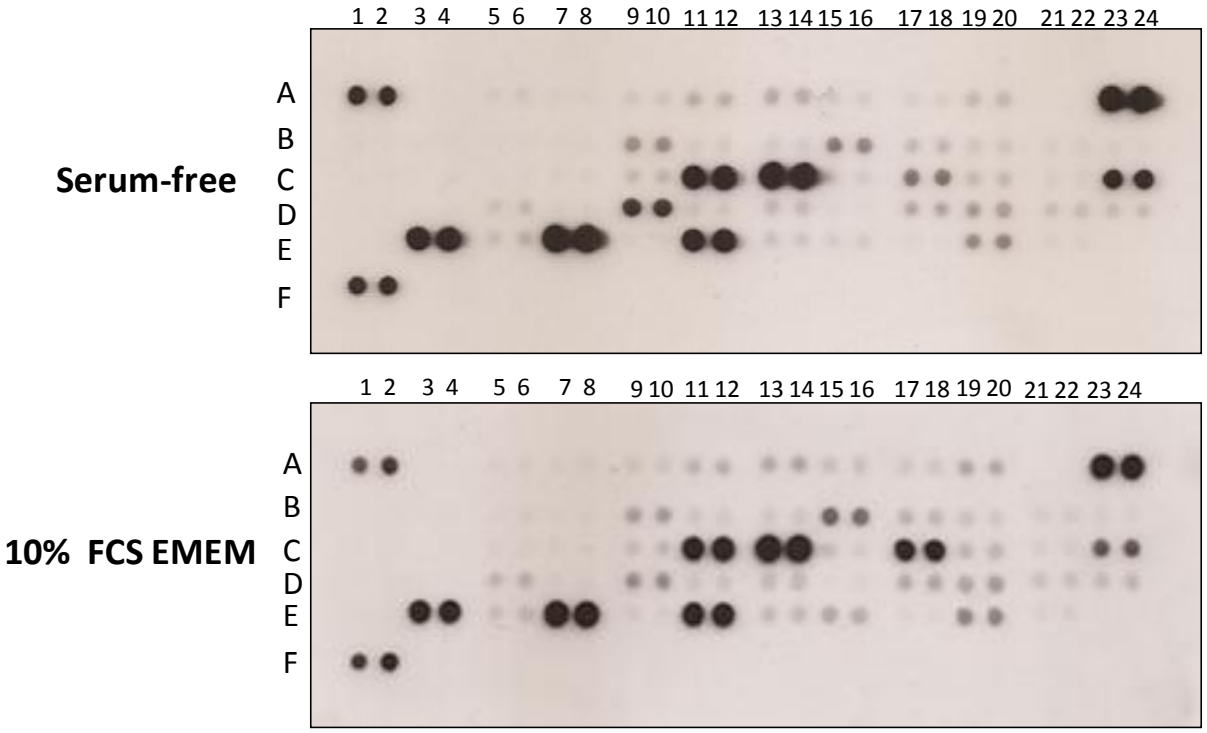
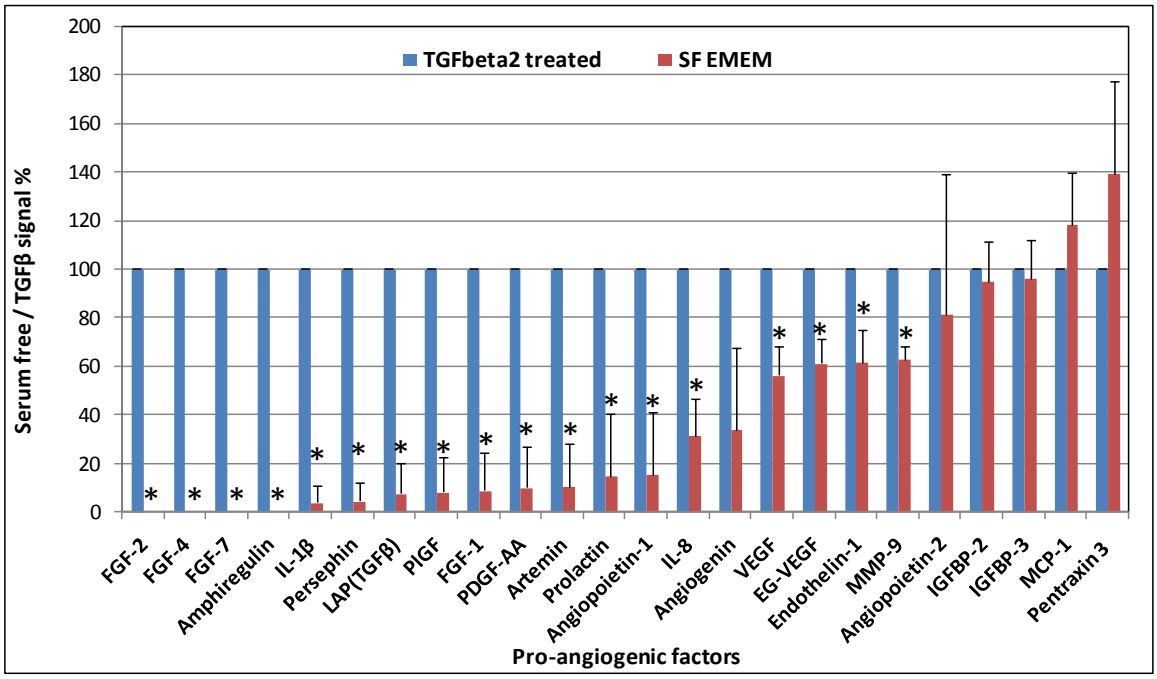


Figure 4.7. The human angiogenesis array detects multiple analytes in serum free and TGFβ2 treated pterygial-derived fibroblasts culture supernatants. Array images were exposure in the X-RAY film and the detectable protein spots were labelled on the film. The coordinate reference for analyte identification is listed in the Methods section, Table 2.2.



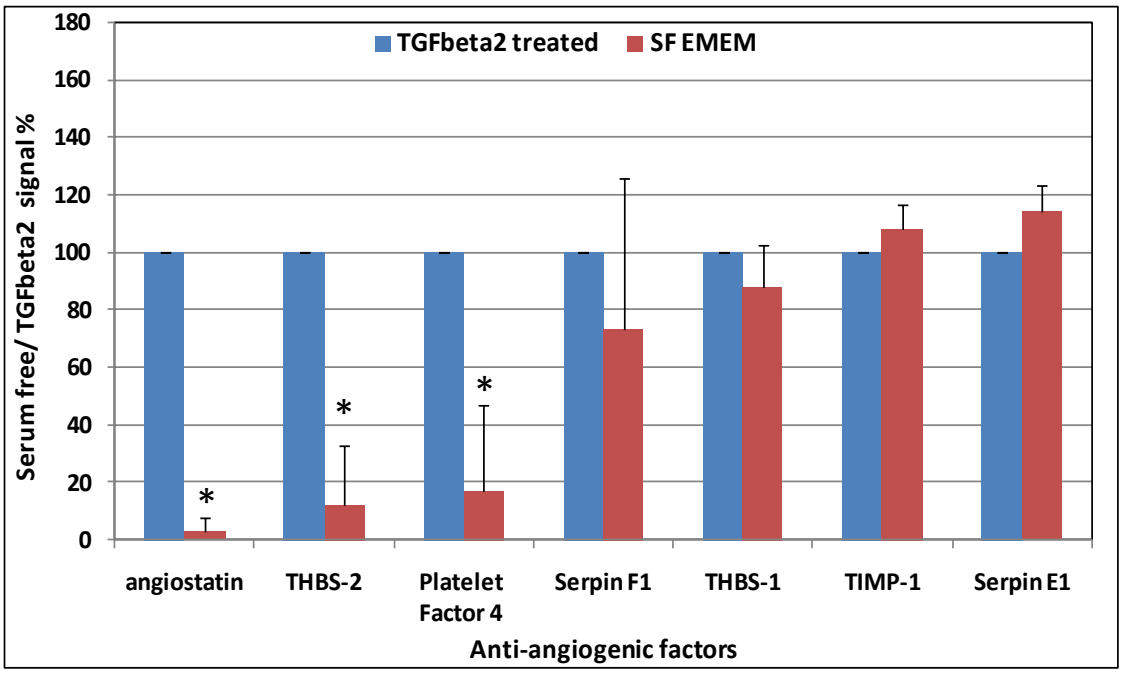


Figure 4.8. Proteome profile of angiogenic factors released from pterygial-derived fibroblasts maintained in the presence or absence of 1ng/ml TGFβ2 for a 24 hour period. The data represent means ± S.D. compiled from three separate experiments. * Indicates a significant difference between TGFβ2 treated and serum free control groups (Student's ttest, $p \leq 0.05$).

4.2.4 Gene array

4.2.4.1 Gene up-regulated and down-regulated by TGFβ2 in pterygial derived fibroblasts

Following treatment with TGFβ2, the differential changes in gene expression detected by Illumina microarrays. A total of 198 genes were up-regulated by more than 2 fold in pterygial fibroblast by TGFβ2 in four biological replicates. Many of these up-regulated genes had biological functions relevant roles in cell growth, proliferation, contraction, transdifferentiation, tissue remodelling, signal transduction and angiogenesis. A total of 197 genes were down-regulated by more than 2 fold in pterygial fibroblast treated with TGFβ2. Many of these down-regulated genes have biological functions including roles in signalling, anti-angiogenesis and as suppressors of cell growth and differentiation. The highest 50 up-regulated and down regulated genes by TGFβ2 were listed with gene information in the Appendix I.

4.2.4.2 Gene expression profile of growth factor and their receptors in pterygial fibroblasts

TGFβR2 and TGFβR3 were detected in pterygial fibroblast (Figure 4.9) giving a higher baseline signal value compared to TGFβR1 while its baseline signal was undetected. TGFβR2 gene expression was down-regulated 1.5 folds following treatment with TGFβ2; in contrast, TGFβR3 gene expression was down-regulated more than two-fold (Figure 4.9).

Of the three bone morphogenic protein (BMP) receptors detected in pterygial fibroblasts BMPR2 had the highest value for baseline signal expression. The gene expression of all three BMP receptors remained unchanged following treatment with TGFβ2. Four fibroblast growth factor (FGF) receptors were detected in pterygial fibroblasts. All FGF receptors demonstrated a very low value for baseline signal expression (Figure 4.9). The gene expression of the four FGF receptors remained unchanged following treatment with TGFβ2 (Figure 4.9). Amongst epidermal growth factor (EGF) receptors family members, ERBB2 was detected slightly higher baseline signal value in pterygial fibroblasts while EGFR, ERBB3 and ERBB4 illustrated low baseline signal value (Figure 4.9). Three EGF receptors expressed in pterygial cells, ERBB2 was negatively affected by TGFβ2 treatment more than 1.5 fold (Figure 4.9).

TGFβ isoforms 1, 2, and 3 were detected in pterygial cells. However, the baseline signal of TGFβ1 was relatively low value (Figure 4.9). TGFβ2 and TGFβ3 gene expressions were up-regulated more than 1.3 fold following addition of TGFβ2. In contrast, the gene expression of TGFβ1 was unchanged (Figure 4.9). Connective tissue growth factor (CTGF) was detected in pterygial fibroblasts with a high baseline signal value (Figure 4.9) and was up-regulated by more than two fold following treatment with TGFβ2 (Figure 4.9). Ten BMP isoforms on the microarray were all detected in pterygial fibroblasts and BMP1, 2, 3 and 6 showed relatively higher baseline than others. Treatment of pterygial fibroblasts with TGFβ2 up-regulated BMP6 gene expression by 2 fold and down-regulated BMP4

gene expression more than 1.5 fold. The gene expression of all other BMPs detected remained unchanged following TGFβ2 treatment. In FGFs family, only FGF2 had a relatively higher baseline signal value and was up-regulated more than 1.5 fold following treatment with TGFβ2. The growth factors, EGF and HB-EGF (heparin binding-EGF), were detected in pterygial fibroblasts but the values were low. The gene expression of HB-EGF was up-regulated more than one and half fold following treatment with TGFβ2. In contrast, EGF gene expression was unchanged by TGFβ2 treatment.

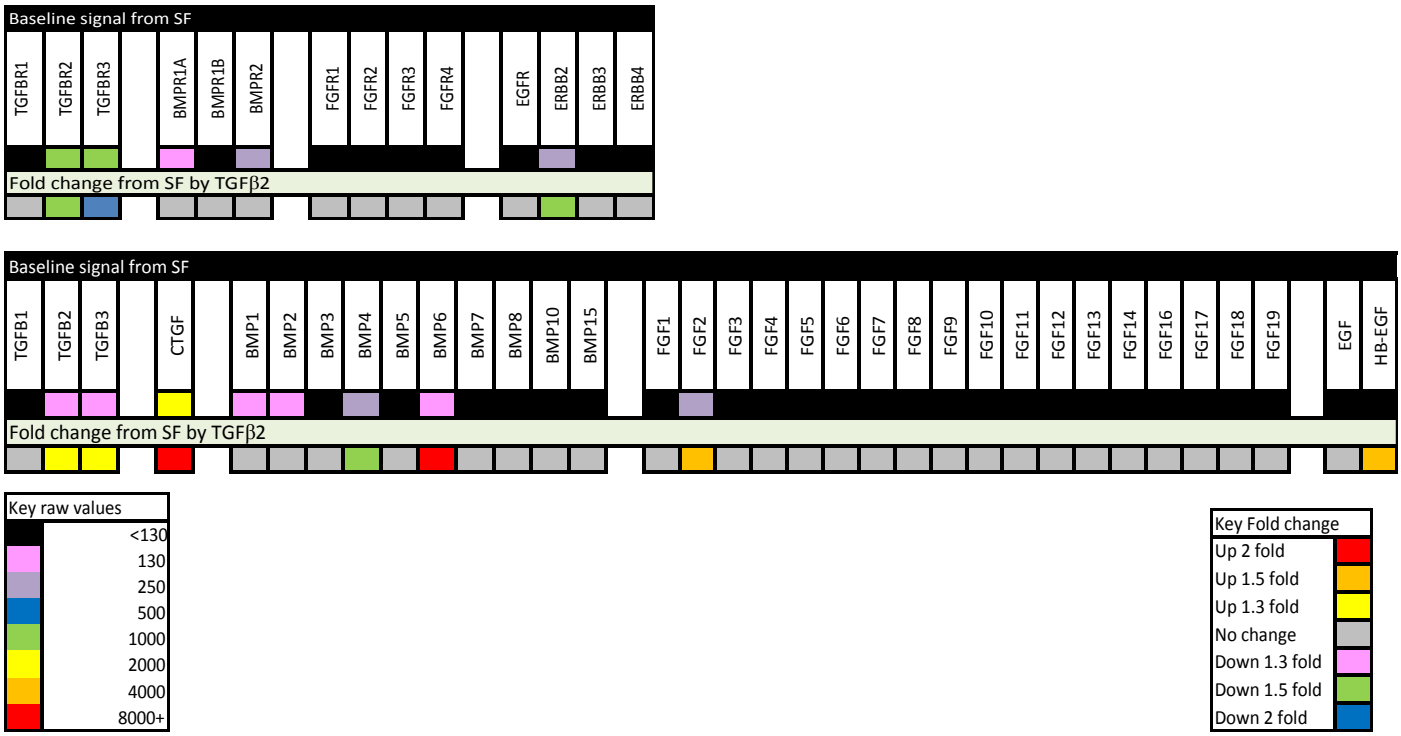


Figure 4.9. A gene expression profile of growth factors and their selected receptors in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 1ng/ml TGFβ2 relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

4.2.4.3 Gene expression profile of signalling components in pterygial fibroblasts

All eight Smad family members present on the microarray, Smad1, 2 and 9 showed detectable but low baseline signal values. Smad3, 4, 5 and 6 had relatively high baseline signal values and Smad3 showed the highest signal value compared with others (Figure 4.10). Smad7 gene expression was up-regulated by 1.5 fold and Smad3 and 6 were down-regulated by 2 fold and 1.5 fold following treatment with TGFβ2 (Figure 4.10). The gene expression of all other Smads detected remained unchanged following TGFβ2 treatment. Both TNF receptor-associated protein (TRAP-1) and SMURF were detected in pterygial fibroblasts and had high baseline signal values (Figure 4.10). But with TGFβ2 treatment, both TRAP-1 and SMURF gene expression were unchanged (Figure 4.11). 12 MAPK family members were present and gave varying baseline signal values with MAPK6 and 9 having higher signal values than others. MAPK6 gene expression was up-regulated 1.5 fold following treatment with TGFβ2 (Figure 4.10). The gene expressions of all other MAPK members remained unchanged following TGFβ2 treatment. Inhibitors of FGF signalling, SPRY1 (sprouty 1), SPRY2 and were detected in pterygial cells and the baseline signal of was slightly higher. SPRY1 gene expression was down-regulated 1.5 fold by TGFβ2 but nothing change of SPRY2 gene expression by TGFβ2 (Figure 4.10).

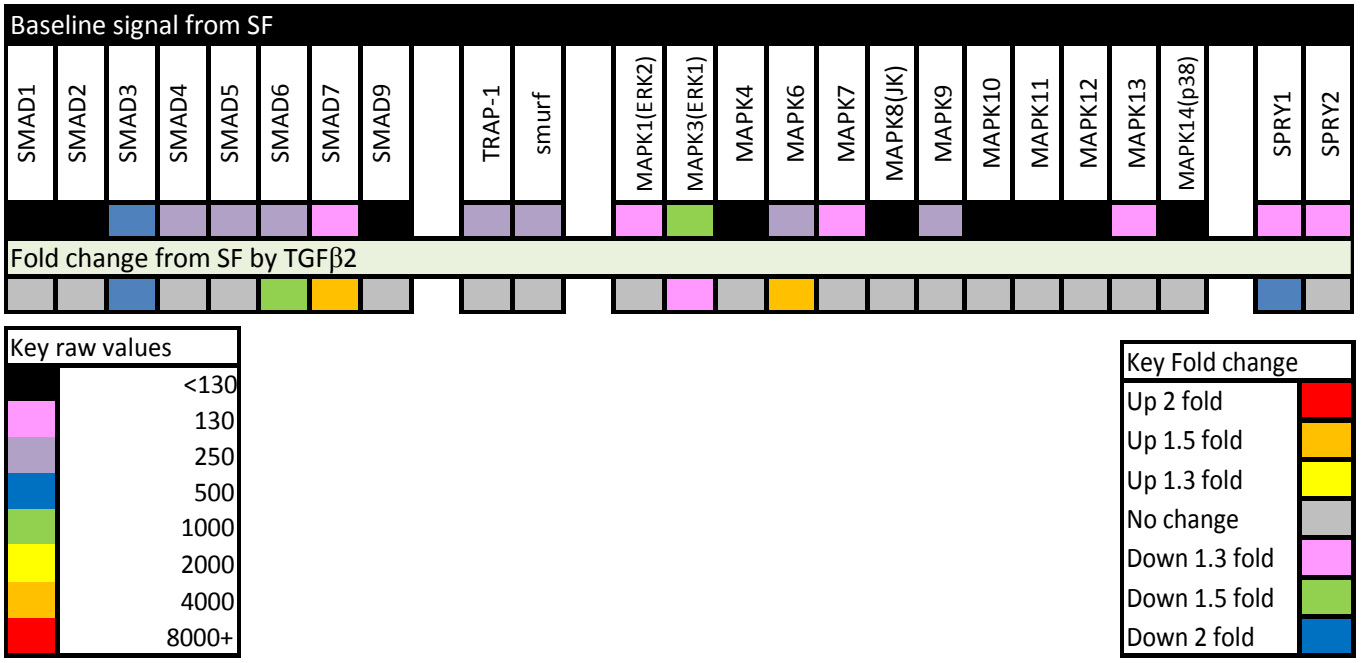


Figure 4.10. A gene expression profile of signalling components in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 1ng/ml TGFβ2 relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

4.2.4.4 Gene expression profile of matrix components in pterygial fibroblasts

33 collagens showed varying baseline signal values that collagen 1α1 (COL1A1) and collagen 1α2 (COL1A2) had the highest signal values. In addition, collagen 4α1 (COL4A1), collagen 5α1 (COL5A1), collagen 6α1 (COL6A1) and collagen 6α3 (COL6A3) showed relatively high baseline values. With respect to TGFβ2 treatment, the gene expression of 7 collagens were up-regulated significantly that collagen 4α1 (COL4A1), collagen 4α2 (COL4A2) and collagen 7α1 (COL7A1) were up-regulated 2 fold (Figure 4.11). The gene expression of collagens 4α5, 6α1, 6α2, 12α1, 15α1 and 18α1 were down-regulated by 1.5 fold following TGFβ2 treatment. The gene expression of all other detected collagens remained unchanged following TGFβ2 treatment. Fibronectin was detected in pterygial fibroblasts with a relatively high baseline signal value (Figure 4.11). But with TGFβ2 treatment, fibronectin gene expression was up-regulated 1.3 fold (Figure 4.11). Of 11 laminin family members present on the microarray, the laminin members showed varying baseline signal values that LAMA4, LAMB1 and LAMB2 had higher baseline value. Only laminin γ1 (LAMC1) gene expression was up-regulated more than two-fold following treatment with both TGFβ2 (Figure 4.11). Moreover, the gene expressions of Laminin α2 to 5 (LAMA), laminin β1 and 2 (LAMB) were down-regulated by at least 1.3 fold following treatment with TGFβ2, The gene expression of all other detected laminins remained unchanged following TGFβ2 treatment.

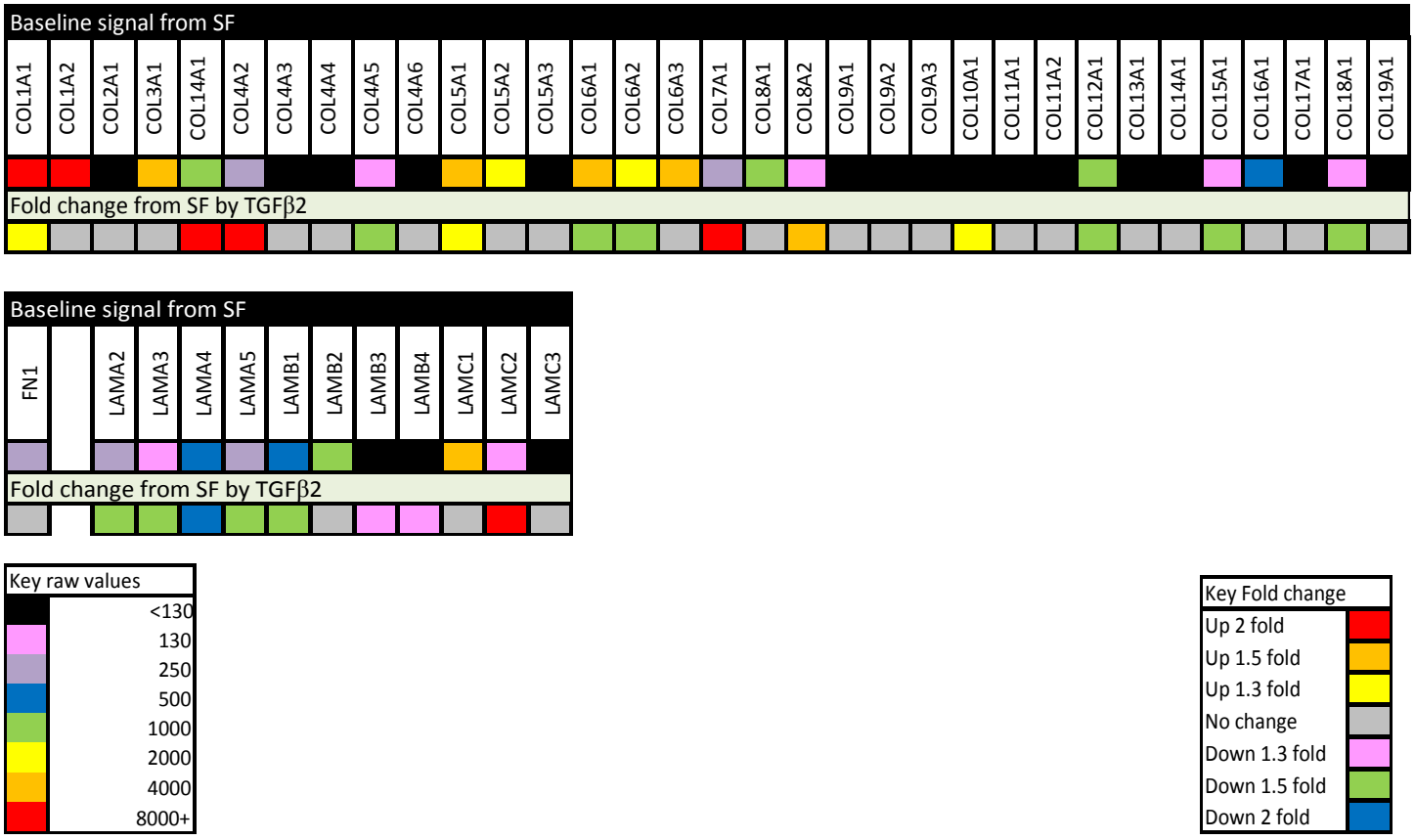


Figure 4.11. A gene expression profile of matrix components in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 1ng/ml TGFβ2 relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

4.2.4.5 Gene expression profile of integrins in pterygial fibroblasts

Of 28 integrins present on the microarray, of 15 integrins baseline signal values were detected in pterygial fibroblasts (Figure 4.12). The integrins showed varying baseline signal values with ITGAV, integrin β5 having high baseline signal values while value of integrin β1 was the highest. With respect to TGFβ2 treatment, the gene expressions of integrins α2 and α5 and β5 were up-regulated 1.5 fold while integrins α1 and α11 and αV were up-regulated 2 fold. However, integrins α8 was down-regulated 1.5 fold by TGFβ2 (Figure 4.12). The gene expressions of the remaining 19 integrins were unchanged.

4.2.4.7 Gene expression profile of myosin light chains and regulatory 6enzymes in pterygial fibroblasts

Gene expression profile of myosin light chains and regulatory enzymes in pterygial fibroblasts: seven myosin light chain family were detected on the microarray. These showed varying baseline signal values with MYL6 having highest baseline signal values whereas MYL1 to MYL4 had low baseline signal values (Figure 4.13). With respect to myosin regulatory enzymes, MYLK (also known as MLCK; myosin light chain kinase) were detected and gene expression of MYLK was up-regulated more than two-fold following the TGFβ2 treatment (Figure 4.13). Expression levels for other genes were unaffected by TGFβ2 treatment.

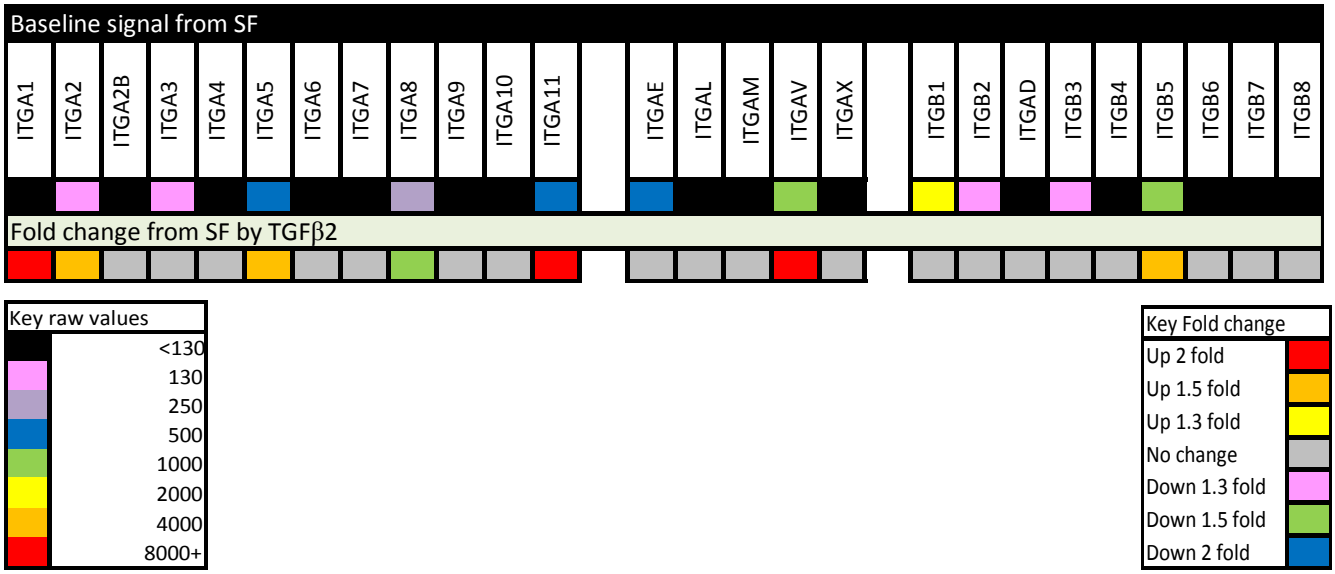
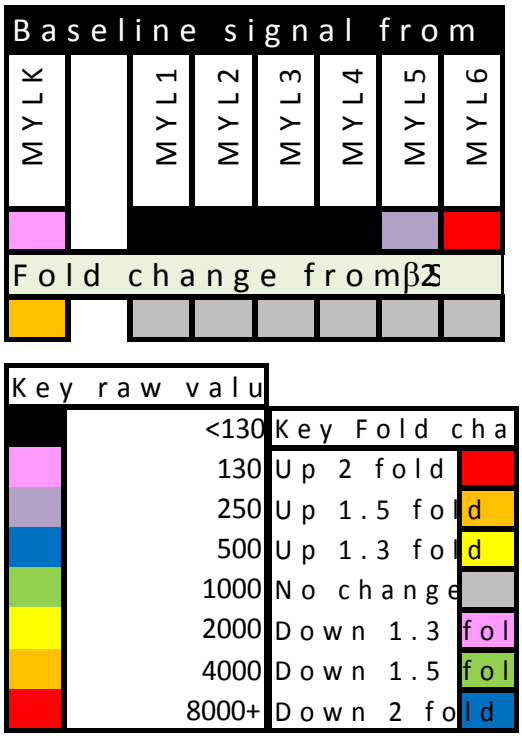


Figure 4.12. A gene expression profile of selected integrins in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 1ng/ml TGFβ2 relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.



4.13. A gene expression profile of myosin light chains and regulatory enzymes in pterygial fibroblasts. The data are presented in a colorimetric form to indicate the relative level of signal detected for each gene in non-stimulated serum-free controls using microarrays. Fold changes were detected in gene expression following 24 hours culture in 1ng/ml TGFβ2 relative to serum-free controls. Data are derived from the mean value of 4 separate experiments.

4.3 Discussion

TGFβ is a potent inducer of transdifferentiation throughout the body (Zavadil and Bottinger 2005). In the current study, TGFβ1 and TGFβ2 actively stimulated Smad2/3 translocation into the nucleus of pterygial fibroblasts. Thus it demonstrates that active TGFβ1 and TGFβ2 receptors are present in pterygial fibroblasts. Cells showed a high degree of sensitivity to TGFβ1 and TGFβ2, such that low concentrations still induced Smad2/3 activation. The data support the notion that TGFβ2 is capable of significantly inducing transdifferentiation of pterygial fibroblasts to a myofibroblast which is a typical index of fibrosis. TGFβ2 significantly stimulated pterygial fibroblasts transdifferentiation while TGFβ1 did not activate the gene expression of αSMA, which is the potent marker of transdifferentiation and fibroblast contractility (Skalli, Pelte *et al*. 1989; Hinz, Celetta *et al*. 2001). However, there was no significant change detected in the cells following the treatment of 10% serum for 24 hours. Validation of TGFβ/Smad signalling in relation to transdifferentiation in excised pterygial tissue also indicates its role in pterygial formation.

Angiogenesis is considered a key process in the promotion of pterygium formation (Marcovich, Morad *et al*. 2002; Aspiotis, Tsanou *et al*. 2007). Proteome Profiler™ Array data revealed that pterygial-derived fibroblasts release angiogenic factors into the bathing medium with TGFβ2 exposure. It resulted in a significant increase in some well

documented angiogenic factors like IL-8, MMP9 and VEGF, endothelin-1 and fibroblast growth factor2 (Shoyab, Plowman *et al*. 1989; Ferrara and DavisSmyth 1997; Bergers, Brekken *et al*. 2000; Dunn, Heese *et al*. 2000; Fujiyama, Matsubara *et al*. 2001; Martin, Galisteo *et al*. 2009; Banerjee, Wu *et al*. 2012). It has been reported that the TGFβ2 mediated MAPK pathway is involved in VEGF secretion in human retinal pigment epithelium (Bian, Elner *et al*. 2007) and TGFβ can also play an important role in VEGF mediated cell migration (Bulut, Pennartz *et al*. 2006). TGFβ is reported to induce VEGF production which leads to vascular remodelling and angiogenesis (Fang, Pentinmikko *et al*. 2012). A study suggested VEGF stimulates FGF-2 release but inhibits the expression of TGF-beta1 in injured endothelial cells (Li, Zhang *et al*. 2009). Interleukin 8 (IL8) is a pro-inflammatory cytokine which could contribute to pterygium (Di Girolamo, Kumar *et al*. 2002). It has been suggested that TGFβ can induce IL-8 production via a connective tissue grow factor independent pathway (Qi, Chen *et al*. 2006) and regulate IL8 mRNA and protein expression (Kumar, Rabadi *et al*. 1996), but TGFβ1 can also inhibit the IL-8-dependent pathway to play anti-inflammatory role (Smith, Noack *et al*. 1996). MMP-9 has been proposed to play a role in the progression of pterygium (Naib-Majani, Eltohami *et al*. 2004; Yang, Lin *et al*. 2009). It was reported that TGFβ1 can promote MMP9 expression through Smad3 recruitment (Chou, Wang *et al*. 2006) and TGFβ/Smad3 signalling may play an important role in MMP9 transcription (Warburton, Shi *et al*. 2013). Another study reported that TGFβ can also increase MMP9 release via the p38 MAPK signalling pathway (Safina, Vendette *et al*. 2005). In addition, it has been reported that

TGFβ1 and 2 may regulate endothelin-1 synthesis and induce its secretion (Schnermann, Zhu *et al*. 1996). It is suggested that FGF2 induces transdifferentiation of retinal pigment epithelium cells by activation of MAPK/ERK kinase pathway (Susaki and Chiba 2007) and synergy of FGF2 and TGFβ could activate human corneal endothelial cells proliferation (Rieck, Oliver *et al*. 1995). Interestingly, with respect to anti-angiogenic factors, TGFβ2 also significantly elevates the levels of angiostatin, thrombospondin-2 and Platelet Factor 4. The overall potential to undergo angiogenesis is established by the net balance of various pro-angiogenic and anti-angiogenic factors (Pepper 1997). In the situation that excessive angiogenic factors are produced, anti-angiogenic factors may also be produced to neutralize these angiogenic factors.

Pterygial fibroblasts express a range of growth factors including members of the TGFβ, CTGF, BMP, FGF, and EGF family and their corresponding receptors, all of them potentially play important roles in the progression of pterygium (Kria, Ohira *et al*. 1998; Nakagami, Murakami *et al*. 1999; Nolan, DiGirolamo *et al*. 2003; van Setten, Aspiotis *et al*. 2003; Bianchi, Scarinci *et al*. 2012). TGFβ isoforms 1, 2, and 3 were detected in pterygial fibroblasts. Both TGFβR1 and TGFβR3 are essential for TGFβ2 signalling, the cells could not respond to TGFβ without their involvement (Massague 2000). Surprisingly, both TGFβR2 and TGFβR3 were down-regulated by TGFβ2. TGFβ2 and TGFβ3 gene expressions were up-regulated following treatment with TGFβ2 while the gene expression of TGFβ1 was unchanged. Connective tissue growth factor (CTGF) showed very high baseline signal

value and was up-regulated 2 fold following treatment with TGFβ2. It has been reported that CTGF can give rise to fibrosis in the epithelium of pterygium (van Setten, Aspiotis *et al*. 2003). CTGF actives the TGFβ/Smad signalling pathway by suppressing Smad7, the antagonist, of Smad2/3 signalling (Wahab, Weston *et al*. 2005). TGFβ2 up-regulated gene expression of BMP6 and down-regulated BMP4. Bone morphogenetic proteins (BMPs) belong to transforming growth factor beta family; they could play various biological functions as cell proliferation, apoptosis, and differentiation in human organs and tissues including eye (Hogan 1996; Massague 1998). It was reported that BMP6 is detected significantly increased in scar conjunctival tissue compared with normal conjunctiva and it may play a role in conjunctival tissue fibrosis and wound healing process (Andreev, Zenkel *et al*. 2006). TGFβ/ BMP signalling pathways were activated by either Smad dependent or p38 MAPK pathway to regulate cellular functions (Chen, Deng *et al*. 2012). Therefore, TGFβ/ BMP signalling pathways in pterygium may play a similar role in tissue fibrosis. In the members of FGF family of growth factors and receptors observed in pterygial fibroblasts, only FGF2 was detected with a relatively high signal value, and gene expression was up-regulated by TGFβ2. A previous study detected the combination of FGF2 and TGFβ could enhance the stimulation of human corneal endothelial cells proliferation (Rieck, Oliver *et al*. 1995). FGF2 plays an important role in cell proliferation and interaction of TGFβ with FGF2 is reported to influence cell differentiation (He, Yu *et al*. 2008). FGF2 can induce TGFβ, but reduces collagen1 secretion and may play a negative feedback mechanism in cell differentiation and ECM accumulation (Unda,

Martin *et al*. 2000; Cross, Reid *et al*. 2006). With respect to growth factors EGF and HB-EGF (heparin binding-EGF), they were detected with a low expression in pterygial fibroblasts. However, TGFβ2 increased HB-EGF gene expression and interestingly, the receptor ERBB2 was down-regulated by TGFβ2. It was reported that Heparin binding EGF-like growth factor can stimulate smooth muscle cell migration (Higashiyama, Abraham *et al*. 1993). It was reported that both TGFβ1 and 2 can stimulate production of HB-EGF during airway epithelial repair (Ito, Harada *et al*. 2011).

Moreover, the signal transduction and regulation molecules of TGFβ, Smad family were investigated. All members of the Smad family and their associated components (TRAP-1, smurf) were expressed in pterygial cells. Moreover, the active signalling was detectable in response to TGFβ. As the major inhibitory regulator of TGFβ/Smads signalling, Smad7 was expressed with a high signal value in pterygial cells and was up-regulated by TGFβ2. The essential signalling Smads 3, 4 showed a relatively high level of expression in the cells. Smads2, 3 and 4 mediated TGFβ signals, the Smad2/3-Smad4 complex can translocate into the nucleus to play a role in transcriptional functions (Massague 1998). Smad7 is a major antagonist of TGFβ signalling and blocks Smad2/3 phosphorylation (Nakao, Afrakhte *et al*. 1997). Mitogen-activated protein kinases signalling family, which includes ERK1 (MAPK3), JNK (MAPK8) and p38 (MAPK14) were investigated in pterygial fibroblasts. In recent research, the active MAPKs signalling pathway was identified in pterygium (Torres, Enriquez-de-Salamanca *et al*. 2011). It may function as the mediator in TGFβ

signalling transduction (Yamaguchi, Shirakabe *et al*. 1995). The MAPK members detected varying baseline signal values with MAPK6 and 9 having relatively high level. MAPK6 gene expression was up-regulated following treatment with TGFβ2 and SPRY1 was down-regulated by TGFβ2. Inhibition of MAPKs signalling can abolish the UVB-induced angiogenic factors release IL6, IL8 and VEGF in pterygial cells (Di Girolamo, Wakefield *et al*. 2006). MAPK6 is a marker of BRAF-induced oncogenic signalling to affect cell proliferation and angiogenesis in melanoma (Hoeflich, Eby *et al*. 2006). SPRY1 is the Inhibitor of FGF signalling (Hanafusa, Torii *et al*. 2002). TGFβ2 suppressed SPRY1 expression may induce FGF signalling and thus impact TGFβ transduction in pterygial fibroblasts. It has been reported that both MAPK and Smad pathways are involved in TGFβ activities and imbalance of them may induce TGFβ tumour promoting effects (Nickl-Jockschat, Arslan *et al*. 2007). The p38 MAPK inhibitor can block TGFβ Smad signalling (Fu, O'Connor *et al*. 2003) and the synergistic activation of Smad and MAPK pathways can induce excess collagen deposition (Tsukada, Westwick *et al*. 2005) and regulate cell differentiation via TGFβ (Akel, Bertolette *et al*. 2003).

Extracellular matrix provides structural support between cells and stimulates the remodelling process, inflammation, and angiogenesis to result in progression of pterygium (Di Girolamo, Chui *et al*. 2004; Naib-Majani, Eltohami *et al*. 2004). All of 33 collagens were detected in pterygial fibroblasts. The collagen 1α1, collagen 4α1, collagen 5α1, collagen 6α1 and collagen 6α3 and collagen 1α2 demonstrated very high signal. The

collagen 4α1 and 2 were highly up-regulated by TGFβ2. Collagen 4 was strongly positive in the wall of pterygial blood vessels (Naib-Majani, Eltohami *et al*. 2004). TGFβ2 also activated the Collagen 1α1, 5α1, 7α1, 8α2 and 10α1 gene expression. Collagen I is involved myofibroblasts synthesis in fibrocontractive disease (Gabbiani 2003). As the main component of connective tissue, the other positively regulated collagens may also be involved in pterygial tissue fibrosis. In addition, with respect to laminin family members, TGFβ2 could up regulate laminin γ1 gene expression. As a noncollagenous glycoprotein from basement membrane, laminins family can interact with collagens to generate extracellular matrix (Kefalides 1973; Timpl, Rohde *et al*. 1979). Laminins have previously been reported to be highly expressed in pterygial vascular walls (Naib-Majani, Eltohami *et al*. 2004).

The gene expression of the members of integrin family was detected in pterygial fibroblasts. An integrin is a cell surface receptor mediate the cell attachment, it is a primary mediator of the extracellular matrix that pass chemical and mechanical signals into the cells (Giancotti and Ruoslahti 1999). It plays many important roles in cell physiology like cell motility and cell cycle (Clark and Brugge 1995; Giancotti and Ruoslahti 1999). In present study, Integrin β1 and Integrin β5 expressed relatively high signal level in pterygial fibroblasts. It is reported that Integrin β1 transmits mechanical signals to the cytoskeleton and produce a stiffening response which correlates with focal adhesion formation (Wang, Butler *et al*. 1993). Various integrins including α1, α5, αV and β5 were

up-regulated by TGFβ2. Integrins consist of 16α and 8β transmembrane subunits and β1 associate with 12 α integrin subunits (Clark and Brugge 1995; Elner and Elner 1996). Therefore, αVβ5 and α5β1 integrins may demonstrate high levels of expression by TGFβ2 stimulation in pterygial fibroblasts. It has been reported that integrin signalling is involved in TGFβ induced myofibroblast differentiation (Jester, Huang *et al*. 2002). Many integrin β subunits can form heterodimers with αV like αVβ5 and αVβ1 (Walker and Menko 2009). It reported that αVβ5 plays a critical role in growth of blood vessels and potentiate angiogenic pathways in ocular angiogenesis (Friedlander, Brooks *et al*. 1995). TGFβ2 is also reported to induce αVβ5 gene expression in lens epithelial cell line (Dawes, Elliott *et al*. 2007). αV integrins may play an important role in mediating the matrix proteins through activation of TGFβ in lens fibrotic diseases (Walker and Menko 2009). It is proposed that α5β1 may regulate the interaction between myofibroblast and fibronectin (Hakkinen, Heino *et al*. 1994). Gene expression of light chains kinase (MYLK) and regulatory enzymes in pterygial fibroblasts were investigated. MYLK was up-regulated more than two-fold by TGFβ2. MYLK is involved in mechanism of regulating smooth muscle contraction via calcium/calmodulin-dependent myosin light chain phosphorylation (Lazar and Garcia 1999).

The current study investigated TGFβ2 induced signalling pathway, transdifferentiation, angiogenesis and matrix contraction processes. TGFβ2/Smad signalling is present in pterygial fibroblasts and TGFβ2 can significantly promote gene expression of αSMA which

is an indicative marker of transdifferentiation. The gene microarrays and Proteome Profiler identified potentially important genes and secreted proteins following application of TGFβ2, which may contribute pathological process in pterygium.

CHAPTER 5

Summary and future work

Pterygium is a proliferative fibrovascular altered conjunctival tissue. Cell proliferation, fibrosis and angiogenesis are the major features of pterygium. Thus, the primary objective of the current thesis was to determine the influences of serum (mimic the blood supply) and TGFβ on cellular proliferation, signalling, transdifferentiation and angiogenesis in pterygial fibroblasts. These processes are likely to contribute to the progression of this disease (Cameron 1983; Karukonda, Thompson *et al*. 1995; Coroneo, Di Girolamo *et al*. 1999; Marcovich, Morad *et al*. 2002; Aspiotis, Tsanou *et al*. 2007). In the laboratory, pure pterygial fibroblast cultures were successfully established from donor pterygium specimens and assessed. Through histological and immunochemical techniques, it was confirmed that pterygium was more compact arrangement of fibroblasts, which demonstrate elevated levels of transdifferentiation markers and more abundant blood vessels relative to normal conjunctiva. Serum supplemented media was commonly used for the cell and tissue culture and the serum may be one of the major environmental factors for pterygium because of the high degree of vasculature associated with this disorder. Serum angiogenic activity

was previously reported in inflammatory diseases (Zielonka, Demkow *et al*. 2007; Zielonka, Demkow *et al*. 2007). The extensive vascular network is likely to significantly contribute to the progression of the disease. An approach adopted in the current study to understand the role a rich blood supply can perform on pterygial cell behaviour was to maintain cells in a serum rich and serum poor/starved environment. This provides a simple model that generates extreme conditions and allows changes that result to be assessed. As predicted pterygial cells are reliant on a supply of growth and survival factors from serum. Little work has been carried out on pterygial cells with regard to calcium signalling and thus increasing our knowledge of this fundamental signalling mechanism was important. Histamine and epidermal growth factor have been shown to stimulate intracellular calcium mobilization in pterygial derived fibroblasts (Maini, Collison *et al*. 2002). However, the mechanisms regulating calcium signalling and its functional importance have not been fully resolved. In current study, serum deprivation suppressed histamine, ATP, acetylcholine and epidermal growth factor mediated calcium signalling in pterygial fibroblast. It demonstrates that these receptors are functional available on the surface of fibroblasts derived from human pterygium. All ligands (histamine, ATP, EGF, acetylcholine) provoke a reduced calcium response in serum deprivation conditions, which suggests that a common factor is changing. Therefore, Illumina gene array was used here to establish profiles of these receptors associated with the test ligands and calcium signalling components. However, maintenance in 10% serum did not elevate

gene expression of histaminergic, purinergic, cholinergic and EGF receptors or activate any important calcium signalling components relative to serum starved cells. To address the importance of the ER store, intracellular Ca^{2+} pump inhibitor thapsigargin was used to observe ER store depletion which increases intracellular Ca^{2+} without generating IP_3 (Parekh and Penner 1997). In the present study, addition of thapsigargin to cells incubated in serum free condition can deplete the calcium store relative to serum maintained cells. Therefore, intracellular calcium stores are sensitive to serum deprivation. Many studies have reported disruption of the ER calcium have influence on cell division (Wang, Wormstone *et al*. 2005) and migration (Duncan, Wormstone *et al*. 1997; Nicola, Timoshenko *et al*. 2005). Persistent depletion of the calcium store play significant role in reducing cell survival, migration, protein synthesis, ER stress and potentially cell death by apoptosis (Shi, Wang *et al*. ; Nicola, Timoshenko *et al*. 2005; Wang, Wormstone *et al*. 2005; Zhang, Duncan *et al*. 2007). Therefore, serum starvation suppressed calcium signalling could affect functions related to the pterygium. Further investigation of these ligands mediated signalling pathways, their functional roles in pterygium and the effects of long-term depletion of the ER store and ER stress related events in cell growth and migration of pterygial fibroblasts will be a valuable topic of investigation.

Investigation of serum versus serum-free responses provides gross changes that are of interest to pterygial cells. A second line of study was to concentrate on one specific

pathway and identify its potential in regulating pterygium formation. As pterygium was reported to possess the hallmarks of fibrosis (Coroneo, Di Girolamo *et al*. 1999), TGFβ was investigated, as this is strongly implicated in fibrotic disorders (Leask and Abraham 2004). Serum contains various growth factors and TGFβ is likely to be present (in a latent or active form) at some level. With regard to pterygium pathologies, serum can produce/release pro-angiogenic factors to induce angiogenic activities in inflammatory disease (Zielonka, Demkow *et al*. 2007; Zielonka, Demkow *et al*. 2007). TGFβ can also play an important role in transdifferentiation and regulation of the extracellular matrix (Miettinen, Ebner *et al*. 1994). The most potent marker of myofibroblast in transdifferentiation is αSMA (Desmouliere, Geinoz *et al*. 1993). Myofibroblasts synthesize extracellular matrix components to play a role in wound healing and fibrocontractive diseases (Gabbiani 2003). The current work demonstrated that active TGFβ1 and TGFβ2 receptors are detected and showed high sensitivities in pterygial fibroblasts. TGFβ2 was capable of significantly inducing transdifferentiation of pterygial fibroblasts to a myofibroblast at the gene level. The data supported that TGFβ2 is a more potent isoform in the promotion of transdifferentiation in the superficial ocular tissues and human aqueous humour (Jampel, Roche *et al*. 1990; Pasquale, Dormanpease *et al*. 1993). 10% serum and TGFβ2 stimulated conditions were highlighted to provide a global profile of gene expression in pterygial fibroblasts. TGFβ has been reported to promote transdifferentiation and regulate extracellular matrix modification in mammary

epithelial cells (Miettinen, Ebner *et al*. 1994), lens capsular bag (Wormstone, Tamiya *et al*. 2002) and human Tenon's fibroblasts (Meyer-Ter-Vehn, Katzenberger *et al*. 2008).

A range of growth factors including TGFβ, CTGF, BMP, FGF, and EGF family and their corresponding receptors were investigated in microarray analysis. They were reported to potentially involve in pterygium progression (Kria, Ohira *et al*. 1998; Nakagami, Murakami *et al*. 1999; Nolan, DiGirolamo *et al*. 2003; van Setten, Aspiotis *et al*. 2003; Bianchi, Scarinci *et al*. 2012). The important finding from the investigation is that connective tissue growth factor (CTGF) showed high expression in pterygial fibroblasts in non-treated condition and was up-regulated following treatment with TGFβ2. CTGF is present in epithelium of pterygium and possibly interact with vascular growth factor (VEGF) to contribute to fibrosis in pterygium (van Setten, Aspiotis *et al*. 2003). It has been reported that CTGF enhances phosphorylation and nuclear translocation of Smad 2/3 and activates TGFβ/Smad signalling pathway by suppressing Smad7 (Wahab, Weston *et al*. 2005). The Smad proteins are essential intracellular effectors of TGFβ/Smad signalling to regulate transcription events in nucleus (Derynck, Zhang *et al*. 1998). Interestingly, in current study TGFβ2 significantly enhanced Smad7 gene expression, this may explain the reason why TGFβ2 down-regulated the essential signalling component Smad3. TGFβ2 also down-regulated the antagonist of TGFβ signalling Smad6 (Imamura, Takase *et al*.

1997). This thus demonstrates that following treatment with TGFβ2, Smad6 was suppressed to activate signalling and Smad7 may participate in a negative feedback mechanism in pterygial fibroblasts. The interaction in Smad proteins to keep balance in signalling needs further investigation. All three isoforms of TGFβ and their receptors were detectable in pterygial fibroblast. This suggests an active TGF/Smad signalling pathway is in place in pterygial fibroblasts. The immunochemical data also supports this issue. Although TGFβ2 and TGFβ3 receptors were negatively regulated by TGFβ2 and TGFβR3 expression were down-regulated following 10% serum treatment. TGFβR2 and 3 complex was reported indispensable in TGFβ signalling (Brown, Boyer *et al*. 1999). Therefore, high levels of TGFβ2 may play an inhibitory role on TGFβR2 and 3 receptor expressions. The suppressed TGFβR3 response by TGFβ2 was also found in lens cells (Dawes, Elliott *et al*. 2007). TGFβ2, 3 and bone morphogenetic protein 6 (BMP6) were up-regulated by TGFβ2. It demonstrates that TGFβ2 in high level could persistently increase the overall pool of TGFβ2 in pterygial fibroblasts. Basic fibroblast growth factor (FGF2) showed relatively high level of gene expression and was up-regulated by TGFβ2. Previous studies showed bFGF expressed high level in the cultured fibroblasts derived from primary and recurrent pterygium (Kria, Ohira *et al*. 1998; Detorakis, Zaravinos *et al*. 2010). It has been reported that synergy of FGF2 and TGFβ could activate human corneal endothelial cells proliferation (Rieck, Oliver *et al*. 1995) and FGF2 induces transdifferentiation of retinal pigment epithelium cells through MAPK/ERK signalling pathway (Susaki and

Chiba 2007). MAPK signalling family include ERK1 (MAPK3), JNK (MAPK8) and p38 (MAPK14) were detected in pterygial fibroblasts and TGFβ2 up-regulated MAPK6 gene expression in pterygial fibroblasts. MAPK6 was reported to play a role in cell proliferation and angiogenesis in tumour cells (Hoeflich, Eby *et al*. 2006). The active MAPKs signalling was identified in pterygium (Torres, Enriquez-de-Salamanca *et al*. 2011) and it reported ERK1/2 MAPK dependant pathway was involved in ultraviolet light induced MMP1 expression in development of pterygium (Di Girolamo, Coroneo *et al*. 2003). MAPK signalling play a role on mediating TGFβ signalling transduction (Yamaguchi, Shirakabe *et al*. 1995). It has been reported that synergy of MAPK and Smad pathways involve TGFβ activities (Nickl-Jockschat, Arslan *et al*. 2007). They induces excess collagen deposition (Tsukada, Westwick *et al*. 2005) and regulates cell differentiation via TGFβ (Akel, Bertolette *et al*. 2003). In addition, SPRY1 is negatively mediated by TGFβ2. It is the Inhibitor of FGF signalling (Hanafusa, Torii *et al*. 2002). TGFβ2 suppressed MAPK inhibitory pathway may stimulate FGF signalling via ERK/MAPK pathway and induce TGFβ signalling in pterygial fibroblasts. The functional role of MAPK in pterygium could be a target in future study.

Extracellular matrix (ECM) constitutes the connective tissues filling the extracellular space. Overexpression of ECM with alteration of the collagen and elastic fibres are contributing factors to pterygium pathological progression (Cameron 1983; Karukonda, Thompson *et al*. 1995; Coroneo, Di Girolamo *et al*. 1999). TGFβ2 can

up-regulate gene expression of collagen 4 which was positively distributed in pterygial vascular walls (Naib-Majani, Eltohami *et al*. 2004). Another important finding is that TGFβ2 enhanced collagen 1 gene expression which was reported to play a role in myofibroblasts synthesis in wound healing and fibrocontractive disease (Gabbiani 2003). TGFβ2 can also simulate main component of connective tissue laminin γ1 gene expression in pterygial fibroblasts. It has been reported that laminins may interact with collagens to generate extracellular matrix in pterygium (Naib-Majani, Eltohami *et al*. 2004). Moreover, a number of cell surface receptor integrin family members were stimulated by TGFβ2. Integrin may transduct signals into pterygial fibroblasts to induce ECM remodelling to contribute transdifferentiation in pterygium. It reported that the synergy of integrin and TGFβ2 may induce transdifferentiation in rabbit keratocytes (Jester, Huang *et al*. 2002). The data demonstrates Integrin β1 is most abundant in pterygial fibroblasts and it was reported that Integrin β1 can associate with any 1 of 10 α subunits to form α-β1 to bind ECM ligands (Clark and Brugge 1995; Elner and Elner 1996). Integrin α1, α5, αV and β5 were up-regulated by TGFβ2. Therefore, α5β1, αVβ5 integrin combinations may demonstrate higher expression as a consequence of TGFβ2 stimulation in pterygial fibroblasts. Integrin signalling is involved in myofibroblast differentiation via TGFβ activities (Jester, Huang *et al*. 2002). Integrin α5β1 is the receptor of fibronectin and it regulates the interaction between myofibroblast and fibronectin (Hakkinen, Heino *et al*. 1994) and may also play a role in ocular angiogenesis and tumour

progression (Danen, Tenberge *et al*. 1994; Muether, Dell *et al*. 2007). TGFβ2 induces αVβ5 gene expression in lens fibrotic diseases (Dawes, Elliott *et al*. 2007; Walker and Menko 2009) and it is feasible that αVβ5 could also play a role in pterygium. αVβ5 may also regulate new growth of blood vessels and potentiate ocular angiogenesis (Friedlander, Brooks *et al*. 1995). In addition, TGFβ2 also up-regulated MYLK which involved in regulation of smooth muscle contraction (Lazar and Garcia 1999).

Pterygium exhibits a high degree of vasculature relative to normal conjunctiva. Angiogenesis plays an important role in promoting pterygium formation (Marcovich, Morad *et al*. 2002; Aspiotis, Tsanou *et al*. 2007). Proteome profiler™ array, data showed that pterygial-derived fibroblasts released angiogenic factors into the bathing medium. A number of important pro-angiogenic factors showed high protein expression in 10% serum and TGFβ2 bathing medium. The important findings include IL-8, MMP9, VEGF were positively mediated by both 10% serum and TGFβ2 conditions in pterygial fibroblasts. This suggests potent angiogenic activities in pterygial fibroblasts and supports the work of previous studies (Di Girolamo, Kumar *et al*. 2002; Marcovich, Morad *et al*. 2002; Reid and Dushku 2010; Liang, Jiang *et al*. 2012). The active involvement of these pro-angiogenic factors may increase cell migration and promoting the formation of blood vessels in the pathogenesis of pterygium (Salcedo, Ponce *et al*. 2000; Low, Drugea *et al*. 2001; Ma, Wang *et al*. 2007). The data demonstrated that pterygial fibroblasts growing in the presence of a

rich blood supply and TGFβ may release VEGF, FGF2, MMP9, IL8 and EN1 pro-angiogenic factors to support pterygial fibroblast cellular angiogenic activities. In addition, angiogenesis is controlled by the net balance between positive and negative regulatory activity. 10% serum significantly suppressed the release of anti-angiogenic factors angiostatin, SerpinE1, TIMP-1 and Thrombospondin-1 (Murphy, Unsworth *et al*. 1993; Cao 2001). The inhibitory functions of these factors may reduce the progressive nature of pterygium. TGFβ2 activated production of angiostatin, Thrombospondin-2 and Platelet Factor 4. All these data illustrate that the synergy between positive and negative angiogenic activities are important in the mechanism of pterygial angiogenesis. Another interesting phenomenon is that serum starvation can also induce the angiogenesis by increasing the production/release of a number of angiogenic factors. This may be because cells in serum free conditions release angiogenic factors to promote new blood vessel formation and enrich their local environment. Hypoxia can activate hypoxia-inducible factor (HIF) to increase VEGF (Risau 1997; Pousa and Gisbert 2006). The hypoxia mediated angiogenesis with a number of angiogenic factors like FGF-2, IGFBP-1 and angiogenin were reported (Tazuke, Mazure *et al*. 1998; Hartmann, Kunz *et al*. 1999; Kroon, Koolwijk *et al*. 2001). However, due to the weak protein expression of these up-regulated angiogenic factors in serum starved condition, the Western blot will be utilized to address this issue in future study.

Fibroblasts derived from pterygium can be successfully cultured and analysed, post-mortem and biopsy tissue can be assessed to provide important data relating to the native tissue. Calcium activities play a role in pterygial fibroblast cellular functions. Cells were sensitive to serum starvation that resulted in reduced growth and impaired calcium signalling. The cells were found to be sensitive to the pro-fibrotic protein TGFβ, which induced Smad signalling and myofibroblast expression. TGFβ was found to induce transdifferentiation and elevate angiogenic factors in pterygial derived fibroblasts. TGFβ2 and serum levels/blood supply can play a key role in pterygial cell responses and on the pathogenesis and development of pterygium.

References

Abbracchio, M. P., G. Burnstock, *et al*. (2006). "International union of pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy." Pharmacological Reviews 58(3): 281-341.

Akel, S., D. Bertolette, *et al*. (2003). "Cross talk between the smad and the mitogen-activated protein kinase (MAPK) pathways regulates erythroid differentiation of erythroleukemic cells induced by TGF-beta, activin, hydroxyurea and butyrate." Blood 102(11): 834A-834A.

Al-Bdour, M. D. and M. M. Al-Latayfeh (2004). "Risk factors for pterygium in an adult Jordanian population." Acta Ophthalmologica Scandinavica 82(1): 64-67.

Andreev, K., M. Zenkel, *et al*. (2006). "Expression of bone morphogenetic proteins (BMPs), their receptors, and activins in normal and scarred conjunctiva: Role of BMP-6 and activin-A in conjunctival scarring?" Experimental Eye Research 83(5): 1162-1170.

Aspiotis, M., E. Tsanou, *et al*. (2007). "Angiogenesis in pterygium: study of microvessel density, vascular endothelial growth factor, and thrombospondin-1." Eye 21(8): 1095-1101.

Baek, J. H., J. E. Jang, *et al*. (2000). "Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis." Oncogene 19(40): 4621-4631.

Banerjee, A., Z.-S. Wu, *et al*. (2012). "ARTEMIN Promotes De Novo Angiogenesis in ER Negative Mammary Carcinoma through Activation of TWIST1-VEGF-A Signalling." PloS one 7(11): e50098.

Bataller, R. and D. A. Brenner (2005). "Liver fibrosis." Journal of Clinical Investigation 115(2): 209-218.

Beard, N. A., D. R. Laver, *et al*. (2004). "Calsequestrin and the calcium release channel of skeletal and cardiac muscle." Progress in Biophysics & Molecular Biology 85(1): 33-69.

Bergers, G., R. Brekken, *et al*. (2000). "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis." Nature Cell Biology 2(10): 737-744.

Bergner, A. and R. M. Huber (2008). "Regulation of the Endoplasmic Reticulum Ca2+-Store in Cancer." Anti-Cancer Agents in Medicinal Chemistry 8(7): 705-709.

Berridge, M. J. (1993). "Inositol trisphosphate and calcium signaling." Nature 361(6410): 315-325.

Bian, Z. M., S. G. Elner, *et al*. (2007). "Regulation of VEGF mRNA expression and protein secretion by TGF-beta 2 in human retinal pigment epithelial cells." Experimental Eye Research 84(5): 812-822.

Bianchi, E., F. Scarinci, *et al*. (2012). "Immunohistochemical profile of VEGF, TGF-beta and PGE(2) in human pterygium and normal conjunctiva: experimental study and review of the literature." Int J Immunopathol Pharmacol 25(3): 607-615.

Bootman, M. D., T. J. Collins, *et al*. (2001). "Calcium signalling—an overview." Seminars in Cell & Developmental Biology 12(1): 3-10.

Border, W. A. and E. Ruoslahti (1992). "Transforming growth-factor-beta in disease - the dark side of tissue-repair." Journal of Clinical Investigation 90(1): 1-7.

Boudier, H. (1999). "Arteriolar and capillary remodelling in hypertension." Drugs 58: 37-40.

Brejc, K., W. J. van Dijk, *et al*. (2001). "Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors." Nature 411(6835): 269-276.

Brew, K., D. Dinakarpandian, *et al*. (2000). "Tissue inhibitors of metalloproteinases: evolution, structure and function." Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1477(1-2): 267-283.

Brown, C. B., A. S. Boyer, *et al*. (1999). "Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart." Science 283(5410): 2080-2082.

Bulut, K., C. Pennartz, *et al*. (2006). "Vascular endothelial growth factor (VEGF(164)) ameliorates intestinal epithelial injury in vitro in IEC-18 and Caco-2 monolayers via induction of TGF-beta release from epithelial cells." Scandinavian Journal of Gastroenterology 41(6): 687-692.

Cameron, M. E. (1983). "Histology of pterygium - an electron-microscopic study." British Journal of Ophthalmology 67(9): 604-608.

Cao, Y. (2001). "Endogenous angiogenesis inhibitors and their therapeutic implications." International Journal of Biochemistry and Cell Biology 33(4): 357-369.

Carmeliet, P. (2003). "Angiogenesis in health and disease." Nature Medicine 9(6): 653-660.

Caulfield, M. P. (1993). "Muscarinic receptors - characterization, coupling and function." Pharmacology & Therapeutics 58(3): 319-379.

Chen, G. Q., C. X. Deng, *et al*. (2012). "TGF-beta and BMP Signaling in Osteoblast Differentiation and Bone Formation." International Journal of Biological Sciences 8(2): 272-288.

Chen, J. K., R. J. F. Tsai, *et al*. (1994). "Fibroblasts isolated from human pterygia exhibit transformed-cell characteristics." In Vitro Cellular & Developmental Biology-Animal 30A(4): 243-248.

Chou, Y. T., H. Wang, *et al*. (2006). "Cited2 modulates TGF-beta-mediated upregulation of MMP9." Oncogene 25(40): 5547-5560.

Clapham, D. E. (1995). "Calcium signaling." Cell 80(2): 259-268.

Clark, E. A. and J. S. Brugge (1995). "Integrins and signal-transduction pathways - the road taken." Science 268(5208): 233-239.

Cohen, M. M. (1997). "Transforming growth factor beta s and fibroblast growth factors and their receptors: Role in sutural biology and craniosynostosis." Journal of Bone and Mineral Research 12(3): 322-331.

Connor, T. B., A. B. Roberts, *et al*. (1989). "Correlation of fibrosis and transforming growth factor-beta type-2 levels in the eye." Journal of Clinical Investigation 83(5): 1661-1666.

Cooke, J. P. and Y. T. Ghebremariam (2008). "Endothelial Nicotinic Acetylcholine Receptors and Angiogenesis." Trends in Cardiovascular Medicine 18(7): 247-253.

Coroneo, M. T. (1993). "Pterygium as an early indicator of ultraviolet insolation - a hypothesis." British Journal of Ophthalmology 77(11): 734-739.

Coroneo, M. T., N. Di Girolamo, *et al*. (1999). "The pathogenesis of pterygia." Current opinion in ophthalmology 10(4): 282-288.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature 420(6917): 860-867.

Cross, N. A., S. V. Reid, *et al*. (2006). "Opposing actions of TGF beta 1 and FGF2 on growth, differentiation and extracellular matrix accumulation in prostatic stromal cells." Growth Factors 24(4): 233-241.

Dameron, K. M., O. V. Volpert, *et al*. (1994). "Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1." Science 265(5178): 1582-1584.

Danen, E. H. J., P. J. M. Tenberge, *et al*. (1994). "Emergence of Alpha-5-Beta-1 Fibronectin-Receptor and Alpha-V-Beta-3 Vitronectin-Receptor Expression in Melanocytic Tumor Progression." Histopathology 24(3): 249-256.

Dawes, L. J., R. M. Elliott, *et al*. (2007). "Oligonucleotide microarray analysis of human lens epithelial cells: TGF beta regulated gene expression." Molecular Vision 13(128-30): 1181-1197.

De Paepe, B., K. K. Creus, *et al*. (2012). "Upregulation of chemokines and their receptors in Duchenne muscular dystrophy: potential for attenuation of myofiber necrosis." Muscle Nerve 46(6): 917-925.

Derynck, R., Y. Zhang, *et al*. (1998). "Transcriptional Activators of TGF-[beta] Responses: Smads." Cell 95(6): 737-740.

Desmouliere, A., A. Geinoz, *et al*. (1993). "Transforming growth-factor-beta-1 induces alpha-smooth muscle actin expression in granulation-tissue myofibroblasts and in quiescent and growing cultured fibroblasts." Journal of Cell Biology 122(1): 103-111.

Detorakis, E. T., E. E. Drakonaki, *et al*. (2000). "Molecular genetic alterations and viral presence in ophthalmic pterygium (review)." International Journal of Molecular Medicine 6(1): 35-41.

Detorakis, E. T., A. Zaravinos, *et al*. (2010). "Growth factor expression in ophthalmic pterygia and normal conjunctiva." International Journal of Molecular Medicine 25(4): 513-516.

Di Girolamo, N., J. Chui, *et al*. (2004). "Pathogenesis of pterygia: role of cytokines, growth factors, and matrix metalloproteinases." Progress in Retinal and Eye Research 23(2): 195-228.

Di Girolamo, N., M. T. Coroneo, *et al*. (2003). "UVB-elicited induction of MMP-1 expression in human ocular surface epithelial cells is mediated through the ERKI/2 MAPK-dependent pathway." Investigative Ophthalmology & Visual Science 44(11): 4705-4714.

Di Girolamo, N., R. K. Kumar, *et al*. (2002). "UVB-mediated induction of interleukin-6 and-8 in pterygia and cultured human pterygium epithelial cells." Investigative Ophthalmology & Visual Science 43(11): 3430-3437.

Di Girolamo, N., N. Tedla, *et al*. (1999). "Culture and characterisation of epithelial cells from human pterygia." British Journal of Ophthalmology 83(9): 1077-1082.

Di Girolamo, N., D. Wakefield, *et al*. (2006). "UVB-mediated induction of cytokines and growth factors in pterygium epithelial cells involves cell surface receptors and intracellular signaling." Investigative Ophthalmology & Visual Science 47(6): 2430-2437.

Duncan, G. and D. J. Collison (2003). "Role of the non-neuronal cholinergic system in the eye: a review." Life Sci 72(18-19): 2013-2019.

Duncan, G., I. M. Wormstone, *et al*. (1997). "Thapsigargin-coated intraocular lenses inhibit human lens cell growth." Nat Med 3(9): 1026-1028.

Dunn, I. F., O. Heese, *et al*. (2000). "Growth factors in glioma angiogenesis: FGFs, PDGF, EGF, and TGFs." Journal of Neuro-Oncology 50(1-2): 121-137.

Dushku, N., M. K. John, *et al*. (2001). "Pterygia pathogenesis - Corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells." Archives of Ophthalmology 119(5): 695-706.

Dushku, N. and T. W. Reid (1994). "Immunohistochemical evidence that human pterygia originate from an invasion of vimentin-expressing altered limbal epithelial basal cells." Current Eye Research 13(7): 473-481.

Eldred, J. A., J. Sanderson, *et al*. (2003). "Stress-induced ATP release from and growth modulation of human lens and retinal pigment epithelial cells." Biochem Soc Trans 31(Pt 6): 1213-1215.

Elner, S. G. and V. M. Elner (1996). "The integrin superfamily and the eye." Investigative Ophthalmology & Visual Science 37(5): 696-701.

Erlinge, D. and G. Burnstock (2008). "P2 receptors in cardiovascular regulation and disease." Purinergic Signalling 4(1): 1-20.

Fang, S. T., N. Pentinmikko, *et al*. (2012). "Dual action of TGF-beta induces vascular growth in vivo through recruitment of angiogenic VEGF-producing hematopoietic effector cells." Angiogenesis 15(3): 511-519.

Ferrara, N. and T. DavisSmyth (1997). "The biology of vascular endothelial growth factor." Endocrine Reviews 18(1): 4-25.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nature Medicine 1(1): 27-31.

Folkman, J. and M. Klagsbrun (1987). "Angiogenic factors." Science 235(4787): 442-447.

Folkman, J. and Y. Shing (1992). "Angiogenesis." Journal of Biological Chemistry 267(16): 10931-10934.

Francis, H., S. Glaser, *et al*. (2008). "Small mouse cholangiocytes proliferate in response to H1 histamine receptor stimulation by activation of the IP3/CaMK I/CREB pathway." American Journal of Physiology-Cell Physiology 295(2): C499-C513.

Friedlander, M., P. C. Brooks, *et al*. (1995). "Definition of 2 angiogenic pathways by distinct alpha(v) integrins." Science 270(5241): 1500-1502.

Fruchtpery, J. and M. Ilsar (1994). "The use of low-dose mitomycin-c for prevention of recurrent pterygium." Ophthalmology 101(4): 759-762.

Fu, Y. X., L. M. O'Connor, *et al*. (2003). "The p38 MAPK inhibitor, PD169316, inhibits transforming growth factor beta-induced Smad signaling in human ovarian cancer cells." Biochemical and Biophysical Research Communications 310(2): 391-397.

Fujiyama, S., H. Matsubara, *et al*. (2001). "Angiotensin AT(1) and AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation." Circulation Research 88(1): 22-29.

Gabbiani, G. (2003). "The myofibroblast in wound healing and fibrocontractive diseases." Journal of Pathology 200(4): 500-503.

Gachet, C. (2006). Regulation of platelet functions by P2 receptors. Annual Review of Pharmacology and Toxicology. Palo Alto, Annual Reviews. 46: 277-300.

Gellatly, S., S. Kalujnaia, *et al*. (2010). "Expression of gene isoforms encoding phospholipase C X-domain containing proteins in fish and humans." FASEB Journal 24.

Giancotti, F. G. and E. Ruoslahti (1999). "Transduction - Integrin signaling." Science 285(5430): 1028-1032.

Guidry, C. (1992). "Extracellular-matrix contraction by fibroblasts - peptide promoters and 2nd messengers." Cancer and Metastasis Reviews 11(1): 45-54.

Hakkinen, L., J. Heino, *et al*. (1994). "Altered interaction of human granulation-tissue fibroblasts with fibronectin is regulated by alpha-5-beta-1 integrin." Biochimica Et Biophysica Acta-Molecular Cell Research 1224(1): 33-42.

Hanafusa, H., S. Torii, *et al*. (2002). "Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway." Nature Cell Biology 4(11): 850-858.

Hartmann, A., M. Kunz, *et al*. (1999). "Hypoxia-induced up-regulation of angiogenin in human malignant melanoma." Cancer Research 59(7): 1578-1583.

He, H. X., J. H. Yu, *et al*. (2008). "Effects of FGF2 and TGF beta(1) on the differentiation of human dental pulp stem cells in vitro." Cell Biology International 32(7): 827-834.

Heeschen, C., J. J. Jang, *et al*. (2001). "Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis." Nature Medicine 7(7): 833-839.

Helenius, A., E. S. Trombetta, *et al*. (1997). "Calnexin, calreticulin and the folding of glycoproteins." Trends in Cell Biology 7(5): 193-200.

Higashiyama, S., J. A. Abraham, *et al*. (1993). "Heparin-binding egf-like growth-factor stimulation of smooth-muscle cell-migration - dependence on interactions with cell-surface heparan-sulfate." Journal of Cell Biology 122(4): 933-940.

Hilgers, J. H. C. (1960). "Pterygium - its incidence, heredity and etiology." American Journal of Ophthalmology 50(4): 635-644.

Hill, J. C. and R. Maske (1989). "Pathogenesis of Pterygium." Eye 3: 218-226.

Hinz, B., G. Celetta, *et al*. (2001). "Alpha-smooth muscle actin expression upregulates fibroblast contractile activity." Molecular Biology of the Cell 12(9): 2730-2741.

Hirst, L. W. (2003). "The treatment of pterygium." Survey of Ophthalmology 48(2): 145-180.

Hoeflich, K. P., M. T. Eby, *et al*. (2006). "Regulation of ERK3/MAPK6 expression by BRAF." International Journal of Oncology 29(4): 839-849.

Hogan, B. L. M. (1996). "Bone morphogenetic proteins: Multifunctional regulators of vertebrate development." Genes & Development 10(13): 1580-1594.

Huang, S. Y., L. Mills, *et al*. (2002). "Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma." American Journal of Pathology 161(1): 125-134.

Ignotz, R. A. and J. Massague (1987). "Cell-adhesion protein receptors as targets for transforming growth-factor-beta action." Cell 51(2): 189-197.

Imamura, T., M. Takase, *et al*. (1997). "Smad6 inhibits signalling by the TGF-beta superfamily." Nature 389(6651): 622-626.

Irigoyen, J. P., P. Munoz-Canoves, *et al*. (1999). "The plasminogen activator system: biology and regulation." Cellular and Molecular Life Sciences 56(1-2): 104-132.

Ito, J., N. Harada, *et al*. (2011). "Wound-induced TGF-beta 1 and TGF-beta 2 enhance airway epithelial repair via HB-EGF and TGF-alpha." Biochemical and Biophysical Research Communications 412(1): 109-114.

Jampel, H. D., N. Roche, *et al*. (1990). "Transforming growth-factor-beta in human aqueous-humor." Current Eye Research 9(10): 963-969.

Jester, J. V., J. Y. Huang, *et al*. (2002). "TGF beta induced myofibroblast differentiation of rabbit keratocytes requires synergistic TGF beta, PDGF and integrin signaling." Experimental Eye Research 75(6): 645-657.

Kadler, K. E., A. Hill, *et al*. (2008). "Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators." Current Opinion in Cell Biology 20(5): 495-501.

Kagami, S., W. A. Border, *et al*. (1994). "Angiotensin-ii stimulates extracellular-matrix protein-synthesis through induction of transforming growth-factor-beta expression in rat glomerular mesangial cells." Journal of Clinical Investigation 93(6): 2431-2437.

Kalimo, H., M. M. Ruchoux, *et al*. (2002). "CADASIL: a common form of hereditary arteriopathy causing brain infarcts and dementia." Brain Pathology 12(3): 371-384.

Kalluri, R. and E. G. Neilson (2003). "Epithelial-mesenchymal transition and its implications for fibrosis." Journal of Clinical Investigation 112(12): 1776-1784.

Karukonda, S. R. K., H. W. Thompson, *et al*. (1995). "Cell-cycle kinetics in pterygium at 3 latitudes." British Journal of Ophthalmology 79(4): 313-317.

Kau, H. C., C. C. Tsai, *et al*. (2004). "Genetic polymorphism of hOGG1 and risk of pterygium in Chinese." Eye 18(6): 635-639.

Kefalides, N. A. (1973). "Structure and biosynthesis of basement membranes." International review of connective tissue research 6: 63-104.

Kellermann, M. G., L. M. Sobral, *et al*. (2008). "Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: Induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation." Oral Oncology 44(5): 509-517.

Kennedy, M., K. H. Kim, *et al*. (1997). "Ultraviolet irradiation induces the production of multiple cytokines by human corneal cells." Investigative Ophthalmology & Visual Science 38(12): 2483-2491.

Khoo, J., S. M. Saw, *et al*. (1998). "Outdoor work and the risk of pterygia: a case-control study." International Ophthalmology 22(5): 293-298.

Kria, L., A. Ohira, *et al*. (1996). "Immunohistochemical localization of basic fibroblast growth factor, platelet derived growth factor, transforming growth factor-beta and tumor necrosis factor-alpha in the pterygium." Acta Histochemica 98(2): 195-201.

Kria, L., A. Ohira, *et al*. (1998). "Growth factors in cultured pterygium fibroblasts: Immunohistochemical and ELISA analysis." Graefes Archive for Clinical and Experimental Ophthalmology 236(9): 702-708.

Kroon, M. E., P. Koolwijk, *et al*. (2001). "Hypoxia in combination with FGF-2 induces tube formation by human microvascular endothelial cells in a fibrin matrix: involvement of at least two signal transduction pathways." Journal of Cell Science 114(4): 825-833.

Kumar, N. M., N. H. Rabadi, *et al*. (1996). "Induction of interleukin-1 and interleukin-8 mRNAs and proteins by TGF beta 1 in rat lung alveolar epithelial cells." J Cell Physiol 169(1): 186-199.

Kwok, L. S. and M. T. Coroneo (1994). "A Model for Pterygium Formation." Cornea 13(3): 219-224.

Lam, D. S. C., A. K. K. Wong, *et al*. (1998). "Intraoperative mitomycin C to prevent recurrence of pterygium after excision - A 30-month follow-up study." Ophthalmology 105(5): 901-904.

Landström, M., N.-E. Heldin, *et al*. (2000). "Smad7 mediates apoptosis induced by transforming growth factor [beta] in prostatic carcinoma cells." Current Biology 10(9): 535-538.

Lazar, V. and J. G. N. Garcia (1999). "A single human myosin light chain kinase gene (MLCK; MYLK) transcribes multiple nonmuscle isoforms." Genomics 57(2): 256-267.

Leask, A. and D. J. Abraham (2004). "TGF-beta signaling and the fibrotic response." FASEB Journal 18(7): 816-827.

Lee, S. C., O. W. Kwon, *et al*. (2001). "Epitheliomesenchymal transdifferentiation of cultured RPE cells." Ophthalmic Research 33(2): 80-86.

Leibovich, S. J., P. J. Polverini, *et al*. (1987). "Macrophage-induced angiogenesis is mediated by tumor-necrosis-factor-alpha." Nature 329(6140): 630-632.

Leippi, S., F. Grehn, *et al*. (2009). "Antiangiogenic therapy for pterygium recurrence." Ophthalmologe 106(5): 413-419.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell 88(3): 323-331.

Li, D. M., C. L. Zhang, *et al*. (2009). "VEGF regulates FGF-2 and TGF-beta 1 expression in injury endothelial cells and mediates smooth muscle cells proliferation and migration." Microvascular Research 77(2): 134-142.

Liang, K., Z. X. Jiang, *et al*. (2012). "The expression of vascular endothelial growth factor in mast cells promotes the neovascularisation of human pterygia." British Journal of Ophthalmology 96(9): 1246-1251.

Lim, S. Y., W. Y. Chung, *et al*. (2008). "Direct and nondestructive verification of PNA immobilization using click chemistry." Biochemical and Biophysical Research Communications 376(4): 633-636.

Lin, C. Q. and M. J. Bissell (1993). "Multifaceted Regulation of Cell-Differentiation by Extracellular-Matrix." FASEB Journal 7(9): 737-743.

Liu, X. J., H. Hu, *et al*. (2006). "Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis." Liver International 26(1): 8-22.

Low, Q. E. H., I. A. Drugea, *et al*. (2001). "Wound healing in MIP-1 alpha(-/-) and MCP-1(-/-) mice." American Journal of Pathology 159(2): 457-463.

Ma, J., Q. Wang, *et al*. (2007). "MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration." Blood 109(3): 987-994.

Maini, R., D. J. Collison, *et al*. (2002). "Pterygial derived fibroblasts express functionally active histamine and epidermal growth factor receptors." Experimental Eye Research 74(2): 237-244.

Mantovani, A., P. Allavena, *et al*. (2008). "Cancer-related inflammation." Nature 454(7203): 436-444.

Marcovich, A. L., Y. Morad, *et al*. (2002). "Angiogenesis in pterygium: Morphometric and immunohistochemical study." Current Eye Research 25(1): 17-22.

Martin, D., R. Galisteo, *et al*. (2009). "CXCL8/IL8 Stimulates Vascular Endothelial Growth Factor (VEGF) Expression and the Autocrine Activation of VEGFR2 in Endothelial Cells by Activating NF kappa B through the CBM (Carma3/Bcl10/Malt1) Complex." Journal of Biological Chemistry 284(10): 6038-6042.

Martin, P. (1997). "Wound healing - Aiming for perfect skin regeneration." Science 276(5309): 75-81.

Massague, J. (1990). "The transforming growth-factor-beta family." Annual Review of Cell Biology 6: 597-641.

Massague, J. (1998). "TGF-beta signal transduction." Annual Review of Biochemistry 67: 753-791.

Massague, J. (2000). "How cells read TGF-beta signals." Nature Reviews Molecular Cell Biology 1(3): 169-178.

McCarthy, D. J. and G. K. Smyth (2009). "Testing significance relative to a fold-change threshold is a TREAT." Bioinformatics 25(6): 765-771.

McCarty, C. A., C. L. Fu, *et al*. (2000). "Epidemiology of pterygium in Victoria, Australia." British Journal of Ophthalmology 84(3): 289-292.

Meredith, J. E., B. Fazeli, *et al*. (1993). "The Extracellular-Matrix as a Cell-Survival Factor." Molecular Biology of the Cell 4(9): 953-961.

Mery, L., N. Mesaeli, *et al*. (1996). "Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx." Journal of Biological Chemistry 271(16): 9332-9339.

Meyer-Ter-Vehn, T., B. Katzenberger, *et al*. (2008). "Lovastatin inhibits TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts." Investigative Ophthalmology & Visual Science 49(9): 3955-3960.

Meyer, M., M. Clauss, *et al*. (1999). "A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases." Embo Journal 18(2): 363-374.

Michalak, M., E. F. Corbett, *et al*. (1999). "Calreticulin: one protein, one gene, many functions." Biochemical Journal 344: 281-292.

Miettinen, P. J., R. Ebner, *et al*. (1994). "TGF-beta induced transdifferentiation of mammary epithelial-cells to mesenchymal cells - involvement of type-i receptors." Journal of Cell Biology 127(6): 2021-2036.

Milner, R. E., K. S. Famulski, *et al*. (1992). "calcium-binding proteins in the sarcoplasmic endoplasmic-reticulum of muscle and nonmuscle cells." Molecular and Cellular Biochemistry 112(1): 1-13.

Mott, J. D. and Z. Werb (2004). "Regulation of matrix biology by matrix metalloproteinases." Current Opinion in Cell Biology 16(5): 558-564.

Muether, P. S., S. Dell, *et al*. (2007). "The role of integrin as alpha(5)beta(1) in the regulation of corneal neovascularization." Experimental Eye Research 85(3): 356-365.

Murphy, A. N., E. J. Unsworth, *et al*. (1993). "TIssue inhibitor of metalloproteinases-2 inhibits bfgf-induced human microvascular endothelial-cell proliferation." Journal of Cellular Physiology 157(2): 351-358.

Naib-Majani, W., I. Eltohami, *et al*. (2004). "Distribution of extracellular matrix proteins in pterygia: an immunohistochemical study." Graefes Archive for Clinical and Experimental Ophthalmology 242(4): 332-338.

Nakagami, T., A. Murakami, *et al*. (1999). "Mast Cells in Pterygium: Number and Phenotype." Japanese Journal of Ophthalmology 43(2): 75-79.

Nakao, A., M. Afrakhte, *et al*. (1997). "Identification of Smad7, a TGF beta-inducible antagonist of TGF-beta signalling." Nature 389(6651): 631-635.

Nickl-Jockschat, T., F. Arslan, *et al*. (2007). "An imbalance between Smad and MAPK pathways is responsible for TGF-B tumor promoting effects in high-grade gliomas." International Journal of Oncology 30(2): 499-507.

Nicola, C., A. V. Timoshenko, *et al*. (2005). "EP1 receptor-mediated migration of the first trimester human extravillous trophoblast: the role of intracellular calcium and calpain." J Clin Endocrinol Metab 90(8): 4736-4746.

Nishimura, Y., A. Nakai, *et al*. (2000). "Long-term results of fractionated strontium-90 radiation therapy for pterygia." International Journal of Radiation Oncology Biology Physics 46(1): 137-141.

Nolan, T. M., N. DiGirolamo, *et al*. (2003). "The Role of Ultraviolet Irradiation and Heparin-Binding Epidermal Growth Factor-Like Growth Factor in the Pathogenesis of Pterygium." The American Journal of Pathology 162(2): 567-574.

Oreilly, M. S., L. Holmgren, *et al*. (1994). "Angiostatin - a novel angiogenesis inhibitor that mediates the suppression of metastases by a lewis lung-carcinoma." Cell 79(2): 315-328.

Otlu, B., S. Emre, *et al*. (2009). "Investigation of human papillomavirus and Epstein-Barr virus DNAs in pterygium tissue." European Journal of Ophthalmology 19(2): 175-179.

Otsuki, M., K. Fukami, *et al*. (1999). "Identification and characterization of a new phospholipase C-like protein, PLC-L-2." Biochemical and Biophysical Research Communications 266(1): 97-103.

Padgett, R. W. and G. I. Patterson (2001). "New Developments for TGF[beta]." Developmental Cell 1(3): 343-349.

Palczewski, K., T. Kumasaka, *et al*. (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science 289(5480): 739-745.

Parekh, A. B. and R. Penner (1997). "Store depletion and calcium influx." Physiol Rev 77(4): 901-930.

Park, J. E., G. A. Keller, *et al*. (1993). "Vascular endothelial growth-factor (vegf) isoforms - differential deposition into the subepithelial extracellular-matrix and bioactivity of extracellular matrix-bound vegf." Molecular Biology of the Cell 4(12): 1317-1326.

Pasquale, L. R., M. E. Dormanpease, *et al*. (1993). "Immunolocalization of tgf-beta-1, tgf-beta-2, and tgf-beta-3 in the anterior segment of the human eye." Investigative Ophthalmology & Visual Science 34(1): 23-30.

Paula, J. S., F. Thorn, *et al*. (2006). "Prevalence of pterygium and cataract in indigenous populations of the Brazilian Amazon rain forest." Eye 20(5): 533-536.

Pepper, M. S. (1997). "Manipulating angiogenesis - From basic science to the bedside." Arteriosclerosis Thrombosis and Vascular Biology 17(4): 605-619.

Potempa, J., E. Korzus, *et al*. (1994). "The serpin superfamily of proteinase-inhibitors - structure, function, and regulation." Journal of Biological Chemistry 269(23): 15957-15960.

Pousa, I. D. and J. P. Gisbert (2006). "Gastric angiogenesis and Helicobacter pylori infection." Revista Espanola De Enfermedades Digestivas 98(7): 527-535.

Qi, W., X. Chen, *et al*. (2006). "TGF-beta1 induces IL-8 and MCP-1 through a connective tissue growth factor-independent pathway." Am J Physiol Renal Physiol 290(3): F703-709.

Reid, T. W. and N. Dushku (2010). "What a study of pterygia teaches us about the cornea? Molecular mechanisms of formation." Eye Contact Lens 36(5): 290-295.

Reiher, F. K., O. V. Volpert, *et al*. (2002). "Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics." International Journal of Cancer 98(5): 682-689.

Rhodes, J. D., S. L. Russell, *et al*. (2009). "Regional differences in store-operated Ca2+ entry in the epithelium of the intact human lens." Invest Ophthalmol Vis Sci 50(9): 4330-4336.

Rieck, P., L. Oliver, *et al*. (1995). "The role of exogenous endogenous basic fibroblast growth-factor (FGF2) And transforming growth-factor-beta (TGF-beta-1) on human corneal endothelial-cells proliferation in-vitro." Experimental Cell Research 220(1): 36-46.

Rim, T. H. T., J. Nam, *et al*. (2013). "Risk factors associated with pterygium and its subtypes in Korea: the korean national health and nutrition examination survey 2008-2010." Cornea 32(7): 962-970.

Risau, W. (1997). "Mechanisms of angiogenesis." Nature 386(6626): 671-674.

Roberts, A. B., M. B. Sporn, *et al*. (1986). "Transforming growth-factor type-beta - rapid induction of fibrosis and angiogenesis invivo and stimulation of collagen formation invitro." Proceedings of the National Academy of Sciences of the United States of America 83(12): 4167-4171.

Rodrigues, F. W., J. T. Arruda, *et al*. (2008). "TP53 gene expression, codon 72 polymorphism and human papillomavirus DNA associated with pterygium." Genetics and Molecular Research 7(4): 1251-1258.

Safina, A. F., E. Vendette, *et al*. (2005). "TGF-beta-p38MAPK signaling contributes to tumor invasion and pulmonary metastases by increasing MMP9 activity and cell

motility." Proceedings of the American Association for Cancer Research Annual Meeting 46: 1028.

Saito, Y., Y. Ihara, *et al*. (1999). "Calreticulin functions in vitro as a molecular chaperone for both glycosylated and nonglycosylated proteins." Embo Journal 18(23): 6718-6729.

Sakuntabhai, A., V. Ruiz-Perez, *et al*. (1999). "Mutations in ATP2A2, encoding a Ca2+ pump, cause Darier disease." Nature Genetics 21(3): 271-277.

Salcedo, R., M. L. Ponce, *et al*. (2000). "Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression." Blood 96(1): 34-40.

Schafer, K. A. (1998). "The cell cycle: A review." Veterinary Pathology 35(6): 461-478.

Schnermann, J. B., X. L. Zhu, *et al*. (1996). "Regulation of endothelin production and secretion in cultured collecting duct cells by endogenous transforming growth factor-beta." Endocrinology 137(11): 5000-5008.

Sherr, C. J. (1996). "Cancer cell cycles." Science 274(5293): 1672-1677.

Shi, Y., Y.-F. Wang, *et al*. (1998). "Crystal Structure of a Smad MH1 Domain Bound to DNA: Insights on DNA Binding in TGF-[beta] Signaling." Cell 94(5): 585-594.

Shoyab, M., G. D. Plowman, *et al*. (1989). "STructure and function of human amphiregulin - a member of the epidermal growth-factor family." Science 243(4894): 1074-1076.

Sime, P. J. and K. M. A. O'Reilly (2001). "Fibrosis of the lung and other tissues: New concepts in pathogenesis and treatment." Clinical Immunology 99(3): 308-319.

Sisakhtnezhad, S. and M. M. Matin (2012). "Transdifferentiation: a cell and molecular reprogramming process." Cell and Tissue Research 348(3): 379-396.

Skalli, O., M. F. Pelte, *et al*. (1989). "Alpha-smooth muscle actin, a differentiation marker of smooth-muscle cells, is present in microfilamentous bundles of pericytes." Journal of Histochemistry & Cytochemistry 37(3): 315-321.

Smith, W. B., L. Noack, *et al*. (1996). "Transforming growth factor-beta 1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium." Journal of Immunology 157(1): 360-368.

Soloman, A., D. Q. Li, *et al*. (2000). "Regulation of collagenase, stromelysin, and urokinase-type plasminogen activator in primary pterygium body fibroblasts by inflammatory cytokines." Investigative Ophthalmology & Visual Science 41(8): 2154-2163.

Song, P. I., T. A. Abraham, *et al*. (2002). "Ultraviolet Irradiation Induces Human Corneal Epithelial Cell EGF Receptor Phosphorylation, NF-kappaB Activation and Proinflammatory Cytokine Production." ARVO Annual Meeting Abstract Search and Program Planner 2002: 2959.

Stamenkovic, I. (2003). "Extracellular matrix remodelling: the role of matrix metalloproteinases." Journal of Pathology 200(4): 448-464.

Sun, Z. and M. Ke (2007). "Expression and function of matrix metalloproteinases and transforming growth factor-beta 1 in pterygium." Wuhan Daxue Xuebao (Yixue Ban) 28(1): 44-46.

Susaki, K. and C. Chiba (2007). "MEK mediates in vitro neural transdifferentiation of the adult newt retinal pigment epithelium cells: Is FGF2 an induction factor?" Pigment Cell Research 20(5): 364-379.

Tazuke, S. I., N. M. Mazure, *et al*. (1998). "Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: A possible model for IGFBP-1 expression in fetal hypoxia." Proceedings of the National Academy of Sciences of the United States of America 95(17): 10188-10193.

Threlfall, T. J. and D. R. English (1999). "Sun exposure and pterygium of the eye: A dose-response curve." American Journal of Ophthalmology 128(3): 280-287.

Timpl, R., H. Rohde, *et al*. (1979). "Laminin - glycoprotein from basement-membranes." Journal of Biological Chemistry 254(19): 9933-9937.

Torres, J., A. Enriquez-de-Salamanca, *et al*. (2011). "Activation of MAPK Signaling Pathway and NF-kappa B Activation in Pterygium and Ipsilateral Pterygium-Free Conjunctival Specimens." Investigative Ophthalmology & Visual Science 52(8): 5842-5852.

Tsai, Y. Y., D. T. Bau, *et al*. (2007). "Pterygium and genetic polymorphism of DNA double strand break repair gene Ku70." Molecular Vision 13(158): 1436-1440.

Tsai, Y. Y., C. C. Chang, *et al*. (2009). "HPV infection and p53 inactivation in pterygium." Molecular Vision 15(114-15): 1092-1097.

Tsai, Y. Y., C. C. Chiang, *et al*. (2008). "Vascular endothelial growth factor gene 460 polymorphism is associated with pterygium formation in female patients." Cornea 27(4): 476-479.

Tsai, Y. Y., C. C. Chiang, *et al*. (2010). "Effect of TIMP-1 and MMP in pterygium invasion." Invest Ophthalmol Vis Sci 51(7): 3462-3467.

Tsukada, S., J. K. Westwick, *et al*. (2005). "SMAD and p38 MAPK signaling pathways independently regulate alpha 1(I) collagen gene expression in unstimulated and transforming growth factor-alpha-stimulated hepatic stellate cells." Journal of Biological Chemistry 280(11): 10055-10064.

Unda, F. J., A. Martin, *et al*. (2000). "Dissection of the odontoblast differentiation process in vitro by a combination of FGF1, FGF2, and TGF beta 1." Developmental Dynamics 218(3): 480-489.

van Setten, G., M. Aspiotis, *et al*. (2003). "Connective tissue growth factor in pterygium: simultaneous presence with vascular endothelial growth factor - possible contributing factor to conjunctival scarring." Graefes Arch Clin Exp Ophthalmol 241(2): 135-139.

Verrecchia, F. and A. Mauviel (2007). "Transforming growth factor-beta and fibrosis." World Journal of Gastroenterology 13(22): 3056-3062.

Wahab, N. A., B. S. Weston, *et al*. (2005). "Modulation of the TGF beta/Smad signaling pathway in mesangial cells by CTGF/CCN2." Experimental Cell Research 307(2): 305-314.

Walker, J. and A. S. Menko (2009). "Integrins in lens development and disease." Experimental Eye Research 88(2): 216-225.

Wang, L., I. M. Wormstone, *et al*. (2005). "Growth factor receptor signalling in human lens cells: role of the calcium store." Exp Eye Res 80(6): 885-895.

Wang, N., J. P. Butler, *et al*. (1993). "Mechanotransduction across the cell-surface and through the cytoskeleton." Science 260(5111): 1124-1127.

Warburton, D., W. Shi, *et al*. (2013). "TGF-beta-Smad3 signaling in emphysema and pulmonary fibrosis: an epigenetic aberration of normal development?" American Journal of Physiology-Lung Cellular and Molecular Physiology 304(2): L83-L85.

Welling, T. H., S. Fu, *et al*. (2012). "Elevated serum IL-8 is associated with the presence of hepatocellular carcinoma and independently predicts survival." Cancer Invest 30(10): 689-697.

Wong, T. Y., P. J. Foster, *et al*. (2001). "The prevalence and risk factors for pterygium in an adult Chinese population in Singapore: The Tanjong Pagar Survey." American Journal of Ophthalmology 131(2): 176-183.

Wormstone, I. M., S. Tamiya, *et al*. (2002). "TGF-beta 2-induced matrix modification and cell transdifferentiation in the human lens capsular bag." Investigative Ophthalmology & Visual Science 43(7): 2301-2308.

Wu, J.-W., M. Hu, *et al*. (2001). "Crystal Structure of a Phosphorylated Smad2: Recognition of Phosphoserine by the MH2 Domain and Insights on Smad Function in TGF-[beta] Signaling." Molecular Cell 8(6): 1277-1289.

Wynn, T. A. (2008). "Cellular and molecular mechanisms of fibrosis." Journal of Pathology 214(2): 199-210.

Yamaguchi, K., T. Shirakabe, *et al*. (1995). "Identification of a member of the mapkkk family as a potential mediator of TGF-beta signal-transduction." Science 270(5244): 2008-2011.

Yang, S. F., C. Y. Lin, *et al*. (2009). "Increased expression of gelatinase (MMP-2 and MMP-9) in pterygia and pterygium fibroblasts with disease progression and activation of protein kinase C." Invest Ophthalmol Vis Sci 50(10): 4588-4596.

Zavadil, J. and E. P. Bottinger (2005). "TGF-beta and epithelial-to-mesenchymal transitions." Oncogene 24(37): 5764-5774.

Zhang, H., G. Duncan, *et al*. (2007). "Arsenic trioxide initiates ER stress responses, perturbs calcium signalling and promotes apoptosis in human lens epithelial cells." Exp Eye Res 85(6): 825-835.

Zhu, B. Q., C. Heeschen, *et al*. (2003). "Second hand smoke stimulates tumor angiogenesis and growth." Cancer Cell 4(3): 191-196.

Zielonka, T. M., U. Demkow, *et al*. (2007). "Modulatory effect of sera from sarcoidosis patients on mononuclear cell-induced angiogenesis." Journal of Physiology and Pharmacology 58: 753-766.

Zielonka, T. M., U. Demkow, *et al*. (2007). "Angiogenic activity of sera from interstitial lung diseases patients in relation to IL-6, IL-8, IL-12 and TNF alpha serum level." Central European Journal of Immunology 32(2): 53-60.

APPENDIX I

Gene Microarray: 10% serum and TGFβ2 regulated gene expression data

The following tables list all genes that are either up-regulated or down-regulated more than 2 fold in pterygial fibroblasts by 10% serum and TGFβ2 in four biological replicates detected by gene microarrays.

Appendix IA. Gene up-regulated more than 2 fold in pterygial fibroblasts by 10% serum

Symbol	Gene	Fold change
ID3	inhibitor of DNA binding 3	9.03
RGS4	regulator of G-protein signalling 4	5.87
ACTG2	actin, gamma 2, smooth muscle, enteric	5.6
ID1	inhibitor of DNA binding 1	4.96
CDC20	cell division cycle 20 homolog	4.03
ACTC1	actin, alpha, cardiac muscle 1	3.86
KRT7	keratin 7	3.53
TUBB3	tubulin, beta 3 class III	3.43
GLIPR1	GLI pathogenesis-related 1	3.19
ATOH8	atonal homolog 8 (Drosophila)	3.13
CEP55	centrosomal protein 55kD	3.12
UBE2C	ubiquitin-conjugating enzyme E2C	3.08
CCNA2	cyclin A2	3.08
ANLN	anillin, actin binding protein	3.02
AURKA	aurora kinase A	3.02
NEFM	neurofilament, medium polypeptide	3.00
PRC1	protein regulator of cytokinesis 1	3.00
SERPINE1	serpin peptidase inhibitor, clade E	2.99

CCNB2	**cyclin B2**	**2.98**
KRTAP1-5	**keratin associated protein 1-5**	**2.96**
KRT34	**keratin 34**	**2.92**
TK1	**thymidine kinase 1, soluble**	**2.92**
PBK	**PDZ binding kinase**	**2.86**
TOP2A	**topoisomerase (DNA) II alpha 170kDa**	**2.85**
CDKN3	**cyclin-dependent kinase inhibitor 3**	**2.77**
PTTG1	**pituitary tumour-transforming 1**	**2.72**
KIFC1	**kinesin family member C1**	**2.64**
KIF20A	**kinesin family member 20A**	**2.63**
DKK1	**dickkopf 1 homolog (Xenopus laevis)**	**2.63**
CDCA8	**cell division cycle associated 8**	**2.63**
HJURP	**Holliday junction recognition protein**	**2.61**
CKAP2L	**cytoskeleton associated protein 2-like**	**2.60**
AURKB	**aurora kinase B**	**2.60**
CDCA3	**cell division cycle associated 3**	**2.52**
IL7R	**interleukin 7 receptor**	**2.51**
CKS2	**CDC28 protein kinase regulatory subunit 2**	**2.49**
RRM2	**ribonucleotide reductase M2**	**2.48**
DLGAP5	**discs, large (Drosophila) homolog-associated protein 5**	**2.47**

LOC651816	ubiquitin-conjugating enzyme E2S	2.47
TRIP13	thyroid hormone receptor interactor 13	2.46
NCAPG	non-SMC condensin I complex, subunit G	2.43
COTL1	coactosin-like 1 (Dictyostelium)	2.40
MELK	Maternal embryonic leucine zipper kinase	2.40
MALL	mal, T-cell differentiation protein-like	2.39
ODC1	ornithine decarboxylase 1	2.39
CENPA	centromere protein A	2.38
PRR11	proline rich 11	2.37
STS-1 UBASH3B	Ubiquitin associated and SH3 domain containing B	2.34
SPOCD1	SPOC domain containing 1	2.32
TPM1	tropomyosin 1 (alpha)	2.32
SPC24	Kinetochore protein Spc24	2.34
LOC731049	similar to Ubiquitin-conjugating enzyme E2S	2.31
NPAS1	neuronal PAS domain protein 1	2.31
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.31
PTTG3P	pituitary tumour-transforming 3, pseudogene	2.30
BDKRB1	bradykinin receptor B1	2.30
LOC92755	tubulin, beta pseudogene 1	2.28
TACC3	transforming, acidic coiled-coil containing protein 3	2.28

CDCA2	**cyclin-dependent kinase inhibitor 2A**	**2.26**
TYMS	**thymidylate synthetase**	**2.24**
ID2	**inhibitor of DNA binding 2,**	**2.24**
CDC45L	**cell division cycle 45**	**2.23**
TNFRSF12A	**tumour necrosis factor receptor superfamily, member 12A**	**2.23**
CENPN	**centromere protein N**	**2.22**
PDCD1LG2	**programmed cell death 1 ligand 2**	**2.22**
ASPM	**asp (abnormal spindle) homolog, microcephaly associated (Drosophila)**	**2.21**
F3	**coagulation factor III (thromboplastin, tissue factor)**	**2.21**
TPX2	**microtubule-associated, homolog (Xenopus laevis)**	**2.20**
UHRF1	**ubiquitin-like with PHD and ring finger domains 1**	**2.18**
LOC730743	**similar to keratin associated protein 1-1**	**2.18**
NUSAP1	**nucleolar and spindle associated protein 1**	**2.18**
LOC731314	**similar to H2A histone family, member X**	**2.17**
GTSE1	**G-2 and S-phase expressed 1**	**2.17**
LOX	**lysyl oxidase**	**2.16**
LOC402221	**actin, beta pseudogene**	**2.16**
RPS7	**ribosomal protein S7**	**2.14**

BIRC5	baculoviral IAP repeat containing 5	2.14
CNN1	calponin 1, basic, smooth muscle	2.14
LOC100133565	ubiquitin-like, containing PHD and RING finger domains, 1 pseudogene	2.13
CDCA5	cell division cycle associated 5	2.12
ELN	Elastin	2.12
RAD51AP1	RAD51 associated protein 1	2.11
SKA1	spindle and kinetochore associated complex subunit 1	2.11
LOC728255	keratin associated protein 1-4	2.10
LOC728285	similar to keratin associated protein	2.08
NEK7	NIMA-related kinase 7	2.07
HS.579631	small ILF3/NF90-associated RNA A1	2.06
TUBB6	tubulin, beta 6 class V	2.06
RACGAP1	Rac GTPase activating protein 1	2.05
FOSL1	FOS-like antigen 1	2.04
KIF2C	kinesin family member 2C	2.04
DDX39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A	2.04
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	2.02
GINS2	GINS complex subunit 2 (Psf2 homolog)	2.01
LPXN	leupaxin	2.00

Appendix IB. Gene down-regulated more than 2 fold in pterygial fibroblasts by 10% serum

Symbol	Gene	Fold change
TMEM119	transmembrane protein 119	0.27
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	0.31
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.31
RCAN2	regulator of calcineurin 2	0.32
APOD	apolipoprotein D	0.33
EPHB6	EPH receptor B6	0.34
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	0.34
TM7SF2	transmembrane 7 superfamily member 2	0.34
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	0.34
ADH1B	alcohol dehydrogenase 1B (class I), beta polypeptide	0.35
SLC2A12	solute carrier family 2, member 12	0.36
ANGPTL2	angiopoietin-like 2	0.36
OLFML2A	olfactomedin-like 2A	0.37
GAS1	growth arrest-specific 1	0.40
LOC731954	unkown	0.40
CTSF	cathepsin F	0.41
TKT	transketolase	0.41
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	0.42

GPNMB	**glycoprotein (transmembrane) nmb**	**0.43**
SCARA5	**Scavenger receptor class A member 5**	**0.43**
COLEC12	**collectin sub-family member 12**	**0.43**
GSTM3	**glutathione S-transferase mu 3 (brain)**	**0.43**
SVEP1	**sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1**	**0.44**
TGFBR3	**transforming growth factor, beta receptor III**	**0.45**
QPRT	**quinolinate phosphoribosyltransferase**	**0.45**
HS.552087	**slit homolog 3 (Drosophila)**	**0.45**
CTSK	**cathepsin K**	**0.45**
SVIL	**supervillin**	**0.46**
DHCR7	**7-dehydrocholesterol reductase**	**0.46**
EPHX1	**epoxide hydrolase 1, microsomal (xenobiotic)**	**0.46**
RAB7B	**RAB7B, member RAS oncogene family**	**0.47**
PLSCR4	**phospholipid scramblase 4**	**0.47**
TSHZ2	**teashirt zinc finger homeobox 2**	**0.47**
INSIG1	**insulin induced gene 1**	**0.47**
LSS	**lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)**	**0.47**
PAMR1	**peptidase domain containing associated with muscle regeneration 1**	**0.48**

LDLR	**low density lipoprotein receptor**	**0.48**
C13ORF15	**Regulator of cell cycle RGCC**	**0.48**
C1S	**complement component 1, s subcomponent**	**0.48**
PCYOX1	**prenylcysteine oxidase 1**	**0.48**
LRP1	**low density lipoprotein receptor-related protein 1**	**0.48**
CMBL	**carboxymethylenebutenolidase homolog (Pseudomonas)**	**0.49**
HCFC1R1	**host cell factor C1 regulator 1 (XPO1 dependent)**	**0.49**
CDKN1C	**cyclin-dependent kinase inhibitor 1C (p57, Kip2)**	**0.49**
PTGES	**prostaglandin E synthase**	**0.49**
SERPING1	**serpin peptidase inhibitor, clade G (C1 inhibitor), member 1**	**0.49**
MXRA5	**matrix-remodelling associated 5**	**0.50**
GSTM5	**glutathione S-transferase mu 5**	**0.50**
MGP	**matrix Gla protein**	**0.50**
AKR1C3	**aldo-keto reductase family 1, member C3**	**0.50**

Appendix IC. Gene up-regulated more than 2 fold in pterygial fibroblasts by TGFβ2

Symbol	Gene	Fold change
ACTC1	actin, alpha, cardiac muscle 1	10.37
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	8.38
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	7.81
C5ORF13	neuronal regeneration related protein homolog (rat)	7.24
ACTG2	actin, gamma 2, smooth muscle, enteric	6.93
MAMDC2	MAM domain containing 2	6.84
IER3	immediate early response 3	6.19
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	5.59
IL11	interleukin 11	5.57
COL4A1	collagen, type IV, alpha 1	4.91
RGS4	regulator of G-protein signalling 4	4.87
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	4.84
CNN1	calponin 1, basic, smooth muscle	4.63
HS.36053	neuronal regeneration related protein homolog (rat)	4.63
ANKRD38 KANK4	KN motif and ankyrin repeat domains 4	4.51

BMP6	**bone morphogenetic protein 6**	**4.47**
TPM1	**tropomyosin 1 (alpha)**	**4.42**
KANK4	**KN motif and ankyrin repeat domains 4**	**4.41**
TMEM2	**transmembrane protein 2**	**4.38**
DACT1	**dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)**	**4.32**
ADAM19	**ADAM metallopeptidase domain 19**	**4.24**
FSTL3	**follistatin-like 3 (secreted glycoprotein)**	**4.15**
TP53I3	**tumor protein p53 inducible protein 3**	**4.05**
ODZ4	**teneurin transmembrane protein 4**	**4.05**
CTPS	**CTP synthase 1**	**4.03**
ESM1	**endothelial cell-specific molecule 1**	**3.97**
FNDC1	**fibronectin type III domain containing 1**	**3.81**
ELN	**elastin**	**3.79**
TSPAN13	**tetraspanin 13**	**3.72**
XYLT1	**xylosyltransferase I**	**3.70**
STS-1	**ubiquitin associated and SH3 domain containing B**	**3.66**
LRRN3	**leucine rich repeat neuronal 3**	**3.63**
LMCD1	**LIM and cysteine-rich domains 1**	**3.60**
CD55	**CD55 molecule, decay accelerating factor for complement**	**3.53**

	(Cromer blood group)	
GADD45B	growth arrest and DNA-damage-inducible, beta	3.53
PI16	peptidase inhibitor 16	3.48
COMP	cartilage oligomeric matrix protein	3.48
COL7A1	collagen, type VII, alpha 1	3.47
APCDD1L	adenomatosis polyposis coli down-regulated 1-like	3.40
ZPLD1	zona pellucida-like domain containing 1	3.39
ACTA2	actin, alpha 2, smooth muscle, aorta	3.39
IVNS1ABP	influenza virus NS1A binding protein	3.33
ENC1	ectodermal-neural cortex 1 (with BTB-like domain)	3.30
KRT7	keratin 7	3.26
CSRP2	cysteine and glycine-rich protein 2	3.24
LAMC2	laminin, gamma 2	3.13
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	3.08
PMEPA1	prostate transmembrane protein, androgen induced 1	3.08
P4HA2	prolyl 4-hydroxylase, alpha polypeptide II	3.08
LOC399942	tubulin, alpha pseudogene 2	3.06
GLS	glutaminase	3.05
CCDC99	spindle apparatus coiled-coil protein 1	3.04
BHLHB2	basic helix-loop-helix family, member e40	3.04

COTL1	coactosin-like 1 (Dictyostelium)	3.01
SGK1	serum/glucocorticoid regulated kinase 1	3.01
ACTN1	actinin, alpha 1	3.00
VCAN	versican	3.00
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	2.99
NOX4	NADPH oxidase 4	2.99
PPME1	protein phosphatase methylesterase 1	2.98
F3	coagulation factor III (thromboplastin, tissue factor)	2.96
LANCL2	LanC lantibiotic synthetase component C-like 2 (bacterial)	2.95
PAWR	PRKC, apoptosis, WT1, regulator	2.94
PPP1R13L	protein phosphatase 1, regulatory subunit 13 like	2.91
ALDH1B1	aldehyde dehydrogenase 1 family, member B1	2.90
UCK2	uridine-cytidine kinase 2	2.90
FZD8	frizzled family receptor 8	2.90
IL6	interleukin 6 (interferon, beta 2)	2.90
CTGF	connective tissue growth factor	2.89
SGK	serum/glucocorticoid regulated kinase	2.88
TUBB6	tubulin, beta 6 class V	2.87
GLIPR1	GLI pathogenesis-related 1	2.85
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	2.81

TUBB3	**tubulin, beta 3 class V**	**2.78**
LOC402221	**actin, beta pseudogene**	**2.76**
EDN1	**endothelin 1**	**2.73**
MTHFD2	**methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase**	**2.70**
C6ORF145	**PX domain containing 1**	**2.66**
DSP	**desmoplakin**	**2.64**
ODC1	**ornithine decarboxylase 1**	**2.63**
DSE	**dermatan sulfate epimerase**	**2.63**
CSRP1	**cysteine and glycine-rich protein 1**	**2.62**
SERTAD4	**SERTA domain containing 4**	**2.60**
MT1X	**metallothionein 1X**	**2.59**
SRM	**spermidine synthase**	**2.58**
SPHK1	**sphingosine kinase 1**	**2.55**
MICAL2	**microtubule associated monooxygenase, calponin and LIM domain containing**	**2.55**
DKK1	**dickkopf 1 homolog (Xenopus laevis)**	**2.55**
C1ORF133	**SERTAD4 antisense RNA 1**	**2.54**
TSPAN2	**tetraspanin 2**	**2.52**
WNT5B	**wingless-type MMTV integration site family, member 5B**	**2.50**

NTM	neurotrimin	2.50
ITGA1	integrin, alpha 1	2.49
ODZ3	teneurin transmembrane protein 3	2.48
DDAH1	dimethylarginine dimethylaminohydrolase 1	2.47
PDLIM7	PDZ and LIM domain 7 (enigma)	2.47
CYR61	cysteine-rich, angiogenic inducer, 61	2.46
GOLSYN	Syntabulin	2.46
BCAT1	Branched-chain-amino-acid aminotransferase, cytosolic	2.45
MGC16121	uncharacterized protein MGC16121	2.44
PFKP	PFKP protein	2.44
DYRK2	Dual specificity tyrosine-phosphorylation-regulated kinase 2	2.41
NP	Transgelin-3	2.41
PRPS1	Ribose-phosphate pyrophosphokinase 1	2.40
SLC7A1	High affinity cationic amino acid transporter 1	2.40
SLC35F2	Solute carrier family 35 member F2	2.39
LOC729768	Uncharacterized protein	2.39
CSMD2	CSMD2 protein	2.38
MYOZ1	myozenin 1	2.37
PGM2L1	Glucose 1,6-bisphosphate synthase	2.36
FABP5L2	fatty acid binding protein 5 pseudogene 2	2.36

TFPI2	Tissue factor pathway inhibitor 2	2.36
NPPB	Natriuretic peptides B	2.34
SERPINB2	Plasminogen activator inhibitor 2	2.32
PDCD1LG2	Programmed cell death 1 ligand 2	2.32
C6ORF85	Solute carrier family 22 member 23	2.30
ITGA11	Integrin alpha-11	2.29
LMO4	LIM domain transcription factor LMO4	2.28
TUBB2A	tubulin, beta 2A class IIa	2.28
ARL4A	ADP-ribosylation factor-like 4A	2.27
NUAK1	NUAK family, SNF1-like kinase, 1	2.26
CHN1	chimerin 1	2.26
DDX21	DEAD (Asp-Glu-Ala-Asp) box helicase 21	2.26
LOXL3	lysyl oxidase-like 3	2.26
GALNT10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	2.26
VIL2	ezrin	2.26
PLAUR	plasminogen activator, urokinase receptor	2.25
SRPX2	Sushi repeat-containing protein SRPX2	2.24
EFHD1	EF-hand domain family, member D1	2.24
TMEM158	transmembrane protein 158 (gene/pseudogene)	2.23

GFPT1	glutamine--fructose-6-phosphate transaminase 1	2.23
EFNB2	ephrin-B2	2.23
RUSC2	RUN and SH3 domain containing 2	2.23
PSME4	proteasome (prosome, macropain) activator subunit 4	2.23
IGFBP3	insulin-like growth factor binding protein 3	2.23
HS3ST3A1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	2.23
SDC1	syndecan 1	2.22
LOC728285	Uncharacterized protein	2.21
CALB2	calbindin 2	2.21
E2F7	E2F transcription factor 7	2.19
GALNTL4	Putative polypeptide N-acetylgalactosaminyltransferase-like protein 4	2.19
GPC4	glypican 4	2.19
EZR	ezrin	2.19
KIAA1539	family with sequence similarity 214, member B	2.18
FERMT2	fermitin family member 2	2.18
LOC441019	Uncharacterized protein	2.17
F2RL1	Proteinase-activated receptor 2	2.16
POTEF	POTE ankyrin domain family, member F	2.16
TAGLN	transgelin	2.16

SCHIP1	schwannomin interacting protein 1	2.15
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.14
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.14
PSMD2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	2.
SRF	serum response factor	2.13
RAB3B	member RAS oncogene family	2.13
LOC100134265	Uncharacterized protein	2.12
ANKRD37	ankyrin repeat domain 37	2.12
CREB3L2	cAMP responsive element binding protein 3-like 2	2.11
MSN	moesin	2.10
ULBP2	UL16 binding protein 2	2.10
SPOCD1	SPOC domain containing 1	2.10
ARMET	mesencephalic astrocyte-derived neurotrophic factor	2.10
UHRF1	ubiquitin-like with PHD and ring finger domains 1	2.10
IL7R	interleukin 7 receptor	2.10
BAIAP2L1	BAI1-associated protein 2-like 1	2.09
PGM3	phosphoglucomutase 3	2.09
SDF2L1	stromal cell-derived factor 2-like 1	2.09
PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	2.08

COL4A2	**collagen, type IV, alpha 2**	**2.08**
DCBLD1	**discoidin, CUB and LCCL domain containing 1**	**2.07**
HYOU1	**hypoxia up-regulated 1**	**2.07**
PCDH19	**protocadherin 19**	**2.07**
LRRC2	**leucine rich repeat containing 2**	**2.06**
TNS1	**tensin 1**	**2.06**
CHSY3	**chondroitin sulfate synthase 3**	**2.06**
LOC92755	**tubulin, beta pseudogene 1**	**2.06**
TDG	**thymine-DNA glycosylase**	**2.05**
PDLIM5	**PDZ and LIM domain 5**	**2.05**
MEX3B	**mex-3 homolog B (C. elegans)**	**2.05**
LOC730417	**Uncharacterized protein**	**2.05**
LARGE	**like-glycosyltransferase**	**2.04**
FAM101B	**family with sequence similarity 101, member B**	**2.04**
BOP1	**block of proliferation 1**	**2.03**
COL22A1	**collagen, type XXII, alpha 1**	**2.03**
CAP1	**CAP, adenylate cyclase-associated protein 1 (yeast)**	**2.03**
MFAP5	**microfibrillar associated protein 5**	**2.02**
RBM24	**RNA binding motif protein 24**	**2.02**
SLC39A14	**solute carrier family 39 (zinc transporter), member 14**	**2.01**

ECGF1	**thymidine phosphorylase**	**2.01**
ACLY	**ATP citrate lyase**	**2.01**
UCN2	**urocortin 2**	**2.01**
SRPRB	**signal recognition particle receptor, B subunit**	**2.01**
MTE	**metallothionein 1I, pseudogene**	**2.00**

Appendix ID. Gene down-regulated more than 2 fold in pterygial fibroblasts by TGFβ2

Symbol	Gene	Fold change
ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.09
KIAA1199	Protein KIAA1199	0.11
RCAN2	regulator of calcineurin 2	0.13
COLEC12	collectin sub-family member 12	0.18
AKR1C3	aldo-keto reductase family 1, member C3	0.18
RAB7B	RAB7B, member RAS oncogene family	0.19
CCL2	chemokine (C-C motif) ligand 2	0.19
GAS1	growth arrest-specific 1	0.20
EPHB6	EPH receptor B6	0.21
ADH1B	alcohol dehydrogenase 1B (class I), beta polypeptide	0.21
ANGPTL2	angiopoietin-like 2	0.22
SLC2A12	solute carrier family 2, member 12	0.22
SDPR	serum deprivation response	0.22
ADM	adrenomedullin	0.22
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	0.23
OSR2	odd-skipped related 2 (Drosophila)	0.25
SVIL	supervillin	0.25
TGFBR3	transforming growth factor, beta receptor III	0.25

RARRES3	**retinoic acid receptor responder (tazarotene induced) 3**	**0.26**
TNFRSF19	**tumor necrosis factor receptor superfamily, member 19**	**0.26**
CLDN11	**claudin 11**	**0.27**
PAMR1	**peptidase domain containing associated with muscle regeneration 1**	**0.27**
ALDH1A3	**aldehyde dehydrogenase 1 family, member A3**	**0.27**
TKT	**transketolase**	**0.27**
NTN4	**netrin 4**	**0.28**
CDC42EP4	**CDC42 effector protein (Rho GTPase binding) 4**	**0.28**
DHOUR3	**dehydrogenase/reductase (SDR family) member 3**	**0.28**
TMEM140	**transmembrane protein 140**	**0.28**
SMAD3	**SMAD family member 3**	**0.29**
AKR1C4	**aldo-keto reductase family 1, member C4**	**0.29**
PDE7B	**phosphodiesterase 7B**	**0.30**
PTGIS	**prostaglandin I2 (prostacyclin) synthase**	**0.30**
SOX15	**SRY (sex determining region Y)-box 15**	**0.31**
RABGAP1	**RAB GTPase activating protein 1**	**0.31**
CAMK2N1	**calcium/calmodulin-dependent protein kinase II inhibitor 1**	**0.31**
GBP2	**guanylate binding protein 2, interferon-inducible**	**0.32**
TMEM119	**transmembrane protein 119**	**0.33**

LDB2	LIM domain binding 2	0.33
APOD	apolipoprotein D	0.33
IFITM1	Interferon-induced transmembrane protein 1	0.34
ALDH3A2	aldehyde dehydrogenase 3 family, member A2	0.34
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.34
TNFRSF14	tumor necrosis factor receptor superfamily, member 14	0.35
DBC1	deleted in bladder cancer 1	0.35
CXCL12	chemokine (C-X-C motif) ligand 12	0.35
C4ORF49 OSAP	mitochondria-localized glutamic acid-rich protein	0.35
CPA4	carboxypeptidase A4	0.35
TCEA3	Transcription elongation factor A protein 3	0.36
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.36
UBA7	ubiquitin-like modifier activating enzyme 7	0.36
CTSK	cathepsin K	0.36
EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	0.36
SLC15A3	solute carrier family 15, member 3	0.36
PTGES	prostaglandin E synthase	0.37
C3ORF72	chromosome 3 open reading frame 72	0.37
ALDH3A1	aldehyde dehydrogenase 3 family, member A1	0.37

PPL	**periplakin**	**0.37**
ADRA1B	**adrenoceptor alpha 1B**	**0.37**
BIN1	**bridging integrator 1**	**0.37**
CYBRD1	**cytochrome b reductase 1**	**0.37**
IFIT2	**interferon-induced protein with tetratricopeptide repeats 2**	**0.37**
SEMA6A	**sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A**	**0.37**
TFPI	**tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)**	**0.38**
RNASET2	**ribonuclease T2**	**0.38**
UBE2L6	**ubiquitin-conjugating enzyme E2L 6**	**0.38**
CD248	**CD248 molecule, endosialin**	**0.38**
PYCARD	**PYD and CARD domain containing**	**0.38**
VIT	**vitrin**	**0.38**
TSHZ2	**teashirt zinc finger homeobox 2**	**0.39**
FLT3LG	**fms-related tyrosine kinase 3 ligand**	**0.39**
S1PR3	**sphingosine-1-phosphate receptor 3**	**0.39**
MRGPRF	**MAS-related GPR, member F**	**0.40**
CRABP2	**cellular retinoic acid binding protein 2**	**0.40**
CMBL	**carboxymethylenebutenolidase homolog (Pseudomonas)**	**0.40**

GYPC	glycophorin C (Gerbich blood group	0.40
PCDH18	protocadherin 18	0.40
HCFC1R1	host cell factor C1 regulator 1 (XPO1 dependent)	0.40
SLC9A9	solute carrier family 9, subfamily A (NHE9, cation proton antiporter 9), member 9	0.40
C13ORF15	regulator of cell cycle	0.40
PLSCR4	phospholipid scramblase 4	0.40
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	0.41
GSTM5	glutathione S-transferase mu 5	0.41
SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	0.42
AKAP12	A kinase (PRKA) anchor protein 12	0.42
MXD4	Max dimerization protein 4	0.42
EDNRA	endothelin receptor type A	0.42
RDH10	retinol dehydrogenase 10 (all-trans)	0.42
NQO1	NAD(P)H dehydrogenase, quinone 1	0.42
CROT	carnitine O-octanoyltransferase	0.42
DYNC2H1	dynein, cytoplasmic 2, heavy chain 1	0.42
CLIC6	chloride intracellular channel 6	0.42
PLEKHG4	pleckstrin homology domain containing, family G	0.43

	(with RhoGef domain) member 4	
SCARA5	scavenger receptor class A, member 5 (putative)	0.43
IGFBP4	insulin-like growth factor binding protein 4	0.43
PPAP2B	phosphatidic acid phosphatase type 2B	0.43
NOV	nephroblastoma overexpressed	0.43
JAG1	jagged 1	0.43
IRF1	interferon regulatory factor 1	0.43
HS.20255	unkown	0.43
LAMA4	laminin, alpha 4	0.43
CAPN5	calpain 5	0.43
TBC1D2B	TBC1 domain family, member 2B	0.44
GPNMB	glycoprotein (transmembrane) nmb	0.44
C10ORF33	pyridine nucleotide-disulphide oxidoreductase domain 2	0.44
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	0.44
PCYOX1	prenylcysteine oxidase 1	0.44
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	0.44
NUDT7	nudix (nucleoside diphosphate linked moiety X)-type motif 7	0.44
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	0.44

ZNF581	zinc finger protein 581c	0.45
ANKRD35	ankyrin repeat domain 35	0.45
C10ORF10	chromosome 10 open reading frame 10	0.45
ZNF395	zinc finger protein 395	0.45
PGBD3	piggyBac transposable element derived 3	0.45
PIR	pirin (iron-binding nuclear protein)	0.45
LPIN1	lipin 1	0.45
TMEM130	transmembrane protein 130	0.45
GSTM3	glutathione S-transferase mu 3 (brain)	0.45
SELENBP1	selenium binding protein 1	0.45
QPRT	quinolinate phosphoribosyltransferase	0.45
CLDN23	claudin 23	0.45
MYLIP	myosin regulatory light chain interacting protein	0.45
CASP1	caspase 1, apoptosis-related cysteine peptidase	0.45
CYB5A	cytochrome b5 type A (microsomal)	0.46
CARD10	caspase recruitment domain family, member 10	0.46
PLEKHA4	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	0.46
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	0.46
B3GALT4	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase,	0.46

	polypeptide 4	
ARL6IP5	ADP-ribosylation-like factor 6 interacting protein 5	0.46
ERRFI1	ERBB receptor feedback inhibitor 1	0.46
DRAM1	DNA-damage regulated autophagy modulator 1	0.46
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	0.46
GMDS	GDP-mannose 4,6-dehydratase	0.46
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	0.46
EXPH5	exophilin 5	0.46
GSTM2	glutathione S-transferase mu 2 (muscle)	0.46
MAOA	monoamine oxidase A	0.46
PARP14	poly (ADP-ribose) polymerase family, member 14	0.47
FNBP1	formin binding protein 1	0.47
MN1	meningioma (disrupted in balanced translocation) 1	0.47
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9	0.47
SNORD13	small nucleolar RNA, C/D box 13	0.47
PARP9	poly (ADP-ribose) polymerase family, member 9	0.47
C5ORF41	CREB3 regulatory factor	0.47
OKL38	oxidative stress induced growth inhibitor 1	0.47

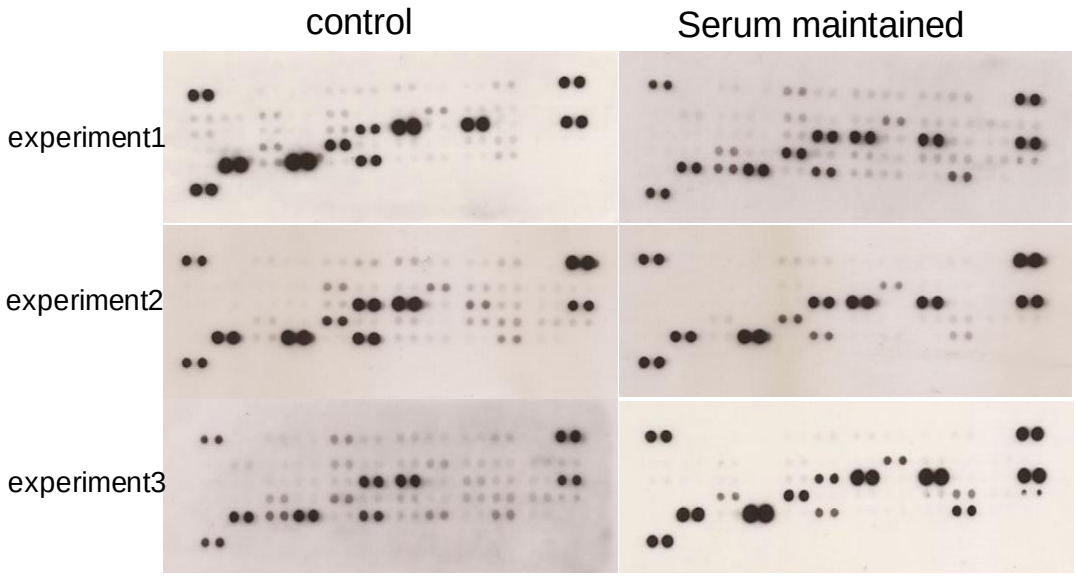
RND3	Rho family GTPase 3	0.47
AKR1C2	aldo-keto reductase family 1, member C2	0.47
C13ORF33	mesenteric estrogen-dependent adipogenesis	0.47
PNMA2	paraneoplastic Ma antigen 2	0.47
HS.552087	slit homolog 3 (Drosophila)	0.47
ANKDD1A	ankyrin repeat and death domain containing 1A	0.47
KRTAP1-5	keratin associated protein 1-5	0.48
ADRA2A	adrenoceptor alpha 2A	0.48
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	0.48
PLEKHA6	pleckstrin homology domain containing, family A member 6	0.48
ZNF423	zinc finger protein 423	0.48
AMOT	angiomotin	0.48
ATP8B4	ATPase, class I, type 8B, member 4	0.48
PSMB10	proteasome (prosome, macropain) subunit, beta type, 10	0.48
GSTM1	glutathione S-transferase mu 1	0.48
GPR177	wntless homolog (Drosophila)	0.48
SULF1	sulfatase 1	0.48
PDE5A	phosphodiesterase 5A, cGMP-specific	0.48

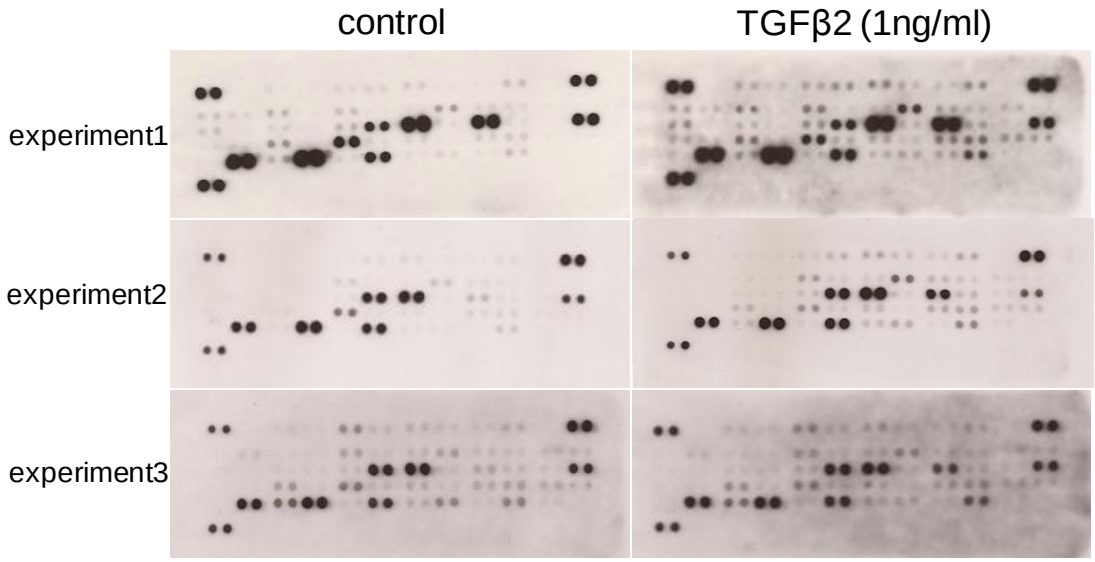
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	0.48
TRIM22	tripartite motif containing 22	0.48
CTSF	cathepsin F	0.48
C5	complement component 5	0.48
CA12	carbonic anhydrase XII	0.48
PTGR1	prostaglandin reductase 1	0.48
CNKSR3	CNKSR family member 3	0.48
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	0.49
TXNRD1	thioredoxin reductase 1	0.49
SLC27A3	solute carrier family 27 (fatty acid transporter), member 3	0.49
CCDC102A	coiled-coil domain containing 102A	0.49
ACOX2	acyl-CoA oxidase 2, branched chain	0.49
RAP1GAP	RAP1 GTPase activating protein	0.49
CPXM2	carboxypeptidase X (M14 family), member 2	0.49
CAT	catalase	0.49
OLFML2B	olfactomedin-like 2B	0.49
REV3L	REV3-like, polymerase (DNA directed), zeta, catalytic subunit	0.49
LOC100133999	unkown	0.49
RHOBTB3	Rho-related BTB domain containing 3	0.49
OLFML1	olfactomedin-like 1	0.49

SPRY1	**sprouty homolog 1, antagonist of FGF signalling (Drosophila)**	**0.50**
OLFML3	**olfactomedin-like 3**	**0.50**
PDGFD	**platelet derived growth factor D**	**0.50**
CDKN1C	**cyclin-dependent kinase inhibitor 1C (p57, Kip2)**	**0.50**
PSMB8	**proteasome (prosome, macropain) subunit, beta type, 8**	**0.50**
CFD	**complement factor D (adipsin)**	**0.50**
STAT1	**signal transducer and activator of transcription 1, 91kDa**	**0.50**

APPENDIX II

Proteome profiler: human angiogenesis array





Array images of detectable protein spots in the X-RAY film. Proteome profile of angiogenic factors released from pterygial-derived fibroblasts maintained in the presence or absence of 10% serum or 1ng/ml TGFβ2 for a 24 hour period.