The influence of low levels of fluid flow on fibroblast behaviour

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List of Publications

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Abstract

Fibroblasts are a type of cell which forms the structural framework of the connective tissue and are key players of wound closure, fibrosis and cancer. These cells experience mechanical perturbations due to matrix remodelling and interstitial fluid movement. Chemical signals from the tissue microenvironment such as Transforming growth factor β1 (TGF-β1) promote differentiation of fibroblasts to a myofibroblastic phenotype marked by enhanced expression of α-smooth muscle actin (α-SMA) rich stress fibres. However most of the evidence for this has been derived in static cultures which do not fully recapitulate the mechanically dynamic environment *in vivo*.

The response of primary human fibroblasts to physiological levels of fluid movement and chemical perturbations from TGF-β1 is examined in this study. Findings show that fluid flow induced widespread changes in gene expression compared to static cultures and up-regulated genes such as α-SMA and Collagen 1A1. Surprisingly the combination of flow and exogenous TGF-β1 resulted in reduced myofibroblast differentiation.

Myofibroblastic differentiation under flow was partially inhibited by follistatin-288 and blocked by TGF-β1 supplementation. This was associated with caveolin-mediated internalisation of TGF-β receptor type II. These findings suggest that fluid flow modulates fibroblast response to pro-differentiation signals such as TGF-β1. We propose that this may be a novel mechanism by which mechanical forces buffer responses to chemical signals *in vivo*, maintaining a context-specific fibroblast phenotype.

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Abbreviations

α-SMA	α-smooth muscle actin.
AMHR-II	Anti-Müllerian hormone type II receptor.
ANGPTL-4	Angiopoietin-like 4
ALK	activin linked kinases.
aRNA	amplified RNA.
BMP	Bone morphogenetic protein.
BMPR-II	Bone morphogenetic protein receptor type II.
CAF	Cancer associated fibroblasts.
CAV1, CAV2, CAV3	caveolin-1, caveolin-2, caveolin-3.
CCL	CC-chemokine ligand.
CDK	Cyclin dependant kinases.
CNS	central nervous system
COL1A1	Collagen I A1
COX-2	Cyclooxygenase-2.
CSF	cerebro spinal fluid
CTGF	Connective tissue growth factor.
E	Tissue stiffness.
ECM	Extra cellular matrix.
ECV	Endosomal carrier vesicles.
EE	Early endosomes.
EEA-1	Early endosomal antigen-1.
EGFR	Epidermal growth factor receptor.
EMT	Epithelial Mesenchymal Transition.
EndoMT	Endothelial Mesenchymal Transition.

FST	Follistatin.
FSTL-3	Follistatin-like 3.
GDF	Growth and differentiation factor.
GO	Gene ontology.
GOTERM	Gene ontology Term enrichment.
HGF	Hepatocyte growth factor.
ICAM-1	Intercellular adhesion molecule 1.
ID	Inhibitor of DNA binding.
IF	interstitial fluid
IFNα	Interferon-α.
IGF-1	Insulin like growth factor -1.
IL4	Interleukin 4.
INHBA	Activin A
KEGG	Kyoto Encyclopaedia of Genes and Genomes.
KGF	Keratinocyte growth factor.
kPa	Kilo Pascal.
LE	Late endosomes.
LTBP	Latent precursor bound protein.
MAPK	Mitogen-activated protein kinases.
MCmB	Multi-compartmental modular bioreactor.
MSC	Mesenchymal stem cell.
MMP3	Matrix metalloproteases.
MVB	Multivesicular bodies.
NFκB	Nuclear factor kappa-B.

NHDFC	Normal human dermal fibroblast cells.
NOF	Normal oral fibroblasts.
NSCLC	Non-squamous cell lung cancer.
PCA	Principal Component Analysis.
PDMS	Poly-dimethyl-siloxane.
PI3K	Phosphoinositide-3- kinase.
PKD1	Polycystin1
PKD2	Polycystin2
PKC delta	Protein Kinase C Delta.
RT-PCR	Reverse transcriptase PCR.
SA-β Gal	Senescence associated-β galactosidase.
SASP	Senescence associated secretory phenotype.
SMC	Smooth muscle cells.
TGF-β1	Transforming growth factor type 1.
TGFβRII	TGF β receptor type II.
TGN	Trans golgi network.
TN-C	Tenascin –C.
TNFα	Tumour necrosis factor α
TRPC1	Transient receptor potential cation channel sub family C
uPA	Urokinase-type plasminogen activator.
VEGFRII	Vascular endothelial growth factor receptor type II.
YAP	Yes associated protein.

Chapter 1- Introduction

1.0 Introduction

Many studies have shown that the architecture of all living cells rely on the tensional integrity in the internal and external environment of cells (Ingber, 1993, Ingber et al., 1981). Cells perform myriad tasks of building tissues and organs in an incredibly complex environment that experiences physical forces, biochemical signals and communications from other cells or regulators due to basic movement like respiration, walking, talking etc. (Mammoto and Ingber, 2010, O'Connor and Gomez, 2014). These processes are critical for normal physiological activities and mediate functions of all tissues including those of the oral cavity. Cells adapt to the rapidly changing environment by mechanisms that modulate their biochemistry and signalling processes (Berg JM, 2002). It is this ability of the cells to respond to perceived signals that forms the basis of all biological processes such as development, wound repair, inflammation and cellular homeostasis.

Chemical signals from the environment are initially transmitted into cells via processes like diffusion and active transport. Subsequently, chemical signals act as ligands and bind to receptors, both surface associated and intra cellular and induce a conformational change in the protein structure of receptors, thus invoking a cascade of signalling events inside a cell (GM., 2000). However, *in vivo* both mechanical forces and chemical signals contribute synchronously all aspects of development (Mammoto and Ingber, 2010), biological processes (Linville et al., 2016) and in disease progression (Janmey and Miller, 2011). Signals can be classified into two types: Mechanical forces and chemical signals.

Mechanical forces comprise of signals that are exerted on (endogenous) and generated by (endogenous) a cell which results in cytoskeletal contractility and activation of various signalling pathways. Examples of exogenous forces include gravity and voluntary actions

whereas endogenous forces include fluid-tissue interactions such as blood circulation induced by pumping of the heart (Barbee et al., 1994). This form of signalling is also known as mechanotransduction. In this type of signalling, cells respond to extrinsically applied forces and to those generated by cell-cell contacts. These signals not only determine its mechanical properties such as matrix stiffness and contractility but also the phenotype of a cell. Numerous studies have shown that cellular mechanics are determinants of the cell cycle (Klein et al., 2009, Winer et al., 2009), transcription (Li et al., 2007, Engler et al., 2007), and disease progression (Georges et al., 2007). When force is exerted on the cell membrane some of the basic cellular processes such as cell migration (Lo et al., 2000, Masuda and Fujiwara, 1993), cell growth (Georges et al., 2006), differentiation (Engler et al., 2006, Engler et al., 2008) and production of proteases (Wall et al., 2009, Bhadal et al., 2008) are altered. These cellular functions are sustained by three processes: detection of signals from the environment, transmission of signals to changes in gene expression and transformation of these changes in gene and or protein expression to mechanical signals for other cells (Miller and Davidson, 2013).

Apart from these forces, the stiffness of extracellular matrix also functions as a crucial mediator of cellular function. Tissue stiffness (E) is defined as a measure of resistance that a material shows to stress and deformation (Handorf et al., 2015). It differs by several orders of magnitude ranging from kilo pascals (kPa) in adipose tissue (Samani and Plewes, 2004) to giga pascals (GPa) in bone (Rho et al., 1993) and is constantly subjected to variability during developmental stages (Handorf et al., 2015). Numerous studies on cellular stiffness revealed that cells can generate their own forces which are then exerted on substrate and surrounding tissues. With increasing stiffness, cells move slowly (Pelham and Wang, 1997), alter the cell-cycle (Klein et al., 2009) and limit cellular homeostasis giving rise to an abnormal tissue

(Discher et al., 2005). Further, single molecule experiments have shown that cytoskeletal proteins like activin (Rief et al., 1999) and filamin (Furuike et al., 2001) can be structurally modified when low-magnitude forces in the order of 50-200 pN act on them. These studies indicate that protein folding and structure associated changes can either relax or stress the cytoskeleton of a cell thus invoking sensor mechanisms that transduce molecular stress.

Chemical signals are generated by chemical molecules that act as ligands and either bind to surface receptors or diffuse through cell membranes to bind to intracellular receptors to cause a conformational change and cascade of signalling events. These messengers either diffuse from their production site to the target cell or are carried to the target locations by interstitial fluid flow and blood circulation (Mammoto and Ingber, 2010). Effects of chemical signalling depend upon factors such as exposure time and concentration of these messengers. For example, when glomerular mesangial cells were treated with TGF-β1, Smad2 mRNA expression was reduced (Poncelet et al., 2007). Further, prolonged exposure of mesangial cells with TGF-β1 for longer period of 5-7 days induced expression of α-smooth muscle actin (α-SMA) and induced epithelial to mesenchymal transition like phenotype in these cells. Numerous studies have reported that concentration of chemicals determine the response of cells. For example, TGF-β1 exerts a concentration dependant effect on immunoglobulin production (McKarns et al., 2003). Reports have also suggested that levels of TGF β receptor type II regulate the biological effect of TGF β signalling (Rojas et al., 2009). These findings establish that it is not just the combined effect of mechanical forces and chemical signals that drive cellular signalling, but magnitude of the forces and signals also play a vital role in all biological processes. Chemical signals however activate different modes of cell- cell signalling which can be broadly classified to four types: autocrine signalling, paracrine signalling, endocrine and direct signalling via gap junctions.

1.1 Types of cell signalling

Cells communicate by various methods that permit chemical signals to reach target locations and invoke a response. Extracellular molecules released by signalling cells recognise and bind to surface receptors of target cells and elicit a cellular response by means of a signalling mechanism. The four types of cell signalling differ from each other in the distance that the signals travel to reach target cell (Figure 1.1).

In **autocrine signalling**, ligands released by cells bind to its own surface receptor suggesting that the signalling cell and the target cell are the same. For example, numerous studies have shown that all cancer cells produce autocrine growth factors for self-sustenance (Hanahan and Weinberg, 2000). One such observation in non-squamous cell lung cancer (NSCLC) revealed the frequent expression of epidermal growth factor receptor (EGFR) (Hirsch et al., 2003) as well as EGF family ligands (Rusch et al., 1997).

Signals acting in localised regions of a tissue between different cell types that lie close to each other is known as **paracrine signalling**. These signals move by diffusion to their target cell via the extracellular matrix and last for a short period of time. The released ligand molecules are then quickly degraded by enzymes. One such example of paracrine signalling is the transmission of signals across synapses between nerve cells (GM, 2000).

In the epidermis, Wnt signalling molecules convey signals to the underlying fibroblasts. These signals act locally via extracellular matrix deposition and by secreting other factors that influence fibroblast behaviour in other regions of the skin (Driskell and Watt, 2015). This suggests that fibroblasts in the skin experience both autocrine and paracrine signalling.

Endocrine signals are signals from hormones that originate from endocrine glands such as thyroid and pituitary glands and act on distant target cells. Response from these type of cells is slow but lasts longer. Ligands released are present in low concentrations when they reach their target cells. For example in neuroendocrine signalling, neurotransmitters released from nerve cells invoke release of hormones into the blood stream (Malenka RC, 2009).

Direct signalling happens via gap junctions which appear as connections between plasma membranes of cells. These gap junction channels allow diffusion of small molecule signalling mediators such as calcium ions (Ca^{2+}) from one cell to another. In the heart, the signal to contract muscle cells is passed through gap junctions (Lorber and Rayns, 1977).

These signalling networks work in association with mechanical forces like blood flow, interstitial fluid flow and chemical signals to contribute to the development of an organism.

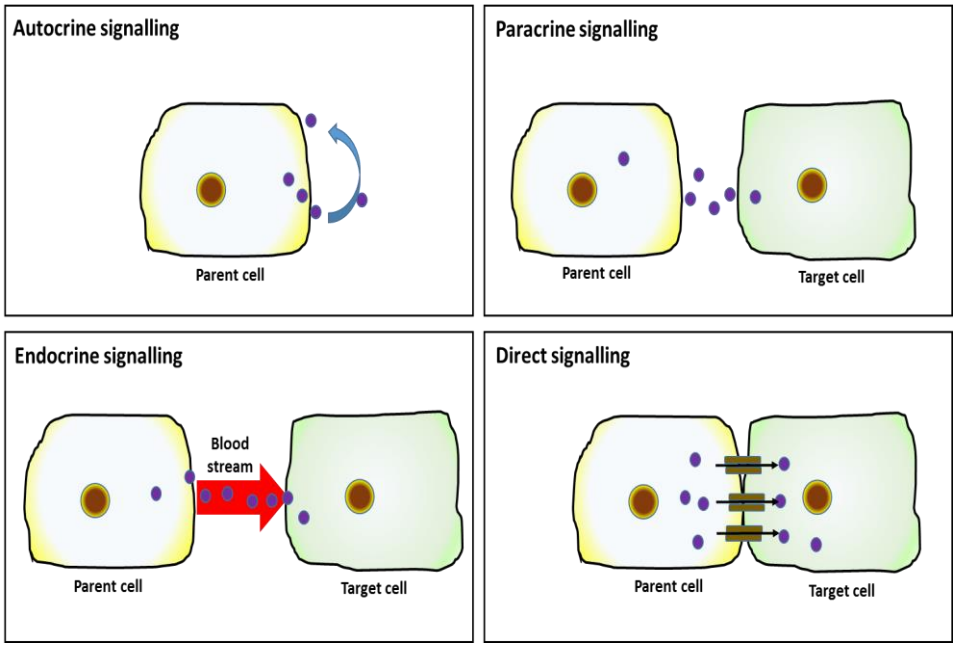

Figure 1.1: Types of chemical signalling i) Autocrine signalling ii) Paracrine signalling iii) Endocrine and iv) Direct signalling by gap junction proteins.

1.2 Mechanics of fluid flow in development and repair.

The most basic mode of transportation of biochemical products in living organisms is via fluids. More complex animals contain multiple networks of tubules that are involved in movement of products to facilitate biochemical reactions and sustenance of life. The most primitive complex forms of life, single celled organisms use fluids for intracellular transport and nutrition (Cartwright et al., 2009). As the complexity of organisms increase, so the complexities of fluid transport system increase. Development of the embryo commences with formation of a mass of cells from a fertilised egg. Subsequently, cavities and tubes are formed in order to carry signalling molecules (Mammoto and Ingber, 2010) and nutrients to enable formation of embryonic axis and growth (Freund et al., 2012). Fluid flow is required at all stages of vertebrate development including morphogenesis of kidney (Serluca et al., 2002), formation of inner ear and otolith (Colantonio et al., 2009), migration of neurons (Sawamoto et al., 2006), formation of the heart (Slough et al., 2008), hematopoiesis (Pardanaud and Eichmann, 2009) and in determining symmetry and positioning of organs (Nonaka et al., 1998) in a developing fetus. This compelling evidence confirms that in a biological system, fluid flow and growth are intertwined right from the beginning of life.

Developmental controls were first explained in mechanical terms by D'Arcy Thompson who defined cellular patterning and signalling as "diagram of underlying forces". He mentioned that changes in three dimensional structure of cells occurs as a result of force acting on a mass (Thompson, 1917). Embryonic development is the vital process of life which directs all processes that sculpt organs and tissues. At this stage the embryo experiences isometric tension due to cytoskeletal contraction (Ingber, 2006), stiffness due to tissue elasticity, pressure caused by fluid flow induced stress acting perpendicular to embryo surface and

shear caused by fluid flow induced friction that acts parallel to the surface (Gjorevski and Nelson, 2010).

Isometric cellular contraction and fluid flow mediated shear stresses regulate cellular structure, behaviour and fate. Extracellular matrix derived mechanical tension passes through membrane bound receptors and is transmitted to the cytoskeleton (Assoian and Klein, 2008) to control transit through G1 phase of the cell cycle (Huang and Ingber, 2002). At this stage signalling is also regulated by the small GTPase Rho and its effectors Rho Kinase (ROCK). Subsequent increase in Rho A activity leads to degradation of cyclin dependant kinase inhibitor $p27^{kip1}$ permitting progression of the G1 cell cycle phase (Mammoto et al., 2004).

Mechanical stresses obtained from tension and fluid flow induced shear also regulate programmed cell death. In these studies the cells rounded up due to decreased cytoskeletal tension arising from ECM scaffold depletion (Chen et al., 1997). Direct evidence of apoptotic death was observed during physiological involution of the mammary gland (Wicha et al., 1980).

Apart from cellular proliferation and programmed cell death, embryonic development largely relies on changes in cellular phenotype wherein progenitor stem cells differentiate into various lineages. Phenotype associated changes are regulated by changes in gene expression like downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as N-cadherin and α- smooth muscle actin (α-SMA) (Kalluri and Neilson, 2003). Recent studies have shown that the differentiation process is tightly regulated by matrix stiffness (Lv et al., 2015) which in turn is modulated by mechanical forces like fluid flow (Kohn J et al., 2015). These findings were further strengthened when researchers

observed *in vitro* that the rhythmic contractility of embryonic cardiomyocytes is similar to *in vivo* only when the extra cellular matrix (ECM) shows similar mechanical properties to that of a heart (Engler et al., 2008).

Transition of an epithelial phenotype to a mesenchymal phenotype, a property termed as epithelial mesenchymal transition (EMT) is marked by the detachment of epithelial cells from each other increased migratory behaviour. EMT in developing embryos is essential for gastrulation and neural crest formation (Thiery et al., 2009). Recent studies have shown that EMT could be induced by transforming growth factor- β1 (TGF-β1) interstitial fluid flow in cancer cells (Shieh and Swartz, 2011, Rizvi et al., 2013) and proteases like matrix metalloproteases (MMP3) (Sternlicht et al., 1999). When mouse mammary epithelial cells were treated with MMP3, EMT was observed to occur with changes in cellular morphology and increased cell spreading (Radisky et al., 2005). These observations suggest that mechanical forces like fluid flow induced changes in cytoskeletal activity resulting in EMT.

Although all cells in the body experience various types of mechanical force induced fluid movement from a nanoscale level (active transport, osmosis and diffusion), microscale level (interstitial fluid flow, intercellular and intracellular) to a mega scale (blood flow), in this review we shall discuss only the role two main types of fluid flow in development and repair: blood flow and interstitial fluid flow.

1.2.1 Blood flow

Blood is a complex fluid which undergoes continuous circulation by directional flow in the cardiovascular system to ensure transportation of nutrients, hormones, metabolic wastes,

drugs, signalling molecules and oxygen to tissues and organs in the body. Blood circulation is one of the most crucial processes for sustenance of all vital processes.

Morphogenesis of heart at the embryo stage is contributed by the combined effect of contraction of heart as well as blood flow. Notably, forces of flow change at every stage of heart development. At the primitive stage of embryonic development, the heart appears as a tube made up of three layers of cells. Later, the heart undergoes a conformational change known as looping to form the chambers. In humans, contraction of heart commences as a peristaltic wave from day 22 post fertilization. At this stage flow is unidirectional. However, as the ventricles of the heart develop ventricular contraction, a significant fraction of blood volume is pushed back into the venous sinus resulting in reverse flow of blood. This flow of blood in both directions confers hemodynamic shear stress on the endocardial cells (Boselli et al., 2015).

Researchers led by Lonard Zon observed that fluid flow induced stress originating from a beating heart triggers haematopoiesis (North et al., 2009). The group found that nitric oxide is one of the chemicals produced under mechanical stress and promotes stem cell production. They used a Zebrafish model to create an embryo with a mutation termed as ‘silent heart’. In this embryo, the heart beat and circulation was absent. These embryos were found to be deficient in hematopoietic stem cells. Nitric oxide supplementation to the mutant embryos restored blood cell production. Conversely, depletion of nitric oxide from these embryos resulted in deficiency of hematopoietic stem cells. The group observed similar results when the same experiments were repeated in mouse models. These findings suggest that blood flow is vital for blood cell production.

Another group validated these findings by studying the effect of fluid flow on mouse embryonic stem cells in a miniaturized flow chamber that mimicked fluid shear stresses in a developing cardiovascular system (Adamo et al., 2009). The group compared blood vessel formation in cells subjected to fluid flow and those that were grown in static conditions. They observed cells that experienced fluid flow mediated shear stress showed elevated levels of CD31 and transcription factor Runx1, the chief regulator of haematopoiesis suggesting that blood flow is essential for angiogenesis.

Besides haematopoiesis and angiogenesis, shear stresses generated due to movement of blood forms the basis for mechanotransduction and is vital for repair processes like wound healing. Cells detect blood flow induced signals via sensing mechanisms as described in section 1.4. As a normal biological process, healing of wounds commences from rapid rushing of blood to the injured site followed by four distinctive processes: haemostasis (blood clotting), inflammation (clearing damaged and dead cells), proliferation (angiogenesis, collagen deposition, formation of granulation tissue, epithelialization and wound contracture) and tissue remodelling (collagen realignment and apoptosis of excess cells) (Gosain and DiPietro, 2004, Mathieu D, 2006). Although each of these phases are clearly distinctive in functions, movement of blood is vital at every stage for wound healing to proceed uninterrupted. For example, the inflammatory response is initiated by changes in blood flow, increase in permeability of blood vessels, migration of T-lymphocytes, white blood cells and proteins (Martin, 1997). Even processes like vascular remodelling largely rely on the flow of blood for efficient completion (Tomlinson and Silva, 2013). Blood is also the carrier of oxygen, an essential nutrient to all cells thus aiding accelerated wound healing (Tandara and Mustoe, 2004). Studies have shown that impairment of blood flow due factors like smoking can delay wound healing (Mosely and Finsett, 1977).

Ageing and disease are the outcome of impaired biological function. All tissues consist of cells that remain in an arrested state which is reversible and may start to proliferate when stimulated, a property often described as quiescence. This short term resting phase of cells often overlaps with senescence where aging cells attain an irreversible growth arrested state (Fridlyanskaya et al., 2015, Dimri et al., 1995). Senescence is characterised by cellular arrest, usually at G1 and sometimes in G2 phases of the cell cycle and is broadly classified into replicative senescence occurring due to loss of telomeric DNA in aging cells (Hodes, 1999) and stress induced premature senescence (SIPS) resulting due to harmful environmental and chemical stresses which halt cell division (Cmielova et al., 2012). Reports show that senescence alters the flow of blood resulting in pathological manifestations (Embleton et al., 2002, Bridgeman and Brookes, 1996). Both senescent and quiescent fibroblasts show variations in gene expression of cell cycle modulators and cyclin dependent kinase inhibitors such as $p21^{Waf\text{-}1}$ and p16Ink4A (Jackson and Pereira-Smith, 2006). Therefore in the presence of proliferative signals such as tissue injury or increased cardiac output as is observed during cardiac disorders, cells exit from their growth arrested state and proliferate for the purpose of cellular replacement(Burton, 2009). For example, endothelial cells in regions of high blood flow proliferate faster to replace damaged cells (Alberts B, 2002a).

It is therefore clear that blood flow and circulation is indispensable for normal functioning of living cells.

1.2.2 Interstitial fluid flow

Apart from blood and lymphatic fluid there are other fluid flows the tissue is exposed to *in vivo*. Fluid that comprises plasma and transcellular fluid and surrounding the cells in a tissue

is known as interstitial fluid (Dorland's, 2012) and comprises approximately 20% of total body weight (Aukland and Reed, 1993). Interstitial fluid flow is associated with lymphatic flow *in vivo* and flows between interstitial cells such as fibroblasts, tumour cells, tissue immune cells and adipocytes. It flows at a much slower velocity that blood due to the resistance imposed by the extracellular matrix (Rutkowski and Swartz, 2007) (Figure 1.2). In normal situations interstitial flow functions as a means to deliver nutrients and signals to cells, remove metabolic waste, induce lymphatic regeneration *in vivo* (Boardman and Swartz, 2003, Rutkowski et al., 2006), regulate normal functioning of osteocytes and chondrocytes (Buschmann et al., 1995, Mow et al., 1984, Grodzinsky et al., 2000), and induce cytokine production by smooth muscle cells (Wang and Tarbell, 2000). Although the exact velocity ranges of this flow is not known, it has been observed that during pathological conditions like cancer and ruptured vasculature, interstitial fluid flow can activate fibroblasts (Ng and Swartz, 2003) (Dan et al., 2010) and smooth muscle cells (Wang and Tarbell, 2000, Shi and Tarbell, 2011) with shear stress values reach up to 0.1-1 dynes/cm2 (0.01-0.1 Pascals) (Shi and Tarbell, 2011).

Further, during conditions like inflammation and acute edema the velocity ranges are increased to 0.1-2 μms-1 (Guyton et al., 1966). Although the flow velocity is miniscule, interstitial fluid flow exerts effects on tissue morphogenesis, cellular migration, differentiation and matrix remodelling (Swartz and Fleury, 2007). Mechanisms by which interstitial fluid flow drives these processes may be dependent on shear stresses induced by physical forces and environmental damage.

Interstitial fluid flow is driven by pressure gradients resulting from hydrostatic pressure arising from differences between blood and the interstitial space (Swartz and Fleury, 2007)

and eventually controls the formation of lymphatic fluid. Pressure gradients between the interstitium and lymphatic capillaries enable flowing of interstitial fluid into the lymphatic system (Swartz and Fleury, 2007). Flow is further maintained by pumping forces in lymphatic capillaries.

Recent studies have shown that interstitial flow in the cartilage is driven by exogenously applied physical forces that act on this tissue (Kwan et al., 1984). Interstitial fluid flow induced in this manner helps to circulate nutrients and biomolecules between cells and transport cellular waste from chondrocytes that are distant from blood capillaries.

Morphogens could be defined as biochemical signals that regulate directional and positioning of organs in an embryo. In the early stage embryonic development, morphogen transport and gradient patterning is known to regulate branching of developing lungs (Warburton et al., 2005). Embryonic lung movement induced interstitial fluid flow influences morphogen distribution which in turn contributes to overall development of the growing foetus (Nonaka et al., 1998). In a skin regeneration model, researchers have shown that lymphatic cells migrate in the direction of interstitial flow and organise themselves around fluid channels. In the absence of flow they are unable to organise themselves into functional capillaries (Boardman and Swartz, 2003, Rutkowski et al., 2006). These studies seem to suggest that interstitial flow itself can act as a morphogenic cue. Wound healing is process that requires migration of epithelial and endothelial (Li et al., 2005) cells which can be activated by shear stress suggesting the interstitial fluid flow promotes blood and capillary formation (Ng et al., 2004).

These compelling evidences suggest that regardless of composition, fluid flow is vital for the normal functioning of life processes and interrupted flow could invoke detrimental responses *in vivo*.

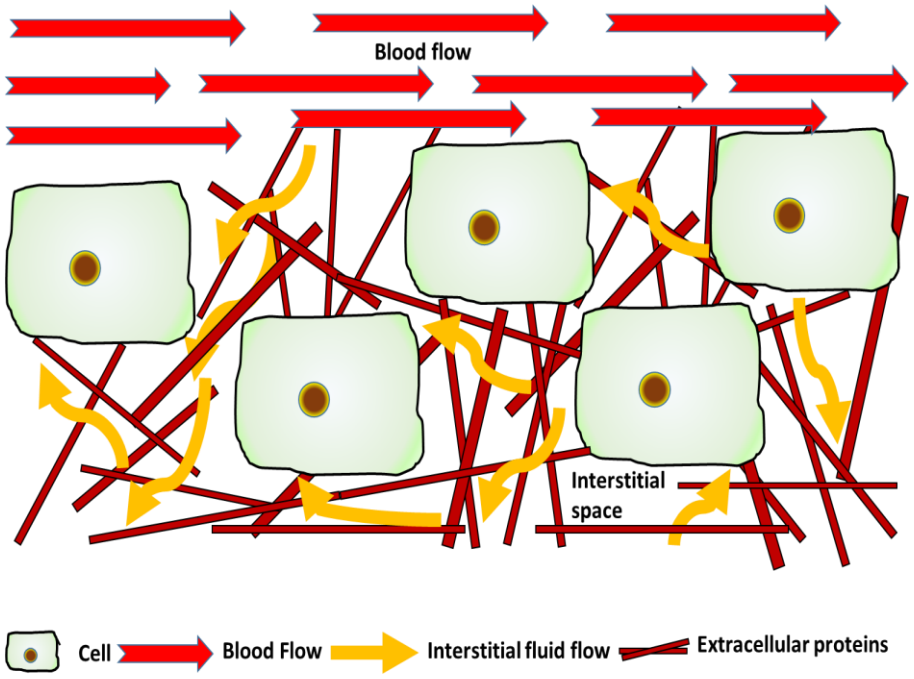

Figure 1.2: Types of fluid flow experienced by living cells.

1.3 Mechanotransduction in living cells

Mechanotransduction describes the cellular processes that translates mechanical stimuli into biochemical signals, thus enabling cells to adapt to their physical surroundings. Cells within tissues are constantly subjected to mechanical forces due to mobility and respiration. Even during normal activities like walking or running, the force acting on the heels can reach several Kilo Newtons, and induce a stress in the order of Mega Pascals. Stress is defined as force per area and it is often measured as Newtons per metre^{-2} or Pascals (Paszek et al., 2005).

Mechanical stresses influence cell shape and cytoskeletal structure (Wang et al., 1993, Wille et al., 2004). This process therefore controls many cellular processes involving tissue development, including migration, growth, and differentiation, apoptosis and stem cell lineage switching (Ingber and Folkman, 1989, McBeath et al., 2004). These stress signals are transmitted to cells embedded in the extracellular matrix (ECM) or are transferred to the contractile cytoskeleton of individual cells. However both types of forces converge on sensory molecules such as primary cilia (Nauli et al., 2008), glycocalyx (Weinbaum et al., 2007), plasma membrane mechanodetectors such as caveolae (Sinha et al., 2011), and transmembrane integrin receptors which connect the ECM to the cytoskeleton (Ingber, 1997, Geiger and Bershadsky, 2001).

1.3.1 Physical force and chemical signal induced mechanotransduction

Cells adapt to forces by increasing ECM rigidity. Therefore, mechanical stiffness of the cell increases linearly with increasing levels of stress (Wang et al., 1993, Galbraith et al., 2002). This enhances the ability of the cell to resist mechanical injury (e.g. prevent membrane tearing) (Wang et al., 2001). There are three types of stress stimuli: compression, shear stress and tension (Figure 1.3). Cells respond to these stimuli and undergo changes in shape, function and behaviour (Vogel and Sheetz, 2006).

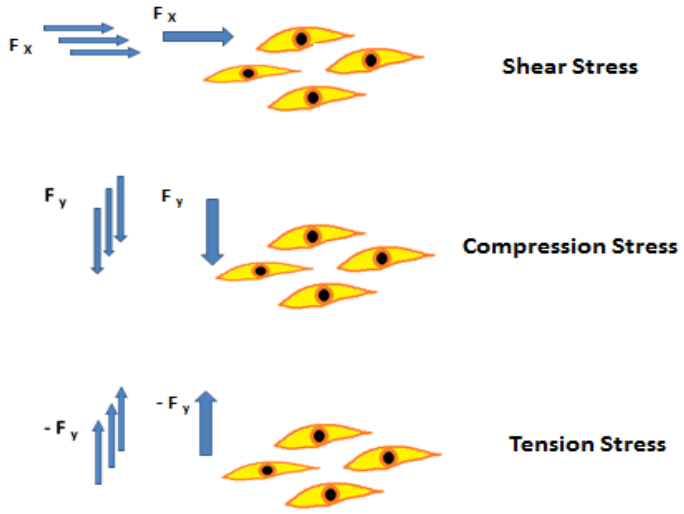

Figure 1.3: Overview of three different types of stress acting on cells plated on a surface i) Fx , stress acting parallel to the surface ii) Fy , stress acting in perpendicular direction towards the surface and iii) –Fy , stress acting perpendicular direction away from the surface.

Chemical responses to stress signals include activation of signalling cascades and / or release of Ca2+ ions from ion channels in the plasma membrane (Butcher et al., 2009). Proteins in the extracellular matrix stretch in response to stress. Furthermore, the nucleus itself acts as a mechanoreceptor and influences chromatin conformation (Butcher et al., 2009).

Studies have revealed the role played by mechanotransduction in a broad range of tissue functions and that this is not limited to a specific type of cell. The process maintains tissue homeostasis and is crucial for development and tissue specific differentiation. Hence it is imperative for all cells to respond to mechanical stress, no matter how primitive they are (Ingber, 2006). For example, stress fibres reorient during bone remodelling after fracture

(Burger and Klein-Nulend, 1999) and cells in the cardiovascular system experience pulsatile shear stress arising from cyclical pressure generated during heartbeat. It is this shear stress which activates the endothelial cells and induces the cytoskeletal network to mature, elongate and align in the direction of blood flow (Pritchard et al., 1995, Takahashi et al., 1997). Reports indicate that mechanical cues are important components of the stem cell niche (Jones and Wagers, 2008) and mechanical tension regulates epithelial morphogenesis in nematodes (Zhang et al., 2011b). The molecular mechanisms by which mechanical forces control stem cell function include chromatin remodelling, nuclear translocation of transcription factors, and modulation of signalling proteins (Christopher B. Wolf, 2009).

Whilst there is compelling evidence for the role of mechanical stress in determining whether the tissue performs its normal function or not, a question arises about how cells of connective tissue respond to mechanical stimuli? All cells respond to stress signals by three methods i) Signalling pathways ii) Co-operation between growth factor and integrin signalling and iii) mechanical control of cell differentiation and growth (Chiquet et al., 2009).

Some of the common events observed to be activated in adherent cell types at the cell-ECM interface when mechanically stressed are i) activation of calcium-dependent signalling due to influx of calcium through ion channels ii) Activation of nuclear factor kappa-B (NFκB) due to generation of Reactive oxygen species iii) Stimulation of small GTPases of the Ras family and of mitogen-activated protein kinases (MAPKs) iv) Altered activity of small GTPases of the Rho family resulting in changes in cytoskeletal dynamics (Chiquet et al., 2009).

It is intriguing to observe that transcription factors are the first to be activated upon mechanical stimulation. Smooth muscle cells subjected to cyclic strain revealed an increase

in promoter activity of profibrotic factor CCN2 due to increased actin polymerisation and NFκ B activation (Chaqour et al., 2006).

A number of reports have focussed on studying the co-ordinated effects of integrins and growth factor receptors in mediating intracellular signalling cascades (Zhang et al., 1995, Miyamoto et al., 1996, Mainiero et al., 1997, Schlaepfer et al., 1998). Both these components function synergistically due to their physical interactions with the ECM. Integrins are cell surface receptors that form structural and functional linkages between the ECM and the intracellular cytoskeletal linker proteins. Binding of integrins with ECM ligands not only leads to clustering and engagement with cytoskeletal proteins, but it also activates signalling cascades such as the MAPKinase pathway which is involved in cell growth and differentiation. Therefore, association of integrins with growth factor receptors enables cells to integrate mechanical and growth factor associated signals and influence subsequent cellular responses. These responses include anchorage dependant growth and Anoikis, a type of programmed cell death triggered when anchorage dependant cells remain suspended for prolonged periods.

Mechanical cues also mediate autocrine signalling in mesenchymal stem cells which are the precursors of fibroblasts. The first evidence of the effect of adhesion mediated autocrine signalling directing stem cell differentiation was provided by C.S.Chen's laboratory. The group demonstrated that mechanical cues created by a micropatterning technique which control cell shape and cell spreading with single-cell precision can act as a switch in directing the differentiation of stem cells into either an osteoblast or adipocyte lineage (McBeath et al., 2004). They plated mesenchymal stem cells on fibronectin squares of different areas (1000–10,000 μm2). Cells which attached on large squares spread extensively and started expressing

alkaline phosphatase, a marker of osteoblastic lineage. Conversely, the cells on smaller squares adopted a rounded shape and started accumulating lipid droplets, as seen in adipocyte differentiation. The authors concluded that mechanical cues experienced by a cell in an autocrine manner due to restrictions in surface area determine the fate of mesenchymal stem cells. In another study, experiments were conducted to study the effect of mechanical strain on protease production by keratinocytes. Lewis et.al (2008) studied the effect of mechanical strain on protease production in normal and transformed keratinocytes. Keratinocytes were seeded on membranes coated with type I collagen or type II collagen. They were then subjected to mechanical strain. Supernatants were then assayed for Urokinase-type plasminogen activator (uPA), a serine protease known to play a critical role in wound healing responses (Jensen and Lavker, 1999). Their results revealed that under static strain, production of uPA was down regulated in both normal human keratinocyte cultures as well as human papilloma virus transformed keratinocytes. Notably, uPA levels returned to resting levels when tension was restored in these cells. Interestingly, this feature was representative of wound healing response *in vivo* wherein wounding upregulates the levels of uPA (Huang et al., 2002). Notably, this study confirms the theory that mechanical forces are an important signal during wound healing. Another study reported that tensile stress in combination with TGF-β (Wipff et al., 2007) is required for the expression of myofibroblast specific markers such as α- Smooth muscle actin (Zhao et al., 2007).

Anchorage substrate dependant mechanical features also play a role in cellular processes like proliferation, both in single cells as well as in tissue aggregates. Previous studies on 3D collagen matrices reveal the dual nature of fibroblasts in switching between quiescent and proliferative phenotypes, depending upon whether matrices are attached or floating during matrix modelling. One of the research groups initially observed a decrease in ERK mediated

cell signalling in floating matrices. Fringer and Grinnell (2003) reported that serum stimulation of fibroblasts in floating matrices does not result in ERK translocation to the nucleus. Another study on fibroblasts in 3D matrices showed that under tension or relaxed conditions these cells respond differently to growth factor stimulation. Furthermore, switching the mechanical environment between loaded and unloaded conditions influences whether cells acquire proliferative or resting phenotypes.

1.3.2 Fluid flow induced mechanotransduction

Endothelial cells line the blood vessels and are adapted to withstand shear stresses generated by movement of blood. Recent evidence that interstitial flow can direct mechanotransduction events came from studies on the endothelial glycocalyx which plays a role in moderating fluid stresses to cell surface (Tarbell and Pahakis, 2006). Components of the glycocalyx include hyaluronan, chondroitin sulphate and heparin sulphate. When these components were selectively degraded and exposed to shear stress, it was observed that responses such as release of nitric oxide were eliminated (Moon et al., 2005). Recently, a group using atomic force and fluorescence microscopy observed that the endothelial surface glycocalyx regulates nitric oxide production by activation of TRP ion channels (Dragovich et al., 2016). In human placental trophoblastic cells, fluid shear stress mediated microvilli formation and flow induced Ca^{2+} influx was regulated by transient receptor potential, vanilloid family type 6 (TRPV6) thus providing an understanding of signal perception and mechanotransduction (Miura et al., 2015).

In cultured MC3T3-E1 osteoblasts, application of fluid shear invoked reorganisation of actin filaments into contractile stress fibers involving recruitment of β1 integrins and α-actinins to focal adhesions (Pavalko et al., 1998). Two proteins related to mechanotransduction *in vivo*

cyclooxygenase-2 (COX-2) and product of c-fos were also observed to be elevated. Chicken osteocytes showed signs of mechanotransduction by increasing their levels of prostaglandin E_2 within 1 hr. following treatment with pulsating fluid flow leading to disruption of the actin cytoskeleton (Ajubi et al., 1996).

In endothelial and epithelial cells the transcription factor Egr-1 gene is rapidly activated following fluid shear stress via integrin-dependent activation of the Ras/Erk-1/2 pathway (Schwachtgen et al., 1998). The group showed that the Egr-1 expression increased by five to nine fold in human aortic endothelial cells and Hela cells when subjected to fluid shear stress. In periodontal fibroblasts c-Fos, an AP-1 transcription factor gets rapidly induced within 30 minutes of exposure to mechanical tension stress (Yamaguchi et al., 2002). Zhao et.al (2007) showed that mechanical stimulation of smooth muscle cells promoted nuclear translocation of MAL, a myocardin-related transcriptional co-activator of serum response factor (SRF). The authors also reported MAL/SRF-dependent activation of the α-SMA gene promoter in these cells.

1.4 Sensory elements of mechanotransduction

An obvious question related to biological flow is what the mechanisms cells use to detect and translate these signals. Since these physical forces differ in strength, it is not surprising that different molecular mechanisms are employed by cells to detect these forces (Freund et al., 2012). Few such components that mediate mechanotransduction in cells are detailed below.

1.4.1 Low-speed flow sensing: This happens via primary cilia **(Figure 4).** Since they protrude into the flow, these structures act as first line detectors of fluid flow associated signals. Ciliary mechanotransduction is associated with embryonic heart development and left to right patterning of heart and during renal development. The mechanosensory complex includes glycoproteins polycystin1 (PKD1) and polycystin2 (PKD2) in renal cells (Nauli et al., 2003), and PKD1 and PKD2 in the LR organizer (Field et al., 2011). PKD1 senses luminal shear stress though it's extracellular domain and regulates the gating of calcium – permeable channel PKD2 in renal epithelial cells (Nauli et al., 2003). In endothelial cells and in kidney epithelial cells, the PKD1-PKD2 complex is involved only in fluid flow associated mechanotransduction and not in mechanical loading associated mechanotransduction (AbouAlaiwi et al., 2009, Poelmann et al., 2008). However, primary cilia are fragile and cannot withstand strong fluid flows (Nauli et al., 2008). For example 2 hours of laminar shear stress at 15 dynes/cm^2 disassembles most of the cilia in human umbilical vein endothelial cells (HUVECs) (Iomini et al., 2004). Surprisingly, lack of cilia is associated with shear induced epithelial to mesenchymal transition, a process that is activated during valvulogenesis (Egorova et al., 2011).

1.4.2 High-speed flow sensing: In regions of high levels of cardiac output, blood flows a higher speed resulting in a higher fluid shear stress (Figure 1.4). Such high levels of flow require other mechanosensitive mechanisms for sustained high shear flows.

1.4.2.1: Glycocalyx: Amongst all the mechanisms, the glycocalyx has received the most attention in the recent years. The glycocalyx is a 3D meshwork of glycoproteins that covers the plasma membrane surface in a bush like pattern. It is seen as a major transducer of

mechanical forces to the underlying endothelial cytoskeleton (Tarbell and Pahakis, 2006). Studies using a hydrodynamic model have shown that the stiff core proteins of the glycocalyx transmit fluid forces at its tips through a localized torque which further transduces into an integrated torque in the underlying cytoskeleton (Weinbaum et al., 2007). The glycocalyx is present in a developing vasculature (Henry et al., 1995), however its role during embryogenesis needs to be further explored.

1.4.2.2: Mechanosensitive membrane channels: These channels exist in almost every organism and are gated by forces that convert mechanical signals to electrical ones or regulate release of secondary messengers which eventually gate the ion channels rapidly with a short latency. These channels are activated by mechanical stretching as is observed in transient receptor potential cation channel sub family C (TRPC1) (Maroto et al., 2005). TRP channels such as PKD1 are mechanosensitive channels that indirectly activate other mechanosensitive channels (Sharif-Naeini et al., 2009). In the cilia of kidney cells, TRPV4 interacts with PKD2 to form a calcium specific channel for mechanotransduction (Kottgen et al., 2008).

1.4.2.3: Plasma membrane mechanodetectors: Cells have different ways to maintain their plasma membrane integrity whilst experiences shear stresses due to fluid movement and mechanical loading. For example, some of the recent studies have shown that endothelial cells can respond rapidly to tension changes in their membrane through membrane invaginations known as caveolae (Sinha et al., 2011).

1.4.2.4: Adhesive mechanosensory receptors: An important component of the mechanoresponsive machinery lies in the cytoskeleton of cells, specifically in the molecules that anchors it to the neighbouring cells (Galbraith et al., 1998). Although changes in cytoskeleton are observed under influence of fluid flow mediated shear stresses, there is little evidence that the cytoskeleton can directly sense flows. The cytoskeleton of an endothelial cell, however contains membrane adhesion receptors which transmits signals from the outside to the inside of the cell. Flow dependant activation of vascular endothelial growth factor receptor type II (VEGFRII) has been observed in these cells (Tzima et al., 2005). Downstream signalling is mediated by phosphoinositide-3- kinase (PI3K) and the Akt complex which eventually phosphorylates endothelial NO synthase, thus promoting vessel relaxation (Fleming et al., 2005). Integrins and small GTPases (Rho, Rac and Cdc42) also support flow dependant cytoskeletal reorganization (Fleming et al., 2005). Coordinated efforts of two transcription regulators, *Yorkie*-homologues YAP and TAZ sense signals from integrins and translocate to the nucleus to promote differentiation in mesenchymal stem cells as well as in survival of endothelial cells (Dupont et al., 2011).

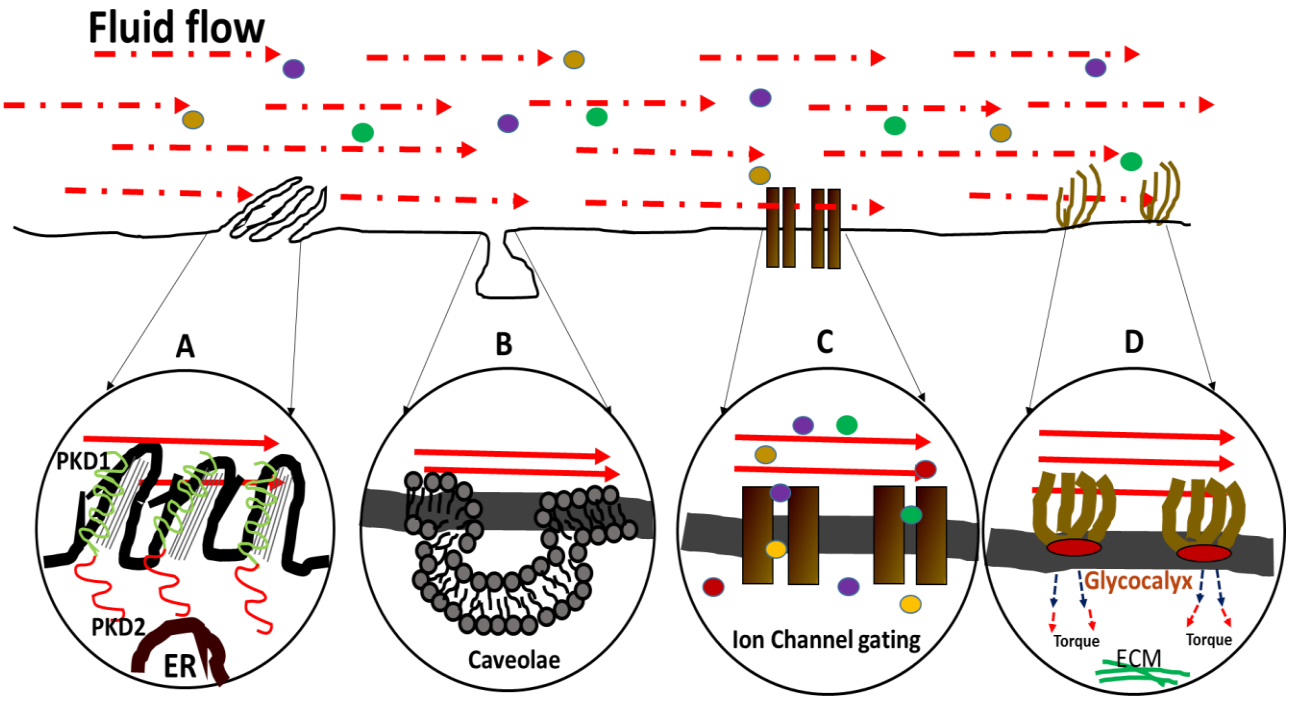

Figure 1.4: Sensory components of flow, A) low speed sensing by primary cilia, B) Plasma membrane bound detectors such as Caveolae, C) Membrane bound ion channels and D) Glycocalyx.

1.5 TGF- β signalling and associated processes

The transforming growth factor beta (TGF-β) pathway is crucial for various cellular functions. TGF-β signalling is known to exert pleiotropic effects on developmental processes including cardiovascular development (Di Guglielmo et al., 2003b), normal tissue homeostasis, cellular differentiation, proliferation (Huang and Huang, 2005), migration, apoptosis, and extracellular matrix production (Runyan et al., 2005). In wound healing, it is known to promote wound closure by increasing production of contractile proteins. However in fibrotic diseases, excessive production results in compromised tissue function (Mitchell et al., 2004a). Given that this pathway is involved in numerous normal biological and

pathologic processes, it is not surprising that the pathway is also involved in mechanotransduction (Chowdhury et al., 2004).

1.5.1: TGF-β ligands- The TGF-β superfamily consists of 30 ligands that are structurally related and interact with cell surface receptors to mediate downstream signalling and transcriptional events. The activin/nodal subfamily and the bone morphogenetic protein (BMP)/ growth and differentiation factor (GDF) also form a part of this signalling family (Ehrlich et al., 2001). In the canonical pathway, there are three variant forms that are conserved through evolution- TGF-β1, TGF-β2 and TGF-β3 (Table 1). The three ligands are produced by different cell types, although TGF-β1 is the predominant type in adults (Moustakas et al., 1993, Huang and Huang, 2005). All these ligands share significant sequence homology of greater than 76% (Derynck and Feng, 1997). Despite similarities in structure, these ligands have different affinities for different receptors. The TGF-β receptor type II (TGFβRII) binds with higher affinity to TGF-β1 and TGF-β3. TGF-β2 needs the presence of TGF-β receptor type III (TGFβRIII) to bind to TGFβRII (Criswell et al., 2008). During wound healing conditions, myofibroblast differentiation and ECM production, TGF-β1 is produced in excess in the tissue microenvironment (Buczek et al., 2016).

1.5.2: TGF-β Receptors- The canonical TGF-β pathway has three types of receptors: TGF-β receptor type I (TGFβRI), TGF-β receptor type II (TGFβRII) and TGF-β receptor type III (TGFβRIII) along with three types of co-receptors known as betaglycan, endoglin and CD109 (Nagaraj and Datta, 2010) (Table 1). TGF-β receptor type III primarily functions to present ligands to TGFβRI and TGFβRII (Nagaraj and Datta, 2010). In the type II receptor subfamily, there are about five types which forms a complex with the ligand: Act- RIIA, Act-RIIB, BMPR-II, AMHR-II and TGFβRII. In the classical pathway, T□RII is the most widely occurring type II receptor (Antony et al., 2010). It is a 62 KDa protein, consisting of a short

cysteine-rich, N-glycosylated extracellular domain. In the type I subfamily there are 7 receptors known as activin linked kinases (ALKS) 1 through 7.

Ligands	TGF-β1, TGF-β2 and TGF-β3.
Receptors	TGF-β receptor type I (TGF-βRI), TGF-β receptor type II (TGF-βRII) and TGF-β receptor type III (TGF-βRIII).
Receptor subtypes	**TGF- β receptor type I** – activin linked kinases (ALKs 1, ALKs 2, ALKs 3, ALKs 4, ALKs 5, ALKs 6 and ALKs 7). **TGF-β receptor type II** – activin receptor (Act- RIIA & Act-RIIB), Bone morphogenetic protein receptor type II (BMPR-II), Anti-Müllerian hormone type II receptor (AMHR-II) and TGFβRII.
Co. receptors	betaglycan, endoglin and CD109.

Table 1.1: Major signalling components of the TGF-β pathway.

1.5.3: TGF- β Signalling cascade-

TGF-β signalling is propagated when the ligand is presented by TGFβRIII to TGFβRII (Papageorgis, 2015). This binding leads to the formation of a receptor- ligand complex of two TGFβRII and one TGFβRI resulting in phosphorylation of TGFβRI which further drives the signal transduction process by activating a group of transcription factors known as Smads. The three classes of Smads transcription factors are regulated by TGF-β superfamily of ligands: the receptor-regulated Smads (R-Smads, Smad 1, 2, 3, 5 and 8) that interact with type I receptor. Smad6 and Smad7 compete with R-Smads for receptor binding and degrade the TGF-β receptors (Gordon and Blobe, 2008). TGF-β1 phosphorylates the R-Smads at the C-terminal of Smad2 and 3. These phosphorylated residues function as docking sites for Smad4 and promote dissociation of Smad2 from TGFβRI. This dissociation results in formation of the heterotrimeric complex with Smad4 (Gordon and Blobe, 2008). In unstimulated conditions Smad2 is located in the cytoplasm.

However upon stimulation, it translocates to the nucleus with Smad4. Inside the nucleus the complex binds to promoters and transcriptional co-activators or co-repressors to induce cell specific transcriptional activity (Pierreux et al., 2000).

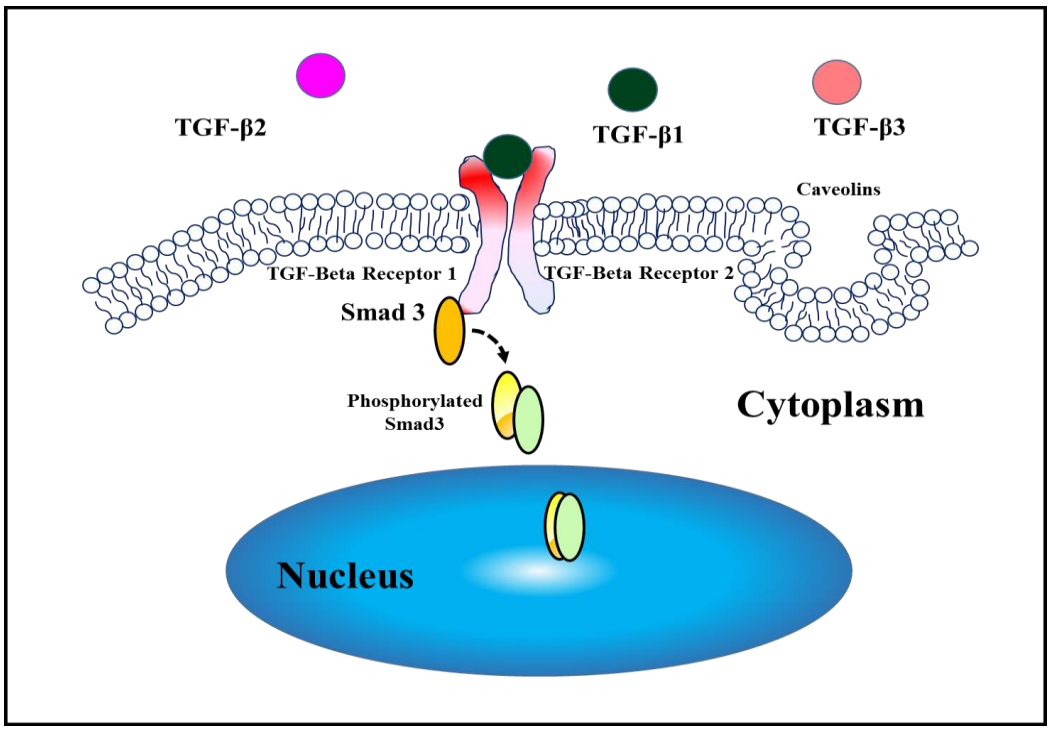

Figure 1.5a: TGF-β receptors and Smad mediated signalling in fibroblasts.

1.5.4 TGF-β pathway associated molecules- The TGF-β superfamily is a family of pleiotrophic ligands and are subdivided into four major sub-classes: the bone morphogenetic proteins (BMPs), activins, NODAL and Growth and differentiation factors (GDFs) (Figure 1.5b). Activins are a homologous family of homodimers and heterodimers and one of the most widely studied members of the TGF- β superfamily. These molecules are known to play crucial roles in early embryonic development and maintenance of tissue homeostasis (Wu and Hill, 2009). An imbalance in their activity can potentially transform the cellular fate to a tumorigenic state (Oshimori and Fuchs, 2012). In this chapter, we shall however discuss only about activins, These dimeric proteins are linked to one another by a single disulphide bond

(Ying, 1987). Both activin A and B can be detected in blood and other biological fluids using ELISAs (Ludlow et al., 2009) and their expression is stimulated via proinflammatory and immunoregulatory pathways. The most common activin complexes identified till date are activin A, activin AB and activin B which differ in their dimer subunits as βA βA , βA βB and βB βB respectively (Kingsley, 1994). Of all the forms, the first-identified and best studied family member is activin A. It was initially identified in ovary, and later from bone marrow cell culture (Brosh et al., 1995). The subunits of activin A and B are products of genes known as inhibin beta A (INHBA) and inhibin beta B (INHBB) respectively (Mason et al., 1986).

Follistatin is an autocrine glycoprotein encoded by the FST gene. It is expressed in all tissues of higher animals as three known isoforms, FS-288, FS-300 and FS-315 (Tortoriello et al., 2001) and is produced by the folliculostellate (FS) cells of the anterior pituitary (Gospodarowicz and Lau, 1989). Its expression can be detected in mid and late stages of embryogenesis (Roberts and Barth, 1994), ovary (Fleming et al., 1995), testis (Michel et al., 1993), vascular smooth muscle cells (Inoue et al., 1995), liver (Kojima et al., 1995) and other tissues. It plays the role of a cellular guard protecting the cells from uncontrolled proliferation and contributes towards tissue building and repair by inhibiting activin binding to type II receptors, thereby abolishing the biological effects of activins (Nakamura et al., 1990). Although it is a product of a single gene, two mRNA transcripts are produced due to alternative splicing, each encoding a 288 amino acid protein (FST 288) and 315 amino acid (FST 315) (Shimasaki et al., 1988). Both proteins clear activins by binding to them which initiates removal through the lysosomal degradation pathway (Hashimoto et al., 1997).

CD109 is a TGF-β binding protein and a member of the α2-macroglobulin family and is expressed as a cell surface antigen on normal hematopoietic cells and hematopoietic tumour cells (Sutherland et al., 1991, Murray et al., 1999). Although the significance of CD109 in the pathology of tumours remain unclear, its expression has been largely studied in tumour tissues including squamous cell carcinoma (SCCs) of oral cavity esophagus, lung, uterus, melanomas and soft tissue sarcomas (Hashimoto et al., 2004). These expression studies suggest that CD109 might be a cancer associated protein. However, it was also found to be a component of the TGF-β receptor system and abolishes TGF-β signalling in human keratinocytes (Finnson et al., 2006).

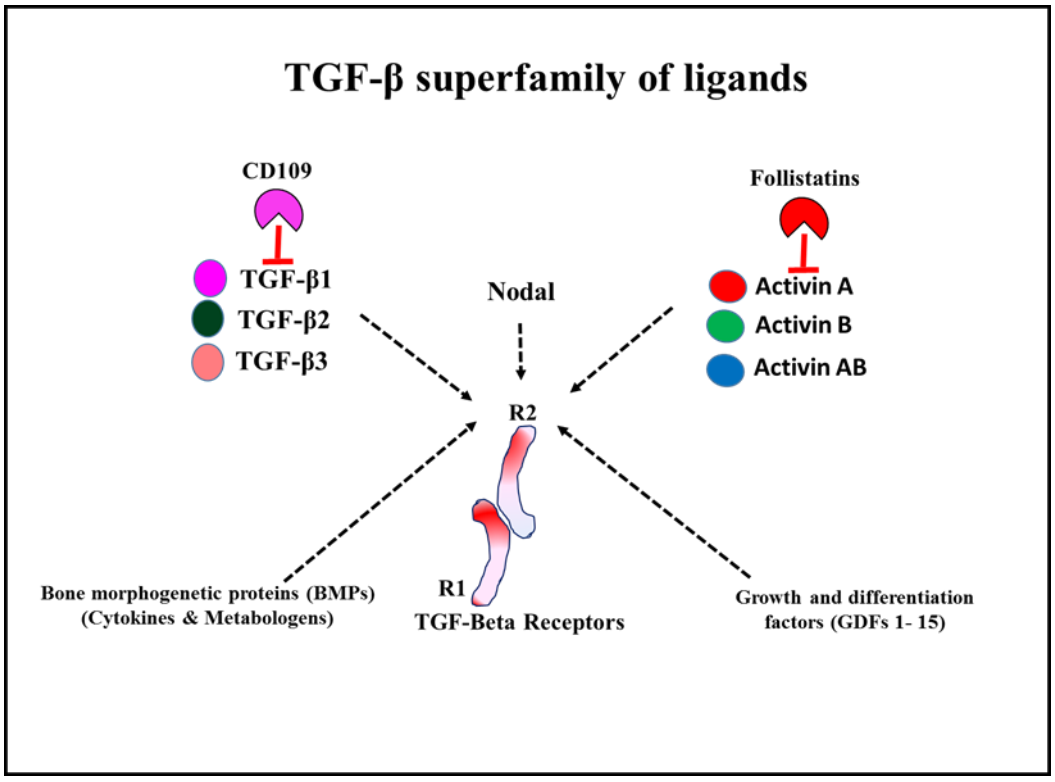

Figure 1.5b: Ligands and signalling molecules associated with TGF-β1 signalling family.

1.5.5 TGF-β pathway associated processes – Although the signalling pathway is associated with numerous biological process, in this review our focus will remain in

describing endocytosis as a crucial regulator of TGF-β signalling. Endocytosis refers to the process by which cell surface associated molecules enter the cell without passing though membrane**.** In this process the plasma membrane invaginates to form a vesicle which contains the internalized cargo (Lodish H, 2000). In mammalian cells, the endocytic pathway consists of specialised compartments that internalise the molecules and either recycle them back to surface or sort them for degradation. These principal components are early endosomes (EE), late endosomes (LE) and Lysosomes (Figure 1.6). EE are the primary compartment where molecules such as ligands, receptors and viruses are engulfed into vesicles. Late endosomes receive the internalised material from EE, trans-Golgi network (TGN) and the phagosomes. Being acidic in nature and composed of numerous vesicles, the late endosomes primarily deliver the ingested material to lysosomes. Lysosomes are the final component of the endocytic pathway. They are acidic in nature and are composed of large vacuoles containing hydrolases. Endocytosis regulates cell signalling via four different mechanisms namely clathrin mediated, caveolae mediated, micropinocytosis and phagocytosis. However, in this review we shall focus only on clathrin and caveolin mediated endocytosis.

Clathrin mediated endocytosis is regulated by a complex of vesicles and pits that are found in in the plasma membrane. Clathrin proteins are known to be involved in two important processes. 1) Fluid phase endocytosis from membrane to endosomes and 2) Transportation from the trans golgi network to the endosomes. These pits can concentrate large molecules and endocytose ligands such as transferrin and low density lipoprotein. (Marsh and McMahon, 1999). Epidermal growth factor receptor (EGFR), G-protein coupled receptors such as β2 adrenergic receptor and TGF-β undergo clathrin dependant endocytosis by direct interaction of their cytoplasmic domains with the clathrin adaptor protein complex AP2 (Sorkin and von Zastrow, 2009, Yao et al., 2002).

Caveolae are flask shaped pits which act as scaffolding proteins within caveolar membranes of mammalian cells and are involved in processes such as lipid regulation, mechanosensing (Parton and Simons, 2007), endocytosis (Kirkham and Parton, 2005) and compartmentalising signalling molecules for membrane trafficking. The caveolins consist of three members: caveolin-1 (CAV1), caveolin-2 (CAV2), and caveolin-3 (CAV3) and display very little difference in their structure (Anderson, 1998). However, they all form hairpin loop like invaginations into the cell membrane. In these proteins, both the C-terminus and the N-terminus face the cytoplasm of the cell. Caveolin-1 has two isoforms: caveolin-1A (CAV-1A) and caveolin-1B (CAV-1B). Occurrence of both CAV1 and CAV2 is predominant in endothelial, fibrous and adipose tissue whereas CAV3 is restricted to striated and smooth muscle cells. They are primarily synthesized as monomers and transported to the golgi apparatus. Subsequently, during the secretory phase when they associate with lipid rafts, they become oligomers to form the hairpin like structure to participate in vesicular transport.

Several studies have shown that caveolins contribute towards the pathology of diseases such as cancer, vascular proliferative disease, cardiac hypertrophy, insulin signalling and diabetes, and muscular dystrophy. CAV1 has been shown to inhibit cellular proliferation suggesting that they may play a role in carcinogenesis (Razani et al., 2000). Analysis of tumour tissue obtained from breast cancer patients showed that nearly 16% of these tumours harbour a dominant negative point mutation in the caveolin-1 gene (Hayashi et al., 2001). Similarly reduced expression of caveolin-1 in proliferating vascular smooth muscle cells of atheroma suggests its expression is inversely correlated in human vasculature (Schwencke et al., 2005) and loss of caveolin-1 promotes changes such as epithelial to mesenchymal transition which further leads to tumourigenesis (Shivshankar et al., 2012). Interestingly, expression of

caveolin-1 is increased in prostate cancer and is expressed even further in metastatic prostrate cells (Yang et al., 1999)

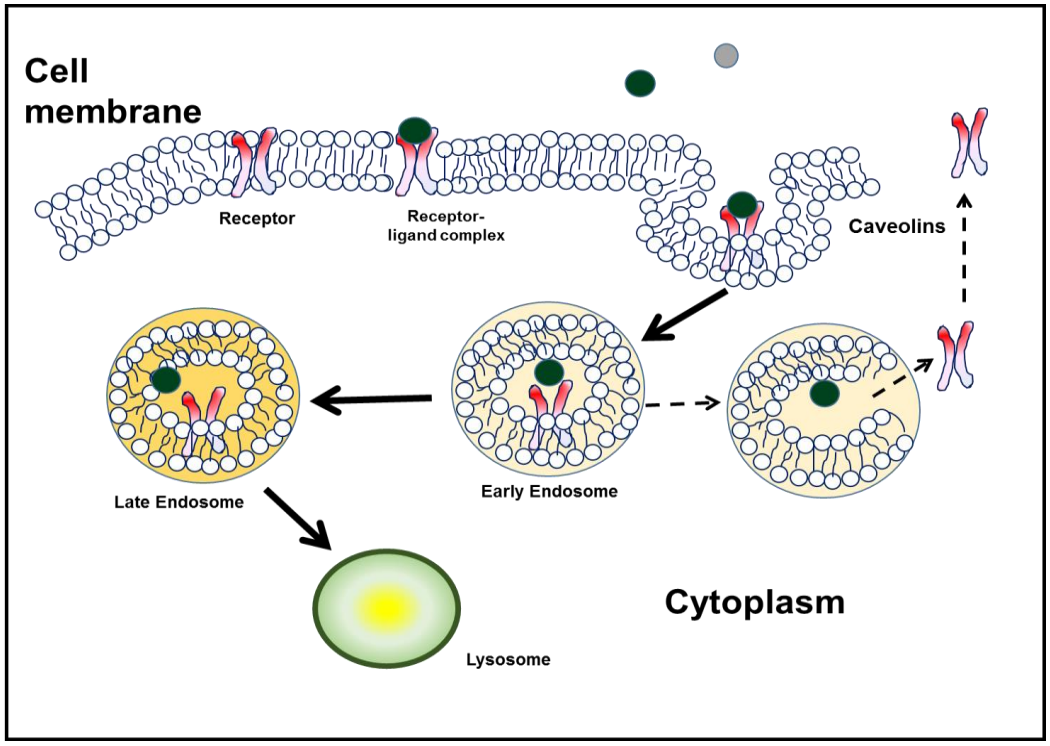

Figure 1.6: Representation of the endocytosis pathway and components involved.

1.6 Fluid flow mediated mechanotransduction in pathological conditions.

Although it seems that developmental mechanisms try to maintain normal rhythms of fluid flow *in vivo*, in certain diseased conditions cellular mechanisms lose direction from their normal path and invoke pathological mechanisms. Blood velocity is highly variable and is largely reliant on cyclical contraction of the heart suggesting that except in smaller vessels, flow is never steady *in vivo* (Freund et al., 2012). Whilst pulsatile contraction associated flow is considered as 'healthy' flow, the heart is also known to experience turbulent flows. In the heart, these turbulent flows in association with other chemical signals give rise to disease conditions such as atherosclerosis in adults (Hahn and Schwartz, 2009) and congenital heart

defects (Armstrong and Bischoff, 2004). Reversal of blood flow can also be observed in developing valves such as mitral valve, the valve between atrium and ventricle. In underdeveloped valves, due to diastolic and systolic contractions lead to blood flows out in a reverse direction from the ventricles to the atrium, a condition known as Ebstein's anomaly (Safi et al., 2016).

Tumours are known to have a highly developed vasculature with leaky and tortuous blood vessels that supply blood and oxygen to the developing cancer cells in a hypoxic microenvironment. Microcirculation of blood and interstitial fluid play and important role in tumour growth, metastasis and in the treatment of tumours. These blood vessels lack the normal architecture of arteries, arterioles and capillaries (Konerding et al., 1999, Warren et al., 1978). Changes in pericytes in these blood vessels leads to changes in stability of the vessel which in turn results in changes in blood flow. Some vessels may not perfuse with blood, whereas other may experience chaotic blood flow along with reverse flow of blood (Baluk et al., 2005) as is observed in Ebstein's anomaly. Recent studies have shown that viral cirrhotic primary liver cancer cells are characterised by hepatic fibrosis that is an outcome of disturbances in blood flow such as lobular vein obstruction, portal vein fistula and portal vein counter current (Liu et al., 2011).

Numerous studies have shown that as well as changes in blood flow, disturbances in interstitial fluid flow also contributes to pathological manifestations such as cancer (Munson and Shieh, 2014), edema (Scallan J, 2010) and central nervous system associated diseases such as Alzheimer disease, multiple sclerosis (Weller, 1998). Tumours are known to display increased mechanical stiffness that exerts pressure on the surrounding tissue. Pressure differences between the tumour and the tissue leads to pressure gradients resulting in

development of interstitial fluid flow through the stroma (Butler et al., 1975). The first evidence of this fluid flow came from murine mammary tumours. Edema is another pathological condition of faulty fluid flow that manifests itself as excessive interstitial fluid accumulation either within the cells (cellular edema) or within interstitial spaces (interstitial edema) distributed within the stroma matrix across the microvascular walls (Dongaonkar et al., 2008, Reed et al., 2010).

Extracellular fluid in the central nervous system (CNS) is made up cerebro spinal fluid (CSF) from the choroid plexus and interstitial fluid (IF) in the gray and white matter. Lymphatic drainage of interstitial fluid contributes to accumulation of prion proteins in the extracellular spaces of gray matter contributing to Alzheimer disease (AD) and Multiple Sclerosis (MS) (Weller, 1998).

1.7 Fibroblasts in normal, diseased and healing tissues.

The functions of fibroblasts include deposition of extracellular matrix, epithelial differentiation and regulation of inflammation in wound healing and other pathological processes that involve uncontrolled growth of cells (Tomasek et al., 2002, Parsonage et al., 2005). Fibroblasts are considered as the main mediators of scar formation and fibrosis and are the primary sources of host derived VEGF (Fukumura et al., 1998), the key molecule responsible for angiogenesis in tumours.

Fibroblasts can trans-differentiate into a specific type of fibroblast which forms part of the tumour stroma with expression of a contractile phenotype (Kalluri and Zeisberg, 2006). This process is known as Mesenchymal Mesenchymal Transition (MMT). These fibroblasts deposit high amounts of collagen and express α- smooth muscle actin upon stimulation with

TGF-β1 (Pellegrin and Mellor, 2007). Accumulation of α-SMA allows fibroblasts to generate increased contractile forces and it is considered a marker for myofibroblast cells. Myofibroblasts usually originate from residing fibroblasts, pericytes, smooth muscle cells from vasculature, mesenchymal stem cells or due to Epithelial Mesenchymal Transition (EMT)/Endothelial Mesenchymal Transition (EndoMT) and play a role in biological processes such as tissue regeneration and cancer progression. Studies have revealed that up to 40% of the fibroblasts in tumours could be EndMT derived and that it plays a key role in angiogenic sprouting (Potenta et al., 2008).

Studies conducted on fibroblasts isolated from healing wounds reveal that they secrete high levels of the extracellular matrix components and are highly proliferative in comparison to their counterparts in normal tissue (Rodemann and Muller, 1991). Such enhanced activity is defined as 'activation' (Castor et al., 1979). Activation of fibroblasts is associated with enhanced capillary density, collagen type 1A, α-smooth muscle actin, ED-A fibrin deposition and increased proliferation (Kohan et al., 2010, Hinz et al., 2001). Recently it was found that migrating epithelial cells in wound margins and deep epithelial ridges gain mesenchymal features like expression of vimentin. Elevated levels of EMT related genes were observed in hypertrophic scars and Tumour Necrosis Factor-α induced EMT in human skin by induction of BMP-2 (Yan et al., 2010).

A number of studies have been conducted to assess the role of fibroblasts in tumours. Studies in chickens infected with Rous sarcoma virus have demonstrated that wounding leads to invasive carcinomas, thus establishing the tumour promoting role of activated stroma (Dolberg et al., 1985, Sieweke et al., 1990). Conditions such as wound healing and fibrosis share a requirement for tissue remodelling. In such conditions, quiescent fibroblasts undergo

activation to become myofibroblasts (Figure 1.7) as originally identified by Gabbiani et al. (1971) in the granulation tissue of healing wounds. These myofibroblasts acquire contractile stress fibres and express proteins such as collagen type I, ED-A splice variant of fibronectin and α- smooth muscle actin, proteins normally expressed in vascular smooth muscle cells and that Upon completion of wound healing, activated fibroblasts undergo a type of programmed cell death known as nemosis. Cancer associated fibroblasts (CAFs) share similarities with myofibroblasts, but they remain activated indefinitely and are not removed by nemosis (Dvorak, 1986). TGF-β1 regulates the levels of expression of activation associated proteins (Varga, 2002, Mori et al., 2003).

TGF-β is a pleiotropic growth factor which plays a tumour supressing role in normal cells and a tumour promoting role in cancer cells by differentiating cells into an invasive phenotype (Massague, 2008). Of all the variants, TGF-β1 has received the most experimental attention for reasons that it is produced by macrophages, fibroblasts, keratinocytes and platelets (Eppley et al., 2004, Lee and Eun, 1999, Mani et al., 2002). It plays a role in inflammation, angiogenesis, reepithelialisation and connective tissue regeneration during wound healing (Kopecki et al., 2007, Kane et al., 1991). This growth factor is chemotactic for fibroblasts, keratinocytes and inflammatory cells. Each isoform performs specific roles in wound healing and cancer (Schrementi et al., 2008). Furthermore, it promotes inflammatory cell recruitment (Moses et al., 1990) and regulates hypertrophic scarring (Russell et al., 1988). Recent studies revealed that SMA-positive, myofibroblastic stroma is the strongest predictor of oral squamous cell carcinoma mortality and αvβ6-dependent TGF-β1 activation mediates myofibroblast trans-differentiation (Marsh et al., 2011). TGF-β1 mediates Epithelial Mesenchymal Transition (EMT) of breast cancer cells to cancer associated fibroblasts (CAFs) by paracrine signalling (Yu et al., 2014).

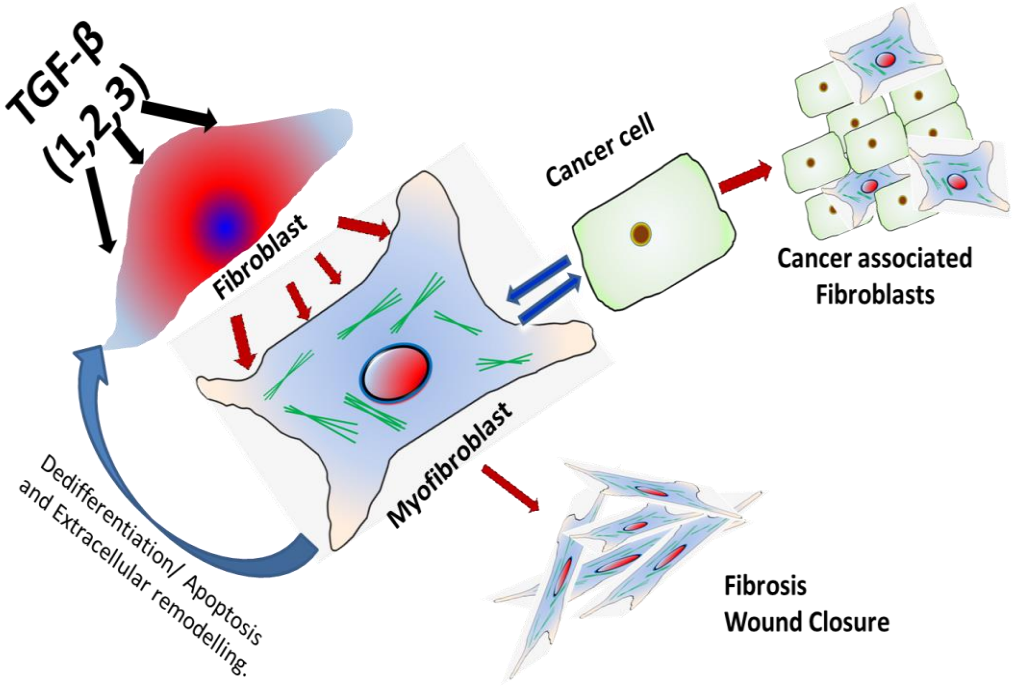

Figure 1.7: Fate of TGF-β stimulated myofibroblasts.

Cancer associated fibroblasts (CAF's) are myofibroblasts which form a crucial part of the tumour's stromal compartment and are more potent in promoting tumour growth when compared to those obtained from normal tissue. This property came to light when researchers studied the growth rate of tumours injected with CAFs and those injected with normal fibroblasts in xenografts. The group found that growth rate was significantly higher in those which were injected with CAFs (Orimo et al., 2005, Ostman, 2004). Subsequent studies from our laboratory also showed that senescent cancer associated fibroblasts develop a unique phenotype known as senescence associate secretory phenotype (SASP) which contributes to cancer progression via a novel miRNA/ PTEN regulated pathway (Kabir et al., 2016).

Studies on humans and 4NQO- induced rat tongue carcinogenesis revealed the presence of myofibroblasts in highly malignant tissues while they were absent in precancerous tissue (Vered et al., 2009). Subsequently, pathological studies have revealed the presence of stromal CAFs is an indicator of poor prognosis of all stages of oral squamous cell carcinoma (Bello et al., 2011, Vered et al., 2010). *In vitro* studies revealed that TGF-β1 released by OSCC's was responsible for the trans-differentiation of normal fibroblasts to CAF's. The CAF's secrete high levels of growth factors that enhance proliferation in OSCC's (Kellermann et al., 2008).

Recently, studies were conducted to analyse a series of matched CAF's and normal fibroblasts for expression of activation markers (α-SMA, SDF-1, FAP, vimentin and cytokeratin). CAFs showed significantly higher α-SMA protein levels, and mRNA levels of α-SMA, SDF1, and FAP. Furthermore, co-cultures of CAFs and normal fibroblasts induced expression of EMT markers like vimentin, fibronectin and enhanced invasion of SCC9 cells (Zhou et al., 2014).

1.8 Parallels between Skin and Oral mucosa.

Based on morphology and differentiation patterns epithelial tissues are broadly classified into three classes: keratinising (cornified) stratified squamous epithelium, non-keratinising stratified epithelium and non –stratified epithelium (Eurell JA, 2006). A critical aspect of stratified keratinising epithelium is that cells undergo a terminal differentiation programme which results in formation of a mechanically tough surface of cells known as corneocytes. These corneocytes are filled with keratin filaments and are devoid of nuclei and cellular organelles.

Keratinocytes are the most abundantly occurring cells in the epithelium. They are tightly connected to each by desmosomes in an organised matrix containing Langerhans cells, melanocytes, Merkel cells, fibroblasts, extracellular matrix proteins like collagen (type I and III). The epidermis is sub-divided into four layers; Stratum Corneum, Stratum granulosum, Stratum spinosum, and stratum basale (McGrath, 2004). During normal tissue turnover, keratinocytes which proliferate in the stratum basale migrate and differentiate to form the stratum corneum**.** Figure 1.7 illustrates the various layers of the epidermis in skin mucosa and their counterpart in oral mucosa. Apart from the presence of four layers as observed in epidermis, oral mucosa also consists of a lamina propria and a sub mucosal layer which is attached to muscle or bone. The submucosal layer is the deepest layer containing fibroblasts, mast cells and adipocytes. Functions of this layer include maintenance of thermoregulation, protection from injury and provision of nutrients.

Oral epithelium could either be keratinised or non-keratinised based on the location. This site specific keratinisation is largely dependent on the functions performed by different parts of the mouth (Squier and Kremer, 2001). Other difference between both mucosa include presence of sweat glands, hair follicles in the skin which are absent in the oral mucosa.

Although both mucosa have similar ECM composition, non-keratinised oral mucosa has a loose ECM structure with presence of elastin which mediates expression of keratins and its variants (Hsieh et al., 2010). Overall, oral and dermal mucosa differs very little in their architecture, inflammatory molecule production, blood vessel density, cell–cell interactions and extracellular components.

Although healing mechanisms are similar in skin and oral mucosa, mechanisms leading to faster wound healing in oral mucosa remain a challenge to understand fully. Many research groups have vested efforts in studying the cause and effect relationship of this variance in both mucosa. Some studies report that one of the key factors which contribute to faster wound healing is saliva (Veerman et al., 2011). Reports show that the humid environment created by the saliva improves survival and functioning of inflammatory molecules that contribute to wound healing (Dawes et al., 2015). Further, saliva contains proteins which accelerate blood clotting and epidermal growth factor which promotes proliferation of epithelial cells (Nagy, 2003). The tissue degrading activity of enzymes such as elastase and trypsin is neutralised by secretory leucocyte protease inhibitor and its absence delays wound healing (Brand and Veerman, 2013).

Under normal conditions similar numbers of blood vessels exist in dermal and oral tissue. However, Wong et al (2009) compared wound healing in pig's oral mucosa to similar experimental wounds created in human subjects. Significantly higher numbers of blood vessels in skin than oral tissue, although number of blood vessels increased in both tissues were observed. Their results concluded wound healing in oral mucosa results in reduced scar formation as compared with skin (Wong et al., 2009).

Another study revealed that under normal conditions there were no differences in VEGF levels in both oral and dermal mucosa. However, in wounded conditions, oral wounds expressed lower levels of VEGF than their dermal equivalents (Szpaderska et al., 2005). These results complement previous research which showed similar numbers of blood vessels in both tissues. Nevertheless, in wounded conditions, skin tissue exhibited more blood vessels (Mak et al., 2009).

Oral and dermal keratinocytes display differences in their responses to inflammatory stimuli which may influence the rate of wound healing. Human oral keratinocytes produce a rapid increase and enhanced expression of IL-6 in response to tumour necrosis factor (TNF)-α, interferon (IFN)-γ, or IL-4 (Li et al., 1996).

Oral wounds in mice reepithelialise and close within 24 hrs of wounding whereas dermal wounds tend to close after 60 hours to 7 days post wounding (Szpaderska et al., 2003). Unlike dermal mucosa, different regions of the oral cavity heal in a different manner. Incisions in the buccal mucosa result in scars whereas gingival grafting heals without scars (Larjava et al., 2011). Numerous molecular alterations are observed in oral versus dermal wound healing (Table 1.2).

Studies of differences between extracellular matrix components in oral and dermal mucosa reveal similar collagen fibril diameter in normal and wounded oral tissue. Nevertheless, in wounded dermal tissue collagen fibres were thinner in diameter (Schrementi et al., 2008). During wounded conditions in pigs, the number of cells expressing pro collagen I was significantly increased in oral wounds than in skin wounds (Wong et al., 2009).

The fibroblast compartment significantly differs in normal and wounded conditions in oral and dermal mucosa. Lee and Eun provided initial evidences of differences between fibroblasts of oral and dermal origin (Lee and Eun, 1999). They showed that oral fibroblasts proliferate at a higher rate than dermal cells. Whilst dermal fibroblasts in a collagen gel had a greater contraction potential than their oral counterparts, oral fibroblasts contracted earlier than the dermal ones. Studies also reported that wound healing with minimal scar formation

may be an outcome of increased matrix metalloproteinase-3 production by oral fibroblasts (McKeown et al., 2007). Transforming growth factor- β1 (TGF-β1) is one of the most studied growth factor in relation to wound healing. It is secreted by the platelets during the initial phases after wounding and acts as a chemo attractant for leukocytes and parenchymatous cells. Although there were no changes in basal collagen synthesis, when fibroblasts from both dermal and oral origin were stimulated by TGF-β1, oral fibroblasts started synthesizing more collagen than the dermal ones. Subsequent findings confirmed that oral fibroblasts displayed accelerated collagen production (Shannon et al., 2006). It was also shown that oral fibroblasts expressed significantly high levels of epithelial growth factors such as keratinocyte growth factor (KGF) and hepatocyte growth factor/scatter factor (HGF) and surprisingly low levels of Alpha smooth muscle actin.

Alpha smooth muscle actin (α-SMA) is used as a marker for myofibroblast identification and is abundantly incorporated into actin stress fibres of fibroblasts under stress conditions and contributes towards increased contractile force. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts (Gabbiani et al., 1971). Interestingly, another study showed that oral fibroblasts express more α-SMA than dermal equivalents. Paradoxically, TGF-β1 stimulated oral fibroblasts to express lower levels of α-SMA than dermal fibroblasts (Lygoe et al., 2007).

Wounds of the oral cavity are known to display healing with little or minimal scarring. When gingival and dermal fibroblasts were induced to express a wound healing transcriptome in 3D cultures, gingival fibroblasts expressed high levels of inflammatory molecules and genes related to ECM remodelling whereas dermal fibroblasts expressed high levels of TGF-β signalling related molecules and contractility related genes. These findings suggested that

increased expression of ECM remodelling associated genes inherent to the phenotype of oral fibroblasts contributed to faster scar free healing of oral wounds (Mah et al., 2014). Subsequently, a comparison of the gene profile of oral & dermal fibroblasts and induced pluripotent stem cells using microarray and *in silico* data analysis revealed that senescence-related genes in the p53 pathway are inactive in human oral fibroblasts (hOFs) (Miyoshi et al., 2015).

	Normal tissue		Wounded tissue	
	Oral	Dermal	Oral	Dermal
Blood Vessels	=	=	↓	↑
Interleukin -6 (in response to tumor necrosis factor (TNF)-α, interferon (IFN)-γ, or IL-4).	↑	ND	↓	ND
Reepithelialise and closure (in mice)	ND	ND	24hrs	60 hours – 7 days
Vascular Endothelial growth factor (VEGF)	=	=	↓	↑
Collagen fibril diameter	=	ND	ND	thinner in diameter
Pro collagen I (in pigs)	ND	ND	ND	ND
Transforming growth factor - β1 (TGF-β1)	ND	ND	ND	ND
Transforming growth factor - β1 (TGF-β1) mediated proliferation in fibroblasts	ND	ND	ND	ND
Alpha – Smooth Muscle Actin (α-SMA)	ND	ND	ND	ND
TGF-β1 stimulated oral fibroblasts	ND	ND	ND	ND
CC-chemokine ligand (CCL) 5, CCL12 and CXC chemokine ligand (CXCL) 10 (mouse)	ND	ND	Present in tongue	ND
CCL3, CCL20, CXCL3, CXCL7 and CXCL13 (mouse)	ND	ND	ND	Present

Table 1.2: Molecular differences between oral and dermal mucosa in normal and wounded conditions. ↑, increased in oral tissue/wounds compared with dermal; ↓, decreased in oral tissue/wounds compared with dermal; =, equal between oral and dermal tissue/wounds. (Glim et al., 2013). 'ND' represents not defined.

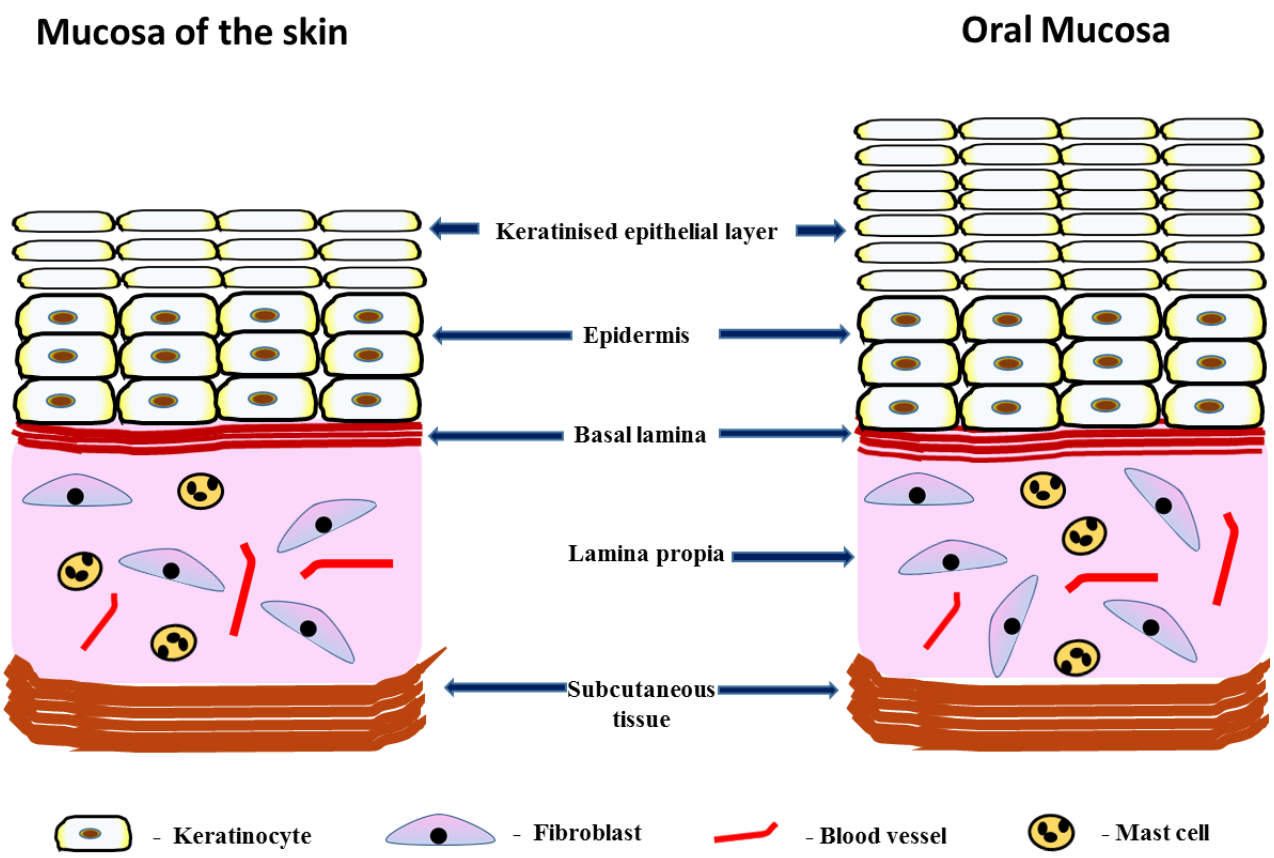

Figure 1.8: Skin and oral mucosa histology. The epidermis and oral epithelium consist of keratinocytes and a few Langerhans cells. Dermis and lamina propria are separated by a basal lamina followed by the subcutaneous layer.

1.9 Immune response in oral and dermal wounds

During wound healing, an inflammatory response is essential as its rapid resolution aids the repair process. In order to determine role of inflammatory molecules, Szpaderska AM et al (2003) compared the inflammatory cell infiltrate and cytokine production in oral and skin wounds. Significantly lower levels of macrophage, neutrophil, and T-cell infiltration were observed in oral wounds than their dermal counterparts. RT-PCR analysis revealed significantly lower IL-6 levels in oral wounds compared to skin wounds. Similarly, levels of the pro-fibrotic cytokine TGF-β1 were lower in oral wounds than in skin wounds. Anti-

inflammatory cytokines like IL-10 and the TGF- β1 modulators did not vary significantly (Szpaderska et al., 2003). Another study in mice revealed that IL-6 or IL-1 receptor antagonist deficiency causes a delay in wound healing (Ishida et al., 2006). IL-1 receptor deficiency delays the wound healing rate in oral wounds but not in dermal wounds (Graves et al., 2001). The effect appeared to be due to limited clearance of infection as administration of antibiotics reversed the wound healing rate.

Chemokines, CC-chemokine ligand (CCL) 5, CCL12 and CXC chemokine ligand (CXCL) 10 appear in mouse tongue wounds whereas CCL3, CCL20, CXCL3, CXCL7 and CXCL13 are present only in dermal wounds (Chen et al., 2010). Functional analysis of these chemokines indicates that oral wounds express chemokines chemotactic for monocytes and dermal wounds mainly express neutrophil attracting chemokines. Mast cells are bone marrow derived immune cells found mainly in the perivascular spaces within skin. These are stimulated following tissue injury and degranulate releasing inflammatory agents such as histamine, proteases, prostaglandins, leukotrienes, inflammatory mediators (such as IL-1, Transforming growth factor–α) and growth factors (TGF-β1, platelet derived growth factor [PDGF]).

Neutrophils are some of the first immune cells which infiltrate the site of injury. In one study, a research group injected mice with antigen ovalbumin (OVA) and made a thoracic incision. This resulted in minimal recruitment of neutrophils, mast cells and lymphocytes to lesion. Further, the OVA tolerant mice showed diminished scarring and normal extracellular matrix deposition (Costa et al., 2011). Another study in mice showed presence of lower numbers of neutrophils in oral wounds than in dermal ones (Szpaderska et al., 2003). These findings

suggest that a reduced number of neutrophils could be responsible for reduced scar formation in oral wounds.

Macrophages are another group of immune cells which respond to tissue injury. These cells produce high amounts of pro inflammatory cytokines and mediate anti-microbial activity. These can be classified into M1 or M2 phenotypes (Mosser and Edwards, 2008). M1 macrophages are the first ones to respond to injury. M2 macrophages are crucial for tissue restoration and express extracellular matrix molecules (ECM) like fibronectin and tenascin – C (TN-C) suggesting that they are involved in ECM deposition and tissue remodelling in the later phase of tissue repair (Deonarine et al., 2007). In an experiment where activated macrophages were co cultured with a fibroblast cell line, M2 macrophages induce fibroblast proliferation and collagen production. On the contrary M1 macrophages reduced collagen production (Song et al., 2000). In other studies reduced numbers of macrophages were found in mouse and pig oral wounds compared to their dermal counterparts (Szpaderska et al., 2003, Mak et al., 2009). These studies strengthen our understanding that macrophages are essential for proper repair. However the presence of lower numbers in oral wounds makes us to contemplate whether a smaller number of these cells promote wound healing and greater numbers promote scarring. Further studies need to be pursued to explore this in dermal and oral wounds.

T-cells play a role in tissue remodelling and potentially increase collagen synthesis and induce α-SMA in dermal fibroblasts. They are found in epidermis and produce the growth factor, Insulin like growth factor -1 (IGF-1) (Toulon et al., 2009) to support keratinocyte survival and proliferation. These experimental findings indicate that reduced inflammation could contribute to scarless and accelerated healing of oral mucosa.

A question now emerges whether wound healing is similar to cancer. Tumours could develop at sites of chronic wounds or untreated mouth ulcers (Dunham, 1972). Liver inflammation due to Helicobacter pylori infection, cirrhosis or hepatitis could lead to cancer (Ruggiero, 2010). Such associations and comparison of histological sections of healing wounds and tumour sections has led to a hypothesis that cancer are wounds that do not heal (Dvorak, 1986, Byun and Gardner, 2013).

1.10 Overview of the bioreactor technology.

A bioreactor is defined as an engineered apparatus which supports live cell culture in a biologically active environment. There are numerous types of bioreactor available on the market, with the majority of them providing media transport and additional benefits for the tissues and cells cultured in them. In bioreactors which display medium movement, a resultant force is applied to the cells due to the movement of fluid over the surface. These bioreactors are engineered such that they provide all the optimal conditions to emulate the *in vivo* environment that the cells would normally be exposed to. Bioreactors are broadly divided into five main categories i) spinner flask, ii) rotating wall, iii) compression, iv) strain, v) hydrostatic pressure and vi) flow perfusion (Plunkett and O'Brien, 2011). Each of these bioreactors differ in their mass transfer characteristics and the physical environment to which cells are exposed. Spinner flask bioreactors are used in applications which require high mass transport, high shear and a turbulent environment whereas rotating wall bioreactors are used in applications which require high mass transport and a low shear environment (Oragui et al., 2011). These bioreactors have limited applications as it cannot be used for differentiating cells into a particular tissue type. Compression bioreactors are used in cartilage engineering

since they are designed such that both static and dynamic loading can be applied (Shahin and Doran, 2015). Tensile strain bioreactors are used to engineer numerous types of tissues. They are designed in a similar way to compression bioreactors, only differing in the way the force is transferred to the construct (Goodhart et al., 2014). Hydrostatic pressure bioreactors are used in applications which require application of a mechanical stimulus to cell-seeded constructs (Elder and Athanasiou, 2009). Scaffolds are cultured in static conditions and then moved to a hydrostatic chamber for loading purposes.

Flow perfusion bioreactors are considered as the best bioreactor for fluid transport. The bioreactor consists of a pump and a scaffold chamber joined together by tubing. A fluid pump forces media to flow through the cell-seeded scaffold. Cells seeded in scaffolds or coverslips are placed in chambers that are designed to direct flow through the interior of the scaffold, thus enhancing fluid transport. The earliest example of a flow bioreactor is the 1951 Hu 'perfusion chamber'. Experiments involved studying the effect of radioactive isotope phosphorous 32 in epidermal cells that were cultured on coverslips (Everett et al., 1951). Media in these bioreactors is continuously pumped through the chambers in which the cells are placed. These bioreactors are often combined as part of a microfluidic device where multiple chambers are connected in series. Hence they serve as an excellent tool to study and model multiple tissue types in a combined culture and represent good *in vitro* models. However, the small scale and size of these bioreactors result in limitations for clinical applications.

Recent studies using flow bioreactors revealed the positive effect of flow in proliferation of mesenchymal stem cells (MSCs) grown in bioreactors compared to those cells grown in static cultures. When cells were treated with osteogenic media, cellular mineralisation was

observed in the bioreactor (Zeng et al., 2006). Another interesting study pursued by Kim and Ma (2012) compared the expansion of MSC's in a flow bioreactor and in a perfusion bioreactor. Cells were expanded in both the bioreactors types for 7 days and then treated with osteogenic media for another 7 days at a flow rate of 0.2ml/min. The study suggested that cells cultured in the flow bioreactor were found to be in at an earlier differentiation stage than cells cultured in the perfusion bioreactor due the reduced shear stress under flow (Kim and Ma, 2012).

Overall the bioreactor technology exposes cells to two key processes, i) mass transport of media and ii) fluid shear forces. Fluid shear forces play a critical role in modulating the response of cells to their environment.

In endothelial cells, laminar flow directly up regulates intercellular adhesion molecule 1 (ICAM-1) expression on cell surface and enhances leucocyte adhesion (Nagel et al., 1994). Other studies suggest a role for shear stress in vascular remodelling and arterial tone. In bovine aortic endothelial cells shear stress induces conformational activation of αvβ3 integrin resulting in increased binding of cells to its extracellular matrix proteins, transient inactivation of Rho and cytoskeletal alignment in the direction of flow (Tzima et al., 2001). Recent studies reveal the role of fluid shear stress in modulating mechanical tension by PECAM-1 initiated signalling (Conway et al., 2013). In Glioma cells fluid shear stress regulates the invasive potential via modulation of migratory activity and matrix metalloproteinase expression (Qazi et al., 2011).

In the current study, efforts were to study the response of cells in a physiologically relevant flow perfusion environment where cells are constantly exposed to chemical signals and fluid

signals arising due to movement of blood, tissue fluid etc. Since the primary objective of the project was to assess if perfusion flow could induce differentiation in fibroblasts. Other bioreactors such as spinner flask bioreactor, tensile strain bioreactor and compression bioreactors have limited applications as described above and hence cannot be used for studying cellular differentiation. Therefore, in this study the Quasi vivo ® bioreactor system (Figure 2.1) originally developed by Dr Ahluwalia et al. at the University of Pisa and commercialised by Kirkstall ltd. was used. The basic design is of a flow perfusion bioreactor. It was adapted from a fully contained Poly-dimethyl-siloxane (PDMS) unit which housed the chambers to a modular system of interconnecting loops which can be setup in a user specific manner (Mazzei et al., 2008). The original design was used for high throughput drug screening and consisted of a simple PDMS circuit with chambers that held different cell types. The design features ensure incorporation of laminar flow at a definable level of shear stress and this was validated with finite element modelling (Mazzei et al., 2008).

In the first experiments with Quasi Vivo® bioreactor, primary murine hepatocytes and human umbilical vein endothelial cells were cultured in separate chambers and connected in series. It was observed that there was no reduction in hepatocyte viability whilst they were cultured in the bio-reactor. The hepatocytes cultured in series with HUVEC's produced higher levels of albumin and urea in comparison to the hepatocytes grown on their own. When the researchers normalised the differences in media volume between both conditions, a 60 fold increase in albumin production compared to static culture was observed. These results prompted the authors to conclude that the bio-reactor and the fluid shear stress induced in the bio-reactor provides an improved cell culture environment (Vozzi et al., 2009).

In 2009 the bio-reactor was re-designed to provide a modular system for cell culture. Before being manufactured the system was modelled for fluid dynamics for shear stress, turbulence and oxygen diffusion using hepatocytes. Shear stress is a physiological state where the stress is parallel to cells or tissues. Maximum wall shear stress for chambers with a 13mm diameter, 3mm distance from the top to the reactor and 1mm fluid outlets with a variable distance "h" between tubes and base at maximal flow rate was calculated as 10^{-5} Pa (Vinci et al., 2011). The design produced optimal fluid dynamic parameters and a turbulence free flow of 500 µL/min. Primary rat hepatocytes were tested in this model and the results indicated that there was no difference in albumin production between static and bio-reactor grown cells. In another study where adipocytes were cultured with HepG2 cells, a significant increase in albumin production, glucose consumption and free fatty acid uptake was observed in cells under flow (Vinci et al., 2010).

Since 2010 there has been activity in the application of the Quasi Vivo ® bio-reactor to a number of cell types and further characterisation of the effect on cells in comparison to a static culture. In recent drug toxicity studies rat hepatocytes and glioblastoma cells were exposed to Diclofenac. Results showed that flow improved toxicity screening, with reduction in concentration of drug causing toxicity suggesting that concentration of drug administered *in vivo* was 10.2x greater (A.Ahluwalia 2011). Vinci et al. (2011) used the modular bio-reactor for primary human hepatocyte culture. The result confirmed previous studies showing that medium flow stimulates expression and activity of detoxification genes in hepatocytes (Vinci et al., 2011).

From the body of documented research using the Kirkstall system benefits of the system over static culture is evident. For these reasons, we suggest that the Quasi vivo® provides an ideal platform to study cell-cell communication studies under medium flow conditions.

1.11: Hypothesis

The tissue dynamics of healing wounds and growing tumors differ from normal tissue where cells such as fibroblasts experience a highly dynamic mechanical environment with increased matrix stiffness and changes in chemical signaling. Whilst the primary inducer of fibroblast-to-myofibroblast differentiation is Transforming growth factor β1 (TGF-β1) (Desmouliere et al., 1993, Evans et al., 2003), it is likely that fibroblasts may respond differently to TGF-β1 under the influence of fluid flow.

We hypothesize that fibroblasts from different tissue origin may be activated by physiologically relevant interstitial fluid flow like signals (Mazzei et al., 2008, Chary and Jain, 1989) and that their response may be modulated differently in the presence of chemical signals such as TGF-β1 and other signaling molecules of the TGF-β pathway.

1.12: Aims of the project

We aimed to study the effect of low levels of fluid movement to model interstitial fluid flow and chemical signals such as TGF-β1 on normal fibroblasts obtained from different tissue origin.

Specific objectives of project are

- To study changes in global gene expression profile of normal fibroblast subjected to fluid flow treatment and compare changes with those treated in static conditions.
- Examine the effect of flow on responsiveness of fibroblasts to TGF-β1by studying the expression of fibroblast activation markers in normal fibroblasts.
- Study the effect of fluid movement on fibroblast associated signalling mechanisms in both normal and TGF-β1 stimulated conditions.

We propose that an in-depth analysis of signaling mechanisms involved in fibroblast differentiation in a physiologically relevant environment that supports both mechanical signals such as interstitial fluid flow and chemical signals such as TGF-β1 may help towards gaining a better understanding of fibroblast behavior in tissues of different origin. Our project will add significantly to the literature on mechanotransduction biology of fibroblasts and will help towards isolating novel molecules which may be targeted for designing anti-fibrotic therapeutics. Further, there is also a potential to identify key genes which may be of use to indicate the effectiveness of skin and oral pharmaceutical agents.

Chapter 2-Materials and Methods

2.1: Cell culture.

Primary human oral fibroblasts, human dermal fibroblasts and human endothelial cells were routinely cultured as described below.

2.2: Fibroblasts.

Normal oral fibroblasts (NOF-320) were obtained from ethically approved waste tissues produced during oral surgical procedures undertaken at the School of Clinical Dentistry, Charles Clifford Dental Hospital, Sheffield, UK. Normal human dermal fibroblasts (NHDFC) were purchased from Promocell. Fibroblasts from both oral and dermal sources were cultured in DMEM (Life Technologies) supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma) and 2.5 mg/L of both penicillin and streptomycin (Sigma). For all experimental purposes, cells at passage numbers ranging from 3 to 5 were used. This study is approved by the University of Sheffield Ethics committee (Ref: 07/H1309/105).

2.3: Quasi-Vivo® bioreactor set-up.

The Quasi-Vivo® bioreactor (Figure 2.1) is composed of chambers of poly-dimethyl-siloxane (PDMS), a biocompatible silicone polymer interconnected in a series. External dimensions of Quasi-Vivo® chambers are 23 mm in height and 37 mm in diameter. Internal volume of each chamber is 2 ml and accommodates 12 mm diameter cover slips. The chambers are connected to a pump (Parker) which circulates media through the chambers. The inlet and outlet tubes of the series are connected to a reservoir bottle which contains minimal media (DMEM containing 0.5% (v/v) FCS).

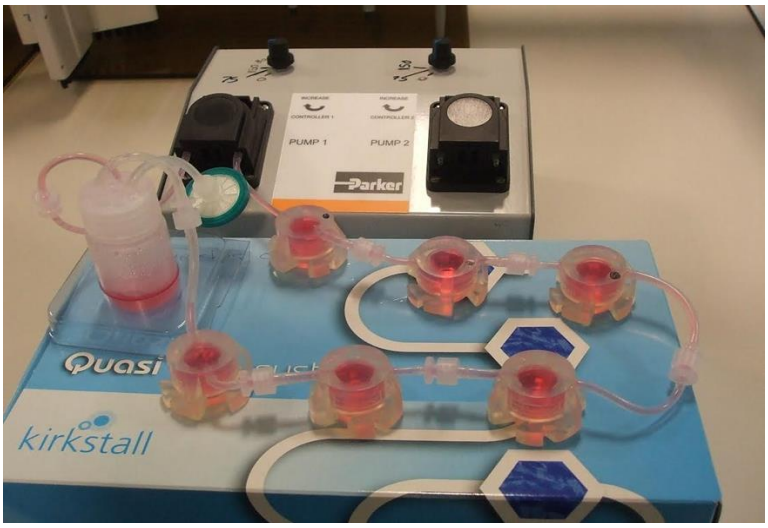

Figure 2.1: The Quasi-vivo® bioreactor set-up.

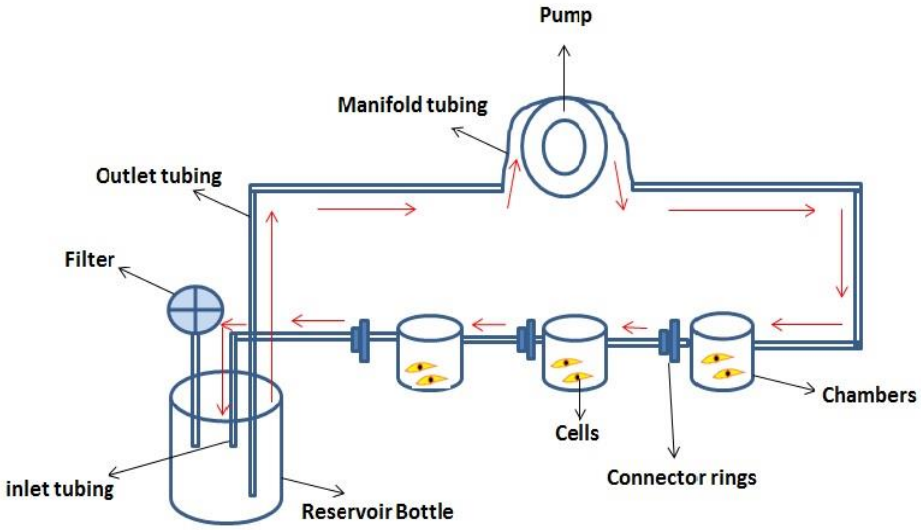

Figure 2.2: Schematic diagram of Quasi-vivo® set up. Red arrows indicate direction of medium flow. Black arrows indicate parts of the set up.

2.4: Preparing the system for use.

The initial set up involves connecting the chambers to connectors, manifold tubing and reservoir bottle as per configuration (Figure 2.2). To ensure consistency, inlet tubing was connected with female connectors and outlet tubing with male connectors. The reservoir bottle has three tubes connected to it, the longest tube being the outlet; while the shorter inlet tube brings in medium from last chamber of loop and finally the shortest tube is connected to a 0.2µm filter which permits gas exchange. The entire set-up is connected under aseptic conditions in a laminar hood. The connected system is then sterilised and prepared for use.

2.5: Disinfecting the Quasi-Vivo® bioreactor.

The chambers were disinfected by treatment with 70% ethanol for 20 mins, PBS for 20 mins and 0.5% serum containing medium for 20 mins. At the end of an experimental run, the chambers were first washed with PBS for 20 mins before sterilising.

2.6: Opening and closing the chamber.

The chambers were opened by gripping the bottom of the chamber between three fingers, and twisting the top of the chamber in an anti-clockwise direction and lifting the top. Chambers were closed by pushing the top chamber onto the bottom and twisting the top in a clockwise direction.

2.7: Determining optimal flow rate.

The system was calibrated by running sterile PBS at various pump settings and collecting the liquid output from the final chamber for a period of 1 minute. The volume of liquid collected

at each speed setting was measured and noted. The whole procedure was repeated three times and mean value was determined. Previous studies in our laboratory revealed that primary fibroblasts are resilient to flow rates from 10 – 150 µL/min. Hence in all our experiments, we used a flow rate of 150 µL/min for primary fibroblasts from both oral and dermal sources.

2.8: Treatment of coverslips.

Cells were plated on two types of cover slips (plain glass or on collagen coated Thermonox cover slips) depending upon experimental needs. Glass cover slips were placed in 24-well plates and sterilised with 70% ethanol prior to plating of cells. Sterile Thermonox cover slips were pre-treated with collagen coating (1:10 dilution; rat tail type I) for a minimum of an hour in 37ºC incubator in 5% CO_2.

2.9: Treating fibroblasts with fluid flow in the Quasi-Vivo® bioreactor.

Primary fibroblasts (55,000 cells/well) from both dermal and oral origin were counted and plated on six cover slips (plain glass or on collagen coated Thermonox cover slips) and allowed to attach in 37ºC incubator in 5% CO_2 for 48 h. Cover slips with cells were transferred carefully into three Quasi-vivo® chambers and 1 ml of minimal media (0.5% serum containing DMEM) was added into each chamber to ensure that cells remain hydrated until all the chambers are filled with the circulating medium. 12 mls of media was added into the reservoir bottle and the entire loop was connected to the pump which circulated minimal medium (0.1% FBS containing DMEM) at flow rate of 150µL/min. Cover slips placed in petridishes and treated with minimal media were used as static controls.

2.10: Stimulation of fibroblasts with TGF-β1.

Cells on cover slips were stimulated with 10ng/ml TGF-β1 (Human recombinant TGF-β1, R&D Systems) in 0.5% fetal bovine serum containing media. Static cultures were incubated with 0.5ml /well of TGF-β1 containing media. Cells subjected to flow were circulated with 18 ml of TGF-β1 containing media. The plates and bioreactor were returned to 37ºC incubator in 5% CO_2 for 24 hrs. Controls were treated with 0.5% fetal bovine serum containing DMEM media. Microarray based studies were pursued on dermal fibroblasts treated in static conditions and under flow in the absence of supplemented TGF-β1.

2.11: Preparation of conditioned medium.

Dermal fibroblasts (55,000 cells/well) were seeded on coverslips placed in a 24 well plate. After incubation for 48h., they were subjected to fluid flow (150µl/min) for 24h. Cells were also treated in static conditions for experimental controls. Medium that was conditioned on dermal fibroblasts that were subjected to fluid flow treatment and static conditions for 24h. was collected and concentrated by 5 times its total volume in centrifugal filter units (Amicon) by centrifugation for 20 mins at 1000 rpm. The concentrated conditioned medium was then used for study purposes.

2.12: Staining dermal fibroblasts for detecting α – smooth muscle actin (α-SMA) expression.

Static and flow treated (150µl/min) cover slips were removed from the chambers of bioreactor and placed in a 24-well plate along with the controls. Medium from the cells were removed and cells were washed twice with PBS. Cells were then washed with 0.5ml of 4 mM

sodium deoxycholate in PBS once and permeabilised in the same solution for 10 minutes. The cover slips were then blocked in 2.5% Bovine Serum albumin (BSA) in PBS for 30 minutes at room temperature followed by incubation with 0.2ml of FITC conjugated anti-alpha smooth muscle actin (α-SMA) antibody (Sigma, Clone1A.4, 1:100 dilution) for one hour at 37ºC. Cells treated with 2.5% BSA were used as a control. Cover slips were then washed twice with PBS and then mounted in DAPI containing antifade reagent (Sigma) and viewed under Plan Neofluar 100X magnification lens with numerical aperture 1.30 (oil immersion) in a fluorescent microscope (Zeiss Axiovert 200). Images were captured with Image proplus software, Version 7.0.1.

2.13: Quantification of cellular fluorescence by Image J.

Images of cells captured by imaging software Image proplus 7.0.1 were loaded on to Image J software and the fluorescence density of each image was determined. A total of three images were analysed for every condition and corrected total cell fluorescence (CTCF) was calculated in accordance with the following formula (McCloy et al., 2014).

CTCF= Integrated density - (selected area * mean florescence of background readings)

2.14: Immunoflorescence analysis of dermal fibroblasts under flow.

Static and flow treated (150µl/min) cover slips were removed from the chambers of bioreactor and placed in a 24-well plate along with the controls. Cells were washed with 1X PBS and fixed cells in 4% paraformaldehyde for 15 mins at room temperature. Coverslips were permeabilized in 0.5% Triton-X 100 for 15 mins. Coverslips were then blocked in 1%

BSA in PBS overnight at 4^0C and incubated with various primary antibodies (Table 2.1). Primary antibodies were detected by FITC conjugated secondary antibodies and slides were mounted DAPI containing antifade reagent (Sigma) and viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging software proplus 7.0.1).

Antigen	Manufacturer	Application
Monoclonal anti-actin, α-smooth muscle - FITC (αSMA) antibody produced in mouse (clone 1A4, F3777)	Sigma-Aldrich	1:100 for IF
Rabbit polyclonal anti-caveolin-1 (ab 2910)	Abcam	1:100 for IF
Rabbit monoclonal anti-Smad3 phospho S423 + S425 antibody (ab52903)	Abcam	1:100 for IF, 1:1000 for WB
Rabbit polyclonal transforming growth factor beta receptor Type II (TGFβRII: L-21: SC-220)	Santa Cruz Biotechnology	1:50 for IF
Mouse monoclonal early endosomal antigen-1 (610456)	B.D Biosciences	1:100 for IF
Anti-rabbit HRP-linked antibody (7074)	New England Biolabs	1:3000 for WB
Donkey anti- rabbit alexa flour CY3 conjugated secondary antibody	Molecular probes	1:200 for IF
Donkey anti- mouse alexa flour FITC conjugated antibody.	Molecular probes	1:200 for IF
Monoclonal anti β actin produced in mouse (A1978)	Sigma	1:10,000 for WB
Anti-mouse HRP conjugated secondary antibody (7076)	Cell signalling	1:3000 for WB
TGF-β1 antibody (aa22-50) LS-C161825	Lifespan Biosciences	1:50 for IF

IF: Immunofluorescence; WB: Western Blot

Table 2.1: Table of antibodies used for immunolabelling studies.

2.15: X-gal staining.

Static and flow treated (150µl/min) cover slips were removed from the chambers of bioreactor and placed in a 24-well plate along with the controls. Cells were washed with 1X PBS and fixed in the fixative provided in the SA-βgal Staining kit (Abcam) for 20 minutes at room temperature. The coverslips were washed with 1X PBS and stained with 20mg/ml of X-gal dissolved in 1x staining solution provided with the kit and incubated overnight at 37ºC. Stained coverslips were visualised under 20X magnification using an inverted microscope (Olympus CKX41). Images were captured and the total number of stained and unstained cells were counted by Image J software.

2.16: pH RODO Dextran staining.

Static and flow treated (150µl/min) cover slips were removed from the chambers of bioreactor and placed in a 24-well plate along with the controls. Cells were washed with 1X PBS and replaced with live cell imaging solution (Molecular probes) containing pHrodo dextran (20µg/ml) for 15 minutes at 37ºC. Cells were washed with live cell imaging solution. Slides were mounted DAPI containing antifade reagent (Sigma) and viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging software proplus 7.0.1).

2.17: Extraction of RNA from fibroblasts.

Total RNA was extracted using the Isolate II RNA mini kit (Bioline) according to the manufacturer's protocol. Briefly the cells were ruptured in the lysis buffer and homogenised

by pipetting up and down. After homogenisation, 70% ethanol was added to the lysate which was further processed through a spin column to which the RNA binds. Additional step of incubating samples with RNase free DNase I eliminates contamination by genomic DNA. Impurities like salt metabolites and cellular components are removed by subsequent washing with different buffers. High quality purified total RNA is finally eluted with RNase –free water.

2.18: cDNA synthesis.

The concentration of the RNA obtained was measured by Nanodrop (ND-1000) Ultra violet visible spectrophotometer (Thermo scientific) at wavelength 260nm/280nm using ND-1000, version 3.7.0 software. A ratio value range from 1.8-1.9 indicates purity of sample. 100ng of pure total RNA was used for cDNA synthesis using Applied Biosystem's 100mM dNTP's (0.8µl), Multiscribe reverse transcriptase (50U/µl;1.0µl), random primers (2.0µl), Reverse transcriptase buffer (2.0µl) and nuclease free water (according to the volume of RNA) to make up a reaction volume of 20µl . The tubes were placed into a thermal cycler (DNA engine DYAD) at 25ºC for 10 minutes, 37ºC for 2 hours, and 85 º C for 5 mins and then held in 4ºC.

2.19: Quantitative PCR for gene expression studies.

The cDNA (0.5µl) was added to qRTPCR mastermix containing 1x SYBR green mixture (Applied Bio systems) with 0.25 µM gene specific primers for α-smooth muscle actin (α-SMA), Collagen IA1 (Col1A1), activin A (INHBA), CD109, Transforming growth factor –β receptor type II (TGFβRII) and Angiopoietin like 4 (ANGPTL-4) (Table 2.2). Assays were performed in triplicates on a 7900HT Fast real time PCR machine (Applied Biosystems) SDS

version 2.4 was used to programme the real time device (95ºC for 15 secs, 60ºC for 60 seconds and 95ºC for 15 seconds) for 40 cycles and RQ Manager version 1.2.1 was used to record the CT values. The values were determined from the software and the delta CT values with reference to endogenous controls (U6) were calculated manually.

Gene	Forward	Reverse
U6	5' CTCGCTTCGGCAGCACA 3'	5′ AACGCTTCACGAATTTGCGT 3'
α-SMA	5' GAAGAAGAGGACAGCACTG3'	5'TCCCATTCCCACCATCAA3'
Collagen 1A1	5'GTGGCCATCCAGCTGACC 3'	5' AGTGGTAGGTGATGTTCTGGGAG 3'
INHBA	5'CCCCTTTGCCAACCTCAAA 3'	5'CATGGACATGGGTCTCAGCTT 3'
CD109	5'AGCTGCTCAAGACAGCATCA3'	5'TTGGGGTCTGATGGAAGAGT3'
ANGPTL-4	5' CTGCAACCAAGCGGGTCTTA3'	5'TCTTAGGTAGCCTGGGAGCG3'
TGFβRII	5'AGTCGGATGTGGAAATGGAGG3'	5'GGAAACTTGACTGCACCGTTGT3'

Table 2.2: Gene expression primers used for RT-PCR.

2.20: Microarray analysis.

RNA was obtained from dermal fibroblasts treated with 75µL/ min and 150µL/ min flow obtained from three different experiments. Linear amplification of the RNA was performed using The GeneChip® 3' IVT Express Kit (Affymetrix) according to the manufacturer's guidelines. Briefly, 200ng mRNA was reverse transcribed using an oligo (dT) primer and then converted to double-stranded cDNA containing a T7 polymerase promoter site. Linear amplification was driven by a T7 promoter for 16 hours at 40ºC and generated labelled aRNA using biotinylated dUTPs. 15µg of aRNA was fragmented in buffer containing Mg2+, at 94 0C for 30 minutes and analysed using an RNA 6000 Nano Chip on the 2100 Bioanalyzer

(Agilent). Hybridisation was carried out using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix). Briefly 12.5 µg of fragmented aRNA was added to the mastermix solution containing pre-labelled hybridization controls (bioB, bioC, bioD, and cre genes) and positive oligonucleotide B2 control (B2 oligo). After 16 hours hybridisation at 42ºC with rotation at 60 rpm, the GeneChips were washed and stained in a GeneChip Fluidics Station 450 (Affymetrix) as per the protocol prescribed in GeneChip® Expression Wash, Stain and Scan User Manual. Scanning was carried out on a GeneChip Scanner 3000 7G (Affymetrix). The scanned image files (.CEL) were processed using the Expression Console software (Affymetrix) for quality control checks and to generate an RMA normalised dataset of the signal intensity for each probeset. Differential gene expression was determined using the Qlucore 'omiics explorer package (Qlucore). DAVID Gene Functional Classification Tool was used to identify genes on Go Term & KEGG pathway maps.

2.21: Western Blot Analysis.

Protein samples were extracted in RIPA lysis buffer containing protease and phosphatase inhibitors (Roche). Protein concentration in each sample was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). 20 µg of total protein extract were reduced in Sample Reducing Agent (10x), loaded with LDS Sample loading buffer (4X) (Nupage) and electrophoresed in 12-15% (v/v) polyacrylamide gels along with EZ-RunTM Prestained Rec Protein Ladder (Fisher Bioreagents). Protein was transferred to a 0.2 µm nitrocellulose membrane (ProTran, GE Healthcare using Trans-Blot® Turbo™ Transfer System (GE Healthcare). The membranes were blocked in 5% Bovine Serum albumin in 1X TBS containing 0.1% tween-20 overnight at 4°C. Membranes were incubated with Rabbit monoclonal Anti-Smad3 phospho S423 + S425 antibody for 1 h at room temperature. Nitrocellulose membranes were washed three times in TBS-Tween and then incubated with

HRP conjugated anti-rabbit secondary antibody for 1 hr. at room temperature and washed three times in TBS-T. Protein bands were visualized using a chemiluminescence detection kit (Pierce ECL Western Blotting Substrate) and developed using XPosure film and X-Ray automatic processor. In order to determine loading efficiency, membranes were routinely stripped with Restore™ Western Blot Stripping Buffer (Thermo Scientific), blocked in TBS Tween for 1 hr. and reprobed with β-actin antibody for 1 hr. Membranes were washed three times with TBS Tween and probed with HRP conjugated secondary antibody for 1hr. The membranes were visualised as described above.

2.22: Detection of TGF-β1 by Enzyme linked immunosorbent assay.

Cell lysate samples was obtained using RIPA lysis buffer containing protease inhibitors & protein concentration was determined as above. TGF-β1 protein was detected in the samples using the TGFβ1 Emax ® ImmunoAssay System according to manufacturer's instructions in a 96-well plate. The plate was coated with TGFβ Coat mAb (provided in kit) and incubated overnight at 4°C. It was then blocked with 5X TGFβ block buffer for 35 minutes at 37°C. Standard curve was prepared by serially diluting the TGF-β1 standard six times in 1X sample buffer. Protein samples were also diluted in the sample buffer and added to the plate. The plate was incubated in the shaker for 1.5 hrs at room temperature. After several washes to remove the unbound protein, 100µl of TGFβ HRP conjugate was added to each well and incubated with shaking for 2 h. at room temperature. 100µl of TMB one solution was added to each well to and incubated without shaking for 15 minutes at room temperature. The reaction was stopped with 100µl of 1N hydrochloric acid. Optical density of each well was using a microplate reader (Tecan) set to absorbance at 450 nm with wavelength correction set

to 570 nm. Absorbance values of the standard curve was plotted on Graphpad prism 7 and total concentration of the protein was estimated by interpolating absorbance values of the test samples.

2.23: Detection of activin A by Enzyme linked immunosorbent assay.

Cellular lysate samples was obtained using RIPA lysis buffer containing protease inhibitors and conditioned medium was obtained from fibroblasts subjected to treatment by fluid flow and static conditions. Samples were also obtained from fibroblasts stimulated with TGF-β1. Prior to adding the protein samples to the streptavidin coated 96-well plate provided in the Quantikine Elisa kit (R&D systems), activin A biotinylated antibody was pipetted into the wells of the plate and incubated for 15 mins at room temperature on an orbital shaker set at 500 rpm. Activin A standards were serially diluted ranging from 1000pg/ml to 15.6pg/ml from the standard provided in the kit. Protein samples were diluted in 100 µl of Assay diluent RD 1-98 (provided in kit) and added to each well. The plates were incubated for 3 h. at room temperature on an orbital shaker set at 500 rpm. Following incubation, plates were washed with wash buffer and incubated with 200 µl of activin A conjugate for 1h. The plate was washed again with wash buffer and 200 µl of substrate solution was added on to the plate. After 30 mins incubation the colour change was stopped by adding 50 µl of stop solution to each well. Optical density of each well was using a microplate reader (Tecan) set to absorbance at 450 nm with wavelength correction set to 570 nm. Absorbance values of the standard curve was plotted on Graphpad prism 7 and total concentration of the protein was estimated by interpolating absorbance values of the test samples.

2.24: Statistical analyses.

Statistical significance of data was tested with one-way ANOVA with multiple comparisons' test using Graph Pad Prism 7.0.

Chapter 3- Optimising cell number and coating methods for use in QV Bioreactor.

3.1 Introduction.

Cellular engineering is an interdisciplinary field which involves culturing primary cells *in vitro* on surfaces that are compatible for optimal growth, differentiation and proliferation prior to using them for various applications and testing purposes. We know that 2 dimensional cell culture does not represent a physiologically relevant cellular environment wherein cells are not only close to each other, but are also mechanically supported by matrix associated proteins and exposed to physical and chemical signals (Jorgensen and Tyers, 2004). Hence these cells that are removed from their natural environment display preferential attachment only to chemically modified surfaces or those that are coated with matrix proteins. It should be noted that surface chemistry associated factors such as stiffness, roughness, microstructure, porosity, surface area, mechanical properties and chemical composition of the surface are known to influence cellular behaviour such as adhesion, growth and differentiation of cells (Wang, 2011). When cells attach to an artificial surface, numerous physico-chemical reactions take place between cell and its surface. Cells attach to their surface and release active compounds for signalling, proliferation and differentiation. Hence in order to attain optimal cellular development, it is vital to understand the factors that influence the adhesion of cells. Some of the known factors that affect focal adhesion of cells are surface hydrophobicity, protein adsorption, surface charge, roughness and stiffness (Wang, 2011). For example, mouse osteoblast-like cell line MC3T3-E1 displayed a fractured morphology on hydrophilic surfaces (Jianhua et al., 2009). A similar phenomenon was observed in neuronal cell spreading on surfaces with reduced hydrophobicity (Khorasani et al., 2008). Hydrophobic surfaces are known to adsorb more proteins whereas hydrophilic surfaces resist adsorption (Xu and Siedlecki, 2007). However, Tamada et.al reported that water wettability does not influence cell adhesion (Tamada and Ikada, 1993). They observed that preadsorption of serum albumin prevented fibroblast adhesion to substrates whereas

fibroblast adhesion was enhanced by preadsorbed fibronectin. Other than hydrophobicity and protein adsorption, cell adhesion is also influenced by surface charges. Studies have shown that the amount of surface charges can also influence cell behaviour (Ishikawa et al., 2007). For example, chondrocytes exhibited enhanced differentiation with increased expression of collagen and glycosaminoglycan expression when cultured on negatively charged oligo polyethylene glycol hydrogel scaffolds compared to neutral or positively charged hydrogels (Dadsetan et al., 2011).

Surface roughness and stiffness are other factors that influence cell adhesion and behaviour (Wang, 2011). Numerous studies have reported that cells that grow on micro-rough surfaces demonstrate enhanced differentiation. For instance, foetal osteoblasts (hFOB 1.19) showed increased spreading and proliferation on rough surfaces but the cellular response to roughness is dependent on the individual cell type (Lim et al., 2005). For example, osteoblastic cells (MC3T3-E1) proliferated at a higher rate on smoother regions (0.55nm) of dendrimer films than on rougher (13nm) regions (Washburn et al., 2004). Stiffness on the other hand is known to be a crucial determinant of cellular differentiation as is observed in fibroblasts. Tan and Teoh observed that 3t3 fibroblasts preferred soft surfaces for proliferation (Zein et al., 2002). Another group used a rheometer to measure adhesion of kidney epithelial (MDCK) cells on surfaces with different softness (O'Brien et al., 2005). Cellular adhesion was seen to increase with decreasing substrate stiffness and this correlated to the area of cell spreading. These observations suggest that properties such as the hydrophilic nature of the surface, composition of the adsorbed protein, surface roughness and stiffness are major factors contributing towards nature of cellular behaviour when they contact different surfaces.

One approach to encourage cells to grow on artificial surfaces is to seed them on glass or plastic surfaces that are pre coated with proteins which resemble matrix proteins. Classically, most primary cells are cultured in tissue culture flasks made of polystyrene. However cells that require special support to attach to the surface can be seeded on surfaces coated with proteins such as poly-lysine, fibronectin, gelatin, and collagen.

Poly-Lysine is a poly-cationic molecule that binds to negatively charged proteins and DNA. It promotes the electrostatic interaction between glass surface and negatively charged membrane bound ions. In other words, it increases the number of positive charges available for binding on the surface of the coverslip. Poly-Lysine does not occur naturally and can be synthesized in 'L' and 'D' forms (Sitterley G, 2008). However, it should be noted that Poly-L-Lysine can be digested by some cells in which case Poly-D-Lysine could be used (Shima et al., 1984). This molecule has been used in culturing neuronal cell lines, glial cells, prostate cancer (PC12) cells etc.

Fibronectin is a high molecular weight glycoprotein which promotes cellular attachment through the association of its cell binding domain sequence to membrane spanning receptor proteins called integrins (Pankov and Yamada, 2002). Endothelial cells, smooth muscle cells, human myeloma cells, Chinese hamster ovary (CHO) cells, fibroblasts and neuroblastoma cells are few of the cell types which attach to fibronectin (Schwarz and Juliano, 1984, Lewandowska et al., 1987).

Collagen is the main extracellular matrix protein associated with the extracellular space of connective tissues of animals and promotes cellular adherence and growth. To date about 28 types of collagen have been identified (Sherman et al., 2015). However, Collagen type I is

the type which is often used for coating coverslips and slides since this form constitutes over 90% of total collagen in body and is readily available commercially. Human umbilical vein endothelial cells (HUVECs), CHO cell lines, muscle cells, fibroblasts are compatible with collagen coated artificial surfaces (Saunders and Hammer, 2010, Gaudet et al., 2003).

3.2. Dermal and oral fibroblasts can attach on glass surfaces.

This chapter describes experiments carried out to optimise the cell number and coverslip coating conditions for optimal growth and proliferation of fibroblasts in the Quasi-vivo® (QV-500) bioreactor.

3.2.1 Methods.

Two types of cover slips (glass & Thermanox) both of 12mm diameter were used to optimise coating method for optimal attachment of cells. Cover slips were treated with different coating proteins namely collagen type I from rat tail (0.2mg/ml, Sigma), fibronectin (0.2mg/ml, Sigma) and poly-l-lysine (0.2mg/ml, Sigma) for 1 hour in 37 ºC incubator. Primary oral fibroblasts (75,000 cells) were plated on cover slips coated with different proteins and incubated at 37 ºC for 24 hours. Cells were also plated on uncoated sterilised glass and Thermanox cover slips as controls. Bright field images were captured using a NIKON Eclipse TS100 camera. Images were analysed by Spot Software Version 5.1.

To optimise cell numbers to be seeded per well, primary dermal fibroblasts (NHDFC) were seeded at varying cell numbers (3125; 6250; 12,500; 25,000; 50,000; 100,000 cells/well) and plates were returned to 37ºC incubator in 5% CO_2 for 24 hrs. Before the end of the incubation period, bright field images were captured using a NIKON Eclipse TS100 camera. Images

were analysed by Spot Software Version 5.1. Approximately 2 h. before the end of the incubation period, cells were treated with CellTiter 96® AQueous One Solution (Promega) as per the manufacturer's instructions and the absorbance was determined at 490nm.

3.2.2 Results

Results revealed that fibroblasts prefer to attach to plain glass cover slips or on collagen coated Thermanox cover slips (Figure 3.1). Whilst they attach to fibronectin coated surfaces, cell spreading and morphology was altered on Poly-L-Lysine coated surfaces. Cells did not attach to ECM protein modified glass surfaces, fibronectin coated glass being an exception. Bright field images (Figure 3.2) and results from CellTiter 96® AQueous One Solution assay (Figure 3.3) suggested that cell numbers greater than 50,000 cells/well would enable optimal spreading and metabolic activity of NHDFC cells.

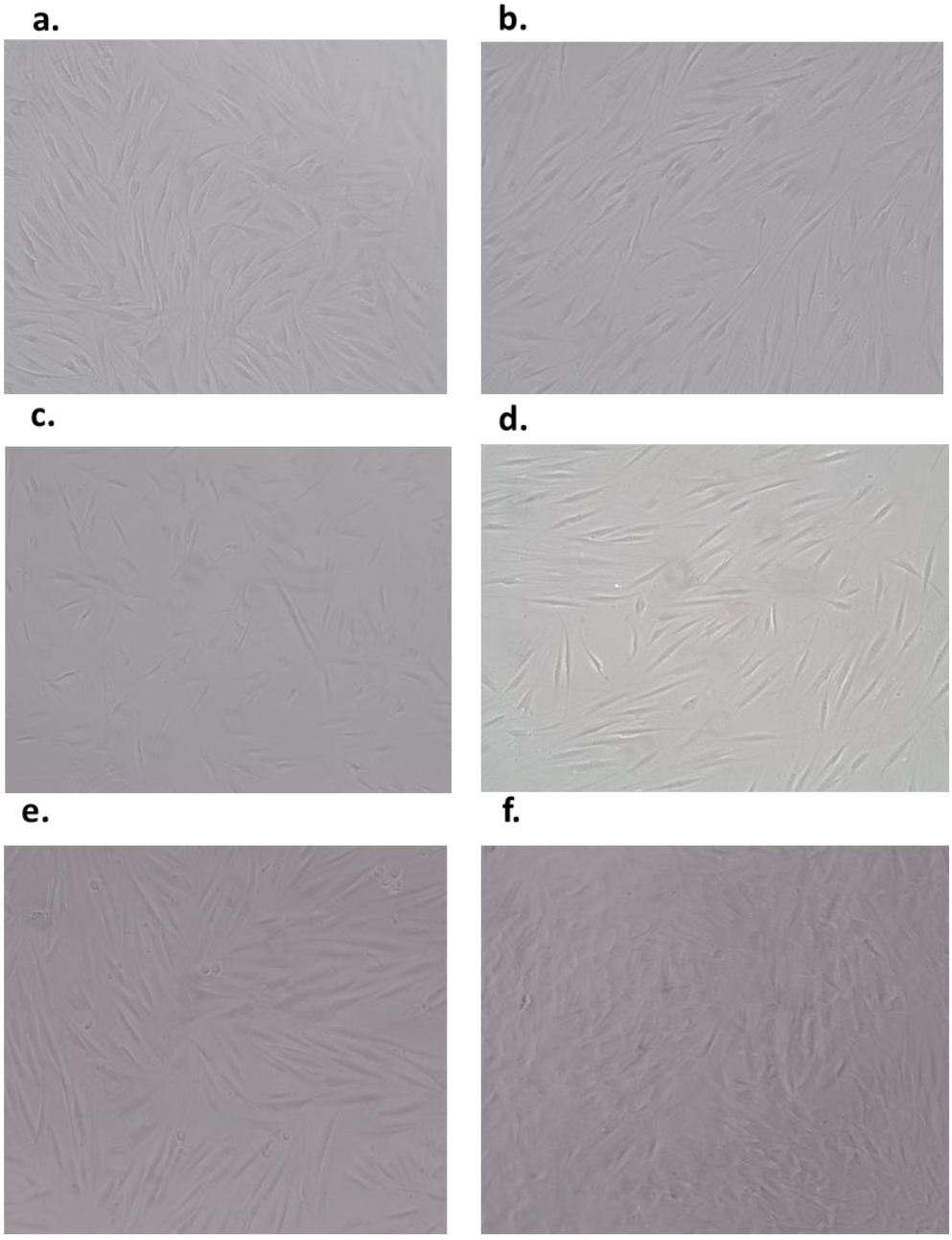

Figure 3.1: Representative images of oral fibroblasts (NOF) (n=3) cultured for 24h. on coverslips coated with different proteins. a) On plain glass; b) on collagen coated Thermonox; c) on fibronectin coated glass; d) on Poly-L-lysine coated glass; e) on fibronectin coated Thermonox; f) on Poly-L-lysine coated Thermonox. Images were captured under 20X magnification lens on an inverted bright field microscope (Olympus CKX41)

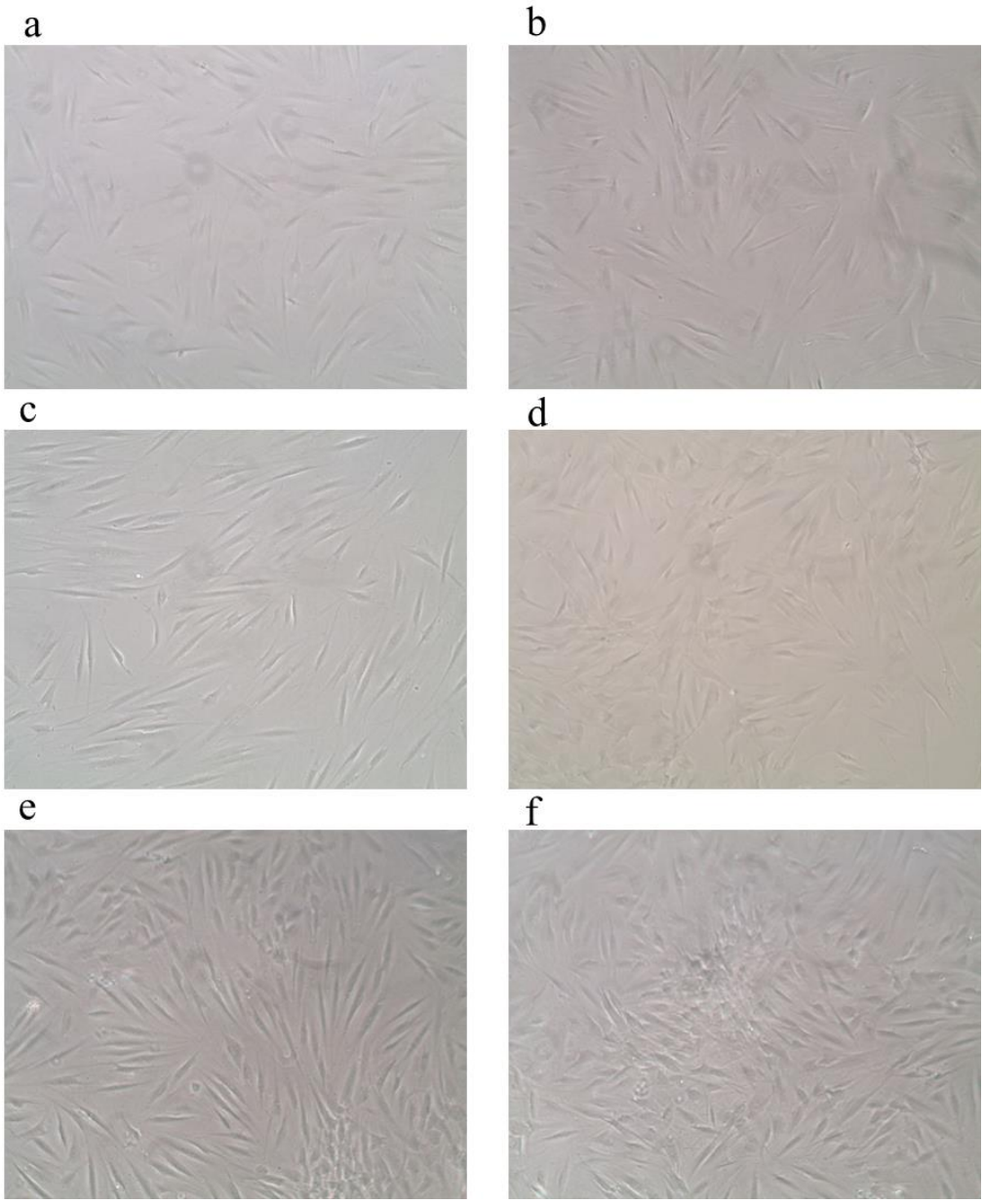

Figure 3.2: Representative images of dermal fibroblasts (NHDFC) (n=3) cultured for 24h. on coverslips seeded on uncoated glass coverslips at different cell numbers a) 3125; b) 6250; c) 12,500; d) 25,000; e) 50,000; f) 100,000 cells/well. Images were captured under 20X magnification lens on an inverted bright field microscope (Olympus CKX41)

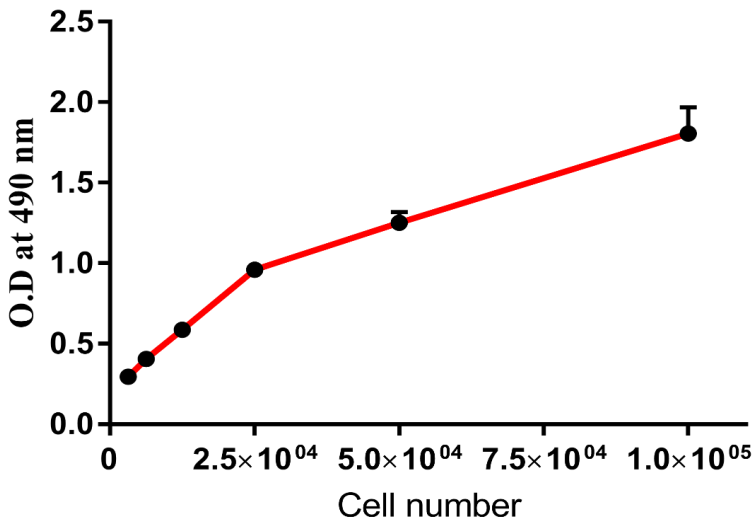

Figure 3.3: Metabolic activity of dermal fibroblasts (NHDFC) on coverslips seeded on uncoated glass coverslips at different cell numbers (3125; 6250; 12,500; 25,000; 50,000; 100,000 cells/well) and treated in static conditions.

3.3: Fibroblasts respond to low levels of fluid flow.

3.3.1 Method.

Fibroblasts (NHDFC's) were plated on coverslips for 24hr. and treated with minimal media at a flow rate of 150 µl/min. At the end of the incubation period, cells were imaged under microscope. Bright field images were captured by NIKON Eclipse TS100 camera. Images were analysed by Spot Software Version 5.1.

3.3.2 Results.

Cells subjected to 150 µl/min remained attached to cover slips and appear stretched in comparison to cells under static conditions (Figure 3.4). Very few cellular debris were also observed.

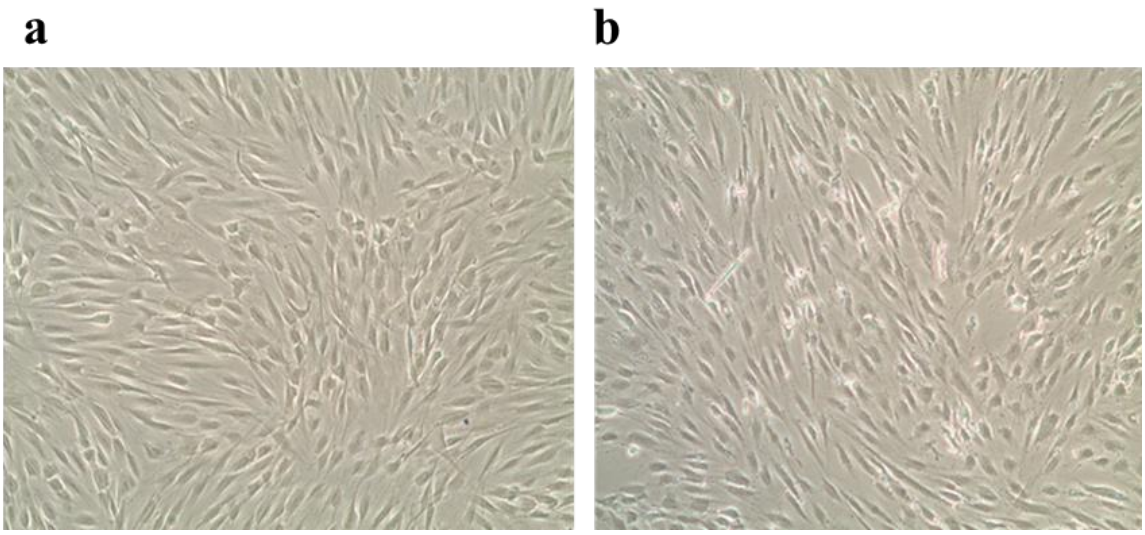

Figure 3.4: Representative images of dermal fibroblasts (55,000 cells/well) (n=3) when subjected to flow (150 µl/min) for 24h. A) Static controls B) flow. Images were captured under 20X magnification lens on an inverted bright field microscope (Olympus CKX41)

3.4: Cellular metabolic activity of fibroblasts is altered at varying flow rates.

3.4.1 Method.

Coverslips with cells (NOF'S and NHDFC's) were transferred carefully into the Quasi-vivo® chambers and 1 ml of minimal media (0.5% serum containing media) was added into each chamber to ensure that cells remain hydrated until all the chambers are filled with the circulating medium. 11mls of media was added to the reservoir bottle and the entire loop was connected to a pump. The pump was run at flow rates 75µL/min and 150µL/min for a period of 24hrs. Control cells were subjected to static culture conditions wherein the cover slips were placed in wells of a 24-well plate and treated with minimal media. The plate was returned to 37ºC incubator in 5% CO_2 for 24 hrs. Approximately 2 hours before the end of

incubation, cover slips from the chambers was transferred into a 24-well plate and Cell Titer 96® AQueous One Solution (Promega) was added. The absorbance was measured at 490nm after 2hrs. Experiments were performed three times on three coverslips per experiment and mean values were plotted.

3.4.2 Results.

Metabolic activity of primary fibroblasts cells from both sources (Dermal and Oral) are reduced at both flow rates of 75µL/min and 150µL/min (Figure 3.5).

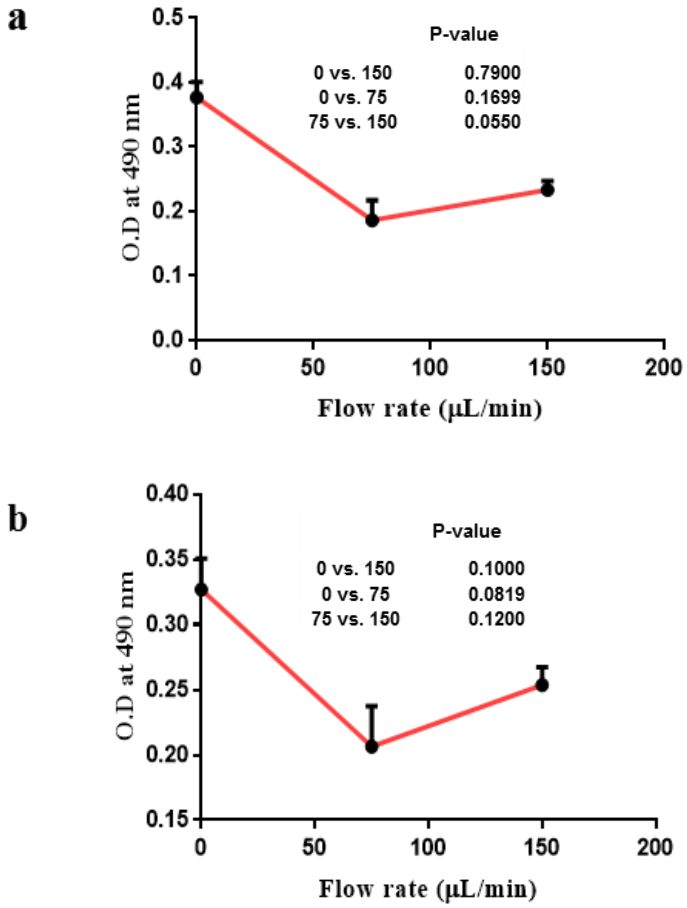

Figure 3.5: Medium flow alters cellular metabolic activity of Primary fibroblasts a) dermal (NHDFC) and b) oral (NOF320) at flow rates 0µL/min, 75µL/min and 150µL/min for 24h. (n=3) (Mean ± SEM).

3.5 Discussion.

Cellular attachment to modified surfaces is particularly important during culturing of cells as poor attachment can lead to apoptosis of unattached cells. Pre-coating of plastic and glass surfaces with ECM proteins to enable optimal attachment of cells has been a common practice in tissue culture (Sherratt et al., 2005). The purpose of these optimisation experiments was to assess a range of ECM proteins for their efficacy in encouraging attachment and growth of primary human fibroblast cells and to study the effect of fluid flow on the metabolic activity of fibroblasts.

3.5.1 Role of surface chemistry and coating proteins on cellular attachment

Collagen is the main structural component of the connective tissue (Di Lullo et al., 2002). It provides the tensile strength of the extracellular matrix (ECM). It is found in abundance in cartilage, bones, blood vessels, intervertebral discs and the dentin in teeth. The first evidence of its role in promoting cell attachment came when George Gey (1956) coated rat tail collagen on glass surfaces. He observed that collagen coating improved cellular attachment and growth. Clinical studies reveal that in the presence of collagen dressings, healthy granulation tissue is able to form in skin burns (Singh et al., 2011). Owing to properties like stability, permeability, hydrophilicity and pore size, collagen scaffolds serve as ideal matrices for deposition of cells. Once human dermal fibroblasts are inserted in these scaffolds, growth continues as normal (Hadjipanayi et al., 2009). Our results complement these findings in that fibroblasts attach well on Thermonox surfaces coated with matrix attachment proteins like collagen I. Fibroblasts preferred to attach to plain glass cover slips or on collagen coated

Thermonox cover slips whereas they failed to attach on glass surfaces which were modified with ECM proteins.

Thermanox coverslips are made of polyester polymer which is highly resistant to most chemicals, alcohols, aldehydes, hydrocarbons, dilute acids (<10%) and dilute alkalis (<2%) and has a low oxygen content. These coverslips offer several advantages of being easy to handle and being packaged in sterile conditions. It is significant to observe that while glass and plastic growth surfaces are flat and optically clear they differ in many ways which affects their performance in various applications. Cellular attachment and spreading on these two growth surfaces may be affected by the chemical composition of surface material.

Glass cover slips are made of German borosilicate glass and offer a hydrophilic surface which enables cells to attach even in the absence of attachment enhancement proteins. Other than fibronectin coated glass coverslips, fibroblasts failed to attach on surfaces coated with Collagen I and Poly-lysine. This suggests that surface chemistry of plain glass is well suited for optimal growth of fibroblasts. However due to technical limitations owing to the fragile nature of cover slips, we considered using modified Thermanox cover slips.

Further, results from optimisation experiments also revealed that cells spread and attach well on collagen coated Thermanox cover slips. Although, cells attached well on Thermanox coverslips coated with fibronectin and poly-L-lysine, cells did not spread uniformly throughout the coverslip. Nonetheless, the Thermanox cover slips had their limitations. Although, they are flat and clear under a bright field microscope, these were not compatible for immunofluorescence studies. It was difficult to image cells under a fluorescent

microscope as the image quality was poor. Hence it was decided to use plain glass cover slips for detection of proteins by immunofluorescence.

It was decided to use a cell seeding density of 55,000 cells per well for reasons that cellular spreading was more uniform over the coverslip, although some cells clustered at the centre of the coverslip at all seeding densities. This clustering almost certainly relates to surface tension forces between the culture medium and plastic culture flasks. It is possible that cells spread more uniformly and that cellular clustering is not likely to occur in 3 dimensional cultures which resemble the *in vivo* environment more closely.

Whilst a strong body of evidence shows that endothelial cell alignment along the direction of laminar fluid flow defines morphological feature of vascular homeostasis (Steward et al., 2015, Wang et al., 2013), fibroblasts also align along the direction of interstitial fluid flow (Ng and Swartz, 2003) and myofibroblast differentiation and collagen alignment along the direction of flow has also been observed (Ng et al., 2005). Subsequent findings showed that fibroblasts, endothelial cells and tumour cells restrict their migration to the direction of interstitial flow in a microfluidic cell culture system (Polacheck et al., 2011). However, it was not obvious from our experiments that dermal fibroblasts aligned along the direction of flow. This may relate to the design of the flow chambers which induce a gradient of shear stress which is maximum at the base of the chamber and the lowest at the edges of the coverslips. These gradients of shear stress may in turn influence cellular alignment as is observed in human mesenchymal stem cells which displayed differentiation and cellular alignment only in flow-induced shear stresses of 0.10 and 0.25 Pa (Jeon et al., 2014).

3.5.2 Fibroblasts respond to medium flow conditions by altering cellular activity

From prokaryotes to higher level organisms, survival is based on methods in which the organism responds to environmental pressures (Farge, 2011). All cells in the human body are subjected to forces acting on them due to breathing, movement and venous blood flow. Almost all cells in multicellular tissues undergo a myriad of forces such as compressive, tensile, fluid and hydrostatic forces each of which play a critical role in the shaping, development and maintenance of the tissue homeostasis (Wozniak and Chen, 2009).

Several studies suggest that stress mediated by fluid flow plays a crucial role in maintaining basic cellular functions. Resnick et al (2011) reported that fluid flow modifies the transepithelial sodium current, cell proliferation, and the actin cytoskeleton in renal epithelium. He aimed to explore whether differentiated epithelial monolayers are sensitive to the flow conditions and whether flow can alter mechanosensation by the cells after differentiation has occurred. Results confirmed that cells differentiated in the presence of flow and displayed a graded response to flow. Changes in flow induced cells to hyperproliferate. Furthermore, the actin cytoskeleton underwent extensive remodelling in the presence of flow. These findings threw light on the role of fluid flow acting via primary cilia to alter epithelial physiology and progression of autosomal dominant polycystic kidney disease (ADPKD) (Resnick, 2011).

Forces acting on and within a cell are defined as stress. Stress is force per unit area and is represented in Newton's metre^{-2} or Pascal. The Quasi Vivo® bioreactor was designed to induce low shear stresses and mimic fluid flow in cells and in tissues. The apparatus permits flow of fluid on cells at set velocities over stipulated intervals of time. Estimation of surface

shear stress values for healing and cancerous tissues exposed to interstitial flow are difficult to calculate, but are relatively low in comparison to those experienced in the vasculature which equates to 4.0 to 30.0 dyn cm^{-2} in the arterial circulation. For interstitial flow rates of 1x 10^{-6} ms^{-1} in tumour cells, one study estimated a fluid shear stress range of 7 x 10^{-3} – 1.5 x 10^{-2} dyn cm^{-2} (Pedersen et al., 2007) which is in orders of magnitude lesser than the maximum maximal flow rates set in the QV-500 bioreactor. Shear stresses in blood vessels aorta and at arterial blood flow separation regions are ~40-50 dynes cm^{-2} (Ku et al., 1985) and during episodes of increased cardiac output it is ~ 100 dynes cm^{-2} (Nerem et al., 1993). Based on studies which characterised the multi compartmental modular bioreactor (MCmB) (Mazzei et al., 2010), the prototype of the Quasi vivo® (QV-500) bioreactor, we estimated that shear stresses produced at a fluid flow rate of 75 & 150 µl/min were 1.8 x 10^{-5} dynes cm^{-2} and 3.6 x 10^{-5} dynes cm^{-2} respectively. The flow velocities generated at a fluid flow rate of 75 & 150 µl/min were 3 x 10^{-7} m/s and 5 x 10^{-7} m/s respectively. Based on previous literature which shows that average interstitial flow velocities in normal and neoplastic tissues is 6 x 10^{-7} m/s (Chary and Jain, 1989), it can be concluded that flow velocities in the same range as those experienced by tumour tissues. Although, fluid flow treated fibroblasts from oral and dermal sources do not display major differences in metabolic profile, dramatic changes can be observed in their global gene expression profile as discussed in chapter 4.

Results from this study conclude fibroblasts of both oral and dermal origin respond to low levels of fluid flow. When fibroblasts were subjected to flow conditions with medium flow rate of 150µl/min they appeared elongated and stretched. It appears that fluid flow induced by the Quasi-Vivo® bioreactor's pump activates the fibroblasts to secrete contractile proteins and initiate cellular signalling mechanisms. This supplements previous studies which claim that the Quasi-Vivo Bioreactor mimics fluid flow in tissues (Vinci et al., 2011). Appearance

of cellular debris suggests that some cells which couldn't withstand flow detached from surfaces of cover slips and started to circulate in the medium. Further analysis of the circulating cells would indicate whether these circulating cells have detached and acquired activation markers like α-SMA, Collagen IA (Pellegrin and Mellor, 2007) or are just dead cells. Notably, fibroblasts from both oral and dermal sources behave in a similar fashion when subjected to varying flow rates.

3.5.3: Cellular activity of primary oral and dermal fibroblasts is altered during flow conditions.

The CellTiter 96 AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt; MTS] and an electron coupling reagent (phenazineethosulfate; PES). This compound is bio reduced by cells into a coloured formazan product and is soluble in tissue culture medium. Tetrazolium dye is reduced in presence of NAD (P) H-dependent oxidoreductase enzymes which are largely present in the cytosolic compartment of the cell. Therefore, reduction of MTS reflects upon cellular metabolic activity due to NAD (P) H flux. Both oral and dermal fibroblasts displayed a reduction in cellular activity when subjected to varying flow rates (75 µl/min and 150 µl/min). In order to understand whether this reduction in cellular activity resulted due to reduced mitochondrial activity or due to loss of cells, we counted the total number of cells present at the end of experiment. Reduction in cellular activity may be due to loss of cells which probably detach from the coverslip and circulate in the flowing medium.

Although fibroblasts from both oral and dermal sources display similar metabolic activity under flow, it appears that the oral and dermal microenvironment may contribute to

differences in fibroblast phenotype and signalling responses. It is highly possible that mechanical forces induced by chewing, action of salivary amylases and inflammatory molecules are crucial contributors to faster and non-fibrotic wound healing responses in the oral cavity. These results suggest that the cellular responses are influenced by mechanical forces as well as chemical signals from the microenvironment *in vivo*. In our efforts to explore differences in fibroblast responses we studied the response of both dermal and oral fibroblasts to both fluid flow induced mechanical signals and chemical signal such as TGF-β1 in the Quasi vivo® bioreactor that mimics physiological interstitial fluid flow like signals.

Chapter 4- Dermal fibroblasts show variations in gene expression profile under flow.

4.1 Introduction.

Cells *in vivo* are constantly exposed to a number of different physical forces such as shear stresses and interstitial fluid flow. These biophysical cues can work concurrently or independently to regulate various signalling activities that define tissue integrity in normal and pathological circumstances, thus controlling cellular processes involving tissue development, migration, growth, and differentiation, apoptosis and stem cell lineage switching (Ingber and Folkman, 1989, McBeath et al., 2004). Fibroblasts *in vivo* will also experience fluid flow induced biophysical cues.

Fibroblasts are mesenchymal cells which synthesize the extracellular matrix including collagen, form the stromal framework of tissues and influence processes such as wound healing and tumorigenesis (Alberts B, 2002b). These cells can alter their phenotype and genotype in response to chemical signals and exist as fibroblasts in 'less active' state or as myofibroblasts in an 'activated' state. It has been known for a century that fibroblasts respond to chemical signals such as transforming growth factor-β1 (TGF- β1) by differentiating to an activated state marked by an increased gene expression of contractile proteins such as α-smooth muscle actin (αSMA) (Hinsley et al., 2012, Das et al., 2013). When a tissue is ruptured, fibroblasts surrounding the wounded site proliferate and migrate into the wound producing huge amounts of matrix which augments repair of damaged tissue.

Arsenite, a widely distributed and ingested compound is known to be involved with many diseases such as atherosclerosis, black foot disease and cancers. When human fibroblasts were exposed to arsenite, abnormal changes were observed in cell cycle, chromosomes and oxidative damage. Microarray studies showed that these cellular changes manifested in gene

expression changes in 94 genes which were associated with processes such as signal transduction, transcriptional regulation, cell cycle control, cellular stress and proteolytic enzymes (Yih et al., 2002). In another study where researchers undertook a systematic microarray based approach to study the gene expression profile of ras v12/ E1A- transformed mouse embryonic fibroblasts, it was observed that 815 genes showed variations in transformed fibroblasts in comparison to controls (Vasseur et al., 2003). Among the known genes 202 genes were up-regulated and 410 genes were down regulated. Notably, genes encoding DNA replication and repair and cell growth related proteins were up-regulated and genes encoding receptor, secretory and structural proteins were down-regulated. These studies suggest that cellular changes invoked by exposure to chemical signals manifest in gene expression changes in fibroblasts.

Fibroblasts not only respond to chemical signals as mentioned above, but they also experience shear stresses induced by fluid flow (Garanich et al., 2007, Steward et al., 2011). Not surprisingly, they display changes in gene expression of actin during conditions such as wound healing and tumours where blood and fluid flow dynamics differ in comparison to normal tissues (Munson and Shieh, 2014). As described earlier, production of extracellular matrix is a major response of fibroblast to shear stresses as is observed in wound healing, fibrosis, tumours etc (Xue and Jackson, 2015, Fan et al., 2012, Nakayama et al., 2016). As a result of dynamic changes in fluid movement over tissues as observed in these diseased conditions, fibroblasts acquire contractile properties similar to that of smooth muscle cells. However, the onset of changes in genes expression is slow and is regulated indirectly possibly by induction of transcription factors which induces changes in regulated genes. For example, in cultured primary fibroblasts, changes in fibronectin gene expression are observed only after 24h. of cyclic tensile strain (Chiquet et al., 2004). Induction of the transcription

factor Egr-1 is an immediate response of fluid shear stress in endothelial cells (Schwachtgen et al., 1998). In glioblastoma cells, Egr-1 is known to transactivate the fibronectin gene by binding to specific sites in its promoter (Liu et al., 2000). Although comparable studies in fibroblasts have not been carried out, it is likely that induction of changes in gene expression of fibronectin is preceded by early changes in Egr-1 transcription in fibroblasts.

From the available body of literature, it is evident that changes in physical stimuli like mechanical strain and chemical signals like TGF-β1 cause variations in gene expression of fibroblasts. Most of these classical studies have been pursued in cells which are treated in static conditions in the absence of *in vivo* like conditions such as blood flow or interstitial fluid flow. The effect of fluid flow in inducing changes in fibroblasts remains to be explored.

This chapter details experiments that aim to explore gene expression associated changes in dermal fibroblasts that are exposed to fluid flow. A systems biology approach using micro array studies was adopted to study global gene variations associated with the fibroblasts subjected to fluid flow treatment in the Quasi vivo® bioreactor compared to those in static culture conditions.

4.2: Fluid flow alters the gene expression profile of dermal fibroblasts.

4.2.1 Methods

Primary dermal fibroblasts were treated with fluid flow as described in section 2.10 for 24 hr and RNA was extracted as described in section 2.17. Three coverslips were used per test condition and the entire experiment was performed three times. RNA samples from three separate experiments were subjected to microarray analysis as described in section 2.19.

4.2.2 Results

Microarray-based genome analysis on dermal fibroblasts subjected to 75 and 150 µl/min fluid flow rate was performed. As shown in the heat map (Figure 4.1a) representing hierarchical cluster analysis of the three study groups, 54700 genes were differentially expressed which includes 28060 overexpressed genes, 26590 under expressed genes and 44 genes that display fold change ≥ 2.0 at 75µl/min flow rate compared to gene expression of fibroblasts at static conditions. At a flow rate of 150µl/min, 25718 genes were overexpressed, 28967 genes were under-expressed with 55 genes that display fold change ≥ 2.0 in comparison to gene expression of fibroblasts at static conditions. 2944 transcripts were identified to display statistical significance score (t-test, $p<0.05$). Factorisation by principal components was performed for fibroblast expression of these 2944 transcripts which are altered under flow (75 and 150 µl/min). Fibroblasts exposed to 75µL/min are plotted in yellow, those exposed to 150 µl/min are plotted in cyan and the ones in static conditions are plotted in blue. This Principal Component Analysis (PCA) plot of the 2944 genes shows that the first principal component containing the majority of variability in these genes clearly separates the samples exposed to static conditions from the others (Figure 4.1b).

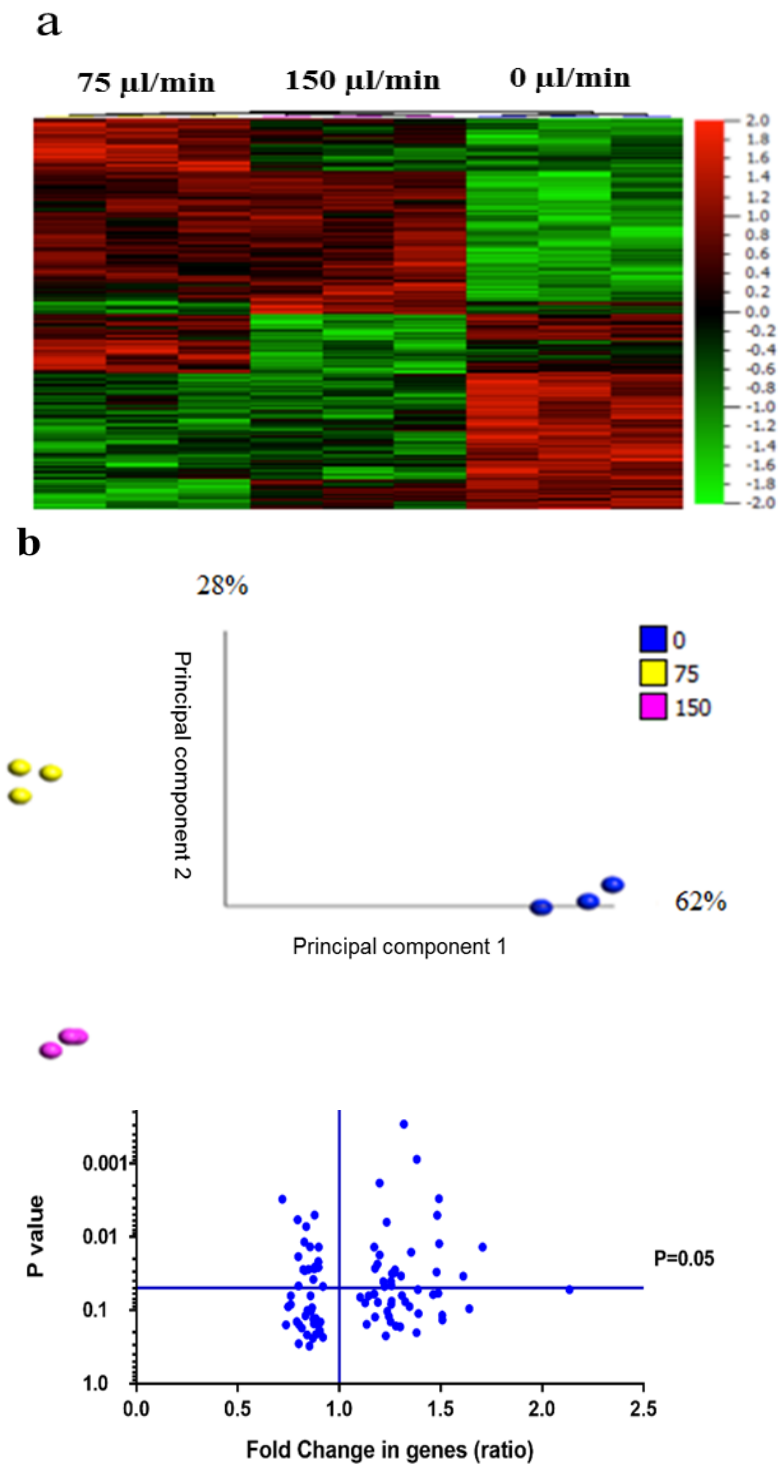

Figure 4.1: Gene expression changes when dermal fibroblasts were subjected to fluid flow (0, 75 and 150 µl/min). a) Heat map of 2944 differentially expressed transcripts (upregulated genes= red, downregulated= green) (t-test, p-value<0.05), b). Principal Component Analysis (PCA) plot of genes altered in dermal fibroblasts exposed to 75µL/min (yellow), 150 µl/min (magenta) and static conditions (blue). c) Volcano plot of the top 100 differentially expressed transcripts in dermal fibroblasts sorted by p-value and fold change.

4.3 Differential gene expression of fibroblasts under flow validates microarray based studies.

4.3.1 Methods

Validation of differential gene expression at different flow rates were performed by studying the expression of collagen IA1 (COL1A1), transforming growth factor receptor type II (TGFβRII) and angiopoietin-like 4 (ANGPTL-4) by quantitative PCR as described in section 2.19. Three coverslips were used per test condition and the entire experiment was performed three times. RNA samples from two separate experiments were subjected to quantitative PCR analysis of genes listed above.

4.3.2 Results

The amplified RNA (aRNA) samples obtained from microarray experiments subjected to gene expression validation studies revealed an increased expression of ANGPTL-4 (Figure 4.2a), COL1A1 (Figure 4.2b) and TGFβRII (Figure 4.2c), and, under flow. In comparison to statics, gene expression of ANGPTL-4 and TGFβRII was increased by 60 fold and 21.45 fold respectively at a flow rate of 75µl/min. Under a flow rate of 150µl/min gene expression of ANGPTL-4, COL1A1and TGFβRII were increased by 16.3 fold, 2.29 fold and 2.15 fold respectively.

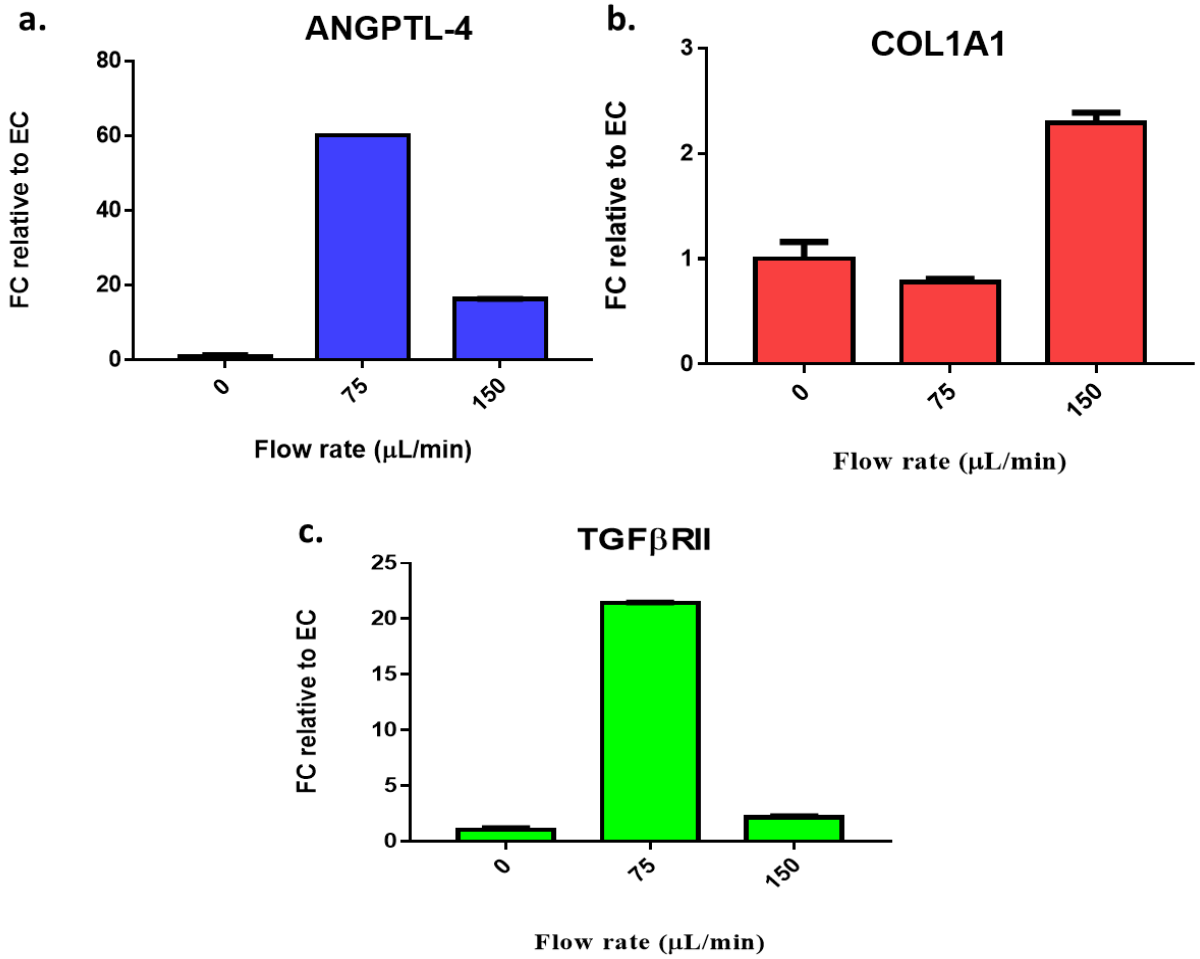

Figure 4.2: Global gene expression plotted as fold change relative to endogenous control in a) Angiopoietin like 4 (ANGPTL-4) (blue) b) Collagen I A1 (COL1A1 (red) and c) Transforming Growth Factor β receptor type II (TGFβRII) (green) in amplified RNA samples obtained microarray analysis performed on fibroblasts subjected to flow rates (0, 75 and 150 µl/min). FC relative to EC represents fold change relative to endogenous control.

4.4: Fluid flow mediated gene alterations are functionally associated with the TGF-β pathway.

4.4.1 Methods

Qlucore software generated 2944 genes ($p<0.05$) obtained from the microarray analysis of dermal fibroblasts subjected to fluid flow were subjected to biological term enrichment by Gene ontology Term enrichment (GOTERM) and the enrichment scores (E. Score) were noted. Highly enriched biological terms were functionally analysed by Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis using the DAVID software.

4.4.2 Results

The GOTERM and KEGG pathways showed that the regulated genes relate to vascular development, angiogenesis, blood vessel morphogenesis, cell morphogenesis, cell motion, cell migration, muscle cell differentiation, regulation of apoptosis, tube development, patterning of blood vessels and TGF-β signal pathway (Figure 4.3). Functional clustering of genes altered under fluid flow (Table 4.1) with enrichment score (E. Score) > 1.5 relate to cell motion migration, programmed cell death, muscle cell differentiation and tissue development, cell morphogenesis, and vascular and blood vessel development. Biological pathways predicted were the pentose phosphate pathway, clathrin and caveolin mediated endocytosis pathway and TGF-beta signalling pathway. Top 20 genes associated with TGF-β pathway and endocytosis altered under both flow rates (75 & 150 µL/min) are shown in Figure 4.4.

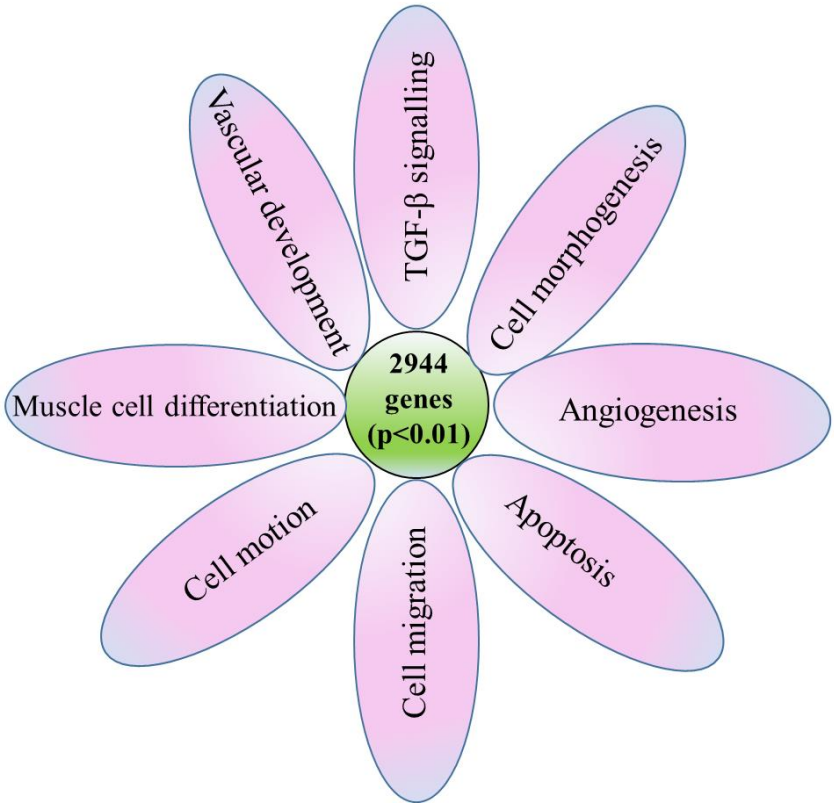

Figure 4.3: Biological functions altered under fluid flow (75 µl/min and 150 µl/min) for 24 h. as assessed by GOTERM and KEGG pathway.

	Biological Process	P_Value	Fold Change
E. Score 2.96	vasculature development	0.0006	1.6
	blood vessel development	0.0007	1.7
	blood vessel morphogenesis	0.0026	1.6
E. Score 2.35	cell morphogenesis involved in differentiation	0.0022	1.6
	cell projection morphogenesis	0.0024	1.6
	cell part morphogenesis	0.0033	1.5
	cell morphogenesis involved in neuron differentiation	0.0040	1.6
	axonogenesis	0.0061	1.6
	neuron projection development	0.0090	1.5
	neuron projection morphogenesis	0.0095	1.5
E. Score 2.28	regulation of striated muscle cell differentiation	0.0021	3
	regulation of skeletal muscle fiber development	0.0050	3
	regulation of skeletal muscle tissue development	0.0130	2.7
E. Score 2.07	regulation of cell death	0.0075	1.3
	regulation of programmed cell death	0.0090	1.2
	regulation of apoptosis	0.0091	1.2
E. Score 1.55	regulation of locomotion	0.0056	1.6
	regulation of cell motion	0.0110	1.5
	regulation of cell migration	0.0420	1.5

Table 4.1: Functional clustering of genes altered under fluid flow (75 µl/min and 150 µl/min) based on enrichment score (E.Score) (Pink), biological process (yellow), P value (green), Fold change (purple).

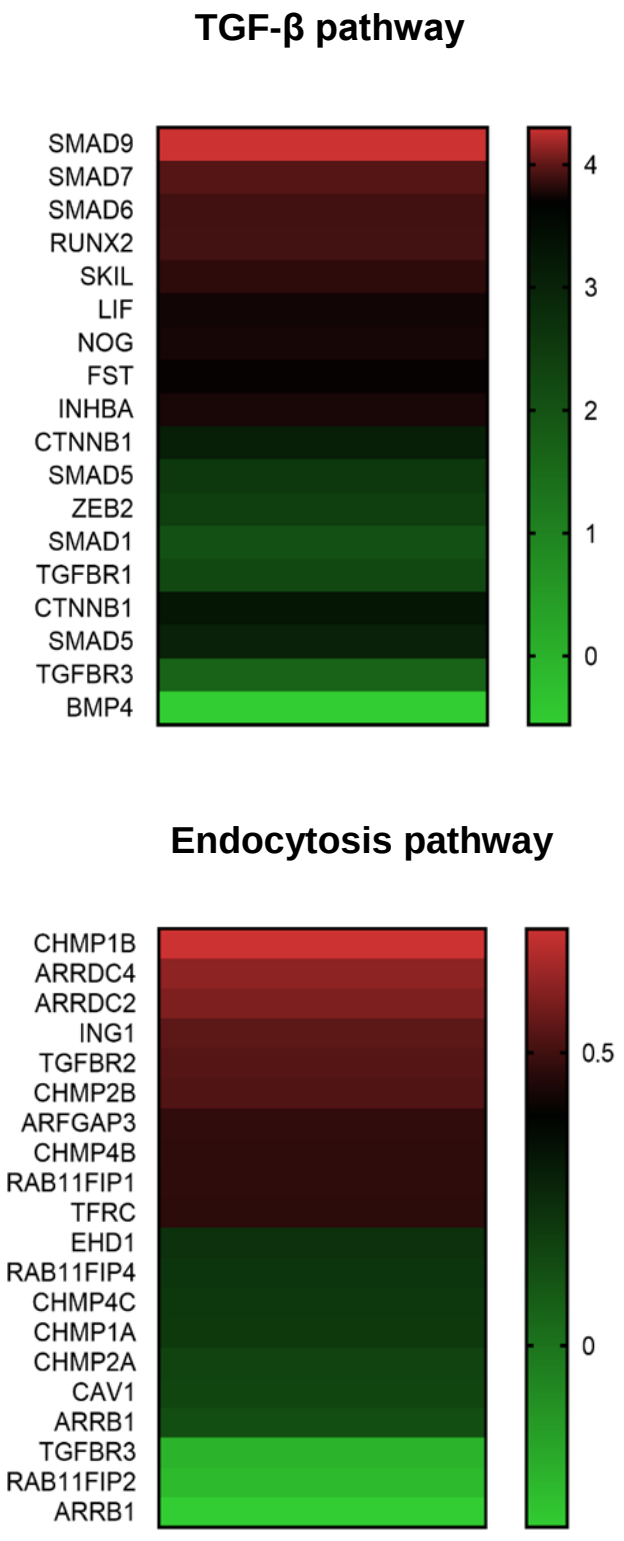

Figure 4.4: Functional analysis of genes altered under both flow rates (75 µl/min and 150 µl/min) for 24 h. in dermal fibroblasts, The TGF-β pathway (top) and the endocytosis pathway (bottom).

4.5 Discussion

As outlined previously, it is known that fibroblasts respond to physical and chemical signals by incorporating contractile stress fibres and acquiring a smooth muscle cell like phenotype and these changes are associated with gene expression changes either mediated directly or indirectly by transactivation of transcription factors. Although a huge body of evidence show that in diseased conditions such as cancer, wound healing and fibrosis cells display changes in gene expression, it is unclear as to the contribution of mechanical cues such as fluid movement in interstitial spaces, blood flow and lymphatic flow. This is partly because most of the studies were either performed on samples obtained from *in vivo* conditions where cells remain embedded in a tissue matrix or in static conditions wherein cells are cultured in 2D cultures in the absence of fluid flow and oxygen conditions that cells experience *in vivo.* In this chapter, gene expression changes in fibroblasts associated with fluid movement that mimics interstitial fluid flow were investigated.

4.5.1 Fluid flow alters gene expression profile of dermal fibroblasts.

Findings from microarray based experiments have shown that fluid flow causes variations in the gene expression profile of dermal fibroblasts. A total of 54700 genes were altered of which 2944 genes were highly significant changes. The heat map shows a visual representation of upregulated genes highlighted in red and downregulated genes in green colour. Notably, it is clear that when dermal fibroblasts experience fluid flow at both flow rates of 75 µl/min and 150 µl/min the same set of genes such as inhibitor of DNA binding (ID3, ID1, and ID4), Smad family member 9 (Smad9), Angiopoietin like-4 (ANGPTL-4) etc. are up-regulated in comparison to statics. Similarly, genes that are upregulated in static

conditions are downregulated under flow treatment. This phenomenon may be because fluid flow acts only on mechanosensory receptors such as primary cilium, receptors, ion channels and caveolae as explained in section 1.6 possibly causing changes in transcription regulators such as the yorkie homologues YAP and TAZ which sense signals from integrins. Recent studies have shown that expression of α-smooth muscle actin (α-SMA) correlated with expression of yes associated protein (YAP) wherein overexpression of α-SMA translocated YAP to the nucleus (Talele et al., 2015). These transcription factors are mechanoregulators of TGF-β signalling (Szeto et al., 2016), a crucial mechanism known to induce fibrosis, fibroblast differentiation etc. (Piersma et al., 2015b).

Other genes which were altered under both fluid flow rates (75 & 150 µL/min) displaying fold change > 4.0 are inhibitor of DNA binding (ID3, ID1, and ID4), solute carrier family 7 (SLC7A11) ($p<0.001$), NmrA-like family domain containing 1 pseudogene (LOC344887) ($p<0.001$), Smad family member 9 (Smad 9), heme oxygenase 1 (HMOX1), FBJ murine osteosarcoma viral oncogene homolog B (FOSB), transmembrane protein 155 (TMEM155) ($p<0.001$), cartilage oligomeric matrix protein (COMP). Although these genes displayed fold changes greater than 4.0 at both flow rates in comparison to fibroblasts treated in static conditions, they were not functionally associated with GOTERM and KEGG analysed TGF-β pathway or the endocytosis pathway. Further, these genes did not relate to biological functions such as cell motion migration, programmed cell death, muscle cell differentiation and tissue development, cell morphogenesis, and vascular and blood vessel development as listed in Table 5. Hence for experimental purposes, genes were subjected to functional annotational analysis by DAVID software and gene enrichment analysis. Genes altered under both flow rates (75 & 150 µL/min) and associated with TGF-β pathway and endocytosis

pathway as analysed by GOTERM and KEGG were isolated and subjected to further analysis as explained in future chapters.

4.5.2 Differential gene expression analysis by quantitative PCR validate microarray based studies.

Validation of microarray studies by analysis of three genes ANGPTL-4, Col1A1 and TGFβRII is underscored by results that show that highly enriched genes with enrichment score were related to Angiogenesis (E. Score = 2.96) and cellular differentiation (E. Score= 2.35) (Table 4.1). ANGPTL-4 is a marker of angiogenesis, Col1A1 is a marker of fibroblast differentiation and TGFβRII is a vital component of TGF-β pathway. Observations show that under a flow rate of 150µl/min gene expression of ANGPTL-4, Col1A1 and TGFβRII were increased by 16.3 fold, 2.29 fold and 2.15 fold respectively as assessed by RT-qPCR. Fold changes in gene expression of ANGPTL-4, COL1A1 and TGFβRII as revealed by the Qlucore software were 5.12, 1.10 and 1.09 respectively at the same flow rate. Both RT-qPCR and Qlucore use different platforms with different technology including sample preparation prior to final quantification and this may account for differences in fold changes of gene expression obtained by two different techniques. However, it is noteworthy to observe that the qPCR results validate Qlucore based findings and show similar pattern of increased gene expression of ANGPTL-4, COL1A1and TGFβRII in dermal fibroblasts treated with fluid flow rate of 150µl/min.

Notably there are profound fold change differences in the chosen genes at two different flow rates. The increase in fold endogenous gene expression with reference to static control is higher for ANGPTL-4 and TGFβRII at a flow rate of 75µl/min than in 150µl/min suggesting

that the same set of genes are predisposed to show changes in different orders of magnitude at different flow rates. This observation is particularly interesting since it shows that fibroblasts are not only sensitive to fluid flow induced shear stresses but gene expression is also directed by the magnitude of shear stresses (Park et al., 2010).

4.5.3 Fluid flow mediated gene alterations are functionally associated with TGF-β pathway.

The gene ontology (GO) defines concepts used to describe gene function and relationships between these concepts. Functions are classified on the basis of three aspects: molecular function of gene product, components where the products are active and biological pathways. GO annotations are statements that describe biological functions of a specified gene. The GO enrichment analysis tool performs an enrichment analysis of GO terms that are over represented for a given set of up-regulated / down-regulated genes and generates a table with following components A) Background frequency: Genes annotated to the GO Term in the background set, B) Over/under represented terms: Over represented and underrepresented genes expressed as '+' and as '-' symbols, C) P-value: probability of observing x number of genes out of total n genes in the background distribution of annotation. Therefore, closer the p-value is to zero, the higher is the significance of the specific GO Term associated group of genes. In the current study GO Term pathways for 2944 transcripts that displayed statistical significance score (t-test, $p<0.01$) were assessed. Of these transcripts, genes which showed high enrichment score greater than 2.3 were associated with TGF-β pathway related processes such as muscle cell differentiation (Owens et al., 2004), vascular development & angiogenesis (Gaengel et al., 2009), apoptosis (Jang et al., 2002), cell morphogenesis (Ogata et al., 2007), cell migration (Vo et al., 2013) and cell motion (Yang and Moses, 1990). Gene

expression of the Smad proteins 1, 5, 6, 7 and 9 are altered under flow (Figure 4.4). Smad proteins are transcription factor which function as pivotal mediators of TGF-β pathway. Interestingly, it was observed that Smad7, an inhibitory Smad protein was also upregulated under both flow rates (75µl/min and 150µl/min) (Figure 4.4). Literature has shown that Smad7 acts as a negative regulator of the TGF-β pathway (Nakao et al., 1997) and its presence calls for further investigation of signalling mechanisms induced by flow in fibroblasts.

The KEGG pathway is the collection of pathway maps that brings together multiple entities such as protein, RNA, genes, chemical compounds, glycans and chemical targets. Pathway maps are classified as separate sections on metabolism, information processing, cellular processes, diseases and drug development. KEGG categories enriched were the pentose phosphate pathway, clathrin and caveolin mediated endocytosis and TGF- β signalling pathway. Pentose phosphate pathway is a metabolic pathway which resembles glycolysis pathway and generates 5-carbon sugars and NADPH (Keller et al., 2014). Changes in cellular metabolism affects migration and invasion of cancer cells (Han et al., 2013). In cancer cells, glycolysis is used to produce ATP and substrates to the pentose phosphate pathway for nucleotide synthesis. Tumours are known to experience insufficient oxygen supply which is incapable of catering to growing demands of the developing vasculature (Vaupel and Mayer, 2007). In such low oxygen conditions, Pyruvate, the final product of glycolysis is converted to lactate by an enzyme called lactate dehydrogenase. In glioma cells, TGF-β2 mediated cell migration is regulated by lactate metabolism (Baumann et al., 2009) suggesting that TGF-β pathway and metabolic pathways such as pentose phosphate pathway are intertwined in disease and health. Similarly, it is also well documented that processes such as clathrin and caveolin mediated endocytosis are known to regulate TGF-β pathway (Chen, 2009). For

example, in human embryonic kidney HEK293 cells, internalisation studies revealed that both TGFβRI and TGFβRII are endocytosed.

The TGF-β pathway has received enormous attention as a signalling process regulating cellular differentiation (Hinz, 2015) in normal fibroblasts, as a promoter of Epithelial to Mesenchymal transition (EMT) (Tchafa et al., 2015, O'Connor and Gomez, 2014) and Endothelial to Mesenchymal Transition (EndoMT) and as a stress induced mechanotransductor in the extracellular matrix (Klingberg et al., 2014, Maller et al., 2013, Hao et al., 2015). Reports show that in dermal fibroblasts cultured in a 3 dimensional environment which mimics interstitial fluid flow, displayed increased fibroblast-to-myofibroblast differentiation, proliferation and collagen alignment even in the absence of exogenous mediators (Ng et al., 2005). The study shows that these remarkable changes observed in fibroblasts under fluid flow were associated with TGF-β1 induction. Subsequent reports showed that interstitial fluid flow and cyclic strain differentially regulate cardiac fibroblast activation via Angiotensin II type I receptor (AT1R) and TGF-β1 (Galie et al., 2012). These studies suggest that the TGF-β pathway functions as a crucial regulator of fluid flow associated changes in gene expression profile of dermal fibroblasts and calls for the exploration of the effect of fluid flow in fibroblasts in the presence of chemical signals like TGF-β1 that are supplemented exogenously.

Chapter 5 - Fluid flow mediated activation of fibroblasts is transient and is regulated by the TGF-β pathway

5.1 Introduction

Fibroblasts are influenced by tissue turnover, repair and homeostasis. The term 'Fibroblast' denotes all stromal cells that which do not express marks of any other mesenchymal cell type such as smooth muscle cells or progenitor cells. However the fibroblast phenotype is highly heterogeneous with the oral cavity itself housing a variety of fibroblast sub populations (Hinz, 2013). A good example of fibroblast heterogeneity is observed in the skin where different layers of the dermis have different fibroblast subpopulations (Nolte et al., 2008, Sorrell and Caplan, 2004). Fibroblast heterogeneity in the oral cavity can be seen in the periodontium where fibroblasts from gingiva and periodontal ligaments are highly heterogeneous in function and contain a fraction of cells with stem cell characteristics (McCulloch and Bordin, 1991, Nanci and Bosshardt, 2006). Fibroblasts from the periodontal ligament orient along the collagen fibres and account for high levels of collagen turnover in the ligament pockets (McCulloch et al., 2000). On the other hand, gingival fibroblasts are responsible for preservation of connective tissue architecture (Coletta and Graner, 2006). A dysregulation in signalling mechanisms leads to gingival overgrowth as is observed in gingival fibromatosis (Trackman and Kantarci, 2004). Oral fibroblasts from gingival and periodontal sources respond to environmental factors such as mechanical stimulation (Pender and McCulloch, 1991) and infection (Scheres et al., 2010).

Fibroblasts respond to mechanical signals by forming stress fibres and developing contractile features which aid mechanotransduction. Cells displaying this type of an 'activated' phenotype are termed as a 'myofibroblasts'. They were first discovered in wound granulation tissue wherein they displayed contractile microfilament bundles and a prominent endoplasmic reticulum (Gabbiani et al., 1971). Another hallmark of these fibroblasts is the expression of

α-smooth muscle actin (α-SMA) in the stress fibres which confers the contractility in these cells (Hinz, 2007). The percentage of fibroblasts which acquire α-SMA positive fibres largely depend on the species and tissue origin (Hinz, 2010, Rohr, 2011). For example, during pathological conditions of a liver disease, it has been reported that myofibroblastic cells expressing α-smooth muscle actin are derived either from hepatic stellate cells or from portal fibroblasts (Desmouliere, 2007).

Transforming growth factor-β1 (TGF-β1) is the pivotal cytokine which induces fibroblast differentiation to a myofibroblast phenotype (Desmouliere et al., 1993). Differentiated myofibroblasts can produce their own TGF-β1 for persistence of fibrosis (Wynn and Ramalingam, 2012). TGF-β1 triggers the expression of ED-A form of fibronectin (Oyama et al., 1989) which supports enhanced expression of α-SMA and collagen type I (Serini et al., 1998). Connective tissue growth factor (CTGF) is another profibrotic protein secreted by endothelial cells and is known to play a role in downstream mediation of TGF-β signalling in fibroblasts (Leask et al., 2004, Grotendorst, 1997). However, in this study focus will largely be on effects of TGF-β signalling in fibroblast differentiation.

TGF-β1, 2 and 3 are the ligands associated with the inhibition of proliferation in most types of cells. However, in mesenchymal cells they induce proliferation, production of extracellular matrix and induce fibrosis *in vivo* (Otranto et al., 2012, McCartney-Francis et al., 1998). The TGF-β isoforms are initially synthesized as latent precursors bound to binding proteins (LTBP-1, 2 and 3). LTBP, which is predominately found in the matrix (Werb, 1997) is proteolytically cleaved and releases the active forms of TGF-β ligands (Annes et al., 2003). Once the ligands are activated, they bind to a heterotrimeric receptor which consists of type I and II receptor to form a TGF-β receptor complex (Heldin and Moustakas, 2016). Upon

activation of the receptor complex, TGF-β functions within the cell through the action of the Smad family of transcription factors (Miyazono et al., 2000). TGF-β receptor complex activation promotes phosphorylation of receptor activated Smads (Smad 2 and 3) by TGF-β receptor I kinase which further bind to Smad4 and translocate to nucleus (Figure 5.1) (Verrecchia and Mauviel, 2002). Recent experiments using microarrays have compared gene expression profiles of fibroblasts obtained from $Smad3^{-/-}$ and $Smad3^{+/+}$ mice. Results showed that TGF-β was unable to induce transcription in $Smad3^{-/-}$ mice (Yang et al., 2003) suggesting that phosphorylation of Smad3 is indispensable for effective functioning of the TGF-β signalling pathway.

This chapter aims to explore the effect of fluid flow and TGF-β1 mediated signals in modifying the phenotype of fibroblasts from different tissue sources.

5.2: Fluid flow enhances gene expression of α-SMA and Collagen IA in Dermal and Oral Fibroblasts.

5.2.1 Methods

Primary dermal fibroblasts (NHDFC) and normal oral fibroblasts (NOF) were treated with fluid flow (150 μL/min) as described in section 2.10 for 24 h and RNA was extracted as described in section 2.17. RNA was extracted from three coverslips per condition and the entire experiment was conducted three times on plain glass and Collagen coated Thermonox coverslips. All three experiments were subjected to quantitative PCR analysis of α-SMA and COL1A1 as described in section 2.19. For immunofluorescence detection of α-SMA protein, coverslips containing dermal fibroblasts subjected to static and fluid flow conditions with and without TGF-β1 were immunolabelled with α-smooth muscle actin (α-SMA) antibody (Sigma) as described in section 2.12.

5.2.2 Results

TGF-β1 supplemented dermal fibroblasts treated in static conditions showed elevated levels of α-SMA and Col IA1 gene expression. Interestingly, fluid flow (150 μl/min) also showed an increased gene expression of α-SMA in dermal fibroblasts grown on glass and collagen type I coated Thermonox surfaces (Figure 5.2 a and c). Similarly, COL1A1expression was also increased in fluid flow treated (150 μL/min) dermal fibroblasts grown on glass and collagen type I coated Thermonox surfaces (Figure 5.3 a and c). However, when TGF-β1 supplemented fibroblasts were subjected to flow treatment, gene expression of both α-SMA and COL1A1were reduced.

Likewise in oral fibroblasts, TGF-β1 supplementation in static conditions increases levels of α-SMA and COL1A1 gene expression. Intriguingly, fluid flow (150 µl/min) also showed an increased gene expression of α-SMA in oral fibroblasts grown on glass and collagen type I coated Thermonox surfaces (Figure 5.2 b and d). Concurrently, Col IA1 expression was also increased in fluid flow treated dermal fibroblasts grown on glass and collagen type I coated Thermonox surfaces (Figure 5.3 b and d). TGF-β1 supplementation in oral fibroblasts subjected to flow treatment reduced gene expression of both α-SMA and COL1A1. Fold changes associated with changes in α-SMA and COL1A1 gene expression under flow treatment are show in table 5.1.

Supplementation and test conditions	Fold change relative to statics	
	α-SMA	Col IA1
Dermal fibroblasts on plain glass		
Flow	1.9	3.4
TGF-β1 supplemented Static	2.3	0.8
TGF-β1 supplemented Flow	0.7	1.2
Oral fibroblasts on plain glass		
Flow	3.9	1.5
TGF-β1 supplemented Static	17.5	3.1
TGF-β1 supplemented Flow	0.5	0.5
Dermal fibroblasts on collagen coated thermonox		
Flow	5.8	3.9
TGF-β1 supplemented Static	1.8	1.3
TGF-β1 supplemented Flow	0.6	0.6
Oral fibroblasts on collagen coated thermonox		
Flow	2.4	3.2
TGF-β1 supplemented Static	3.4	1.5
TGF-β1 supplemented Flow	0.4	0.4

Table 5.1: Fold changes in the gene expression of α- SMA and Coll IA1 in fluid flow (150 µL/min) treated dermal and oral fibroblasts grown on glass and collagen coated Thermonox surfaces compared to static controls (n=3).

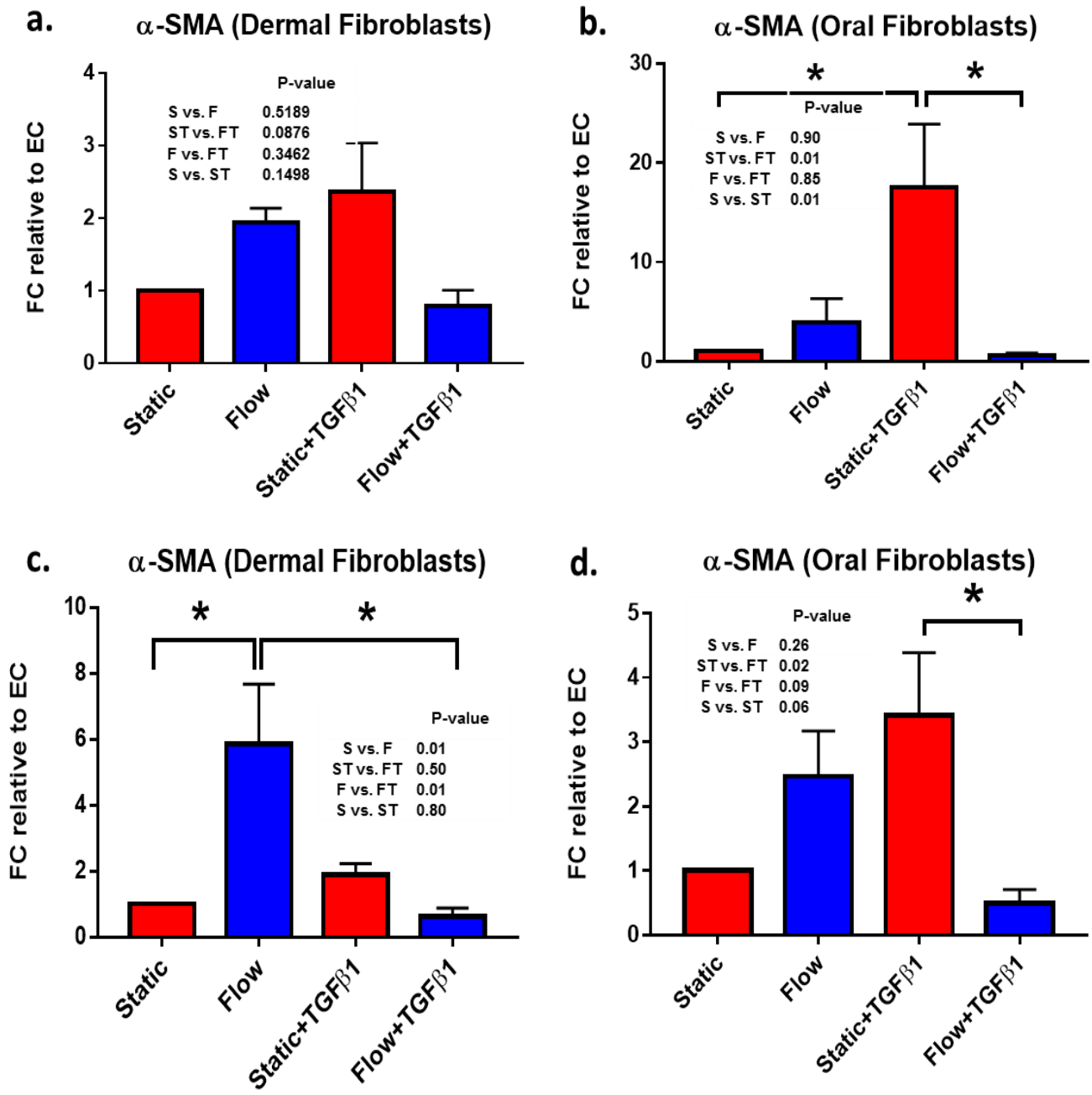

Figure 5.2: Changes in α-SMA gene expression of fibroblasts under flow (150 µL/min) for 24 h. Dermal fibroblasts cultured on glass coverslips (a) and collagen type I coated Thermonox coverslips (c), oral fibroblasts cultured on glass coverslips (b) and collagen type I coated Thermonox coverslips (d), (n=3) (* represents $p < 0.05$). Error bars represent mean ±SD. FC relative to EC represents fold change relative to endogenous control. (S= Static, F= Flow, ST= Static+TGF-β1 and FT= Flow+TGF-β1, vs= versus).

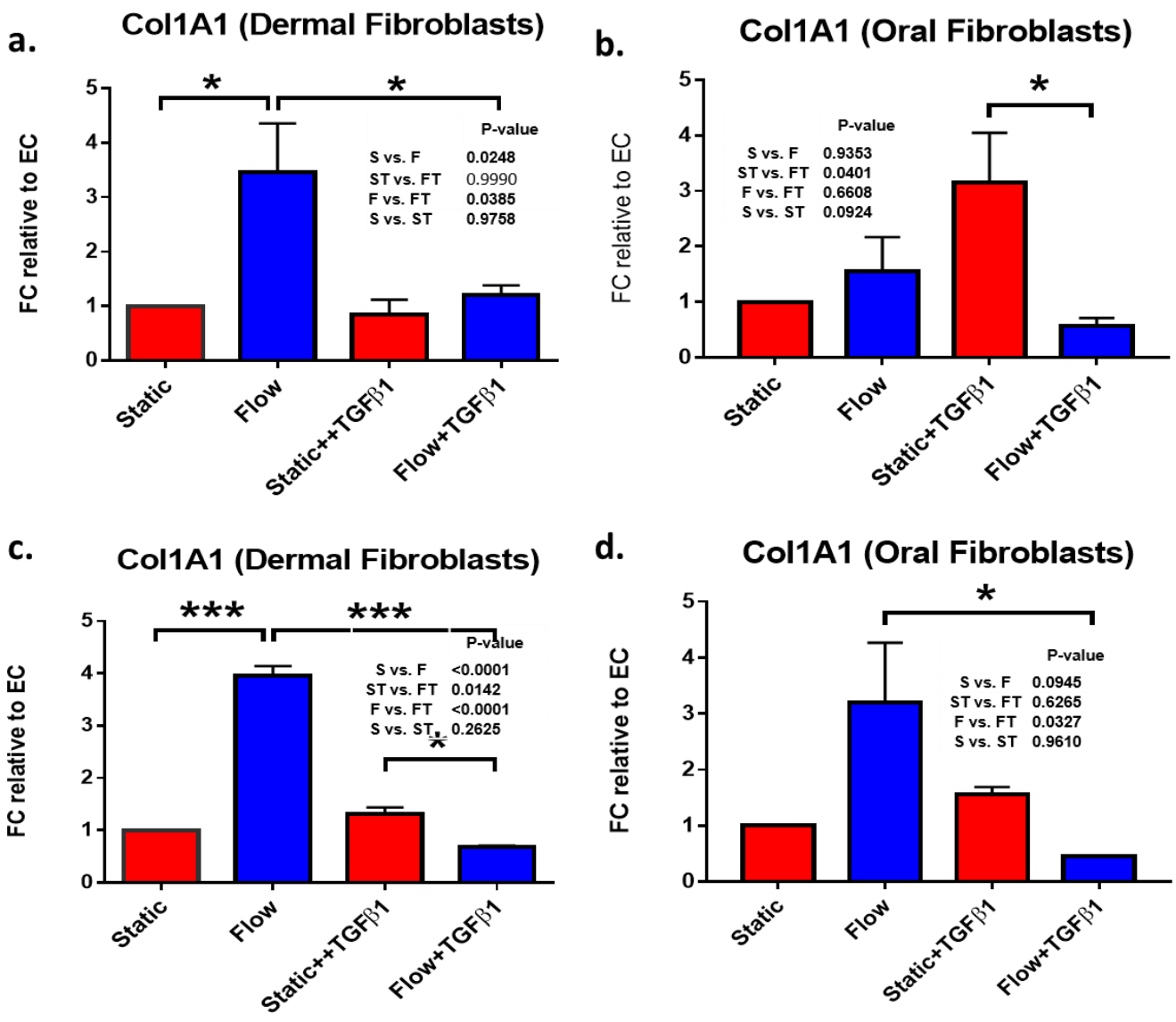

Figure 5.3: Changes in Collagen I A1 (COL1A1) gene expression of fibroblasts under flow (150 µL/min) for 24 h. Dermal fibroblasts cultured on glass coverslips (a) and collagen type I coated Thermonox coverslips (c), oral fibroblasts cultured on glass coverslips (b) and collagen type I coated Thermonox coverslips (d), (n=3) (* represents $p < 0.05$, *** represents $p< 0.001$). Error bars represent mean ±SD. FC relative to EC represents fold change relative to endogenous control. (S= Static, F= Flow, ST= Static+TGF-β1 and FT= Flow+TGF-β1, vs= versus).

Immunofluorescence studies revealed intense staining of α-SMA protein fibres in TGF-β1 stimulated dermal fibroblasts grown in static conditions. As observed in gene expression studies, fluid flow also increases staining intensity of α-SMA protein fibres in dermal fibroblasts (Figure 5.4). However, the staining intensity decreased in TGF-β1 supplemented and flow treated dermal fibroblasts.

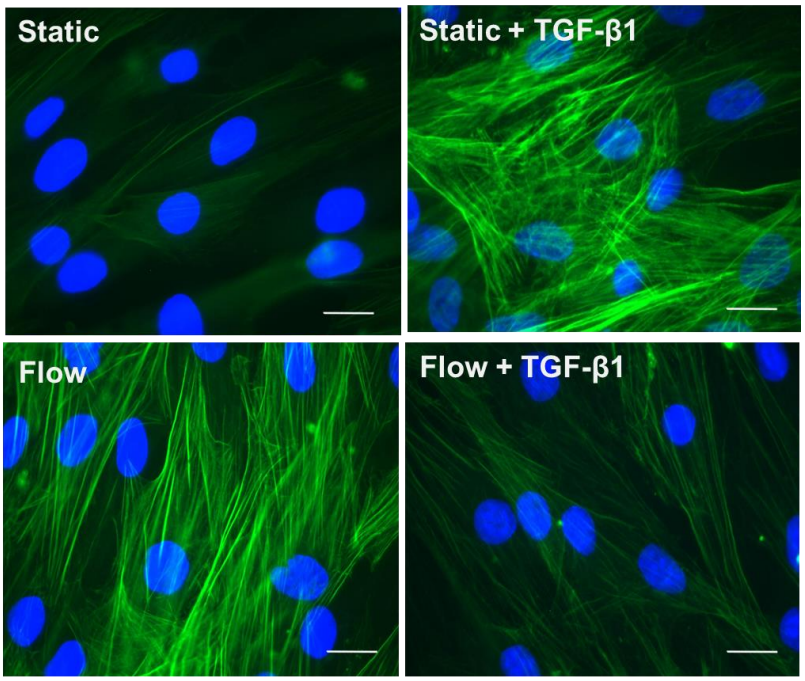

Figure 5.4: Expression of α- smooth muscle actin in dermal fibroblasts subjected to fluid flow (150 μL/min) and TGF-β1 treatment for 24h. All images were captured using 100X magnification lens using the same exposure time. (Scale bar =10 microns).

5.3: Phosphorylation of Smad3 modulates fibroblast activation by fluid flow.

5.3.1 Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 2 h and immunolabelled with anti-Smad3 (phospho S423 + S425) antibody (Abcam) as described in section 2.14. Slides were viewed under a 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging with software proplus 7.0.1). Phosphorylated Smad3 protein was also detected by treating dermal fibroblasts with fluid flow as described in section 2.10 for 2 h, extracting the protein and performing a western blot as explained in section 2.21. Protein concentration was quantified by Image J software and expression change relative to β-actin control and was plotted using graph pad software.

5.3.2 Results

Immunofluorescence studies revealed that in dermal fibroblasts treated in static conditions there is little translocation of phosphorylated Smad3 protein to the nucleus. In TGF-β1 supplemented static conditions, expression of phosphorylated Smad3 was observed in the nucleus. We observed similar intensity of phosphorylated Smad3 positive nuclei in fluid flow (150 μL/min) treated fibroblasts. When fibroblasts were supplemented with TGF-β1 and subjected to fluid flow, all cells displayed phosphorylated Smad3 positive nuclei with intense staining (Figure 5.5 a).

Western blots (Figure 5.5 b) and quantification by Image J (Figure 5.5 c) revealed that that fluid flow increased the level of phosphorylated Smad3 from 15% in static to 44.7% relative

to the β-actin control in comparison to static treatment. When TGF-β1 supplemented fibroblasts were subjected to fluid flow, phosphorylated Smad3 increased 98.9% of the β-actin control which was similar to levels seen in fibroblasts treated with TGF-β1 in static conditions.

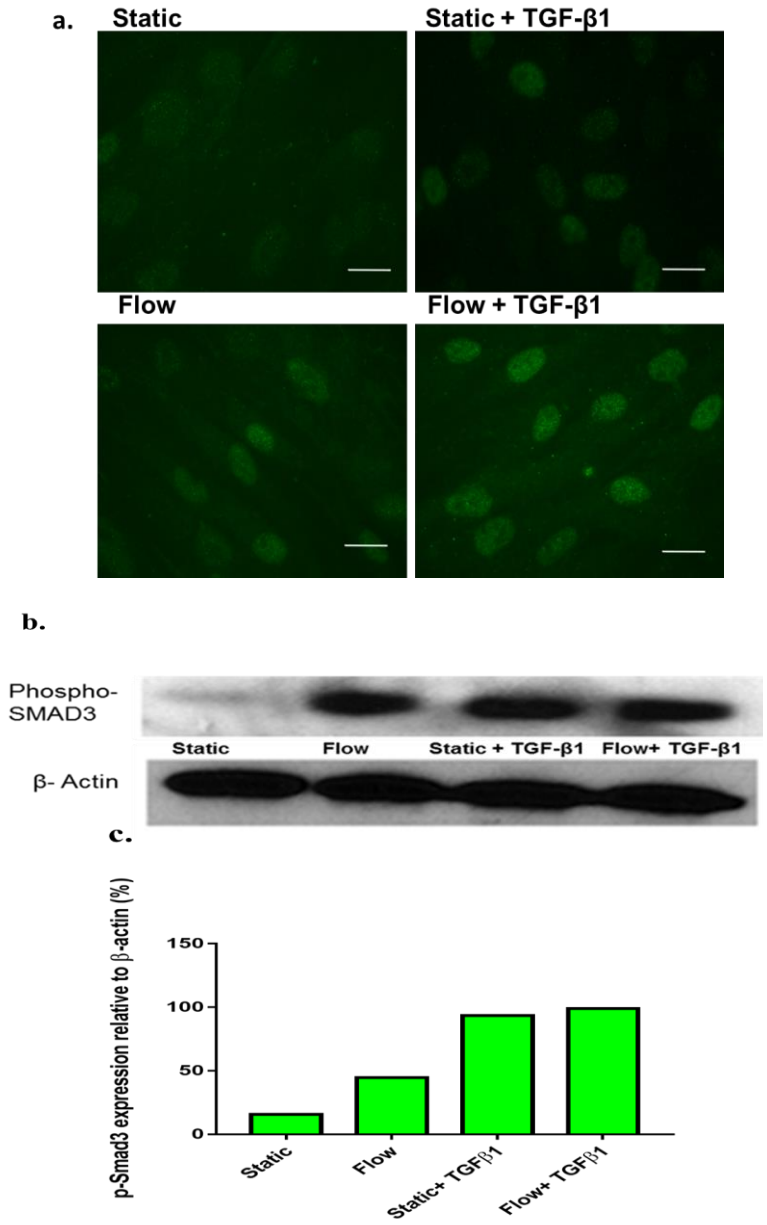

Figure 5.5: Phospho Smad3 expression in dermal fibroblasts subjected to flow (150 µL/min) for 2h.(n=3) a) nuclear localization of phospho-Smad3 in stained dermal fibroblasts subjected to fluid flow and TGF-β1 supplementation b) Expression of phospho-Smad3 protein relative to β-actin expression in dermal fibroblasts. All images were captured at 100X magnification lens using the same exposure time. (Scale bar =10 microns). C) Quantification of phospho-Smad3 protein blots by image J.

5.4: Fluid flow increases expression of TGF-β1 in dermal fibroblasts.

5.4.1 Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 24 h and immunolabelled with TGF- β1 antibody (Lifespan Biosciences) as explained in section 2.15. Slides were viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging with software proplus 7.0.1).

Cell associated and secreted TGF- β1 was determined in dermal fibroblasts subjected to fluid flow treatment for 24 h. using a Quantikine ELISA kit (Abcam). The assay was performed as described in section 2.22.

5.4.2 Results

Dermal fibroblasts subjected to flow treatment revealed intense staining of TGF-β1 in the perinuclear region in comparison to the fibroblasts treated in static conditions. (Figure 5.6 a). Secreted TGF-β1 in circulating medium (Figure 5.6 b) and cell associated TGF-β1 (Figure 5.6 c) were elevated in comparison to statics with secreted TGF-β1 being significantly ($p < 0.01$) higher under flow.

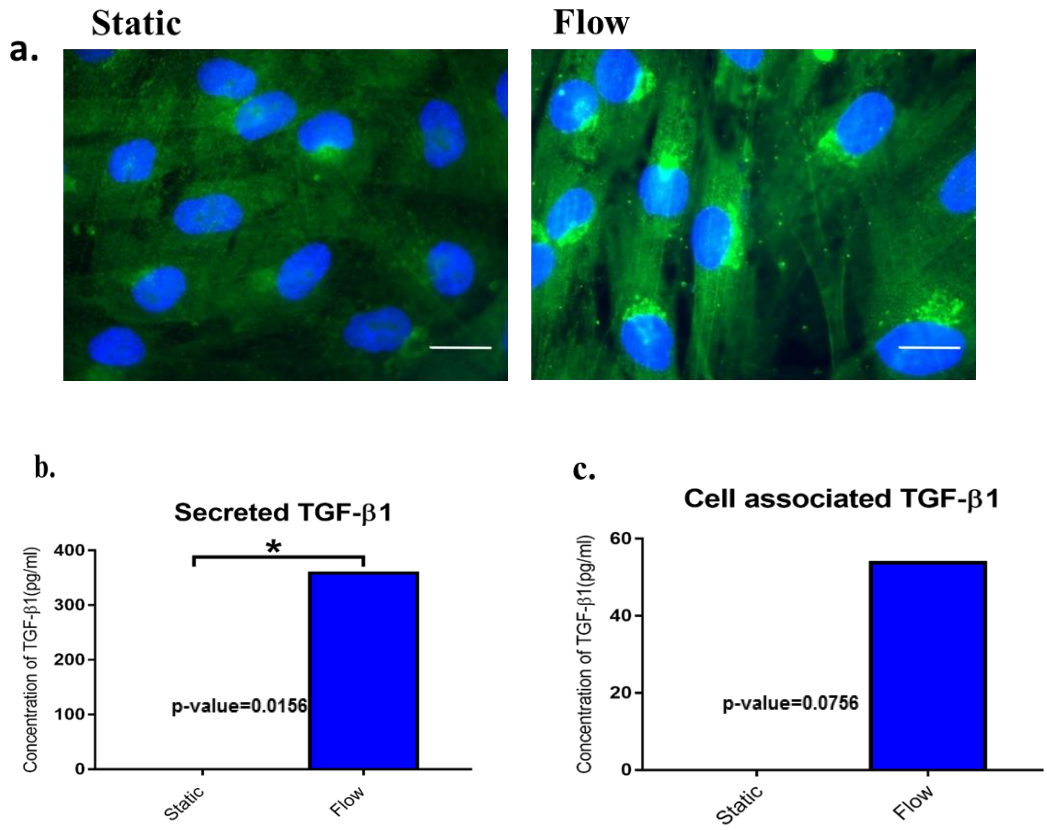

Figure 5.6: Expression of transforming growth factor β1 in dermal fibroblasts subjected to flow (150 µl/min) for 24h. Immunofluorescence detection of TGF-β1 in a) dermal fibroblasts treated in static conditions and flow (n=3). All images were captured using 100X magnification lens using the same exposure time. (Scale bar =10 microns). b) Secreted TGF-β1 protein expression in dermal fibroblasts subjected to fluid flow at 150 µl/min (n=3) (* represents $p < 0.05$), c) Protein expression of cellular TGF-β1 in dermal fibroblasts subjected to fluid flow at 150 µl/min (n=2).

5.5: Fluid flow associated fibroblast differentiation is transient.

5.5.1 Methods

Primary dermal fibroblasts (NHDFC) and normal oral fibroblasts (NOF) were treated with fluid flow (150 µL/min) as described in section 2.10 for 24 h and allowed to remain in static

conditions for 24h. following which α-SMA expression was studied by immunofluorescence staining of the coverslips with α-smooth muscle actin (α-SMA) antibody (Sigma) as described in section 2.12.

5.5.2 Results

In line with previous observations, fluid flow (150µl/min) treatment for 24h. increases expression of α-SMA positive protein fibres. Interestingly, when fluid flow differentiated fibroblasts were allowed to remain in static conditions for 24h., expression of α-SMA positive protein fibres decreased (Figure 5.7).

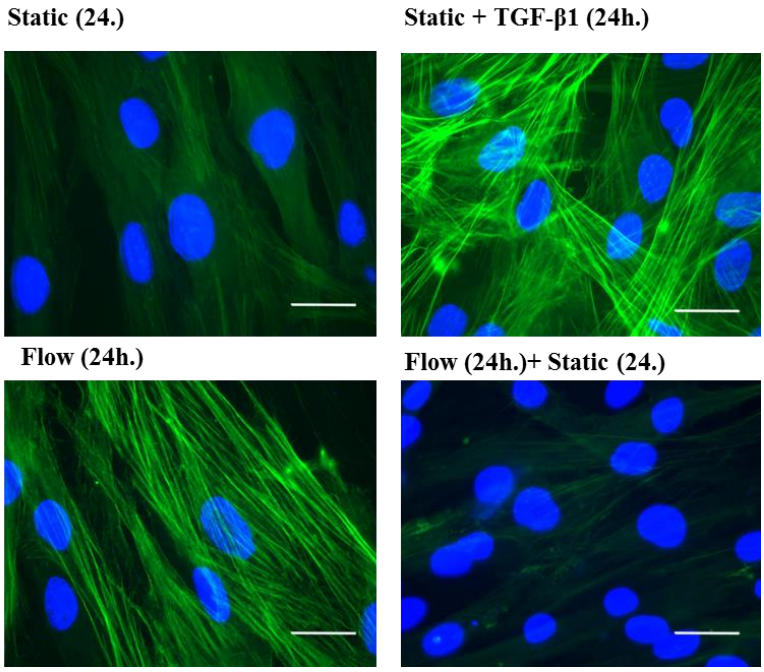

Figure 5.7: Reversal of α- smooth muscle actin (αSMA) expression in dermal fibroblasts subjected to fluid flow (150 µL/min) for 24h. followed by static treatment for 24h. All images were captured using 100X magnification lens using the same exposure time. (Scale bar =10 microns).

5.6: Discussion:

5.6.1: Fluid flow enhances expression of α-SMA and Collagen IA in Dermal and Oral Fibroblasts.

As discussed in the previous sections, myofibroblasts are a variant form of fibroblasts that acquire a smooth muscle phenotype. During wound healing, they contract by using smooth muscle type actin-myosin complex and promote wound contracture. They have been shown to originate from bone marrow derived circulating cells known as fibrocytes (Abe et al., 2001), stromal cells from epithelial tumours (Ishii et al., 2003, Kalluri and Zeisberg, 2006), and epithelial mesenchymal transition (EMT) (Potenta et al., 2008). Hence injured tissue attracts myofibroblasts from different sources to meet the high demands of cells during wound healing activity.

In a tissue, normal fibroblasts lie embedded within the fibrillar extracellular matrix (ECM) of connective tissue, which consists largely of type I collagen and fibronectin (Baum and Duffy 2011). Fibroblasts interact with their surrounding microenvironment through integrins such as the α1β1 integrin. Typically, fibroblasts appear as fusiform cells with a prominent actin cytoskeleton and vimentin intermediate filaments. When these fibroblasts are challenged with mechanical stress such as injury or aberrant fluid flow, mechanoreceptors on the cell surface invoke a variety of cellular responses to instruct the cell to differentiate to myofibroblasts and acquire contractile properties (Duscher, Maan et al. 2014). α-smooth muscle actin (α-SMA) is an isoform typical of smooth muscle cells (SMC) (Skalli et al., 1989) and a hallmark of myofibroblasts (Skalli et al., 1986). Expression of α-SMA in stress fibers confers a two fold increase in contractile activity of myofibroblasts compared to α-SMA negative fibroblasts (Hinz et al., 2001). Although the detection of fibroblasts can be challenging because most

known markers are not specific to fibroblasts, fibroblast-specific protein 1 (FSP1), a member of the family of S100 Ca^{2+} binding proteins, however specifically detects fibroblasts in normal tissues.

Mechanotransduction is a process wherein cells convert mechanical signals into biochemical signals which mediate cellular responses. Myofibroblasts employ actin based motors to generate contractile forces and mediate tissue remodelling by traction (Tomasek et al., 2002). Mechanical force induced SMA uses a feed-forward amplification loop involving a priori SMA in focal adhesions, binding of p38 MAP kinase to SMA filaments, activation of Rho and binding of serum response factor to the CArG-B box of the SMA promoter (Wang et al., 2006a). Therefore, SMA acts a mechanotransductor which receives messages from mechanosensors in focal adhesions and transmits these signals to downstream kinases.

Our results complement previous findings that TGF-β1 is a potent activator of myofibroblasts and it causes changes in the expression of alpha-smooth muscle actin (alpha-SMA) (Honda et al., 2010) and Collagen IA (Cutroneo et al., 2007, Pan et al., 2013).

Results obtained from qPCR based expression analysis of cells cultured on glass, Thermonox cover slips, when subjected to flow conditions in stimulated and unstimulated cultures show significantly increased expression of α-SMA and Collagen IA in both oral and dermal fibroblasts when subjected to flow. The results showed similar patterns of increased gene expression in glass and collagen coated Thermonox cover slips subjected to flow. However, in both dermal and oral fibroblasts, when cells were stimulated with TGF-β1 and subjected to flow, the expression of α-SMA decreased due to unknown reasons. It is possible that the combinatorial effect of TGF-β1 and flow mediated expression of α-SMA switches on

alternative biochemical processes such as membrane trafficking of receptors (Chen, 2009), lysosome mediated endocytosis of receptor bound TGF-β ligands etc. In our efforts to address possible mechanisms involved in downregulation of α-SMA and Col IA1 expression in TGF-β1 stimulated fibroblasts treated by fluid flow, endocytosis related experiments were performed in the sections described further.

One study reported that in human lung fibroblasts, TGF-β1 treatment induces expression of both α-SMA and p38 MAP kinase. The MAP Kinase pathway is known to play a crucial role stress induced senescence (Debacq-Chainiaux et al., 2010, Maruyama et al., 2009). Surprisingly, they also showed that TGF-β1 induced α-SMA can be suppressed by p38 kinase inhibitor (SB203580), Erk inhibitor (PD98059) and the AP-1 inhibitor curcumin suggesting that molecules such as p38 kinase, Erk, and AP-1 can be responsible for the α-SMA expression induced by TGF-β1 in human fetal lung fibroblasts (Hu et al., 2006). This evidence suggests that apart from signalling processes such as endocytosis, receptor regulation and membrane trafficking of receptors TGF-β1 induced α-SMA expression is influenced by other regulatory pathways as well.

5.6.2: Dermal fibroblasts are activated by flow in a phospho-Smad3 dependant manner.

Expression of α-SMA in myofibroblasts and quiescent fibroblasts is induced by TGFβ-1 (Desmouliere et al., 1993). TGFβ-1 is a well-studied factor known to play active roles in recruitment of fibroblasts, conferring smooth muscle phenotype to myofibroblasts (Honda et al., 2010), induction of EMT and metastasis (Siegel and Massague, 2003). TGFβ signalling involves Smads, and Smad3 in particular, is the principal determinant of EMT, a process

which mediates epithelial to mesenchymal transition in pathological conditions like cancer (Mani et al., 2008).

In order to begin to examine the signalling mechanisms underlying our observation that fluid flow can induce fibroblast differentiation, Smad3 phosphorylation was analysed. Smad3 is a transcription factor, which remains localised to the cytoplasm in un-stimulated cells. Upon activation of TGF-β receptors, Smad3 is phosphorylated by TGFβR1, Alk4 and Alk5 (Lin et al. 2009) and translocates to the nucleus. Nuclear localization of phosphorylated Smad3 in dermal fibroblasts subjected to TGF-β1 stimulation and fluid flow suggests activation of the TGF-β pathway even within 2 h of flow treatment. Contrastingly, fibroblasts treated with TGF-β1 and fluid flow also showed nuclear localization of phosphorylated Smad3. It is likely that Smad3 is phosphorylated transiently due to elevated levels of TGF-β1 in the circulating medium even within 2 h flow and is dephosphorylated with time due to caveolin mediated membrane trafficking of TGF-β1 bound receptor complex into the nucleus.

Caveolins coat membrane bound pits that contribute to receptor mediated endocytosis of ligands such as transferrin and low density lipoprotein (Marsh and McMahon, 1999). They exist in three isoforms namely caveolin-1 (Cav-1), caveolin-2 (Cav-2), and caveolin-3 (Cav-3). TGF-β1 inhibits proliferation of Cav-2 knockout endothelial cells (ECs) (L Xie, 2011) via suppression of Alk5-Smad2/3 pathway. This inhibition manifests itself as a reduced magnitude and length of TGF-β-induced Smad2/3 phosphorylation in Cav-2-positive ECs. Subsequent reports showed that N-terminal tyrosine phosphorylation is an essential molecular event which allows Cav2 to inhibit TGF-β-induced stimulation of Smad3 phosphorylation (Abel et al., 2012) thus making it plausible that fluid flow treated dermal fibroblasts enhance the expression of caveolins and TGF-β1 supplementation could reduce the length and

magnitude of SMAD3 phosphorylation after 2h., thus reducing the expression of α-SMA after 24h.

5.6.3: Transient activation of dermal fibroblasts is regulated by TGF-β1.

Our observations from ELISA experiments have shown that TGF-β1 can be detected in cell associated fractions and is also secreted by the cell into the circulating medium. This prompts conclusion that fluid flow mediated activation of fibroblasts is controlled by the TGF-β pathway by release of TGF-β1 which may induce an autocrine and paracrine signalling effect. Previous research by others reveal that interstitial fluid flow can activate dermal fibroblasts by increasing expression of TGF-β1 when subjected to fluid flow at rate of 120µl/min (Ng et al., 2005). The group demonstrated that such low levels of fluid flow stimulated autocrine upregulation of TGF-β1 which in turn induced fibroblast to myofibroblast transition and collagen alignment in vitro and that these effects could be eliminated with TGF-β1 blocking antibodies. These findings suggest that even in the absence of TGF-β1, factors secreted by inflammatory cells and epithelial cells, interstitial fluid flow can induce fibroblast activation and sustain differentiation. Results reveal that similar levels of fluid flow (150 µl/min) can invoke TGF-β1 expression in fibroblasts of both dermal and oral origin suggesting that these mechanical signals evoke similar differentiation responses irrespective of tissue origin.

Immunofluorescence images of flow activated fibroblasts validate our findings further. Observations in the current study show that TGF-β1 is intensely stained in the perinuclear region only in fibroblasts under flow when compared to fibroblasts in static conditions. It highly likely that 24 h. since induction of flow, dermal fibroblasts adapt themselves to an

alternate signalling mechanism which may bring TGF-β1 bound receptor complexes toward the nucleus due to mechanisms such as endocytosis and membrane trafficking (Chen, 2009) as described in Chapter 6. A recent study has shown that TGF-β displays concentration dependant switching of molecular signalling and biological specificity in podocytes (Wu et al., 2005) and that lower concentrations of TGF-β1 induced tubulogenesis in epithelial cells (Montesano et al., 2007). These reports suggest that the combined effect of TGF-β1 supplementation and fluid flow induced release of TGF-β1 leads to supra physiological concentration which may cause an inhibitory effect in expression of α-SMA.

Intriguingly, when fluid flow activated fibroblasts were allowed to remain in static conditions for 24h., these cells reverted to a resting state phenotype. This suggests that fluid flow mediated fibroblast activation is regulated transiently by TGF-β pathway and is influenced by other mechanisms that enable reversal to native state phenotype upon withdrawal of the mechanical signal. Prostaglandin E2 (PGE2) is one such molecule which unambiguously demonstrates reversal of established fibroblast differentiation to normal fibroblasts (Garrison et al., 2013). Fetal and adult lung fibroblasts were first stimulated with TGF-β1 for 24h. to differentiate into myofibroblasts. Cells were then treated without or with PGE2 for various intervals and α-SMA expression was assessed. Findings showed that in the absence of PGE2 treatment, TGF-β1 induced α-SMA expression was stable for up to 8 days. Contrastingly, PGE2 treatment resulted in dose-dependent decrease in α-SMA and collagen I expression within 2 days post treatment and persisted for 8 days in culture. Surprisingly, when TGF-β1 was reintroduced 2 days after addition of PGE2, fibroblasts started to re-express α-SMA, indicating differentiation to myofibroblasts.

Chapter 6 - Caveolin mediated membrane trafficking of TGFβ receptor regulates fluid flow activated fibroblasts.

6.1: Introduction

Cells sense and communicate with the environment by interacting with ligands and activating the signalling receptors at the surface via processes such as endocytosis. The term 'endocytosis' was first coined by Christian deDuve in 1963 to define an energy dependant process wherein a portion of the cellular plasma membrane ingests macromolecules from the surrounding medium and forms a vesicle known as an Endosome.

Early Endosomes (EE) are the first endocytic compartment that receives incoming cargo and are often located in the cellular periphery. They have a mildly acid pH and appear as tubule-vesicular structures. They are composed of regions of thin tubular extensions and large vesicles (Gruenberg, 2001). The ingested receptor-ligand complex is dissociated in the acidic lumen of these endosomes and many of the receptors recycle to the cell surface. Some of these endosomes form aggregates known as multivesicular bodies (MVB) or endosomal carrier vesicles (ECV). The pH in the lumen of these multivesicular bodies ranges from 5.5-6.5. The primary function of the EE is breakdown of cellular waste, fats, proteins and other macromolecules into simple compounds and the simple compounds are later released into the cytoplasm. Interstingly, TGF-β receptors may also enter cells via cholesterol-rich membrane microdomain lipid rafts/caveolae and are found in CAV1 positive vesicles (Chen, 2009). A study reports that TGF-β receptors localize to the ciliary tip and endocytic vesicles at the ciliary base in fibroblasts and TGF-β1 supplementation increases receptor localization and activation of SMAD2/3 and ERK1/2 at the ciliary base (Clement et al., 2013).

Caveolae are a type of membrane raft made of flask shaped invaginations of diameter ranging from 60-80 nm. Of the three variants as described in chapter 1, caveolin-1 and caveolin-2 are found in non-muscle cells whereas caveolin-3 is predominantly expressed in muscle.

Caveolin-1 is shown to be important in membrane raft dependant endocytosis via its interactions with the actin cytoskeleton. Caveolin dependant endocytosis plays a role in biological processes like uptake of viruses, nutrients and cell membrane receptors. Studies have shown that mice deficient in caveolin IA harbour significant vascular defects, and increased deposition of contractile proteins in the lungs suggesting that loss of caveolin-1 may promote fibrosis in lungs. These molecules have been implicated in membrane compartmentalization wherein proteins and lipids accumulate in these membrane microdomains and transmit specific signalling cascades (Boscher and Nabi, 2012). Caveolin-1 is required for signaling and membrane targeting of EphB1 receptor tyrosine kinases which are key players during the development of the embryonic vasculature (Vihanto et al., 2006). Specific components of the TGF-β cascade are associated with caveolin-1 in caveolae with Cav-1 interacting with the TGF-β receptor type I (Razani et al., 2001) and caveolin I mediated TGF-β receptor internalization contributes to the pathogenesis of systemic sclerosis and idiopathic pulmonary fibrosis.

Whilst it is increasingly evident that endocytosis casts an effect on the trafficking of TGF-β receptors and that both membrane-raft and caveolin mediated endocytosis regulate TGF-β signalling (Di Guglielmo et al., 2003a), whether this process is involved in negative regulation of the signalling in TGF-β1 supplemented fibroblasts under flow has not been previously studied. Hence the expression of endocytosis associated markers such as early endosomal antigen (EEA-1) and caveolin-1A (CAV-1A) by immunofluorescence were studied.

6.2: Fluid flow alters the localization of caveolin-1 in dermal fibroblasts.

6.2.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 2 h. In order to detect cytoplasmic expression of caveolin, three coverslips were permeabiilised with 0.1% Triton X-100 in PBS whereas the other three coverslips were not permeabilised to detect surface expression. Cells were immunolabelled with rabbit polyclonal anti-caveolin-1 (Abcam) as described in section 2.14. Mounted slides were viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging with software proplus 7.0.1).

6.2.2: Results

To assess whether the decrease in the activation response in TGF- β1-stimulated fibroblasts subjected to fluid flow was mediated by changes in endocytosis (Chen, 2009), both membrane bound and cytoplasmic protein expression of caveolin-1A under flow were detected. Fibroblasts in static culture conditions had intense staining for caveolin-1A on the cell surface (Figure 6.2a) and lesser cytoplasmic expression (Figure 6.2b). Cytoplasmic expression of caveolin 1A was increased in fibroblasts subjected to flow in both TGF- β1 stimulated and unstimulated conditions suggesting that under the influence of fluid flow, membrane bound caveolin 1A was internalised into the cell.

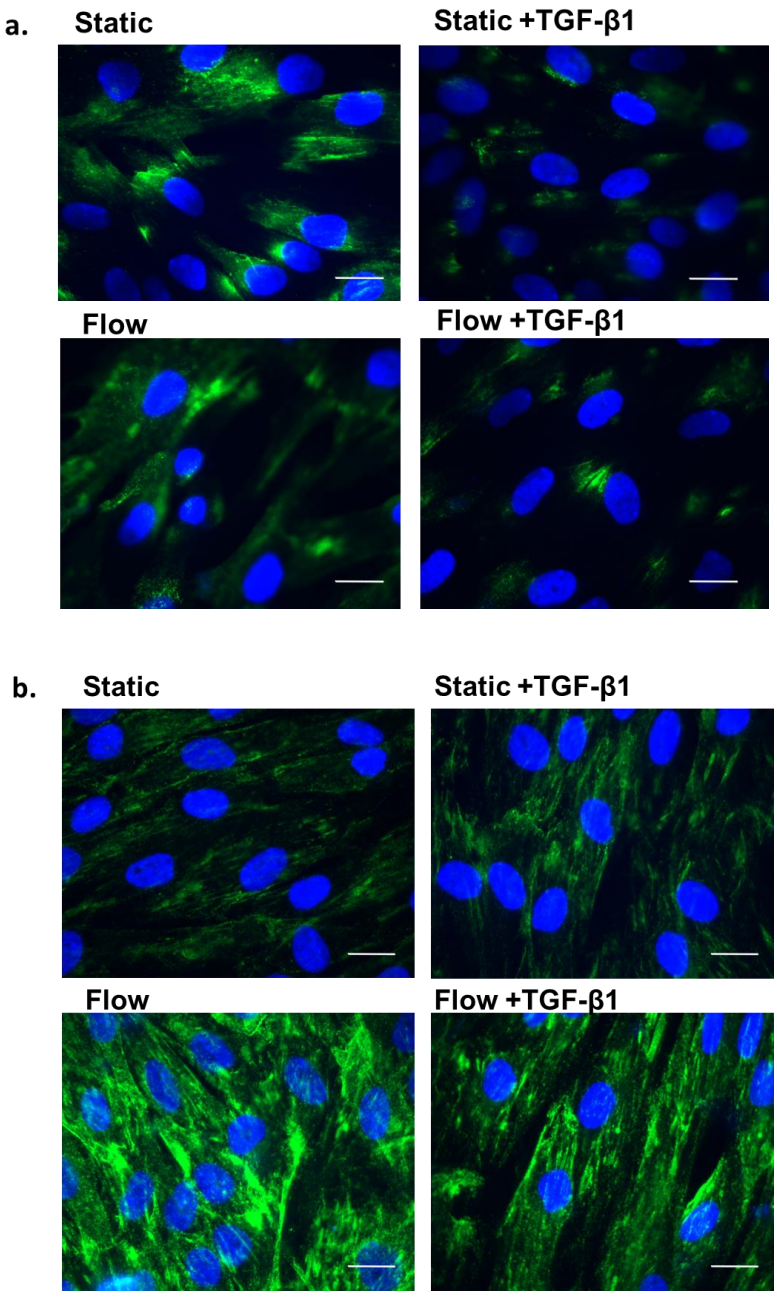

Figure 6.1: Changes in expression of caveolin 1A in dermal fibroblasts subjected to fluid flow (150µl/min) for 2h. a) Cell surface protein expression of caveolin 1A (green) in unpermeabilised dermal fibroblasts and b) cytoplasmic expression of caveolin 1A protein in permeabilised fibroblasts. All images were captured using 100X magnification lens using the same exposure time. (Scale bar =10 microns).

6.3: Fluid flows promotes endocytosis and translocates the TGF-β receptor to the nucleus of dermal fibroblasts.

6.3.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 2 h. and immunolabelled with rabbit polyclonal transforming growth factor β receptor type II (TGFβRII)) (Santa Cruz Biotechnology) and mouse monoclonal early endosomal antigen-1 (EEA-1) (B.D Biosciences) as explained in section 2.14. Mounted slides were viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging with software proplus 7.0.1).

6.3.2: Results

Fluid flow treated fibroblasts were double immunolabelled to simultaneously detect early endosomal antigen-1 (EEA-1) a marker of endosomal vesicle, and transforming growth factor beta receptor type II (TGFβRII). Little expression of EEA-1 (green colour) and TGF-βRII (red colour) was detected in fibroblasts in basal static conditions whereas TGF-β1 stimulation in static cultures resulted in intense staining of EEA-1 (Figure 6.2). Fluid flow treatment triggered not only a similar increase in expression of EEA-1 but also promoted internalisation and nuclear translocation of TGF-βRII (Figure 6.2). Similarly, the combination of TGF- β1 stimulation and fluid flow also induced nuclear translocation of TGF-βRII (Figure 6.2).

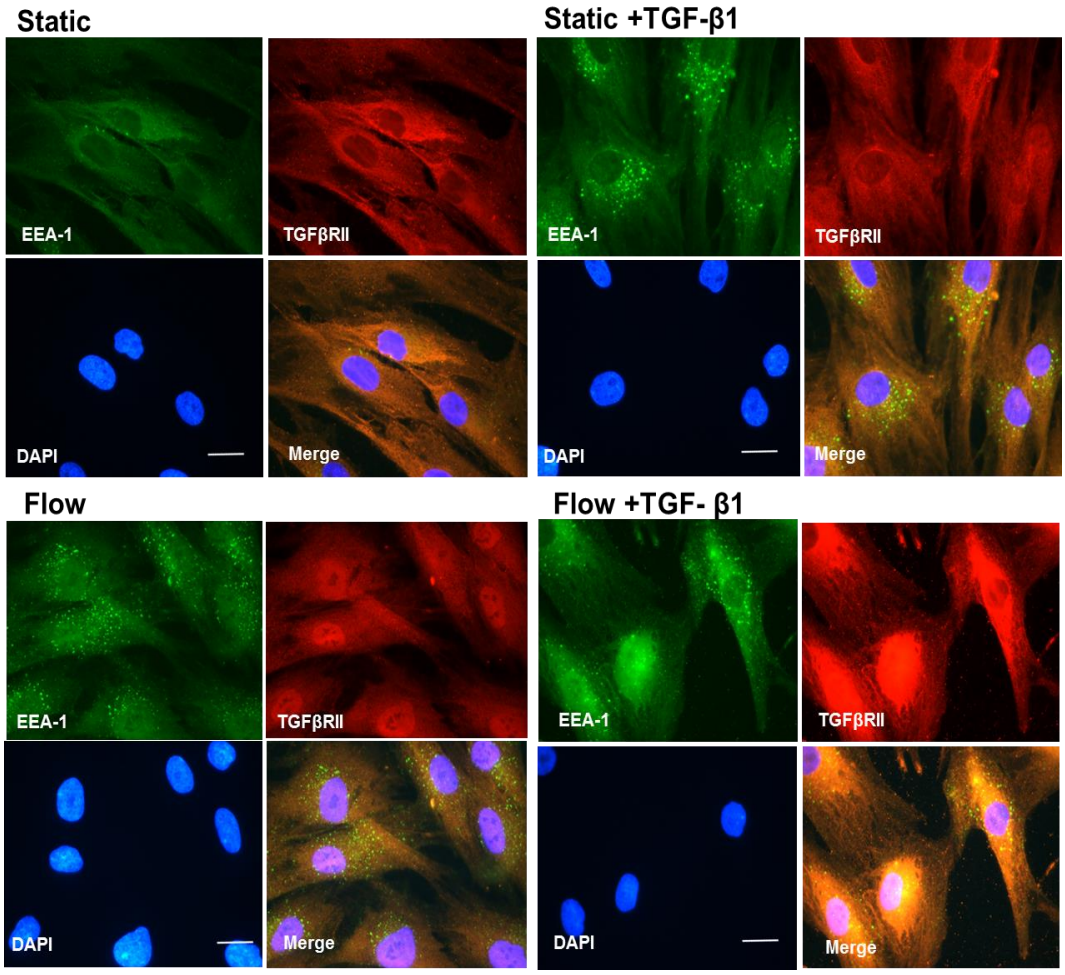

Figure 6.2: Expression of transforming growth factor β receptor type II and early endosomal antigen-1 in dermal fibroblasts subjected to flow (150 μL/min) for 2 h. Cells are stained with (a) EEA-1 (green), (b) TGF-βRII (red), and (c) DAPI (blue) and (d) merged images (red, blue, green) in fibroblasts treated in static conditions, static conditions stimulated with TGF-β1, fluid flow and fluid flow stimulated with TGF-β1. All images were captured using 100X magnification lens and are representative images from experiment of n=3.

6.4: Fluid flows enhances endocytosis in dermal fibroblasts.

6.4.1 Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 2 h. and were stained by pH RODO dextran (70µg/ml) (Molecular Probes) in cell imaging buffer as described in section 2.16. Mounted slides were viewed under 100X magnification lens (oil immersion) in a fluorescent microscope. (Zeiss Axioplan2, Imaging with software proplus 7.0.1).

6.4.2 Results

Little staining of pH RODO dextran was observed in static conditions in both unstimulated and TGF-β1 stimulated fibroblasts. However, findings revealed dramatic increase in pH RODO dextran staining (Pink colour) in both unstimulated and TGF-β1 stimulated fibroblasts which were subjected to flow treatment suggesting that fluid flow increased endocytosis of pH RODO Dextran into endosomes due to endocytosis (Figure 6.3).

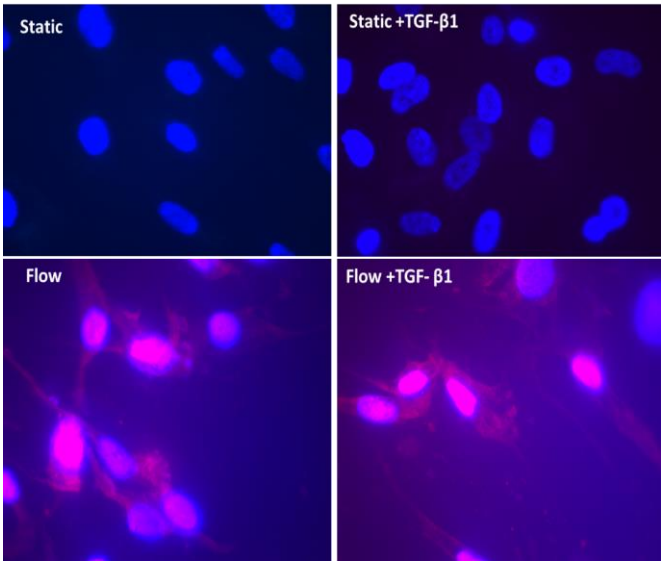

Figure 6.3: Staining of endosomal compartment by pH RODO dextran in dermal fibroblasts subjected to static treatment ± TGF-β1 and fluid flow ±TGF-β1 at 150 µl/min. All images were captured using 100X magnification lens and are representative images from experiment of n=3.

6.5: Discussion.

Various studies in the literature show that receptor mediated endocytosis exerts positive and negative effects on activation of signalling receptors such as G- protein coupled receptors (Sorkin and von Zastrow, 2009). The TGF-β receptors are no exception in that they are sequestered to the vesicular compartments undergoing both clathrin mediated (Chen, 2009) as well as caveolin mediated endocytosis (He et al., 2015). In this chapter, efforts were made to study the effect of fluid flow in modulating the endocytic response of TGFβ Receptor II (TGFβRII) in TGF-β1 stimulated fibroblasts as a possible explanation of the downregulation in response to TGFβ under flow conditions (Chapter 5).

6.5.1: Fluid flow enhances the expression of caveolin-1 in dermal fibroblasts.

As described in the previous pages, caveolins form a crucial component of the endocytic machinery and exist as three separate members. Recent studies have shown that caveolin-1 is a modulator of fibroblast activation and serves as a biomarker for tumours such as gastric cancer (Shen et al., 2015). Findings also showed that CAV-1 was a key regulator of paracrine activation in gastric cancers. Contrastingly, other studies showed that caveolin-1 null mammary stromal fibroblasts share characteristics with cancer associated fibroblasts (CAF) from breast cancer patients (Sotgia et al., 2009) and that a loss of CAV-1 expression in CAFs is a predictor of poor prognosis in breast cancer (Simpkins et al., 2012). Apart from its role in promoting tumourigenesis, caveolin-1 is also known to exert an antifibrotic effect in diseases such as lung fibrosis (Wang et al., 2006b), scleroderma (Tourkina et al., 2008), keloids (Zhang et al., 2011a) and cardiac fibrosis (Miyasato et al., 2011). It was observed that in all these cases, reintroduction of caveolin-1 antagonised the effect of TGF-β signalling by

decreasing expression of Collagen 1AI and fibronectin. Consistent with these observations, several other findings also reported that caveolin-1 is a negative regulator of TGF-β1 signalling in urethral smooth muscle cells (Stehr et al., 2004), leucocytes (Tourkina et al., 2010), and hepatocytes (Mayoral et al., 2010). Caveolins were initially detected at the inner membrane surface of fibroblast Caveolae (Rothberg et al., 1992) and the Golgi apparatus (Dupree et al., 1993) using immunogold cytochemistry. Intense expression of caveolin-1A on the cellular surface of un-stimulated fibroblasts in static conditions confirm that these molecules are membrane bound in conditions devoid of physical forces such as interstitial fluid flow. However, intense cytoplasmic staining of caveolin-1A in both unstimulated and TGF-β1 stimulated fibroblasts suggest that fluid flow treatment for a short period of 2 h can stimulate translocation of these membrane bound molecules to the cytoplasm. Cytoplasmic translocation of caveolin-1 is likely to result in endocytic trafficking of TGF-β1 bound receptor complexes that are then engulfed into caveolar vesicles. This has been observed in previous studies which showed internalisation of TGF-B receptor type I (TGF-βRI) into caveolin-1 and early endosomal antigen-1 (EEA-1) double positive vesicles (He et al., 2015). Our findings of cytoplasmic translocation of caveolin-1 were strengthened by another study which showed that mechanical stretch induces translocation of caveolin from caveolae to non-caveolar sites in vascular smooth muscle cells (Kawabe et al., 2004). The group showed that this translocation is crucial for mechanical stretch induced activation of the ERK signalling cascade, an important mitogenic pathway in vascular smooth muscle cells. Subsequently it was showed that soluble caveolin remains embedded in a lipid particle and can travel to the endoplasmic reticulum, mitochondria and various other locations in the cytosol (Li et al., 2001). Our findings suggest that previous studies describing caveolins as membrane bound molecules (Parton and Simons 2007) must consider incorporating the physiological fluid flow component in studies. Notably, if exposure of fibroblasts to fluid

flow for time periods as short as 2h. can induce cytoplasmic translocation of caveolin-1A, then it is highly likely that longer exposure to flow for over 24h. could possibly translocate all of the caveolins to non-caveolar locations as shown in previous studies (Kawabe et al., 2004), thus carrying vesicle bound TGF-β receptors along with them to either non- caveolar locations or recycling them back to the cellular surface.

6.5.2: Fluid flow promotes endocytosis and translocates the TGF-β receptor to the nucleus of dermal fibroblasts.

The TGF-β receptors are known to be constantly undergoing changes such as activation and engulfment into lipid vesicles either by clathrin-dependent or caveolin-dependent endocytosis (Di Guglielmo et al., 2003b, He et al., 2015). Notably in this study, genes associated with endocytosis were modified by fluid flow in fibroblasts. Since it is know that clathrin–dependent trafficking of TGF-β receptors facilitates TGF-β signalling whereas caveolin-dependent internalisation of the receptors exerts an inhibitory effect (Anders et al., 1998, Di Guglielmo et al., 2003b), whether reduced expression of α-SMA in TGF-β1 stimulated fibroblasts subjected to fluid flow was explored (Figure 5.2) was due to caveolin mediated internalisation of TGFβ receptors. In line with previous studies that show that fluid flow induces endocytosis in the proximal kidney tubule (Raghavan and Weisz, 2015, Raghavan et al., 2014), observations show nuclear and cytoplasmic staining of early endosomal antigen (EEA-1) in fibroblasts subjected to fluid flow and double immunolabelled for EEA-1 and TGFβRII. Interestingly, staining intensity of both nuclear and cytoplasmic EEA-1 was high in TGF-β1 stimulated cultures subjected to fluid flow for 2h (Figure 6.2).

In the unstimulated resting state, TGF-β receptors exist as monomers on the membrane of cells. Upon binding of TGF-β1, the receptors undergo dimerization (Zhang et al., 2009) and the TGF-β co-receptor, betaglycan presents the ligands to TGFβRII (Lopez-Casillas et al., 1993). The ligand bound TGFβRII phosphorylates the GS domain of TGFβRI, thus promoting its interaction with Smad 2/3 and initiation of the signalling cascade (Kang et al., 2009). Fibroblasts that were stained for both EEA-1 and TGFβRII not only reveal that flow promotes endocytosis but also nuclear translocation of TGFβRII (Figure 6.2). Notably, nuclear staining of translocated TGFβRII was notably high in TGF-β1 stimulated cultures subjected to flow. Recent reports have shown that TGFβRI can be cleaved at residue G120 by TNF-α converting enzyme (TACE) and the released intracellular domain enters the nucleus to associate with p300 and regulate the expression of genes such as Snail and MMP2 (Mu et al., 2011). Subsequent papers showed that nuclear transport of TGFβRI is ligand inducible and requires cellular transformation as is observed in breast cancer cells (Chandra et al., 2012) . We report here that TGFβRII can translocate to the nucleus under the influence of TGF-β1 stimulation and low levels of fluid flow. Our findings suggests that even short-term TGF-β1 stimulation and fluid flow treatment for 2h. can augment caveolin mediated endocytic trafficking of the TGFβRII to the nucleus, thus antagonising the TGF-β pathway and expression of α-SMA.

6.5.3: Fluid flows enhances endocytosis in dermal fibroblasts.

One of the characteristic properties of the endosome is its acidic nature. Apart from permitting the selective function of lysosomal enzymes, the acidic profile plays other vital roles such as dissociating the ligand-receptor complexes (Brown et al., 1983). For example, reports have shown that binding of epidermal growth factor is highly pH sensitive that the receptor bound complex dissociates at a pH of 6 (Nunez et al., 1993). This high sensitivity to

pH is generally the rule of ligand dissociation of receptor ligand complexes during membrane trafficking. The trafficking of transferrin is another example which uses the unique pH sensitive membrane trafficking mechanism to achieve its biological function of delivering iron to intracellular sites. Here, the iron-transferrin complex binds to the cell surface receptor and is internalised to the acidic endosomes. Due to the sudden decrease in pH the iron dissociates but the receptor complex remains intact and rapidly returns to the cell surface. At the cell surface, the pH becomes neutral. This sudden change dissociates the apotransferrin complex (May et al., 1984).

pH RODO Dextran is a an alternative to fluorescent conjugates used for live cell imaging of endocytosis. It possesses a pH sensitive fluorescence emission which emits fluorescence only in an acidic profile. Essentially, it is non-fluorescent in the extracellular compartment. However upon internalization, the acidic environment emits a bright red signal from the dextran conjugate. It emits little signal at neutral pH and hence pH RODO dextran prevents detection of non-internalised and non-specific binding of conjugates. Our findings reveal little florescence from the dextran conjugates in static conditions. On the other hand, fluorescence signals were intensely emitted by fibroblasts treated by flow suggesting that flow enhances overall endocytosis and internalisation of ligand bound complexes and that flow induced endocytosis is independent of the highly complex fibroblast activation process. Recent studies which investigated the effect of fluid shear stresses on cellular uptake and endocytosis of nanoparticles in a biomimetic microfluidic system showed that under the influence of fluid flow, cancer cells exhibited higher cellular uptake of positively charged amino modified polystyrene nanoparticles which were delivered to cells in both static and dynamic conditions (Kang et al., 2016). These findings suggest that biomimetic dynamic conditions stimulated specific endocytosis and cellular uptake.

It is also possible that there are other relevant processes which use the endosomal environment to stimulate a chemical signal involved in receptor activation. For example, it has been shown in the past that the epidermal growth factor has to be continually present for a minimum of 8-10 h. to obtain a mitogenic reponse after 20 h. (Jorissen et al., 2003). Subsequently, it was shown that the biological effect of epidermal growth factor can be blocked by compounds such as methylamine and dansylcadaverine which can neutralise the endosomal system (Haigler et al., 1980). In line with these findings, it may be possible that treatment of fibroblasts with components that neutralise the endosomal system may influence the activation of fibroblasts. Whether endocytosis is vital for fluid flow mediated fibroblast activation is an area which requires further exploration.

Chapter 7- Fluid flow mediated fibroblast activation is buffered by TGF-β pathway associated molecules.

7.1: Introduction

The TGF-β superfamily is composed of a numerous signalling molecules interacting with other signalling pathways, thus contributing versatility to its response to various biological processes. In this study we explored flow mediated effects on some of the widely described molecules: activin A, follistatin and CD109.

Activin A's role as a proliferative and a differentiation factor was first studied in human lung fibroblasts (Ohga et al., 1996b). Findings revealed that activin A showed maximal effect on cellular proliferation of lung fibroblasts at levels as low as 10^{-11} M. Activin A also stimulated differentiation of human lung fibroblast to myofibroblast phenotype (Ohga et al., 1996a). Notably, the proliferative effect of activin A was abolished by follistatin, an activin binding protein. Activin A is also a crucial regulator of fibrosis in cardiac fibroblasts and that Angiotensin II enhanced expression of activin A was reversed by follistatin (Hu et al., 2016). Interestingly, follistatin could reverse activin A mediated responses such as cellular proliferation, differentiation, collagen type I expression and activin A activated pathways such as ERK1/2 and p38-MAP kinase pathways (Hu et al., 2016). Hashimoto et.al (1997) reported that the binding of activin to follistatin-288 (FS-288) is an irreversible process since the follistatin-activin complexes are rapidly endocytosed or degraded by lysosomal enzymes (Hashimoto et al., 1997). Reports have shown that follistatin can block the mesoderm inducing property of activins with other studies showing that it can also bind to bone morphogenic proteins (BMP-4 and 7) (Fainsod et al., 1997). The activin A–follistatin signalling system (Figure 7.1) is also a crucial determinant of extracellular matrix mineralization. Activin A induced inhibition of mineralization in osteoblast cultures was reversed by follistatin treatment (Eijken et al., 2007).

Exosomes are membrane vesicles released by epithelial cells, mesenchymal stem cells and tumour cells (Verma et al., 2015) and are known to contribute in cell-cell communication by removing dispensable proteins and cellular debris (Pan and Johnstone, 1983). These vesicles retain mRNAs, proteins and microRNAs and are released from the cells into the culture medium. CD 109 (cluster of differentiation 109) is a glycosylphosphatidylinositol anchored protein and is a crucial component of the exosomal factory. Recent findings revealed that membrane bound CD109 binds to TGF-β1 and promotes TGF-β receptor degradation via Smad7/Smurf2 mediated mechanism. Studies using the HEK293 human embryonic kidney cell line revealed that release of CD109 in the circulating medium is vital in the inhibition of TGF-β signalling (Sakakura et al., 2016).

Based on the available body of literature, it could be hypothesized that these molecules could contribute to fluid flow induced fibroblast differentiation. Hence studies were focussed on activins, antagonists of activins and TGF-β binding proteins that are known to exert both positive and negative regulatory effects on the signalling cascade.

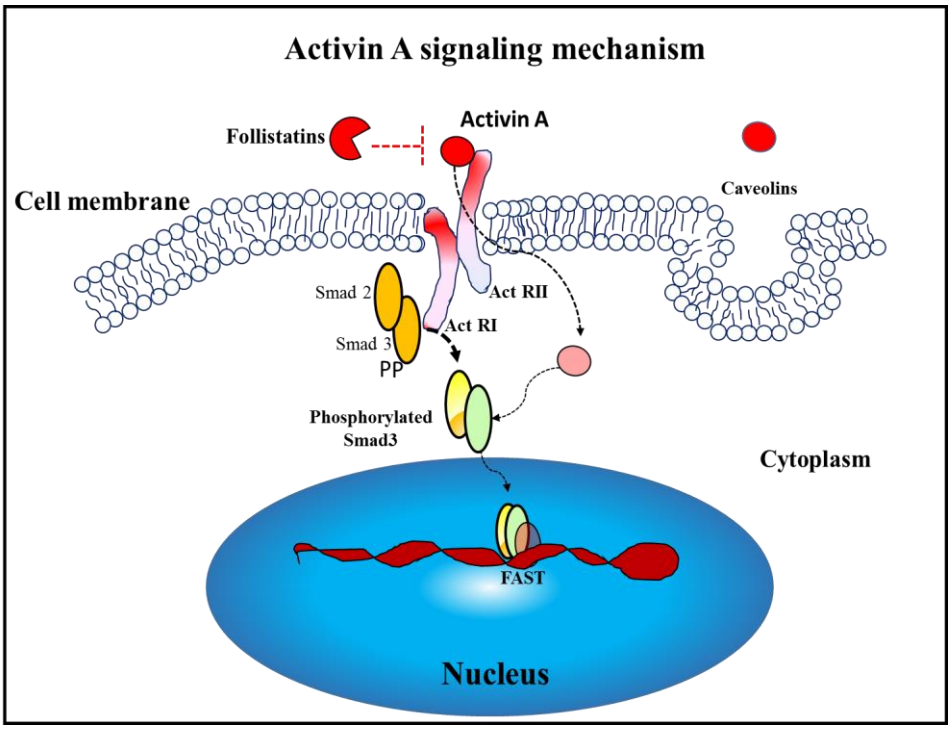

Figure 7.1: Activin A associated signalling mechanism. (Red dotted line indicates follistatin mediated antagonism of the pathway).

7.2: Fluid flow mediated activation of fibroblasts is modulated by activin A.

Although previous studies have shown that one of the widely described functions of activins are to invoke differentiation in fibroblasts, it is not clear yet whether these molecules contribute to fluid flow induced fibroblast activation. Our microarray analysis revealed that activin A is one of the top 20 genes associated with the TGF-β pathway increased under fluid flow, suggesting that activin A may play a role in fluid flow-mediated fibroblast activation and buffering of responses to TGF-β1. In this chapter, the gene and protein expression of activin A in unstimulated and TGF-β1 stimulated fibroblasts subjected to fluid flow were studied.

7.2.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 24 h and RNA was extracted as described in section 2.17. Experiments were performed three times and all replicates were subjected to analysis by quantitative PCR of activin A (INHBA) as described in section 2.19. Both cellular and secreted activin A were studied by subjecting dermal fibroblasts to flow treatment as above and performing ELISA as described in section 2.23.

7.2.2: Results

TGF-β1 supplemented dermal fibroblasts treated in static conditions showed elevated levels of activin A gene expression. Interestingly, activin A transcript levels were increased by 2.5 fold in dermal fibroblasts subjected to fluid flow for 24 h (Figure 7.2A). This was reduced to 0.8 fold in TGF-β1 stimulated fibroblasts subjected to fluid flow as compared to those under fluid flow only.

Concurrently, both cell-associated (Figure 7.2B) and secreted (Figure 7.2C) activin A protein were also measured by ELISA in cell lysates and conditioned medium. Results revealed that activin A was significantly ($p < 0.01$) increased in the conditioned culture medium and in cell extracts of fibroblasts subjected to flow. TGF-β1 stimulated fibroblasts in static and fluid flow conditions also showed elevated levels of cell-associated, but not secreted, activin A.

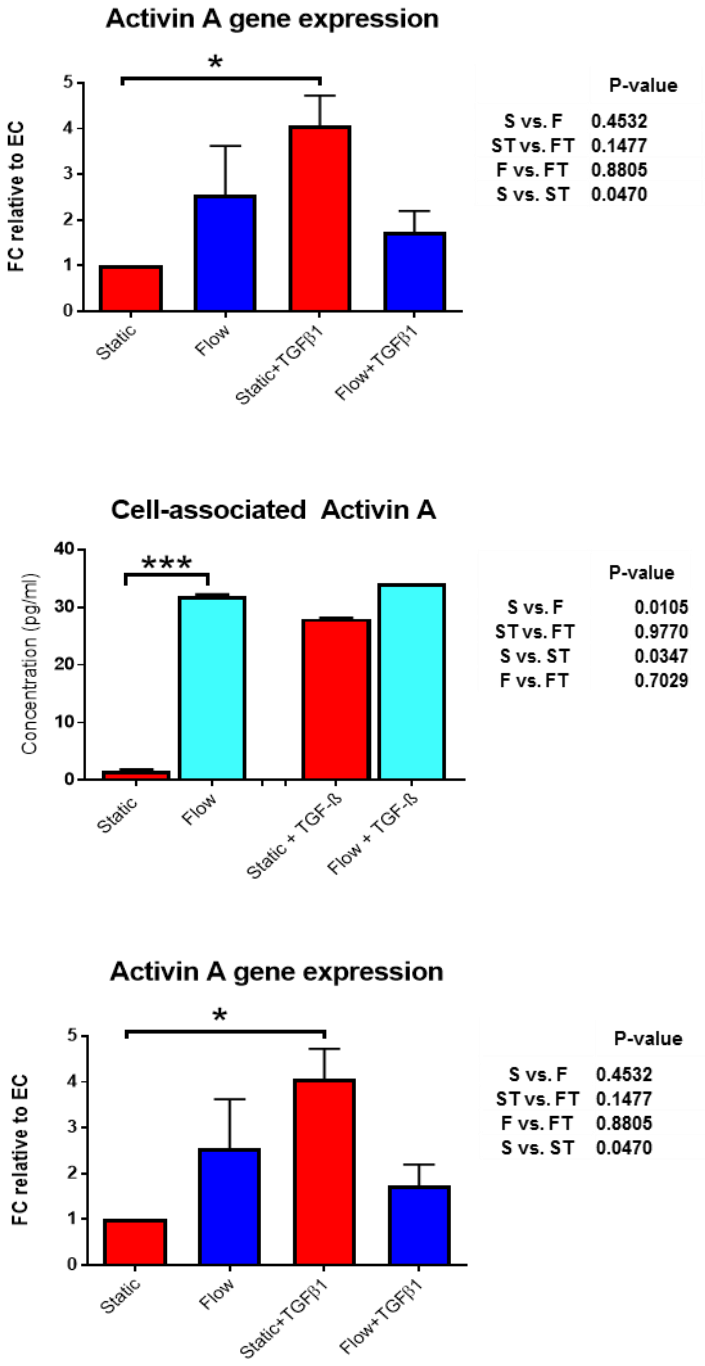

Figure 7.2: Expression of activin A in dermal fibroblasts subjected to fluid flow (150 µl/min) treatment for 24h. a) Gene expression of activin A with fold change relative to endogenous control in dermal fibroblasts subjected to flow (n=3) (* represents $p < 0.05$), b) Protein expression of cell associated A (n=3) (*** represents $p < 0.0001$) and c) Protein expression of secreted activin A in dermal fibroblasts subjected to fluid flow (n=3) (*** represents $p < 0.0001$). FC relative to EC represents fold change relative to endogenous control. (S= Static, F= Flow, ST= Static+TGF-β1 and FT= Flow+TGF-β1, vs= versus).

7.3: Fibroblast activation is attenuated by inhibition of activin A.

Follistatins are activin binding proteins and inhibit the cellular effects mediated by Activin. The role of activins in modulating fluid flow mediated responses was tested by treating fibroblasts with follistatins.

7.3.1: Methods

For immunofluorescence detection of α-SMA, dermal fibroblasts (NHDFC) grown on glass coverslips were subjected to following treatments in static conditions: TGF-β (10ng/ml), activin A (1ng/ml) and flow (150µl/min). follistatin (1ng/ml) inhibited cultures were also subjected to the same treatment as stated above for 24h. Post treatment, coverslips were immunolabelled with α-smooth muscle actin (α-SMA) (Sigma) antibody as described in section 2.12. Experiment was performed three times and staining intensity was quantified by Image J.

7.3.2: Results

To study whether activation of fibroblasts is regulated by activin A, dermal fibroblasts were treated with 1 ng/ml activin A and α-SMA protein was detected by immunofluorescence. Activin A stimulation in static conditions increased α-SMA expression (Figure 7.3a) to a similar level observed in fibroblasts stimulated with TGF-β1. When activin A stimulated fibroblasts were treated with 1 ng/ml follistatin-288 (activin A antagonist), protein expression of α-SMA decreased in comparison to cells treated with activin A alone. We also observed that follistatin treatment in TGF-β1 treated cells reduces the expression of α-SMA. Quantification of staining intensity by Image J analysis software also revealed that follistatin decreases α-SMA expression in both TGF-β1 and activin A stimulated cultures (Figure 7.3

b&c). Notably, it was observed that follistatin treatment caused very little decrease in α-SMA protein in flow activated cultures.

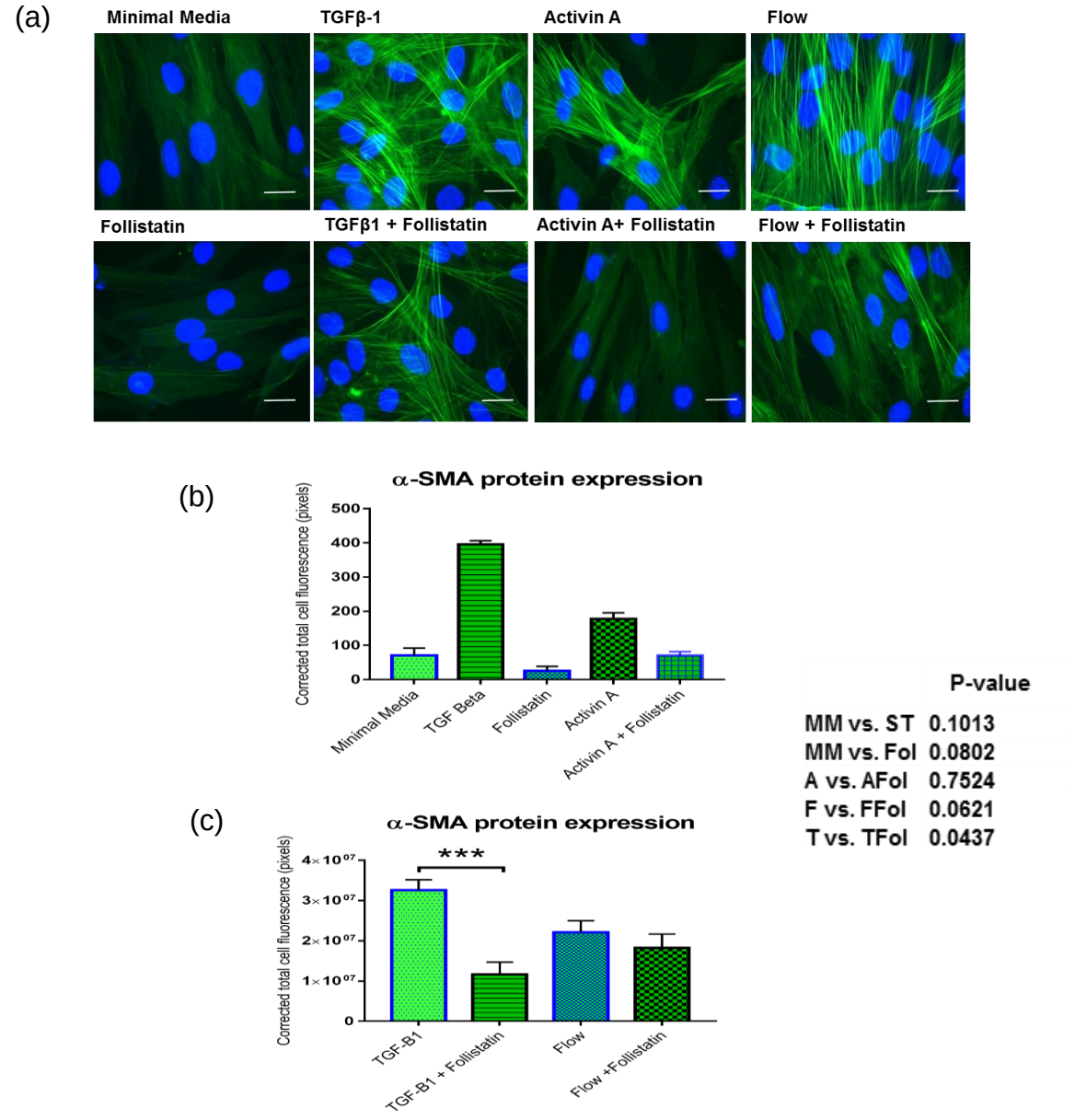

Figure 7.3: Protein expression of α-SMA protein in dermal fibroblasts subjected to fluid flow (150 µl/min), follistatin-288, TGF-β1 and activin A treatment for 24h. (n=3) Top panel: TGF-β1, activin A, fluid flow and Bottom panel: follistatin, TGF-β1 treated with follistatin, activin A treated with follistatin, Fluid flow treated with follistatin. b) Image J based quantification of α-SMA protein in dermal fibroblasts subjected to fluid flow (150 µl/min) for 24h (n=3), treated with minimal medium, TGF-β1, follistatin (Fol), activin A (A) and activin A with follistatin (AFol), TGF-β1 (T), TGF-β1 with follistatin (1 ng/ml) (TFol), flow (F) and flow with follistatin (FFol). (Scale bar =10 microns).

7.4: Fluid flow regulates gene expression of CD109 in fibroblasts.

Although studies have shown that CD109 is a TGF-β binding protein known to negatively regulate the TGF-β signalling pathway, little is known of its role in flow mediated fibroblast differentiation. It is hypothesized that CD109 may contribute to negative regulation of the TGF-β pathway in TGF-β1 supplemented fibroblasts subjected to fluid flow.

7.4.1: Methods

Dermal fibroblasts (NHDFC) seeded on glass coverslips were treated with fluid flow as described in section 2.10 for 24 h and RNA was extracted as described in section 2.17. Experiments were performed three times and all the replicates were subjected to quantitative PCR analysis of CD109 as described in section 2.19.

7.4.2: Results

Gene expression of CD109 was studied by quantitative PCR analysis of cDNA samples obtained from dermal fibroblasts subjected to flow in both TGF-β1 stimulated and unstimulated conditions. CD109 was significantly ($p < 0.01$) decreased by 0.5 fold in dermal fibroblasts treated by fluid flow. Interestingly, TGF- β1 stimulation under flow induces a significant ($p< 0.03$) increase in CD109 gene expression by 0.5 fold (Figure 7.4). Findings suggest that flow treatment in TGF- β1 supplemented cultures enhances expression of CD109, the TGF-β co-receptor and antagonises the fluid flow mediated activation of TGF-β pathway.

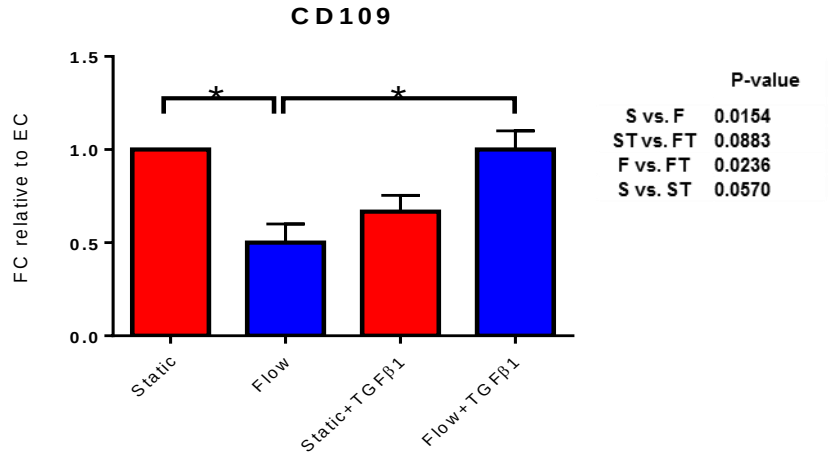

Figure 7.4: Gene expression of CD109 with fold change relative to endogenous control in dermal fibroblasts subjected to flow (150 μL/min) ($p < 0.05$) (n=3). FC relative to EC represents fold change relative to endogenous control.

7.4: Discussion

7.4.1: TGF-β1 regulates fluid flow mediated activation of fibroblasts via activin A.

Activin A is a known member of TGF-β superfamily and is neutralised by binding of two follistatin molecules (Thompson et al., 2005). The first evidence of activin's role in modulating fibroblast activity came from studies which showed that human lung fibroblasts proliferated in the presence of low levels of activin A (Ohga et al., 1996b). Another study showed that activin A promotes proliferation of fibroblast- like synoviocytes (Ota et al., 2003). Subsequently, it was shown that activin A is a potent activator of renal interstitial fibroblasts (Yamashita et al., 2004). Their findings revealed that activin A promoted cell proliferation, enhanced expression of type I collagen mRNA and α-SMA expression in rat kidney fibroblast cells. Recently, activin A was shown to promote profibrotic responses in interstitial fibroblasts during renal fibrosis and administration of recombinant follistatin

alleviated the profibrotic response (Maeshima et al., 2014a). Epithelium derived activin A is known to increase tumour promoting properties in mammary fibroblasts in a Cox-2 dependent manner differentiating these fibroblasts to a cancer associated fibroblast like phenotype (Fordyce et al., 2012). Whilst, literature suggests that activin A is mediator of fibroblast activation, whether supplementation of recombinant activin A is capable of differentiating normal fibroblasts to a myofibroblast like phenotype remains to be established. Findings report here that recombinant activin A can activate dermal fibroblasts by increasing α- SMA protein expression, thereby promoting differentiation to a myofibroblast like phenotype in static conditions.

To observe whether fluid flow mediated fibroblast activation is regulated by activin A, gene and protein expression of activin A was studied in TGF-β1 stimulated and un-stimulated dermal fibroblasts in both static and fluid flow conditions. In line with gene expression profile of α-SMA and Collagen IA1, activin A is also increased under flow. However, TGF-β1 supplementation decreases its expression under flow treatment. These findings suggest that fluid flow mediated activation of fibroblasts is modulated by activin A and are regulated by processes discussed in chapter 6.

Our findings also showed that secreted activin A is detected in high levels only in conditioned medium of fibroblasts subjected to flow treatment whereas cell associated activin A is high in TGF-β1 stimulated fibroblasts in static conditions and in fibroblasts treated with fluid flow in both TGF-β1 stimulated and un-stimulated conditions. This suggests that TGF-β1 supplementation regulates release of ectopic activin A under the influence of fluid flow. It is possible that secreted activin A competitively binds to TGF-β receptor type II and is then internalised into the nucleus. Our findings correlate with a recent paper which shows that

activin A can competitively bind to TGF-β receptor type 2 and BMP receptor type 2 to antagonise the signalling pathway (Aykul and Martinez-Hackert 2016) and undergo proteolytic degradation.

Fumagalli et al. 2007 have shown that in tissue sections of hypertrophic scars, there were high numbers of α-SMA(+) myofibroblasts which co-express activin A. Furthermore, treatment of fibroblasts with activin A induced Akt phosphorylation, promoted cell proliferation, and enhanced α-SMA and type I collagen expression. Follistatin reduced proliferation and suppressed activin-induced collagen expression suggesting that inhibition of activin A can potentially attenuate α-SMA expression (Fumagalli et al., 2007).

7.4.2: TGF-β & activin A induced response is inhibited by follistatin.

Follistatin is an extracellular regulatory protein of activin A and exerts isoform specific inhibitory effects on the biological activity of activin A and other members of the TGF-β superfamily. It is a secreted protein which binds to activins with high affinity and promotes binding to high affinity trans-membrane receptors. A number of studies have shown that expression of follistatins can reduce activin A mediated proliferation of fibroblast- like synoviocytes (Ota et al., 2003), modifies inflammatory cytokine cascade (de Kretser et al., 2012), prevent renal fibrosis *in vivo* (Maeshima et al., 2014b), is inversely proportional to expression of activin A (Forrester et al., 2013) and is highly expressed in all fibroblast strains with similar expression profile regardless of donor species (human or animals) (Kawakami et al., 2001). Since expression of activin A is observed in almost all tissues and of all the isoforms, follistatin 288 (FS-288) is known to show high cell surface binding and ligand

binding affinity to activin A (Sidis et al., 2006). Hence it was decided that FS-288 is used for study purposes.

Findings from our immunofluorescence studies showed that F-288 inhibition in both activin A treated and TGF-β1 stimulated dermal fibroblasts decreased α- SMA protein expression suggesting that a) activin A by itself is capable of differentiating fibroblasts to a pro-myofibroblast like phenotype and b) this biological effect can be inhibited by recombinant follistatin 288. Previous, reports show that cell associated FS-288 accelerates uptake of activin A, into cells which is then degraded by lysosomal enzymes (Hashimoto et al., 1997). Our findings strengthen previous research on the role of follistatin in modulating the response of activin A mediated fibroblast differentiation. It is possible that the under the influence of TGF-β1, fluid flow enhances expression and release of follistatins which may in turn bind to cell associated activin A leading to endocytic degradation.

Follistatin-like 3 (FSTL-3), FLRG, and FSRP are another group of proteins which show similarity with the biochemical, molecular and biosynthetic functions of follistatins. These proteins are known to neutralize ligands of the TGF-β superfamily such as activin A and bind to Myostatin with a 3-5 fold lower affinity (Schneyer et al., 1994, Tortoriello et al., 2001, Sidis et al., 2006). FSTL-3 is a potent inhibitor of activin signalling, negates the cardioprotective role of activin A in cardiomyocytes and is known to regulate stress induced hypertrophy via Smad7 signalling (Shimano et al., 2011). Subsequent studies showed that expression of FSTL-3 is indispensable for regulation of paracrine fibroblast activation by cardiomycocytes (Panse et al., 2012). Based on our microarray analysis which shows that Smad7 is upregulated by 4 fold, there exists a possibility that TGF-β1 stimulation under flow enhances expression of both isoforms of FST and FSTL-3 in fibroblasts and that the

cumulative effect of both FST and FSTL-3 augments binding to activin A promoting its degradation by endosomal enzymes.

7.4.3: Fluid flow mediated activation of fibroblasts regulates gene expression of CD109.

CD109 is a glycosylphosphatidylinositol- anchored protein first identified in primitive hematopoietic stem and progenitor cells (Sutherland et al., 1991). It is a membrane anchored protein and a member of α-2 macroglobulin family of thioester containing proteins (Lin et al., 2002). Expression of CD109 was found to be high in NIH3T3 cells with multiple endocrine neoplasia 2B mutation (Watanabe et al., 2002) and in some tumour cell lines such as squamous cell carcinoma and glioblastomas. Hence it is considered as an important molecular target for development of novel therapeutics for malignant tumours such as squamous cell carcinoma (Hashimoto et al., 2004). CD109 functions as a TGF-β co receptor and an inhibitor of TGF-β pathway in keratinocytes (Finnson et al., 2006) and is known to enhance binding of TGF-β to its receptors, promote TGF-β bound receptor internalization via the Caveolin pathway in a ligand dependant manner (Bizet, Liu et al. 2011) and attenuate ECM production in skin fibroblasts (Man, Finnson et al. 2012).

Our results reveal that gene expression of CD109 (TGF-β co-receptor) is reduced under flow and TGF- β1 supplementation under flow returns levels to that of statics suggesting that CD109 may antagonise the TGF-β pathway and function as an attenuator of extracellular matrix production as is observed previously in scleroderma skin fibroblasts (Man et al., 2012). Observations from studies of endocytosis suggest that TGF-β1 supplementation in the presence of fluid flow enhances expression of CD109. It is possible that CD109 binds to

excess TGF- β1 and enhances caveolin mediated endocytosis of TGF- β1 and negatively regulate the TGF- β pathway (Bizet et al., 2011).

Findings prompt to conclude the negative regulation of the TGF- β pathway as is observed in TGF- β1 supplemented cultures subjected to fluid flow which may be due to the combined effect of multiple processes. Two such proposed mechanisms are a) release of follistatins or follistatin like proteins which bind to activin A and enhance lysosomal degradation and b) release of CD109 which binds to TGF- β1 and promotes caveolin mediated internalization of the TGF- β receptor into endosomes. Since effects observed when normal fibroblasts were subjected to fluid flow are dramatically different to those under flow in TGF-β1 stimulated conditions, suggests that this may be a novel mechanism which regulates the homeostatic balance of fibroblasts during abnormal conditions such as wound healing and cancer.

Chapter 8 – Fluid flow modulates cellular growth arrest in fibroblasts.

8.1: Introduction.

Cellular growth is directed by the cell cycle which has a number of checkpoints to allow the cell to decide whether to continue dividing or stop. The cell cycle commences with G1 phase when the cell prepares to duplicate itself. It remains active in the S phase by DNA replication. Damage to the newly formed DNA are repaired prior to the G2 checkpoint before the cell proceeds to the mitotic M phase where cell division occurs. Cells now return to the G1 phase to continue the process or halt. Cellular checkpoints are influenced by environmental factors such as mechanical forces, radiation and drugs which could either arrest cellular growth, initiate apoptosis or allow the cell to remain in a state of quietness or inactivity (Huselstein et al., 2006, Mundt AJ, 2003, Shiff et al., 1996).

Quiescence is a cell cycle state in which cells do not proliferate. Instead, they retain the ability to proliferate but can remain quiescent for long intervals (Campisi and d'Adda di Fagagna, 2007). Such growth arrest can be caused by withdrawal of serum growth factors. Absence of growth leads to low metabolism, very little protein synthesis and cellular functions. Cells in a quiescent state invoke a program which protects them from programmed cell death, differentiation and oxidative stress (Naderi et al., 2003, Coller et al., 2006, Sang and Coller, 2009). Such programming in tumour cells allows them to survive from apoptosis induced by anti-cancer agents. Nevertheless, in the case of long-lived cells such a protected state may be beneficial. These cells use lysosomal and autophagy associated pathways for protein degradation and protein turnover (Ciechanover, 2005).

On the other hand, senescence can also be defined as cellular aging wherein DNA damage harbouring cells or aging cells are maintained in a cellular state which is irreversible and permanent (Fridlyanskaya et al., 2015, Dimri et al., 1995). It is characterised by growth arrest

usually at G1 and sometimes in G2 phases of the cell cycle. Senescence is broadly classified into two types: replicative senescence occurring due to long-term cultivation of cells in culture resulting in loss of telomeric DNA (Hodes, 1999) and stress induced premature senescence (SIPS) which results due to the effects of radiation, osmotic stress, mechanical stress and drugs which inhibits cell division (Cmielova et al., 2012). Senescent fibroblasts show variations in gene expression of cell cycle modulators and cyclin dependent kinase inhibitors such as p21 $^{Waf-1}$ and p16 Ink4 A (Jackson and Pereira-Smith, 2006). Initially discovered as the terminal replicative stage in mitotic cells, senescence was linked to loss of telomeres (Hayflick, 1965). Later, studies showed that senescence need not be associated with telometric attrition and cell division, particularly in fibroblasts which reach confluency in static cell culture (Munro et al., 2001). Classical studies have shown that activated fibroblasts maintain their phenotype in static cell culture even when they are isolated from the tissue of origin suggesting that they maintain their phenotype by a mechanism which is irreversible and permanent as is observed during senescence. Senescent cancer associated fibroblasts develop a secretory phenotype known as senescence associated secretory phenotype (SASP) (Coppe et al., 2010) and regulate tumour cell adhesion and invasion via TGF-β dependent pathways (Hassona et al., 2015, Calon et al., 2014). TGF-β1 induces senescence of bone marrow mesenchymal stem cells by increasing senescence-associated-β galactosidase (SA-β Gal) activity and p16/INK4 expression (Wu et al., 2014). Co-culture of senescent fibroblasts from tissues of different origin such as prostate or lung with non-malignant epithelial counterparts promotes cell growth, migration and invasion showing that that senescent fibroblasts contribute to augment growth of tumours of epithelial origin (Krtolica et al., 2001). Interestingly, numerous reports showed that genotoxic stress can also invoke senescence of cells (Matos et al., 2015). Recent reports show that differentially expressed micro RNAs such as miR-210 and miR-146a are involved in both fibroblast

activation and senescence (Yao et al., 2011, Bhaumik et al., 2009). Research has also reveal widespread changes in miRNA expression of both cancer associated fibroblasts and senescent fibroblasts (Prime et al., 2016). This compelling evidence suggests that there are common links between fibroblast activation and senescence and it is therefore possible that fluid flow induced fibroblast differentiation is associated with a cell cycle arrest which may result from senescence or quiescence.

8.2: Fluid flow alters gene expression of p16 INK4a in dermal fibroblasts.

8.2.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 24 h. and RNA was extracted as described in section 2.17. Experiments were performed three times and all the replicates were subjected to quantitative PCR analysis of p16 expression.

8.2.2 Results

TGF-β1 treated dermal fibroblasts seeded on glass coverslips revealed an increased expression of p16 INK4a (Figure 8.1a) in static conditions. Interestingly, flow treatment decreases the expression of p16 INK4a. Similarly, when TGF-β1 supplemented fibroblasts were treated by fluid flow, gene expression of p16 INK4a decreased significantly in comparison to fibroblasts stimulated with TGF- β1 in static conditions. Contrastingly, flow treated dermal fibroblasts seeded on collagen IA coated Thermonox coverslips revealed an increased expression of p16 INK4a (Figure 8.1b). Nevertheless, TGF-β1 supplementation

significantly reduces p16 INK4a expression under flow than static in fibroblasts seeded on both glass and collagen coated Thermonox coverslips.

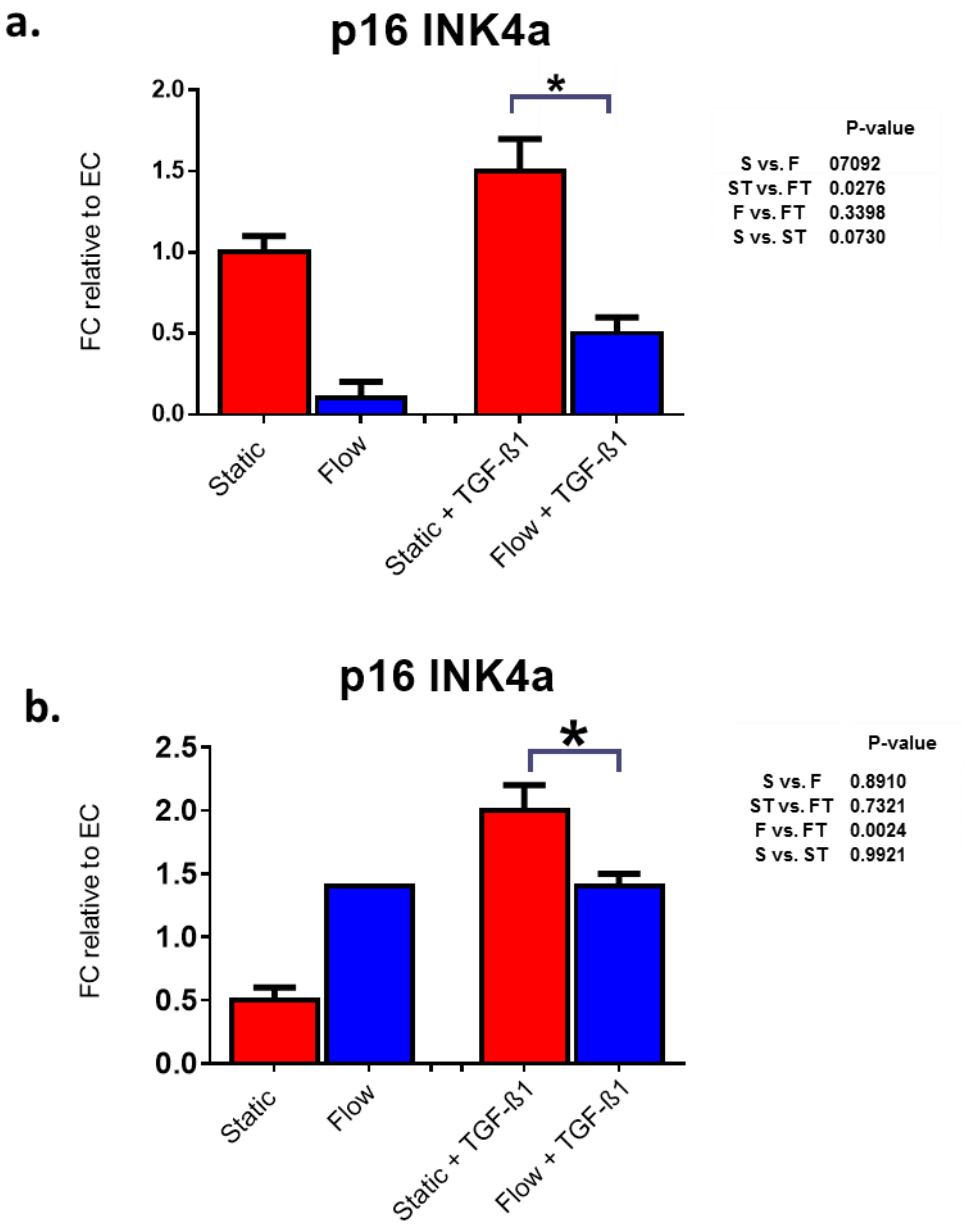

Figure 8.1: Changes in gene expression of p16 in dermal fibroblasts under flow (150 µL/min) for 24 h. Dermal fibroblasts cultured on (a) glass coverslips and (b) collagen type I coated Thermonox coverslips. FC relative to EC represents fold change relative to endogenous control. (S= Static, F= Flow, ST= Static+TGF-β1 and FT= Flow+TGF-β1, vs= versus).

8.3 Fluid flow modulates gene expression of p21^{Waf-1} in dermal fibroblasts.

8.3.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 24 h and RNA was extracted as described in section 2.17. Experiments were performed three times and all the replicates were subjected to quantitative PCR analysis of p21 $^{Waf-1}$ gene expression as described in section 2.19.

8.3.2 Results

TGF-β1 treatment in static conditions decreases expression of p21 $^{Waf-1}$ in dermal fibroblasts that were seeded on glass coverslips. Interestingly, flow treatment enhances the expression of p21 $^{Waf-1}$ in both TGF-β1 supplemented and fibroblasts (Figure 8.2). In line with data obtained from fibroblasts on glass coverslips, static fibroblasts seeded on collagen IA coated Thermonox coverslips showed a decrease in gene expression of p21 $^{Waf-1}$ whereas flow treatment increases the expression in both TGF-β1 supplemented and untreated fibroblasts.

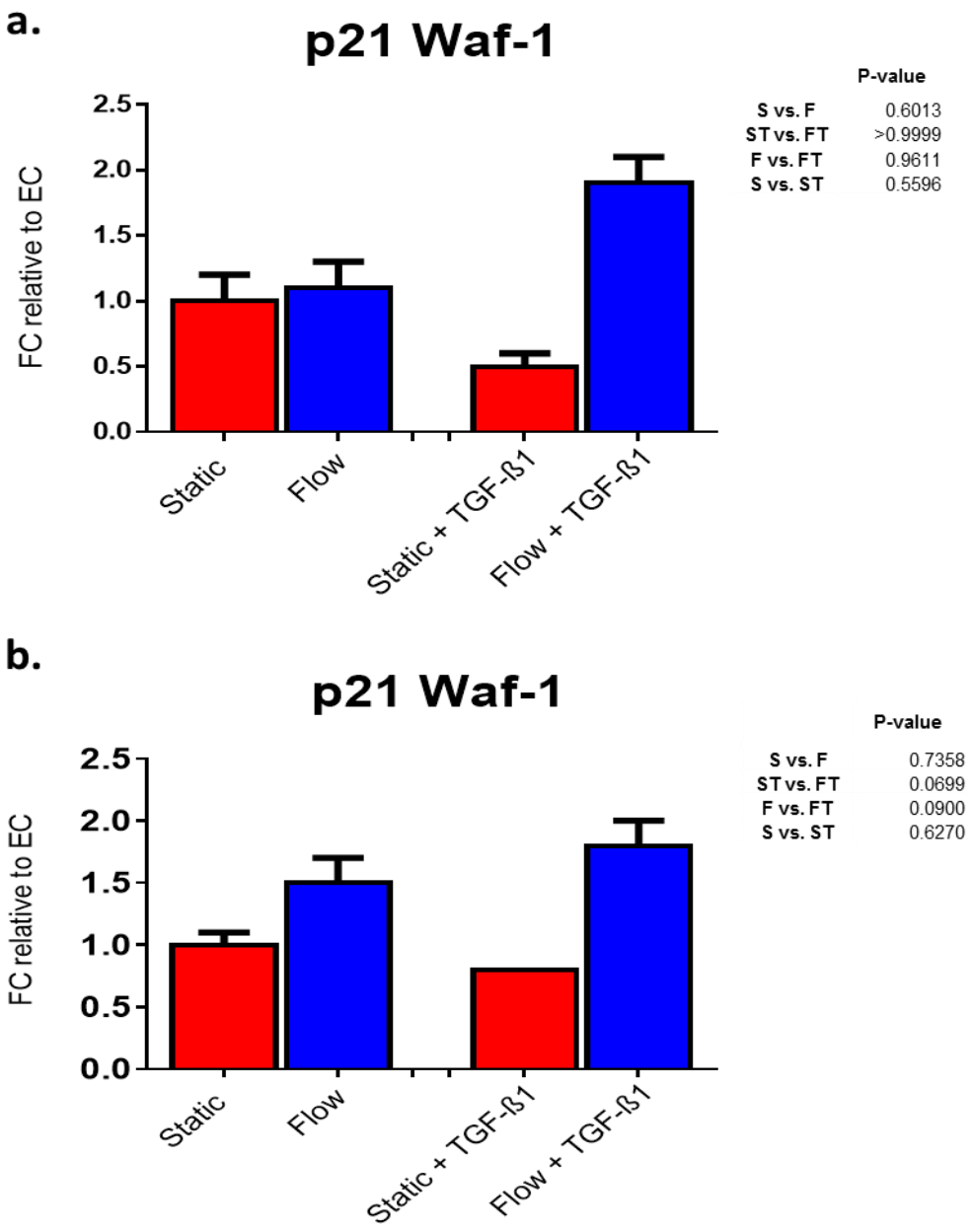

Figure 8.2: Changes in gene expression of $p21^{Waf-1}$ in dermal fibroblasts under flow (150 µL/min) for 24 h. (a) Dermal fibroblasts cultured on glass coverslips and (b) collagen type I coated Thermonox coverslips. FC relative to EC represents fold change relative to endogenous control. (S= Static, F= Flow, ST= Static+TGF-β1 and FT= Flow+TGF-β1, vs= versus).

8.4: Fluid flow decreases senescence in dermal fibroblasts.

8.4.1: Methods

Primary dermal fibroblasts (NHDFC) were treated with fluid flow as described in section 2.10 for 24 h and coverslips were stained for detecting SA β Galactosidase activity using the X-Gal staining kit (Abcam) as explained in section 2.15.

8.4.2 Results

Senescence associated-β galactosidase (SA-β Gal) activity was detected as a blue colour in a few dermal fibroblasts grown on glass coverslips in static conditions. TGF-β1 supplementation in static conditions revealed very little β galactosidase activity in fibroblasts. However, flow treated fibroblasts display little activity of SA-β Gal in both TGF-β1 supplemented and untreated conditions (Figure 8.3). In addition, flow treated fibroblasts display changes in shape in comparison to those in static conditions.

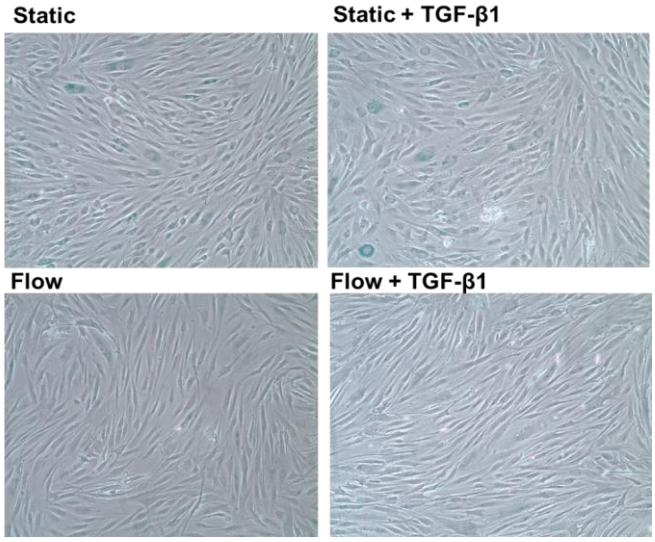

Figure 8.3: SA-β-Galactosidase staining of dermal fibroblasts on glass coverslips under flow (150 μL/min) for 24 h. All images were captured using 10X magnification lens at the same exposure time. Representative images from three independent experiments.

8.5: Discussion

Compelling evidence has shown that normal human fibroblasts gradually lose their proliferative capacity to undergo senescence whereas other studies show that fibroblasts undergo stress induced senescence as an adaptive mechanism to environmental factors and mechanical stresses (Prime et al., 2016). Whilst it is understood that experiments involving senescence and quiescence should be pursued over prolonged exposure times to gain a deeper understanding, in this chapter efforts were focussed to understand if fluid flow invokes stress induced proliferative senescence in dermal fibroblasts during shorter incubation periods of about 24h.

8.5.1: Fluid flow alters the expression of p16 INK4a in dermal fibroblasts.

Whilst senescence is reflected by changes in the phenotypic profile of various organelles, cyclin-dependent kinase inhibitors such as p21 $^{Waf-1}$ and p16 INK4a and p53 pathways are the known molecular mechanisms that mediate senescence (Kabir et al., 2016). Transition from G1 to S phase is controlled by the p16-Rb pathway, binding of p16 INK4a to CDK4/6 prevents phosphorylation of Rb and transcription E2F1 genes that are crucial for G1 to S transition. Studies have also reported that premature senescence could be induced by Rb-p16-19 Arf proteins in the presence of E2F activation in a mouse pituitary tumour model (Lazzerini Denchi et al., 2005). Despite that, cellular proliferation was invoked by E2F and cells underwent senescence through formation of repressor complexes between Rb family members and E2F in the nucleus (Rowland and Bernards, 2006). Rb is therefore a crucial tumour suppressor gene and its inactivation by p16 INK4a promotes proliferation of the

reprogrammed cells (Li et al., 2009). Although there exists a feedback loop mechanism between p16 INK4a and Rb wherein phosphorylation of Rb results in increased inhibition of CDK4/6 by p16 INK4a, various reports have shown that levels of p16 INK4a do not vary appreciably during cell cycle (Li et al., 1994, Hara et al., 1996). However, during aging and in conditions of exposure to DNA damaging agents, expression of p16 INK4a is increased resulting in senescence (Meng et al., 2003, Wang et al., 2006c). Finally, reports have shown that the p16/Rb pathway activates protein kinase C delta (PKC delta) via induction of reactive oxygen species (ROS) leading to an irreversible cell cycle arrest (Takahashi et al., 2006). Our findings show that gene expression of p16 INK4a is altered under flow. In fibroblasts grown on glass surfaces, flow treatment reduces the expression of p16 INK4a whereas those fibroblasts grown on collagen coated Thermonox coverslips reveal an increase in expression of p16 INK4a. These findings seem to suggest that in an *in vivo* like condition where cells experience fluid flow, matrix proteins such as collagen IA1 influence the gene expression of p16 INK4a and may contribute to premature cellular aging. Regardless of the surface treatment when TGF-β1 treated fibroblasts are exposed to fluid flow, expression of p16 INK4a is decreased suggesting that a) despite the presence of endocytosis and ligand trafficking, TGF-β1 can reduce the expression of p16 INK4a under fluid flow and b) The influence of matrix proteins such as collagen I A1 can be antagonised by TGF-β1. It is reported that senescent cells which do not express p16 INK4a can resume growth after genetic interventions that inactivate the p53 tumor suppressor (Beausejour et al., 2003) suggesting that fibroblasts which show reduced expression of p16 INK4a under flow can potentially resume proliferation and regain expression of p16 INK4a upon withdrawal of flow.

Apart from the p16/Rb pathway, the p38 MAP Kinase pathway is known to play a role in premature senescence via the Ets family of transcription factors (Lin et al., 1998, Foos et al., 1998, Freund et al., 2011). Studies have shown using reporter assays that these transcription factors (Ets 1 and Ets 2) enhance transcription of p16 INK4a gene and transcriptional repression of p16 INK4a could be prevented by mutating the binding site of Ets 1 (Ohtani et al., 2001). Data obtained from microarray based global gene expression analysis of fibroblasts subjected to two different flow rates reveal that alterations are observed in the MAP kinase pathway (Chapter 4). This finding suggests that flow mediated stresses causes variations in the MAP kinase which may contribute to p16 INK4A induced premature senescence in flow activated fibroblasts cultured on collagen coated Thermonox coverslips.

Despite evidence that senescence is associated with changes in both p16 INK4a and p21 $^{Waf-1}$, numerous studies have shown that p16 INK4a is not commonly expressed by quiescent or terminally differentiated cells (Hara et al., 1996, Stein et al., 1999). These reports call in to question observations on differential expression of p16 INK4a in plain glass and collagen coated Thermonox coverslips. In line with observations that flow induced fibroblast activation is transient and reversed upon withdrawal of flow (Section 5.5), suggests that the observed effects are the result of a short term cell cycle arrested state which may be reversed and may be an indicator of a quiescent phenotype rather than that of senescence.

8.5.2: Fluid flow alters the expression of p21^{Waf-1} in dermal fibroblasts.

Cellular quiescence is defined as a reversible non-proliferative state. In contrast to terminally differentiated cells which are senescent and irreversibly arrested, quiescent cells can re-enter

the proliferative cell cycle in response to growth signals. Quiescence protects cells against stresses and toxicities in long lived cells such as stem cells (Cheung and Rando, 2013). Studies on quiescent fibroblasts show that whilst these cells are viable, they are protected from proteasome inhibition mediated toxicity (Legesse-Miller et al., 2012). In addition to down-regulation of proliferation related genes, quiescent cells express a group of cyclin-dependent kinase inhibitors (CDKs) p21 $^{Waf-1}$ or p27 that are distinct from cell cycle arrested cells (Liu et al., 2007). In contrast to the senescence pathway, quiescent cells feature high levels of Rb-E2F pathway repressors and Cdk inhibitors such as p21 $^{Waf-1}$ (Cheng et al., 2000) and p27 (Coats et al., 1996). Other studies show that down regulation of Cdk inhibitor p21 $^{Waf-1}$ can reprogram the cells to re-enter the cell cycle (Nakanishi et al., 1995). The study revealed that expression of antisense p21 $^{Waf-1}$ RNA in G0 arrested cells resulted in induction of DNA synthesis and entry of cells into the mitotic phase. These findings suggest that p21^{Waf-1} functions in the G0 and the early G1 phase and a decreased expression of the gene is necessary for cell cycle progression. A recent report revealed that the proliferation – quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit and that this exit is guarded by p21 $^{Waf-1}$ expression (Spencer et al., 2013).

In the face of a huge body of literature which shows that the CDK inhibitor p21 $^{Waf-1}$ can induce growth arrest, senescence and quiescence, its involvement as a promoter of quiescence still remains debated. Studies conducted by Perucca et.al (2009) provide the first evidence showing that high levels of p21^{Waf-1} protein are essential to enter and maintain the quiescence state. Our findings show that gene expression of p21^{Waf-1} is increased under flow. In fibroblasts, flow treatment increases the expression of p21^{Waf-1} in both TGF-β1 treated as well as in untreated cells suggesting that fluid flow may induce a short lived state of quiescence in fibroblasts and that this state may be guarded by increasing levels of p21 $^{Waf-1}$. Quiescent

fibroblasts are known to exhibit high metabolic activity (Lemons et al., 2010). However, results in the current study reveal that fluid flow reduces the metabolic activity of fibroblasts (Section 3.4). Although this observation contradicts existing literature, it is possible that under the influence of fluid flow and high levels of p21, the cellular machinery maintains a low metabolic profile and the cellular machinery focusses only on processes involved in differentiation such as synthesis of fibrous proteins.

Recent studies have shown that the multi-functional cytokine activin A potently inhibits cellular proliferation and tube formation of endothelial cells (HUVECs) by induction of $p21^{Waf-1}$ (Kaneda et al., 2011). Notably, stable $p21^{Waf-1}$ knockdown significantly enhanced cellular proliferation of HUVECs suggesting that $p21^{Waf-1}$ induction has a key role in activin A mediated growth inhibition of endothelial cells. Our observations on flow mediated increase in expression of cell associated and secreted activin A (Section 7.2) validates existing literature. Enhanced expression of $p21^{Waf-1}$ in both normal and TGF-β1 stimulated fibroblasts subjected to flow suggests that fluid flow induces a short term growth arrested quiescent state by a) reducing the mitochondrial activity of fibroblasts and b) activin A mediated increase in the expression of $p21^{Waf-1}$ which inhibits cellular proliferation in fibroblasts.

8.5.3: Fluid flow decreases senescence in dermal fibroblasts.

Morphological changes are one of the most evident changes that occurs when cells undergo senescence. Cells increase in volume and often reach double the size of a presenescent cell (Hayflick, 1965). Upon senescence, cells switch their phenotype from a matrix producing to a matrix degrading phenotype. Production of elastin and collagen are decreased whereas increased production of fibronectin and matrix metalloproteinases- 1 and 3 (MMP-1 and

MMP-3) (Hornebeck and Maquart, 2003) occurs which regulate the activity of factors in the senescence associated secretory phenotype (SASP) and degrade the stromal fibres and the extracellular matrix. Senescent fibroblasts secrete pro-inflammatory cytokines such as IL-6 (Kuilman et al., 2008), IL-1 (Kumar et al., 1992) and numerous epithelial cell growth factors and hence adopt a phenotype which resembles a wounding response. Flatter and irregular shaped cellular architecture, distinct intracellular actin fibres, enhanced nuclear and nucleolar size, multinucleated cells, prominent golgi apparatus, increased number of vacuoles in endoplasmic reticulum and cytoplasm and large lysosomal bodies are other morphological changes which accompany senescence (Cristofalo and Pignolo, 1993). Bright field images of flow treated fibroblasts appear elongated instead of having a flat and irregular shaped morphology as is observed in senescence. However immunofluorescence imaging show that flow enhances expression of the fibrous protein α-SMA in fibroblasts (section 5.2). These observations suggest that fibroblasts treated with flow over a short period of 24h. are not senescent. Instead they acquire a growth arrested, presenescent phenotype which is marked by low mitochondrial activity and enhanced expression of contractile proteins such as α-SMA and Col IA1.

Senescence associated beta galactosidase (SA-β-gal) is a β-galactosidase enzyme which can be detected only in cells undergoing induced or replicative senescence (Dimri et al., 1995). SA-β-gal could be detected by in-situ staining using a chromogenic substance known as X-gal which yields a blue precipitate (Miller, 1972). Since early reports, SA-β-gal activity has been widely used as a biomarker for senescence. Its activity was detected at pH 6.0 detected in organs of old animals suggesting that ageing tissue accumulates senescent cells and is generated by the β-galactosidase that is localised to lysosomes of mammalian cells. Based on indirect physiological experiments, it was initially proposed that increased lysosomal-β-

galactosidase activity in senescent cells accounts for SA-β-gal activity (Kurz et al., 2000). Lee et.al (2006) later showed that GLB1 gene encoded activity of SA-β-gal is typically measured only at a pH of 4.5 (Lee et al., 2006). They showed that fibroblasts from patients with a defective lysosomal β-galactosidase did not express SA-β-gal even when they underwent replicative senescence. In addition, late passage normal fibroblasts that underwent senescence but failed to express SA-β-gal. These findings seem to suggest that the increase in β-galactosidase activity is an outcome of senescence and that SA-β-gal activity cannot stand alone to define a senescent phenotype. Bright field images of fluid flow treated fibroblasts reveal little activity of SA-β-gal compared to static cultures. This observation seemingly suggests that fibroblasts do not undergo senescence under the influence of fluid flow.

p16 INK4a and $p21^{Waf-1}$ are classically characterised as cellular markers of growth arrest. However, from the body of existing literature on the overlapping roles of p16 INK4a and $p21^{Waf-1}$, our findings prompt evaluation of other senescence associated markers and analysis cell cycle analysis by techniques such as flowcytometry and BrDU based detection of cells in S- phase. Nonetheless, results from cumulative experiments on gene expression of p16 INK4a, $p21^{Waf-1}$ and SA-β-gal staining suggest that fluid flow induced differentiation of fibroblasts is more transient and may induce growth arrest that is reversible in nature.

Chapter 9 - Discussion of findings

All living cells are constantly exposed to a number of different physical forces such as shear stresses, interstitial fluid flow, blood flow and tissue fluid flow due to mobility. The microenvironment of growing tumours and healing wounds are known to experience a highly dynamic environment with increased mechanical stiffness due to accumulation and reorganisation of the extracellular proteins and interstitial fluid flow (Butcher et al., 2009). Whilst 3D tissue culture models are a recent advancement and help towards building realistic models incorporating more than one cell type to form a tissue, these models do not easily lend themselves to studying the effect of interstitial fluid flow like mechanical signals on individual cell types or cells grown in a matrix. Furthermore, studies have shown that the oxygen concentrations within the central regions of 3D scaffolds is low in static cultures resulting in a gradient of cellular attachment from the periphery towards the centre. The introduction of fluid perfusion in bioreactors results in an improved oxygen supply to cells (Volkmer et al., 2012, Sugiura et al., 2011, Weyand et al., 2015). Previous studies show that oxygen levels in static cultures drop to 0% within 5 days of culture whereas perfusion bioreactors prevented cell death by sustaining 4% oxygen levels throughout the study (Volkmer et al., 2008).

The Quasi vivo® bioreactor used in this study is a multi-compartmental modular bioreactor (MCmB 2.0) consisting of chambers made of polydimethylsiloxane (PDMS) (Mazzei et al., 2010). The Quasi vivo® system (QV-500) was developed as a dynamic fluid model and was designed initially with assessment of the minimum concentration of oxygen allowed near the cell surface with hepatocytes as a reference cell type. Optimal chamber size enabling both adequate oxygen diffusion and low shear stress near the cell surface were also studied.

Results showed that the total amount of oxygen available is greater in the chambers and that the bioreactor chambers can enhance or maintain cell viability and function when the flow rates are below 500 µL/min, corresponding to wall shear stresses of 10^{-5} Pa or less at the cell culture surface (Mazzei et al., 2010). When human umbilical endothelial cells (HUVECs) and hepatocytes were grown in connected chambers of the Quasi Vivo® bioreactor, they displayed increased endothelial cell viability, elevated levels of hepatic glucose, albumin and urea in hepatocytes (Vozzi et al., 2009). These findings suggest that, unlike the hypoxic environments experienced by cells in static culture, the perfusion flow in multi-compartmental bioreactor supplements the cells with sufficient oxygen levels for improved function. Current study shows that fibroblasts from both oral and dermal tissues can withstand flow conditions. Further, absence of SA-β Galactosidase positive cells in fluid flow treated dermal fibroblasts suggest that the perfused oxygen contributes to decreased expression of SA-β Galactosidase in these cells.

Living cells experience shear stresses due to different types of fluid movement such as blood flow, interstitial fluid flow and that these are highly dependent on the tissue and pathological conditions. It is difficult to determine actual shear stresses and fluid flow velocities experienced by cells. Literature shows that average interstitial flow velocities in normal and neoplastic tissues is 6×10^{-7} m/s (Chary and Jain, 1989), the flow velocities generated at a fluid flow rate of 75 & 150 µl/min were 3×10^{-7} m/s and 5×10^{-7} m/s respectively suggesting that flow velocities experience by fibroblasts in this study are comparable those experienced tissues *in vivo*. These values are many orders of magnitude lower than those present in the vascular system *in vivo*. In this thesis, efforts were focussed to model in *vivo* like interstitial fluid movement on fibroblast and study their responses. The study compares the differences in cellular response, signalling changes and the effect of growth factor supplementation in

fibroblasts subjected to static and dynamic treatment conditions from both dermal and oral sources.

Fibroblasts serve as building blocks of the extracellular matrix in connective tissues. These cells secrete different types of Collagen, glycoproteins, Matrix Metalloproteases and fibrous proteins such as α-smooth muscle actin. They can differentiate into myofibroblasts when stimulated by TGF-β1 (Hinz et al., 2012). Myofibroblasts are a variant form of activated fibroblasts that acquire a smooth muscle phenotype and promote wound contracture. During a wound healing process, cytokines and chemokines released by epithelial, endothelial and mesenchymal cells work in a paracrine manner to stimulate fibroblasts to proliferate and migrate into the granulation tissue matrix. Once they embed themselves into the matrix, they transform into an activated phenotype which is characterised by enhanced expression of α-Smooth Muscle Actin (Honda et al., 2010), and Collagen IA1 (Cutroneo et al., 2007, Pan et al., 2013). Upon completion of wound healing, these fibroblasts undergo apoptosis. However, in pathological conditions such as cancer which is marked by high levels of interstitial fluid flow, angiogenesis and blood vessel formation (Thannickal et al., 2004) and systemic sclerosis (Varga and Jimenez, 1995), these fibroblasts remain activated indefinitely. Hence activated fibroblasts are often used as a marker for poor prognosis in cancer. Recent studies revealed that SMA-positive, myofibroblastic stroma is the strongest predictor of Oral Squamous cell carcinoma mortality (Marsh et al., 2011).

Mechanobiology refers to the method by which living cells sense signals from surroundings, process these signals; modulate structural and mechanical integrity inside cells and in their microenvironment. The extracellular matrix was once thought to solely provide structural support to tissue architecture. However, recent literature suggests that ECM also mediates a range of cellular activities like migration, myofibroblast differentiation, matrix stiffness and

cellular survival. For example studies have shown that myofibroblasts employ actin based motors to generate contractile forces and mediate tissue remodelling by traction (Tomasek et al., 2002). The aim of this study was to investigate the response of fibroblasts to mechanical forces and chemical signals in a physiologically relevant dynamic environment. preliminary experiments showed that that interstitial fluid flow can activate fibroblasts when subjected to fluid flow at rate of 120µl/min (Ng et al., 2005). Results show that such low levels of shear stresses of $3.61x10^{-6}$ Pa at a flow rate of 150 µL/min can induce significant changes in the global gene expression profile of dermal fibroblasts. The changes in gene expression profile was dependant on the fluid flow rate suggesting that fluid flow acts as a mechanotransducer invoking various signalling responses in fibroblasts. Go Term and KEGG pathways analysis also revealed that the genes altered under fluid flow were associated with the TGF-β pathway and biological processes like endocytosis and angiogenesis. Interestingly, when gene expression changes were sorted on the basis of p-value ($p \leq 0.01$), gene expression variations under flow were all associated to the TGF-Beta pathway. The Transforming growth factor β family of ligands includes TGF-β 1-3, Bone Morphogenic Proteins (BMP) and activins (Schmierer and Hill, 2007). Surprisingly, changes were observed in activin A (INHBA), Smad9, and TGF-β pathway antagonists like Smad7, TGFBRIII (Betaglycan) and CD109 that are members of the TGF-β sub family. These findings suggest that the mechanobiology of fibroblasts is highly influenced by the components of TGF-β pathway *in vivo* and their responses may be modified by changes in fluid movement as is observed in pathological conditions such as fibrosis, EMT as discussed in previous sections.

Activins are proteins known to play a role in cellular proliferation, differentiation, apoptosis and wound repair (Chen et al., 2006, Sulyok et al., 2004). Activin A is a potent activator of renal interstitial fibroblasts and enhances the expression of both collagen I A and α-smooth

muscle actin (Yamashita et al., 2004). Smad9 is a transcriptional regulator otherwise known as inhibitory Smad (ISmad) and belongs to the Bone morphogenetic protein (BMP) family. It is known to play a crucial role in phospho-control of TGF-β signalling (Wrighton et al., 2009). CD109 is cell surface antigen (Lin et al., 2002) observed to be highly expressed in cancer (Hashimoto et al., 2004) and known to degrade TGFβ receptors (Bizet et al., 2011) to attenuate extracellular matrix production in scleroderma skin fibroblasts (Man et al., 2012). Interestingly we observed that although activin A, SMAD9 and CD109 do not display fold change of more than 2.0, they were altered under flow suggesting that fluid flow acts as mechanical signal inducing variations in genes listed above, which in turn alter the functioning of normal fibroblasts.

In accordance with fold changes observed in microarray analysis, TGF-β1 stimulation increases expression of α-SMA in fibroblasts of both dermal and oral origin in static conditions. Fluid flow also activates fibroblasts of both dermal and oral origin by enhancing gene expression of α-SMA and Collagen IA. Contrastingly, TGF-β1 stimulation under flow decreases the activation response suggesting that fibroblast mechanobiology is prone to chemical alterations in the microenvironment. Although we had used 10 ng/ml of TGF-β1 in all our experiments, higher doses may result in a different effect. Our findings correlate to the highly heterogeneous profile of fibroblast in tissues *in vivo* where the microenvironment is dynamic in nature. It is possible that levels of TGF-β1 experienced by cells *in vivo* are much higher than those used in this study which may in turn influence the mechanobiology of these fibroblasts.

Smad proteins are regulatory proteins that are responsible for not only transducing the signals from the membrane into the nucleus, but also initiate downstream responses by directly

regulating gene expression. Activated Smads regulate diverse biological functions by partnering with transcription factors and translocation to the nucleus. Upon activation of the TGF- β receptors within a short time period of 1h., the highly homologous Smad2 and Smad3 are phosphorylated by the TGF-β receptor in the C-terminal SXS motif and in the linker region at Ser^{208}, Ser^{204}, and Thr^{179} sites and transduce the signal to the nucleus (Wang, 2009). Smad3 can also be phosphorylated by Alk4 and Alk5 (Attisano and Wrana, 2002). Our studies revealed that protein expression of phosphorylated-Smad3 is elevated by two fold in dermal fibroblasts treated by fluid flow for 2h. Further, we also observed high levels of cell associated and secreted TGF-β1 protein in fibroblasts that were subjected to fluid flow but not in those treated in static conditions. These results support previous findings from microarray data which shows that a) fluid flow stimulates expression of TGF- β1 in dermal fibroblasts and b) fluid flow causes variations in the TGF- β pathway. These results prompt us to conclude that in the presence of fluid flow TGF- β1 is released into the conditioned medium which may in turn cause an autocrine effect leading to activation of fibroblasts.

Homeostasis is maintained by positive and negative feedback mechanisms that respond to signals from the microenvironment and restores normalcy to cells. However in most pathological conditions like systemic fibrosis, cancer and wound healing this feedback mechanism fails and hence a homeostatic imbalance occurs. Studies on mechanobiology of fibroblasts in normal and diseased conditions have attracted a lot of research interest in recent years. These studies not only help towards understanding the heterogeneous nature of fibroblasts but also explain mechanisms involved in regulating 'tensional homeostasis' of fibroblasts, a term recently coined by Brown (Mouw et al., 2014). The group showed that fibroblasts embedded in 3D collagen gels antagonize the effect of external force application in an attempt to maintain the original state of the cell as it was before application of external

force. Although ‘tensional homeostasis’ is a ubiquitous concept with no direct evidence of cell’s ability to respond to mechanical loading by maintaining tensional homeostasis, recently a group set out to look for direct evidence of tensional homeostasis in single fibroblast cells using single-cell contraction force microscopy (Marenzana et al., 2006). They reported that fibroblasts respond to external loading by a process called ‘tensional buffering’ and respond to physical forces. Whether the cell reverts to its original state depends largely on the cell’s previous mechanical state and the force applied on it. Although previous studies showed that interstitial fluid flow invokes fibroblasts to differentiate to an activated phenotype with alignment of collagen fibres *in vitro* (Ng et al., 2005), it was not clear if the cell reverts to its original resting state when the ‘flow’ is withdrawn. Hence, we explored whether fibroblasts respond to fluid flow mediated physical stimuli and whether these signals could be altered to revert the fibroblasts to its original state. Findings in this study show that flow-activated fibroblasts can revert to a resting state phenotype when allowed to remain in static conditions. This transient activation of fibroblast under the influence of fluid flow provides an evidence of ‘tensional buffering’ in fibroblasts treated with physical forces. Although we have shown that fluid flow mediated activation of fibroblasts is regulated by TGF-β pathway, this property of transient activation may be also influenced by other signalling pathways that contribute to maintaining cellular homeostasis in normal and diseased tissues. Our findings prompt further exploration of flow mediated mechanisms that contradict classical pro-fibrotic effects of TGF-β1 in fibroblasts.

In the current study, the effect of other regulatory molecules in driving the molecular events involved in fluid flow mediated fibroblast differentiation were also explored. Analysis of microarray-based global gene expression variations under flow revealed that caveolins are one among the top 20 genes altered under flow. These molecules are membrane bound

proteins with a hairpin like structure that remains embedded in the plasma membrane. These molecules were initially detected in human prostate cancer (Yang et al., 1999) and contribute to cell transformation (Razani et al., 2000). They regulate TGF-β signalling through interactions with the TGF-β type I receptor (Razani et al., 2001) and endocytic pathways (Di Guglielmo et al., 2003b). Caveolins possess anti-fibrotic properties (Tourkina et al., 2008) and are a crucial regulator of lung fibrosis in idiopathic pulmonary fibrosis (Wang et al., 2006b). Deficiency of caveolins in mice modulates cellular senescence in epithelial cells and exerts a protective effect in pulmonary fibrosis (Shivshankar et al., 2012). They are also known to internalise the TGF-β receptor in lipid rafts and degrade the receptors to negatively regulate the pathway (Del Galdo et al., 2008) and hence are considered as modulators of fibroblast activation and biomarkers of gastric cancers (Shen et al., 2015). Immunostaining results revealed high levels of membrane bound caveolin-1A in fibroblasts treated in static conditions whereas cytoplasmic protein expression of caveolin-1A is high under the influence of fluid flow in both TGF-β1 stimulated and unstimulated conditions suggesting that fluid flow internalises membrane bound caveolin 1A into the cytoplasm.

Classical studies have shown that TGFβ receptors are internalized by both lipid raft bound (caveolins) and non-lipid raft (endosomal) pathways (Mitchell et al., 2004b, Di Guglielmo et al., 2003b) involving caveolin-1 and early endosomal antigen-1 (EEA-1) double positive vesicles (He et al., 2015). Further, numerous reports have also shown that the SARA/Smad2/3 complex which initiates TGFβ signalling was found to be localized in the EEA-1 positive endosome whereas the SMURF/Smad7 complex that initiates degradation of TGFβ receptors was localized in caveolin-1 positive lipid rafts (Hayes et al., 2002, Runyan et al., 2005). These studies also showed that disruption to localization of SARA to endosomes disrupts TGFβ signalling and localization of TGFβ receptor type II in caveolin positive lipid

rafts and prevents nuclear translocation of Smad2/3 suggesting that nuclear translocation of p-Smad3 would be abrogated in TGF-β1 stimulated fibroblasts treated with flow. However, in this study, immunofluorescence images and western blot samples revealed nuclear translocation of p-Smad3 in TGF-β1 stimulated fibroblasts treated with flow. There are a number of possible explanations for this. Firstly the western blot samples were taken after 2 hours whereas α-SMA expression was determined after 24 hours. Hence it is possible that nuclear translocation of pSmad3 is disrupted over time in TGF-β1 stimulated fibroblasts treated with flow. Secondly, it is possible that other pathways/mediators are involved and finally the concurrent translocation of the receptor complex and Smad3 under flow conditions may result in a reduced capacity for p-Smad3 binding to promoter regions.

To analyse whether the combined effect of TGF-β1 stimulation and fluid flow internalises the TGFβ receptor type II by enhancing endocytosis, fibroblasts were immunolabelled with both early endosomal antigen 1A (EEA-1) and TGFβ receptor type II (TGFβRII). Immunofluorescence images revealed that TGF-β1 stimulation increases expression of early endosomal antigen-1 in dermal fibroblasts under static conditions. When these fibroblasts were treated with fluid flow, expression of EEA-1 was not only stimulated but the TGF-β receptor type 2 translocated to the nucleus. TGF-β1 stimulation under flow further accelerates nuclear localisation of TGF-β receptor type II even within 2h. of flow treatment. This indicates that rapid localisation of the TGF-β receptor type II reduces its availability to bind to ligands like TGF-β1, TGF-β3 and is unable to form receptor-ligand complex, thus negatively regulating the TGF-β signalling mechanism. Therefore, abrogation of α-SMA expression in TGF-β1 stimulated fibroblasts under flow may occur due to four possible mechanisms: a) TGF-β Receptor type II localizes itself to caveolin-1 and early endosomal antigen-1 (EEA-1) double positive vesicles as is shown in previous studies (He et al., 2015)

and is degraded by endocytosis, b) internalization of TGF-β receptor type II into EEA-1 positive compartments may lead to internalization of Smad7 containing caveolins resulting in nuclear localisation of Smad7 and binding to transcriptional machinery for disruption of TGF-β signalling, c) the cumulative effect of other signalling mechanisms, endocytosis of TGF-β receptor type II into EEA-1 positive compartment and Smad7 signalling leading to disruption of TGF-β signalling and d) release of endogenous TGF-β1 in fibroblasts during TGF-β1 supplemented fluid flow conditions may result in supra physiological levels to promote distinct endocytic pathways, accelerated trafficking of the receptor bound ligand, and inhibition of the activation response.

Activin A is a member of the TGF-β superfamily and is known to be neutralised by binding of two follistatin molecules (Thompson et al., 2005). Recently, activin A was shown to promote profibrotic responses in interstitial fibroblasts during renal fibrosis and administration of recombinant follistatin alleviates the profibrotic response (Maeshima et al., 2014a). Epithelium derived activin A is known to increase tumour promoting properties in mammary fibroblasts in a Cox-2 dependent manner by enhancing incorporation of ECM components and promoting differentiation of fibroblasts to a cancer associated fibroblast like phenotype (Fordyce et al., 2012). Whilst there is a large body of literature demonstrating that TGF-β1 induces fibroblast differentiation to a myofibroblast phenotype with increased expression of α-SMA rich stress fibres, this study has shown that activin A is a potent activator of fibroblasts.

To observe whether fluid flow mediated fibroblast activation is regulated by activin A, gene and protein expression of activin A was studied in TGF-β1 stimulated and unstimulated

dermal fibroblasts in both static and fluid flow conditions. Results show that gene expression of activin A is increased under flow whereas its expression is down regulated in TGF-β1 stimulated flow conditions. Exogenous activin A is detected in high levels only in conditioned medium of fibroblasts subjected to flow treatment whereas endogenous activin A is high in TGF-β1 stimulated fibroblasts in both static and flow conditions. This suggests that fluid flow regulates release of ectopic activin A and this mechanically induced biological release of activin A is negated by TGF-β1 supplementation, possibly because exogenously released activin A competitively binds to TGF-β receptor type II and is internalised into the nucleus. Our findings correlate with a recent paper which shows that activin A can competitively bind to TGF-β receptor type II and BMP receptor type II to antagonise the signalling pathway (Aykul and Martinez-Hackert 2016).

Findings from microarray based analysis of global gene expression profile shows that activin A is one of the top 20 genes altered under flow. Additionally, protein expression of both cell associated and secreted activin A was observed to be increased under flow. To explore whether supplementation of recombinant activin A is capable of differentiating normal fibroblasts to a myofibroblast like phenotype. Findings reveal that recombinant activin A can activate dermal fibroblasts by increasing α- SMA protein expression, thereby promoting differentiation to a myofibroblast like phenotype in static conditions. Follistatin is an extracellular regulatory protein of activin A and exerts isoform specific effect on biological activity of activin A. Of all the isoforms, follistatin 288 (FS-288) is known to show high cell surface binding and ligand binding affinity to activin A (Sidis et al., 2006) and inhibition of exogenous and endogenous activin A is regulated differentially by various isoforms. Follistatin 288 inhibition in both activin A stimulated dermal fibroblasts and TGF-β1 stimulated dermal fibroblasts show decreased α- SMA protein expression suggesting that

activin A on its own is capable of differentiating fibroblasts to a pro- myofibroblast like phenotype and this biological effect can be negated by recombinant follistatin 288. Our data confirms the potent stimulatory effect of TGF-β1 on α-SMA expression and that activin A is intimately linked with this pathway by stimulating α-SMA expression. Findings also show that both these agonists (TGF-β1 and activin A) are sensitive to inhibition by follistatin. Furthermore, TGF-β1 enhances expression of endogenous activin A in dermal fibroblasts.

CD109 is a glycosylphosphatidylinositol- anchored protein and is known to function as a TGF-β co receptor and inhibitor of the TGF-β pathway in keratinocytes. Recently it has been shown that CD109 can attenuate ECM production in scleroderma skin fibroblasts. CD109 is known to enhance binding of TGF-β to its receptors and promote TGF-β bound receptor internalization via a caveolin pathway in a ligand dependant manner (Bizet, Liu et al. 2011) and attenuate ECM production in skin fibroblasts (Man, Finnson et al. 2012). Our results reveal that gene expression of CD109 (TGF-β co-receptor) is increased in TGF-β1 stimulated fluid flow conditions in dermal fibroblasts suggesting that supplementation of TGF-β1 in dermal fibroblasts followed by fluid flow treatment increases expression of CD109 and antagonises the TGF-β pathway. Results on CD109 validate recent studies that show that CD109 increases binding of TGF-β1 to its receptor, enhances caveolin mediated endocytosis of the TGF-β ligand–receptor complex to negatively regulating the TGF- β pathway (Bizet et al., 2011).

Since compelling evidence has shown that cellular activation and senescence are stress responses and share common pathways, we decided to explore if fluid flow induces senescence in fibroblasts. Our findings show that markers of senescence and growth arrest,

p16 INK4a and p21^{Waf-1} are altered in fibroblasts under flow suggesting that fluid flow induced differentiation of fibroblasts is transient and may induce growth arrest that is reversible in nature. However, since existing literature on cellular senescence shows that the CDK inhibitors p16 INK4a and p21 $^{Waf-1}$ have overlapping roles in inducing quiescence and senescence, our findings call in to evaluate other markers of senescence and phases of cell cycle. Quiescent fibroblasts are known to exhibit high metabolic activity (Lemons et al., 2010). However, our findings reveal that fluid flow reduces the metabolic activity of fibroblasts. Although this observation contradicts existing literature, it is possible that in the presence of fluid flow, the cellular machinery focussed its functions on differentiation associated processes such as synthesis of fibrous proteins.

In summary, fluid flow acts as a critical regulator in modulating the phenotype of fibroblasts from both dermal and oral origin and the combined effect of mechanical signals and pro-fibrotic signals like TGF- β1 negatively regulates myofibroblast differentiation. In the absence of regulatory signals like TGF- β1, fluid flow promotes fibroblast differentiation into a myofibroblast phenotype. This biological effect is a cumulative response of sensor-like components such as high levels of ectopic activin A, exogenous and endogenous TGF-β1 and low levels of TGF- β antagonists like CD109 that promote the activation response (Figure 9.1). However in the presence of pro-fibrotic signals like TGF-β1, fluid flow negatively regulates differentiation into a myofibroblast phenotype possibly because ligand bound TGF-β Receptor type II localizes itself to caveolin-1 and early endosomal antigen-1 (EEA-1) double positive vesicles as is shown in previous studies (He et al., 2015) and gets translocated to the nucleus for endocytic degradation (Figure 9.2). Although our results prompt thorough assessment of endocytic regulation of ligand bound TGFβRII to lipid rafts, our

immunofluorescence results clearly reveal the nuclear localization of TGFβRII in EEA-1 positive cells in TGF-β1 stimulated fibroblasts subjected to flow.

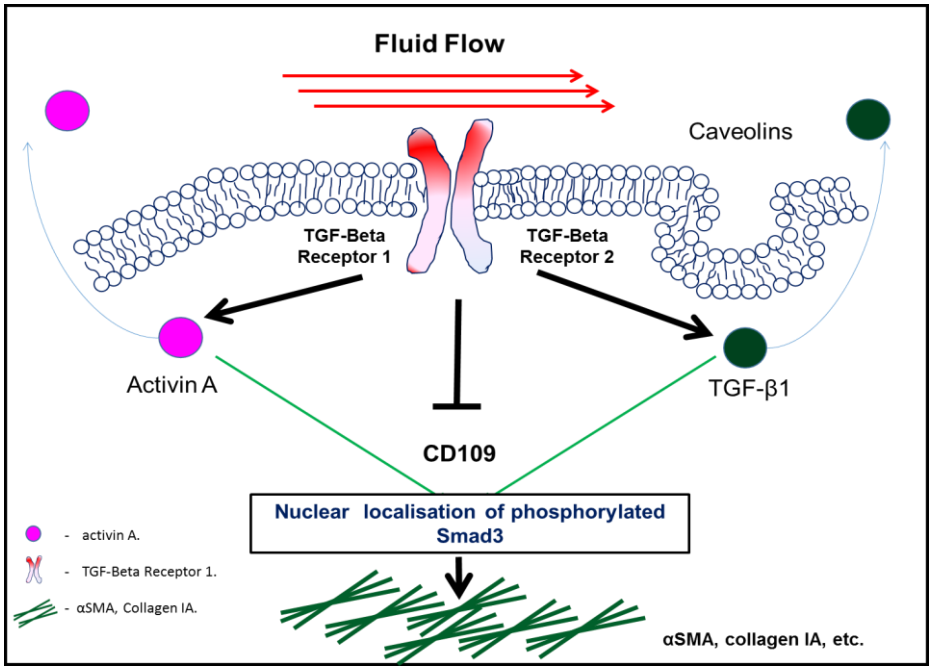

Figure 9.1: Mechanobiology of fibroblasts subjected to fluid flow.

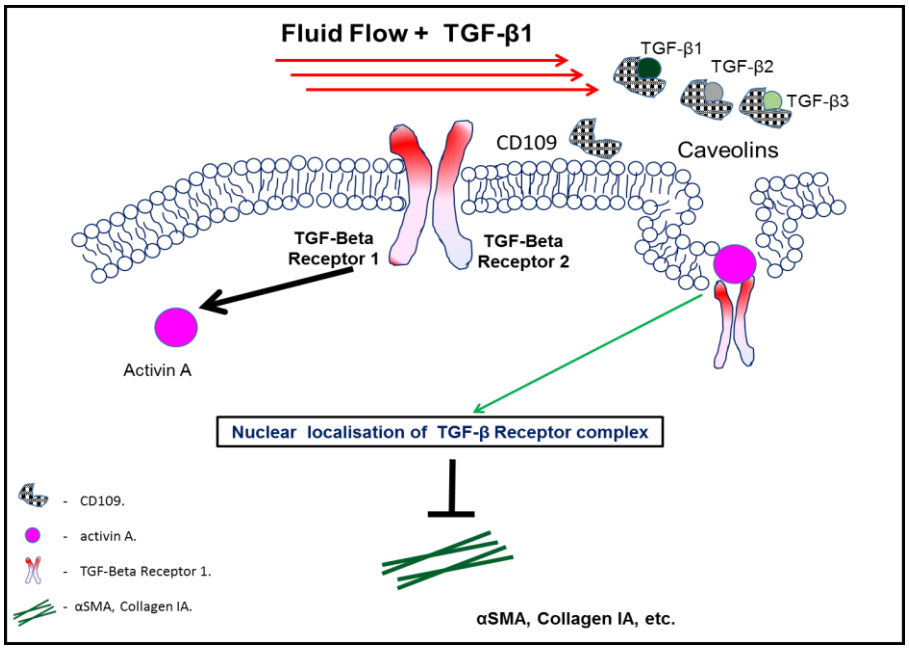

Figure 9.2: Mechanobiology of fibroblasts subjected to TGF-β1 supplementation under fluid flow.

This study shows that a physiologically relevant fluid flow mediated fibroblast response is influenced by the cumulative effect of reduced activin A released into the circulating medium and caveolin mediated internalisation of TGF-β1 bound TGFβRII receptor-ligand complex. Our findings provide first direct evidence of fluid flow mediated 'tensional homeostasis' in fibroblasts.

Surprisingly, our findings show that both dermal and oral fibroblasts respond to flow in a similar manner by showing increase in gene and protein expression of α- SMA and an opposite effect in the presence of TGF-β1. This in particular is an interesting observation since most studies to date have shown that fibroblasts from different origin show phenotypic differences (Shannon et al., 2006). Our findings suggest that differences in phenotype and cellular response of fibroblasts from oral and dermal sources are influenced by other cell types and signalling molecules present in the tissue origin. Although this observation contradicts our hypothesis that cells from different tissue sources may respond differently, results show that factors such as presence of *in vivo* like fluid movement, cellular communication from other cell types and profibrotic molecules contribute to molecular pathology of fibrotic responses. Findings in this study also suggest that care should be observed in extrapolating results from *in-vitro* experiments which lack the presence of these factors in a physiologically relevant condition.

Future work

Microarray based global gene expression analysis revealed changes in YAP (Yes associated protein) (enrichment score: 1.55), a transcriptional co activator that is sensitive to mechanical stress (Szeto et al., 2016) and a regulator of proliferation, differentiation (Piersma et al., 2015a) and senescence (Xie et al., 2013). Following experimental approaches are planned for further studies.

1) Findings have shown that when fluid flow activated fibroblasts are allowed to remain in static conditions for 24h., they revert to resting state phenotype with loss of α-SMA protein expression. Assessing the effect of both fluid flow and chemical signals such as TGF-β1 in inducing changes in expression and localization of YAP1 in fibroblasts will provide a better understanding of the nature and magnitude of stress that can invoke a mechanotransduction response in fibroblasts. Assessing the effect of different fluid flow rates in inducing nuclear translocation and changes in gene expression of YAP1 would further help towards establishing minimal shear stress needed to invoke a stress response in fibroblasts.

2) Recent reports have shown that fluid flow induced YAP1 activation promotes cancer cell motility (Lee et al., 2017). However, the effect of both fluid flow and chemical signals such as TGF-β1 in inducing cellular motility of myofibroblasts remains to be assessed. We aim to study this using the Quasi-vivo® bioreactor and by assessing the gene and protein expression of YAP1. Cellular motility will assessed by wound scratch assay and migration inserts.

3) Experiments stated in points above will be pursued on normal keratinocytes and human umbilical vein endothelial cells (HUVECs) to analyse the effect of fluid flow and chemical

signals in inducing a stress response in cells of both the epithelial and endothelial compartment. The Quasi-vivo® bioreactor provides an advantage wherein multiple cell types could be cultured in the chambers and held together in series of inter connected loops, thus promoting flow of medium between different cell types. Previous studies from our laboratory have shown that when normal fibroblasts and keratinocytes were grown in connected cultures, notable changes in global gene expression were observed in both cell types.

4) Interestingly, experiments in the current study on influence of different flow rates on fibroblasts and former studies on effect of flow on fibroblast-keratinocyte connected cell culture have both shown that the GoTERM enriched gene set are functionally associated with processes such as angiogenesis and cell motion. Future studies aim to explore the functional effect of fibroblast conditioned media and fibroblast-keratinocyte conditioned media in inducing angiogenic response in endothelial using tubule formations assays.

Studies planned for future will provide deeper insight into cell-cell communication mechanisms and novel molecules which can be selectively targeted for anti-fibrotic therapeutic purposes.

References

Fibroblast *Genetics Home Reference* [Online].
A.AHLUWALIA , F. V., F.MONTEMURRO AND M.WILKINSON 2011. Hepatotoxicity of diclofenac in a Quasi-Vivo(TM) multicompartment bioreactor *Toxicology letters,* 205**,** 270.
ABE, R., DONNELLY, S. C., PENG, T., BUCALA, R. & METZ, C. N. 2001. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol,* 166**,** 7556-62.
ABEL, B., WILLOUGHBY, C., JANG, S., COOPER, L., XIE, L., VO-RANSDELL, C. & SOWA, G. 2012. N-terminal tyrosine phosphorylation of caveolin-2 negates anti-proliferative effect of transforming growth factor beta in endothelial cells. *FEBS Lett,* 586**,** 3317-23.
ABOUALAIWI, W. A., TAKAHASHI, M., MELL, B. R., JONES, T. J., RATNAM, S., KOLB, R. J. & NAULI, S. M. 2009. Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. *Circ Res,* 104**,** 860-9.
ADAMO, L., NAVEIRAS, O., WENZEL, P. L., MCKINNEY-FREEMAN, S., MACK, P. J., GRACIA-SANCHO, J., SUCHY-DICEY, A., YOSHIMOTO, M., LENSCH, M. W., YODER, M. C., GARCIA-CARDENA, G. & DALEY, G. Q. 2009. Biomechanical forces promote embryonic haematopoiesis. *Nature,* 459**,** 1131-1135.
AJUBI, N. E., KLEIN-NULEND, J., NIJWEIDE, P. J., VRIJHEID-LAMMERS, T., ALBLAS, M. J. & BURGER, E. H. 1996. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes--a cytoskeleton-dependent process. *Biochem Biophys Res Commun,* 225**,** 62-8.
ALBERTS B, J. A., LEWIS J, ET AL. 2002a. *Blood Vessels and Endothelial Cells.,* New York.
ALBERTS B, J. A., LEWIS J, ET AL. 2002b. Fibroblasts and Their Transformations: The Connective-Tissue Cell Family. Molecular Biology of the Cell.: New York: Garland Science.
ANDERS, R. A., DORE, J. J., JR., ARLINE, S. L., GARAMSZEGI, N. & LEOF, E. B. 1998. Differential requirement for type I and type II transforming growth factor beta receptor kinase activity in ligand-mediated receptor endocytosis. *J Biol Chem,* 273**,** 23118-25.
ANDERSON, R. G. 1998. The caveolae membrane system. *Annu Rev Biochem,* 67**,** 199-225.
ANNES, J. P., MUNGER, J. S. & RIFKIN, D. B. 2003. Making sense of latent TGFbeta activation. *J Cell Sci,* 116**,** 217-24.
ANTONY, M. L., NAIR, R., SEBASTIAN, P. & KARUNAGARAN, D. 2010. Changes in expression, and/or mutations in TGF-beta receptors (TGF-beta RI and TGF-beta RII) and Smad 4 in human ovarian tumors. *J Cancer Res Clin Oncol,* 136**,** 351-61.
ARMSTRONG, E. J. & BISCHOFF, J. 2004. Heart valve development: endothelial cell signaling and differentiation. *Circ Res,* 95**,** 459-70.
ASSOIAN, R. K. & KLEIN, E. A. 2008. Growth control by intracellular tension and extracellular stiffness. *Trends Cell Biol,* 18**,** 347-52.
ATTISANO, L. & WRANA, J. L. 2002. Signal transduction by the TGF-beta superfamily. *Science,* 296**,** 1646-7.
AUKLAND, K. & REED, R. K. 1993. Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiological Reviews,* 73**,** 1-78.
BALUK, P., HASHIZUME, H. & MCDONALD, D. M. 2005. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev,* 15**,** 102-11.
BARBEE, K. A., DAVIES, P. F. & LAL, R. 1994. Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy. *Circ Res,* 74**,** 163-71.
BAUMANN, F., LEUKEL, P., DOERFELT, A., BEIER, C. P., DETTMER, K., OEFNER, P. J., KASTENBERGER, M., KREUTZ, M., NICKL-JOCKSCHAT, T., BOGDAHN, U., BOSSERHOFF, A. K. & HAU, P. 2009. Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. *Neuro Oncol,* 11**,** 368-80.
BEAUSEJOUR, C. M., KRTOLICA, A., GALIMI, F., NARITA, M., LOWE, S. W., YASWEN, P. & CAMPISI, J. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *Embo j,* 22**,** 4212-22.

BELLO, I. O., VERED, M., DAYAN, D., DOBRIYAN, A., YAHALOM, R., ALANEN, K., NIEMINEN, P., KANTOLA, S., LAARA, E. & SALO, T. 2011. Cancer-associated fibroblasts, a parameter of the tumor microenvironment, overcomes carcinoma-associated parameters in the prognosis of patients with mobile tongue cancer. *Oral Oncol,* 47**,** 33-8.

BERG JM, T. J., STRYER L. 2002. *Cells Can Respond to Changes in Their Environments.*, New York: W H Freeman.

BHADAL, N., WALL, I. B., PORTER, S. R., BROAD, S., LINDAHL, G. E., WHAWELL, S. & LEWIS, M. P. 2008. The effect of mechanical strain on protease production by keratinocytes. *Br J Dermatol,* 158**,** 396-8.

BHAUMIK, D., SCOTT, G. K., SCHOKRPUR, S., PATIL, C. K., ORJALO, A. V., RODIER, F., LITHGOW, G. J. & CAMPISI, J. 2009. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. *Aging (Albany NY),* 1**,** 402-11.

BIZET, A. A., LIU, K., TRAN-KHANH, N., SAKSENA, A., VORSTENBOSCH, J., FINNSON, K. W., BUSCHMANN, M. D. & PHILIP, A. 2011. The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. *Biochim Biophys Acta,* 1813**,** 742-53.

BOARDMAN, K. C. & SWARTZ, M. A. 2003. Interstitial flow as a guide for lymphangiogenesis. *Circ Res,* 92**,** 801-8.

BOSCHER, C. & NABI, I. R. 2012. Caveolin-1: role in cell signaling. *Adv Exp Med Biol,* 729**,** 29-50.

BOSELLI, F., FREUND, J. B. & VERMOT, J. 2015. Blood flow mechanics in cardiovascular development. *Cellular and Molecular Life Sciences,* 72**,** 2545-2559.

BRAND, H. S. & VEERMAN, E. C. 2013. Saliva and wound healing. *Chin J Dent Res,* 16**,** 7-12.

BRIDGEMAN, G. & BROOKES, M. 1996. Blood supply to the human femoral diaphysis in youth and senescence. *J Anat,* 188 (Pt 3)**,** 611-21.

BROSH, N., STERNBERG, D., HONIGWACHS-SHA'ANANI, J., LEE, B.-C., SHAV-TAL, Y., TZEHOVAL, E., SHULMAN, L. M., TOLEDO, J., HACHAM, Y., CARMI, P., JIANG, W., SASSE, J., HORN, F., BURSTEIN, Y. & ZIPORI, D. 1995. The Plasmacytoma Growth Inhibitor Restrictin-P Is an Antagonist of Interleukin 6 and Interleukin 11: IDENTIFICATION AS A STROMA-DERIVED ACTIVIN A. *Journal of Biological Chemistry,* 270**,** 29594-29600.

BROWN, M. S., ANDERSON, R. G. & GOLDSTEIN, J. L. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell,* 32**,** 663-7.

BUCZEK, M. E., MILES, A. K., GREEN, W., JOHNSON, C., BOOCOCK, D. J., POCKLEY, A. G., REES, R. C., HULMAN, G., VAN SCHALKWYK, G., PARKINSON, R., HULMAN, J., POWE, D. G. & REGAD, T. 2016. Cytoplasmic PML promotes TGF-[beta]-associated epithelial-mesenchymal transition and invasion in prostate cancer. *Oncogene,* 35**,** 3465-3475.

BURGER, E. H. & KLEIN-NULEND, J. 1999. Mechanotransduction in bone--role of the lacuno-canalicular network. *FASEB J,* 13 Suppl**,** S101-12.

BURTON, D. G. A. 2009. Cellular senescence, ageing and disease. *Age (Dordr),* 31**,** 1-9.

BUSCHMANN, M. D., GLUZBAND, Y. A., GRODZINSKY, A. J. & HUNZIKER, E. B. 1995. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J Cell Sci,* 108 (Pt 4)**,** 1497-508.

BUTCHER, D. T., ALLISTON, T. & WEAVER, V. M. 2009. A tense situation: forcing tumour progression. *Nat Rev Cancer,* 9**,** 108-22.

BUTLER, T. P., GRANTHAM, F. H. & GULLINO, P. M. 1975. Bulk transfer of fluid in the interstitial compartment of mammary tumors. *Cancer Res,* 35**,** 3084-8.

BYUN, J. S. & GARDNER, K. 2013. Wounds that will not heal: pervasive cellular reprogramming in cancer. *Am J Pathol,* 182**,** 1055-64.

CALON, A., TAURIELLO, D. V. & BATLLE, E. 2014. TGF-beta in CAF-mediated tumor growth and metastasis. *Semin Cancer Biol,* 25**,** 15-22.

CAMPISI, J. & D'ADDA DI FAGAGNA, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol,* 8**,** 729-740.

CARTWRIGHT, J. H., PIRO, O. & TUVAL, I. 2009. Fluid dynamics in developmental biology: moving fluids that shape ontogeny. *Hfsp j,* 3**,** 77-93.
CASTOR, C. W., WILSON, S. M., HEISS, P. R. & SEIDMAN, J. C. 1979. Activation of lung connective tissue cells in vitro. *Am Rev Respir Dis,* 120**,** 101-6.
CHANDRA, M., ZANG, S., LI, H., ZIMMERMAN, L. J., CHAMPER, J., TSUYADA, A., CHOW, A., ZHOU, W., YU, Y., GAO, H., REN, X., LIN, R. J. & WANG, S. E. 2012. Nuclear translocation of type I transforming growth factor beta receptor confers a novel function in RNA processing. *Mol Cell Biol,* 32**,** 2183-95.
CHAQOUR, B., YANG, R. & SHA, Q. 2006. Mechanical stretch modulates the promoter activity of the profibrotic factor CCN2 through increased actin polymerization and NF-kappaB activation. *J Biol Chem,* 281**,** 20608-22.
CHARY, S. R. & JAIN, R. K. 1989. Direct measurement of interstitial convection and diffusion of albumin in normal and neoplastic tissues by fluorescence photobleaching. *Proc Natl Acad Sci U S A,* 86**,** 5385-9.
CHEN, C. S., MRKSICH, M., HUANG, S., WHITESIDES, G. M. & INGBER, D. E. 1997. Geometric control of cell life and death. *Science,* 276**,** 1425-8.
CHEN, L., ARBIEVA, Z. H., GUO, S., MARUCHA, P. T., MUSTOE, T. A. & DIPIETRO, L. A. 2010. Positional differences in the wound transcriptome of skin and oral mucosa. *BMC Genomics,* 11**,** 471.
CHEN, Y. G. 2009. Endocytic regulation of TGF-beta signaling. *Cell Res,* 19**,** 58-70.
CHEN, Y. G., WANG, Q., LIN, S. L., CHANG, C. D., CHUANG, J. & YING, S. Y. 2006. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Exp Biol Med (Maywood),* 231**,** 534-44.
CHENG, T., RODRIGUES, N., SHEN, H., YANG, Y., DOMBKOWSKI, D., SYKES, M. & SCADDEN, D. T. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science,* 287**,** 1804-8.
CHEUNG, T. H. & RANDO, T. A. 2013. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol,* 14**,** 329-40.
CHIQUET, M., GELMAN, L., LUTZ, R. & MAIER, S. 2009. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim Biophys Acta,* 1793**,** 911-20.
CHIQUET, M., SARASA-RENEDO, A. & TUNC-CIVELEK, V. 2004. Induction of tenascin-C by cyclic tensile strain versus growth factors: distinct contributions by Rho/ROCK and MAPK signaling pathways. *Biochim Biophys Acta,* 1693**,** 193-204.
CHOWDHURY, T. T., SALTER, D. M., BADER, D. L. & LEE, D. A. 2004. Integrin-mediated mechanotransduction processes in TGFbeta-stimulated monolayer-expanded chondrocytes. *Biochem Biophys Res Commun,* 318**,** 873-81.
CHRISTOPHER B. WOLF, M. R. K. M. 2009. Mechanotransduction and Its Role in Stem Cell Biology. *Trends in Stem Cell Biology and Technology***,** 389-403.
CIECHANOVER, A. 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat Rev Mol Cell Biol,* 6**,** 79-87.
CLEMENT, C. A., AJBRO, K. D., KOEFOED, K., VESTERGAARD, M. L., VELAND, I. R., HENRIQUES DE JESUS, M. P., PEDERSEN, L. B., BENMERAH, A., ANDERSEN, C. Y., LARSEN, L. A. & CHRISTENSEN, S. T. 2013. TGF-beta signaling is associated with endocytosis at the pocket region of the primary cilium. *Cell Rep,* 3**,** 1806-14.
CMIELOVA, J., HAVELEK, R., SOUKUP, T., JIROUTOVÁ, A., VISEK, B., SUCHÁNEK, J., VAVROVA, J., MOKRY, J., MUTHNA, D., BRUCKOVA, L., FILIP, S., ENGLISH, D. & REZACOVA, M. 2012. Gamma radiation induces senescence in human adult mesenchymal stem cells from bone marrow and periodontal ligaments. *International Journal of Radiation Biology,* 88**,** 393-404.
COATS, S., FLANAGAN, W. M., NOURSE, J. & ROBERTS, J. M. 1996. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. *Science,* 272**,** 877-80.

COLANTONIO, J. R., VERMOT, J., WU, D., LANGENBACHER, A. D., FRASER, S., CHEN, J. N. & HILL, K. L. 2009. The dynein regulatory complex is required for ciliary motility and otolith biogenesis in the inner ear. *Nature,* 457**,** 205-9.

COLETTA, R. D. & GRANER, E. 2006. Hereditary gingival fibromatosis: a systematic review. *J Periodontol,* 77**,** 753-64.

COLLER, H. A., SANG, L. & ROBERTS, J. M. 2006. A New Description of Cellular Quiescence. *PLOS Biology,* 4**,** e83.

CONWAY, D. E., BRECKENRIDGE, M. T., HINDE, E., GRATTON, E., CHEN, C. S. & SCHWARTZ, M. A. 2013. Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr Biol,* 23**,** 1024-30.

COPPE, J. P., DESPREZ, P. Y., KRTOLICA, A. & CAMPISI, J. 2010. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol,* 5**,** 99-118.

COSTA, R. A., RUIZ-DE-SOUZA, V., AZEVEDO, G. M., JR., GAVA, E., KITTEN, G. T., VAZ, N. M. & CARVALHO, C. R. 2011. Indirect effects of oral tolerance improve wound healing in skin. *Wound Repair Regen,* 19**,** 487-97.

CRISTOFALO, V. J. & PIGNOLO, R. J. 1993. Replicative senescence of human fibroblast-like cells in culture. *Physiol Rev,* 73**,** 617-38.

CRISWELL, T. L., DUMONT, N., BARNETT, J. V. & ARTEAGA, C. L. 2008. Knockdown of the transforming growth factor-beta type III receptor impairs motility and invasion of metastatic cancer cells. *Cancer Res,* 68**,** 7304-12.

CUTRONEO, K. R., WHITE, S. L., PHAN, S. H. & EHRLICH, H. P. 2007. Therapies for bleomycin induced lung fibrosis through regulation of TGF-beta1 induced collagen gene expression. *J Cell Physiol,* 211**,** 585-9.

DADSETAN, M., PUMBERGER, M., CASPER, M. E., SHOGREN, K., GIULIANI, M., RUESINK, T., HEFFERAN, T. E., CURRIER, B. L. & YASZEMSKI, M. J. 2011. The effects of fixed electrical charge on chondrocyte behavior. *Acta Biomaterialia,* 7**,** 2080-2090.

DAN, L., CHUA, C. K. & LEONG, K. F. 2010. Fibroblast response to interstitial flow: A state-of-the-art review. *Biotechnol Bioeng,* 107**,** 1-10.

DAS, R. K., ANURA, A., PAL, M., BAG, S., MAJUMDAR, S., BARUI, A., CHAKRABORTY, C., RAY, A. K., SENGUPTA, S., PAUL, R. R. & CHATTERJEE, J. 2013. Epithelio-mesenchymal transitional attributes in oral sub-mucous fibrosis. *Exp Mol Pathol,* 95**,** 259-69.

DAWES, C., PEDERSEN, A. M., VILLA, A., EKSTROM, J., PROCTOR, G. B., VISSINK, A., AFRAMIAN, D., MCGOWAN, R., ALIKO, A., NARAYANA, N., SIA, Y. W., JOSHI, R. K., JENSEN, S. B., KERR, A. R. & WOLFF, A. 2015. The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI. *Arch Oral Biol,* 60**,** 863-74.

DE KRETSER, D. M., O'HEHIR, R. E., HARDY, C. L. & HEDGER, M. P. 2012. The roles of activin A and its binding protein, follistatin, in inflammation and tissue repair. *Mol Cell Endocrinol,* 359**,** 101-6.

DEBACQ-CHAINIAUX, F., BOILAN, E., DEDESSUS LE MOUTIER, J., WEEMAELS, G. & TOUSSAINT, O. 2010. p38(MAPK) in the senescence of human and murine fibroblasts. *Adv Exp Med Biol,* 694**,** 126-37.

DEL GALDO, F., LISANTI, M. P. & JIMENEZ, S. A. 2008. Caveolin-1, transforming growth factor-beta receptor internalization, and the pathogenesis of systemic sclerosis. *Curr Opin Rheumatol,* 20**,** 713-9.

DEONARINE, K., PANELLI, M. C., STASHOWER, M. E., JIN, P., SMITH, K., SLADE, H. B., NORWOOD, C., WANG, E., MARINCOLA, F. M. & STRONCEK, D. F. 2007. Gene expression profiling of cutaneous wound healing. *J Transl Med,* 5**,** 11.

DERYNCK, R. & FENG, X. H. 1997. TGF-beta receptor signaling. *Biochim Biophys Acta,* 1333**,** F105-50.

DESMOULIERE, A. 2007. Hepatic stellate cells: the only cells involved in liver fibrogenesis? A dogma challenged. *Gastroenterology,* 132**,** 2059-62.

DESMOULIERE, A., GEINOZ, A., GABBIANI, F. & GABBIANI, G. 1993. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol,* 122**,** 103-11.

DI GUGLIELMO, G. M., LE ROY, C., GOODFELLOW, A. F. & WRANA, J. L. 2003a. Distinct endocytic pathways regulate TGF-[beta] receptor signalling and turnover. *Nat Cell Biol,* 5**,** 410-421.

DI GUGLIELMO, G. M., LE ROY, C., GOODFELLOW, A. F. & WRANA, J. L. 2003b. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol,* 5**,** 410-21.

DI LULLO, G. A., SWEENEY, S. M., KORKKO, J., ALA-KOKKO, L. & SAN ANTONIO, J. D. 2002. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *J Biol Chem,* 277**,** 4223-31.

DIMRI, G. P., LEE, X., BASILE, G., ACOSTA, M., SCOTT, G., ROSKELLEY, C., MEDRANO, E. E., LINSKENS, M., RUBELJ, I., PEREIRA-SMITH, O. & ET AL. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A,* 92**,** 9363-7.

DISCHER, D. E., JANMEY, P. & WANG, Y. L. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science,* 310**,** 1139-43.

DOLBERG, D. S., HOLLINGSWORTH, R., HERTLE, M. & BISSELL, M. J. 1985. Wounding and its role in RSV-mediated tumor formation. *Science,* 230**,** 676-8.

DONGAONKAR, R. M., QUICK, C. M., STEWART, R. H., DRAKE, R. E., COX, C. S. & LAINE, G. A. 2008. Edemagenic gain and interstitial fluid volume regulation. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 294**,** R651-R659.

DORLAND'S 2012. Dorland's medical dictionary Elsevier.

DRAGOVICH, M., CHESTER, D. & ZHANG, X. F. 2016. Mechanotransduction of the Endothelial Glycocalyx Mediates Nitric Oxide Production through Activation of TRP Channels. *Biophysical Journal,* 110**,** 23a.

DRISKELL, R. R. & WATT, F. M. 2015. Understanding fibroblast heterogeneity in the skin. *Trends Cell Biol,* 25**,** 92-9.

DUNHAM, L. J. 1972. Cancer in man at site of prior benign lesion of skin or mucous membrane: a review. *Cancer Res,* 32**,** 1359-74.

DUPONT, S., MORSUT, L., ARAGONA, M., ENZO, E., GIULITTI, S., CORDENONSI, M., ZANCONATO, F., LE DIGABEL, J., FORCATO, M., BICCIATO, S., ELVASSORE, N. & PICCOLO, S. 2011. Role of YAP/TAZ in mechanotransduction. *Nature,* 474**,** 179-83.

DUPREE, P., PARTON, R. G., RAPOSO, G., KURZCHALIA, T. V. & SIMONS, K. 1993. Caveolae and sorting in the trans-Golgi network of epithelial cells. *Embo j,* 12**,** 1597-605.

DVORAK, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med,* 315**,** 1650-9.

EGOROVA, A. D., KHEDOE, P. P., GOUMANS, M. J., YODER, B. K., NAULI, S. M., TEN DIJKE, P., POELMANN, R. E. & HIERCK, B. P. 2011. Lack of primary cilia primes shear-induced endothelial-to-mesenchymal transition. *Circ Res,* 108**,** 1093-101.

EHRLICH, M., SHMUELY, A. & HENIS, Y. I. 2001. A single internalization signal from the di-leucine family is critical for constitutive endocytosis of the type II TGF-beta receptor. *J Cell Sci,* 114**,** 1777-86.

EIJKEN, M., SWAGEMAKERS, S., KOEDAM, M., STEENBERGEN, C., DERKX, P., UITTERLINDEN, A. G., VAN DER SPEK, P. J., VISSER, J. A., DE JONG, F. H., POLS, H. A. & VAN LEEUWEN, J. P. 2007. The activin A-follistatin system: potent regulator of human extracellular matrix mineralization. *Faseb j,* 21**,** 2949-60.

ELDER, B. D. & ATHANASIOU, K. A. 2009. Hydrostatic Pressure in Articular Cartilage Tissue Engineering: From Chondrocytes to Tissue Regeneration. *Tissue Eng Part B Rev,* 15**,** 43-53.

EMBLETON, S. J., HOSKING, S. L., ROFF HILTON, E. J. & CUNLIFFE, I. A. 2002. Effect of senescence on ocular blood flow in the retina, neuroretinal rim and lamina cribrosa, using scanning laser Doppler flowmetry. *Eye,* 16**,** 156-162.

ENGLER, A. J., CARAG-KRIEGER, C., JOHNSON, C. P., RAAB, M., TANG, H. Y., SPEICHER, D. W., SANGER, J. W., SANGER, J. M. & DISCHER, D. E. 2008. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *J Cell Sci,* 121**,** 3794-802.

ENGLER, A. J., SEN, S., SWEENEY, H. L. & DISCHER, D. E. 2006. Matrix elasticity directs stem cell lineage specification. *Cell,* 126**,** 677-89.

ENGLER, A. J., SWEENEY, H. L., DISCHER, D. E. & SCHWARZBAUER, J. E. 2007. Extracellular matrix elasticity directs stem cell differentiation. *J Musculoskelet Neuronal Interact,* 7**,** 335.

EPPLEY, B. L., WOODELL, J. E. & HIGGINS, J. 2004. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg,* 114**,** 1502-8.

EURELL JA, F. B. 2006. Epithelium. In:. *Dellmann's Textbook of Veterinary Histology Blackwell Publishing;***,** 17–30.

EVANS, R. A., TIAN, Y. C., STEADMAN, R. & PHILLIPS, A. O. 2003. TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. *Exp Cell Res,* 282**,** 90-100.

EVERETT, E. T., POMERAT, C. M., HU, F. N. & LIVINGOOD, C. S. 1951. Tissue culture studies on human skin. I. A method of evaluating the toxicity of certain drugs employed locally on the skin; skin toxicity methods. *Tex Rep Biol Med,* 9**,** 281-91.

FAINSOD, A., DEISSLER, K., YELIN, R., MAROM, K., EPSTEIN, M., PILLEMER, G., STEINBEISSER, H. & BLUM, M. 1997. The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech Dev,* 63**,** 39-50.

FAN, D., TAKAWALE, A., LEE, J. & KASSIRI, Z. 2012. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair,* 5**,** 15.

FARGE, E. 2011. Mechanotransduction in development. *Curr Top Dev Biol,* 95**,** 243-65.

FIELD, S., RILEY, K. L., GRIMES, D. T., HILTON, H., SIMON, M., POWLES-GLOVER, N., SIGGERS, P., BOGANI, D., GREENFIELD, A. & NORRIS, D. P. 2011. Pkd1l1 establishes left-right asymmetry and physically interacts with Pkd2. *Development,* 138**,** 1131-42.

FINNSON, K. W., TAM, B. Y., LIU, K., MARCOUX, A., LEPAGE, P., ROY, S., BIZET, A. A. & PHILIP, A. 2006. Identification of CD109 as part of the TGF-beta receptor system in human keratinocytes. *Faseb j,* 20**,** 1525-7.

FLEMING, I., FISSLTHALER, B., DIXIT, M. & BUSSE, R. 2005. Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci,* 118**,** 4103-11.

FLEMING, J. S., GREENWOOD, P. J., HEATH, D. A., HUDSON, N. L., LUN, S., SHAW, L. & MCNATTY, K. P. 1995. Expression of gonadotrophin subunit genes in sheep that were homozygous carriers and non-carriers of the Booroola fecundity gene FecB. *J Reprod Fertil,* 103**,** 315-21.

FOOS, G., GARCIA-RAMIREZ, J. J., GALANG, C. K. & HAUSER, C. A. 1998. Elevated expression of Ets2 or distinct portions of Ets2 can reverse Ras-mediated cellular transformation. *J Biol Chem,* 273**,** 18871-80.

FORDYCE, C. A., PATTEN, K. T., FESSENDEN, T. B., DEFILIPPIS, R., HWANG, E. S., ZHAO, J. & TLSTY, T. D. 2012. Cell-extrinsic consequences of epithelial stress: activation of protumorigenic tissue phenotypes. *Breast Cancer Res,* 14**,** R155.

FORRESTER, H. B., IVASHKEVICH, A., MCKAY, M. J., LEONG, T., DE KRETSER, D. M. & SPRUNG, C. N. 2013. Follistatin Is Induced by Ionizing Radiation and Potentially Predictive of Radiosensitivity in Radiation-Induced Fibrosis Patient Derived Fibroblasts. *PLoS One,* 8.

FREUND, A., PATIL, C. K. & CAMPISI, J. 2011. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. *Embo j,* 30**,** 1536-48.

FREUND, J. B., GOETZ, J. G., HILL, K. L. & VERMOT, J. 2012. Fluid flows and forces in development: functions, features and biophysical principles. *Development,* 139**,** 1229-45.

FRIDLYANSKAYA, I., ALEKSEENKO, L. & NIKOLSKY, N. 2015. Senescence as a general cellular response to stress: A mini-review. *Experimental Gerontology,* 72**,** 124-128.

FUMAGALLI, M., MUSSO, T., VERMI, W., SCUTERA, S., DANIELE, R., ALOTTO, D., CAMBIERI, I., OSTORERO, A., GENTILI, F., CAPOSIO, P., ZUCCA, M., SOZZANI, S., STELLA, M. & CASTAGNOLI, C. 2007. Imbalance between activin A and follistatin drives postburn hypertrophic scar formation in human skin. *Exp Dermatol,* 16**,** 600-10.

FURUIKE, S., ITO, T. & YAMAZAKI, M. 2001. Mechanical unfolding of single filamin A (ABP-280) molecules detected by atomic force microscopy. *FEBS Lett,* 498**,** 72-5.

GABBIANI, G., RYAN, G. B. & MAJNE, G. 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia,* 27**,** 549-50.

GAENGEL, K., GENOVÉ, G., ARMULIK, A. & BETSHOLTZ, C. 2009. Endothelial-Mural Cell Signaling in Vascular Development and Angiogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 29**,** 630-638.

GALBRAITH, C. G., SKALAK, R. & CHIEN, S. 1998. Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. *Cell Motil Cytoskeleton,* 40**,** 317-30.

GALBRAITH, C. G., YAMADA, K. M. & SHEETZ, M. P. 2002. The relationship between force and focal complex development. *J Cell Biol,* 159**,** 695-705.

GALIE, P., RUSSELL, M., WESTFALL, M. & STEGEMANN, J. 2012. Interstitial fluid flow and cyclic strain differentially regulate cardiac fibroblast activation via AT1R and TGF-β1. *Exp Cell Res,* 318**,** 75-84.

GARANICH, J. S., MATHURA, R. A., SHI, Z. D. & TARBELL, J. M. 2007. Effects of fluid shear stress on adventitial fibroblast migration: implications for flow-mediated mechanisms of arterialization and intimal hyperplasia. *Am J Physiol Heart Circ Physiol,* 292**,** H3128-35.

GARRISON, G., HUANG, S. K., OKUNISHI, K., SCOTT, J. P., KUMAR PENKE, L. R., SCRUGGS, A. M. & PETERS-GOLDEN, M. 2013. Reversal of myofibroblast differentiation by prostaglandin E(2). *Am J Respir Cell Mol Biol,* 48**,** 550-8.

GAUDET, C., MARGANSKI, W. A., KIM, S., BROWN, C. T., GUNDERIA, V., DEMBO, M. & WONG, J. Y. 2003. Influence of Type I Collagen Surface Density on Fibroblast Spreading, Motility, and Contractility. *Biophys J,* 85**,** 3329-35.

GEIGER, B. & BERSHADSKY, A. 2001. Assembly and mechanosensory function of focal contacts. *Curr Opin Cell Biol,* 13**,** 584-92.

GEORGES, P. C., HUI, J. J., GOMBOS, Z., MCCORMICK, M. E., WANG, A. Y., UEMURA, M., MICK, R., JANMEY, P. A., FURTH, E. E. & WELLS, R. G. 2007. Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol,* 293**,** G1147-54.

GEORGES, P. C., MILLER, W. J., MEANEY, D. F., SAWYER, E. S. & JANMEY, P. A. 2006. Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys J,* 90**,** 3012-8.

GJOREVSKI, N. & NELSON, C. M. 2010. The mechanics of development: models and methods for tissue morphogenesis. *Birth Defects Res C Embryo Today,* 90**,** 193-202.

GLIM, J. E., VAN EGMOND, M., NIESSEN, F. B., EVERTS, V. & BEELEN, R. H. 2013. Detrimental dermal wound healing: what can we learn from the oral mucosa? *Wound Repair Regen,* 21**,** 648-60.

GM, C. 2000. *Signaling Molecules and Their Receptors.*, Sunderland (MA): Sinauer Associates;.

GM., C. 2000. *Signaling Molecules and Their Receptors.*

GOODHART, J., COOPER, J., SMITH, R., WILLIAMS, J., HAGGARD, W. & BUMGARDNER, J. 2014. Design and Validation of a Cyclic Strain Bioreactor to Condition Spatially-Selective Scaffolds in Dual Strain Regimes. *Processes,* 2**,** 345.

GORDON, K. J. & BLOBE, G. C. 2008. Role of transforming growth factor-β superfamily signaling pathways in human disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1782**,** 197-228.

GOSAIN, A. & DIPIETRO, L. A. 2004. Aging and wound healing. *World J Surg,* 28**,** 321-6.

GOSPODAROWICZ, D. & LAU, K. 1989. Pituitary follicular cells secrete both vascular endothelial growth factor and follistatin. *Biochem Biophys Res Commun,* 165**,** 292-8.

GRAVES, D. T., NOOH, N., GILLEN, T., DAVEY, M., PATEL, S., COTTRELL, D. & AMAR, S. 2001. IL-1 plays a critical role in oral, but not dermal, wound healing. *J Immunol,* 167**,** 5316-20.
GRODZINSKY, A. J., LEVENSTON, M. E., JIN, M. & FRANK, E. H. 2000. Cartilage tissue remodeling in response to mechanical forces. *Annu Rev Biomed Eng,* 2**,** 691-713.
GROTENDORST, G. R. 1997. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev,* 8**,** 171-9.
GRUENBERG, J. 2001. The endocytic pathway: a mosaic of domains. *Nat Rev Mol Cell Biol,* 2**,** 721-30.
GUYTON, A. C., PRATHER, J., SCHEEL, K. & MCGEHEE, J. 1966. Interstitial fluid pressure. IV. Its effect on fluid movement through the capillary wall. *Circ Res,* 19**,** 1022-30.
HADJIPANAYI, E., MUDERA, V. & BROWN, R. A. 2009. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *J Tissue Eng Regen Med,* 3**,** 77-84.
HAHN, C. & SCHWARTZ, M. A. 2009. Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol,* 10**,** 53-62.
HAIGLER, H. T., MAXFIELD, F. R., WILLINGHAM, M. C. & PASTAN, I. 1980. Dansylcadaverine inhibits internalization of 125I-epidermal growth factor in BALB 3T3 cells. *J Biol Chem,* 255**,** 1239-41.
HAN, T., KANG, D., JI, D., WANG, X., ZHAN, W., FU, M., XIN, H. B. & WANG, J. B. 2013. How does cancer cell metabolism affect tumor migration and invasion? *Cell Adh Migr,* 7**,** 395-403.
HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.
HANDORF, A. M., ZHOU, Y., HALANSKI, M. A. & LI, W. J. 2015. Tissue stiffness dictates development, homeostasis, and disease progression. *Organogenesis,* 11**,** 1-15.
HAO, J., ZHANG, Y., JING, D., SHEN, Y., TANG, G., HUANG, S. & ZHAO, Z. 2015. Mechanobiology of mesenchymal stem cells: Perspective into mechanical induction of MSC fate. *Acta Biomater,* 20**,** 1-9.
HARA, E., SMITH, R., PARRY, D., TAHARA, H., STONE, S. & PETERS, G. 1996. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol Cell Biol,* 16**,** 859-67.
HASHIMOTO, M., ICHIHARA, M., WATANABE, T., KAWAI, K., KOSHIKAWA, K., YUASA, N., TAKAHASHI, T., YATABE, Y., MURAKUMO, Y., ZHANG, J. M., NIMURA, Y. & TAKAHASHI, M. 2004. Expression of CD109 in human cancer. *Oncogene,* 23**,** 3716-20.
HASHIMOTO, O., NAKAMURA, T., SHOJI, H., SHIMASAKI, S., HAYASHI, Y. & SUGINO, H. 1997. A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell-surface heparan sulfate. *J Biol Chem,* 272**,** 13835-42.
HASSONA, Y., CIRILLO, N., PARKINSON, K. & PRIME, S. 2015. Senescent Cancer Associated Fibroblasts Regulates Oral Tumour Cell Adhesion and Invasion Via TGF-B Dependent Pathways. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology,* 119**,** e204-e205.
HAYASHI, K., MATSUDA, S., MACHIDA, K., YAMAMOTO, T., FUKUDA, Y., NIMURA, Y., HAYAKAWA, T. & HAMAGUCHI, M. 2001. Invasion Activating Caveolin-1 Mutation in Human Scirrhous Breast Cancers. *Cancer Research,* 61**,** 2361-2364.
HAYES, S., CHAWLA, A. & CORVERA, S. 2002. TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *J Cell Biol,* 158**,** 1239-49.
HAYFLICK, L. 1965. The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research,* 37**,** 614-636.
HE, K., YAN, X., LI, N., DANG, S., XU, L., ZHAO, B., LI, Z., LV, Z., FANG, X., ZHANG, Y. & CHEN, Y.-G. 2015. Internalization of the TGF-[beta] type I receptor into caveolin-1 and EEA1 double-positive early endosomes. *Cell Res,* 25**,** 738-752.
HELDIN, C. H. & MOUSTAKAS, A. 2016. Signaling Receptors for TGF-beta Family Members. *Cold Spring Harb Perspect Biol,* 8.
HENRY, C. B., KLEINSTEIN, E., SHUM, W. & DEFOUW, D. O. 1995. Glycoconjugate expression in the chick embryonic chorioallantoic membrane: comparisons of the chorionic ectoderm and allantoic endoderm. *Anat Rec,* 241**,** 411-6.

HINSLEY, E. E., KUMAR, S., HUNTER, K. D., WHAWELL, S. A. & LAMBERT, D. W. 2012. Endothelin-1 stimulates oral fibroblasts to promote oral cancer invasion. *Life Sci,* 91**,** 557-61.
HINZ, B. 2007. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol,* 127**,** 526-37.
HINZ, B. 2010. The myofibroblast: Paradigm for a mechanically active cell. *Journal of Biomechanics,* 43**,** 146-155.
HINZ, B. 2013. Matrix mechanics and regulation of the fibroblast phenotype. *Periodontol 2000,* 63**,** 14-28.
HINZ, B. 2015. Myofibroblasts. *Exp Eye Res*.
HINZ, B., CELETTA, G., TOMASEK, J. J., GABBIANI, G. & CHAPONNIER, C. 2001. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell,* 12**,** 2730-41.
HINZ, B., PHAN, S. H., THANNICKAL, V. J., PRUNOTTO, M., DESMOULIERE, A., VARGA, J., DE WEVER, O., MAREEL, M. & GABBIANI, G. 2012. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am J Pathol,* 180**,** 1340-55.
HIRSCH, F. R., VARELLA-GARCIA, M., BUNN, P. A., JR., DI MARIA, M. V., VEVE, R., BREMMES, R. M., BARON, A. E., ZENG, C. & FRANKLIN, W. A. 2003. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol,* 21**,** 3798-807.
HODES, R. J. 1999. Telomere Length, Aging, and Somatic Cell Turnover. *The Journal of Experimental Medicine,* 190**,** 153-156.
HONDA, E., YOSHIDA, K. & MUNAKATA, H. 2010. Transforming growth factor-beta upregulates the expression of integrin and related proteins in MRC-5 human myofibroblasts. *Tohoku J Exp Med,* 220**,** 319-27.
HORNEBECK, W. & MAQUART, F. X. 2003. Proteolyzed matrix as a template for the regulation of tumor progression. *Biomed Pharmacother,* 57**,** 223-30.
HSIEH, P. C., JIN, Y. T., CHANG, C. W., HUANG, C. C., LIAO, S. C. & YUAN, K. 2010. Elastin in oral connective tissue modulates the keratinization of overlying epithelium. *J Clin Periodontol,* 37**,** 705-11.
HU, J., WANG, X., WEI, S. M., TANG, Y. H., ZHOU, Q. & HUANG, C. X. 2016. Activin A stimulates the proliferation and differentiation of cardiac fibroblasts via the ERK1/2 and p38-MAPK pathways. *Eur J Pharmacol,* 789**,** 319-27.
HU, Y., PENG, J., FENG, D., CHU, L., LI, X., JIN, Z., LIN, Z. & ZENG, Q. 2006. Role of extracellular signal-regulated kinase, p38 kinase, and activator protein-1 in transforming growth factor-beta1-induced alpha smooth muscle actin expression in human fetal lung fibroblasts in vitro. *Lung,* 184**,** 33-42.
HUANG, E. Y., WU, H., ISLAND, E. R., CHONG, S. S., WARBURTON, D., ANDERSON, K. D. & TUAN, T. L. 2002. Differential expression of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 in early and late gestational mouse skin and skin wounds. *Wound Repair Regen,* 10**,** 387-96.
HUANG, S. & INGBER, D. E. 2002. A discrete cell cycle checkpoint in late G(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. *Exp Cell Res,* 275**,** 255-64.
HUANG, S. S. & HUANG, J. S. 2005. TGF-beta control of cell proliferation. *J Cell Biochem,* 96**,** 447-62.
HUSELSTEIN, C., DE ISLA, N., KOLOPP-SARDA, M. N., KERDJOUDJ, H., MULLER, S. & STOLTZ, J. F. 2006. Influence of mechanical stress on cell viability. *Biorheology,* 43**,** 371-5.
INGBER, D. E. 1993. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci,* 104 (Pt 3)**,** 613-27.
INGBER, D. E. 1997. Tensegrity: the architectural basis of cellular mechanotransduction. *Annu Rev Physiol,* 59**,** 575-99.
INGBER, D. E. 2006. Cellular mechanotransduction: putting all the pieces together again. *FASEB J,* 20**,** 811-27.

INGBER, D. E. & FOLKMAN, J. 1989. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J Cell Biol,* 109**,** 317-30.
INGBER, D. E., MADRI, J. A. & JAMIESON, J. D. 1981. Role of basal lamina in neoplastic disorganization of tissue architecture. *Proc Natl Acad Sci U S A,* 78**,** 3901-5.
INOUE, S., ORIMO, A., HOSOI, T., MATSUSE, T., KOZAKI, K., OUCHI, Y., MURAMATSU, M. & ORIMO, H. 1995. Follistatin as an activin-binding protein expressed in arteriosclerotic lesions. *Ann N Y Acad Sci,* 748**,** 530-3.
IOMINI, C., TEJADA, K., MO, W., VAANANEN, H. & PIPERNO, G. 2004. Primary cilia of human endothelial cells disassemble under laminar shear stress. *J Cell Biol,* 164**,** 811-7.
ISHIDA, Y., KONDO, T., KIMURA, A., MATSUSHIMA, K. & MUKAIDA, N. 2006. Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-kappaB activation and a reciprocal suppression of TGF-beta signal pathway. *J Immunol,* 176**,** 5598-606.
ISHII, G., SANGAI, T., ODA, T., AOYAGI, Y., HASEBE, T., KANOMATA, N., ENDOH, Y., OKUMURA, C., OKUHARA, Y., MAGAE, J., EMURA, M., OCHIYA, T. & OCHIAI, A. 2003. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem Biophys Res Commun,* 309**,** 232-40.
ISHIKAWA, J., TSUJI, H., SATO, H. & GOTOH, Y. 2007. Ion implantation of negative ions for cell growth manipulation and nervous system repair. *Surface and Coatings Technology,* 201**,** 8083-8090.
JACKSON, J. G. & PEREIRA-SMITH, O. M. 2006. p53 is preferentially recruited to the promoters of growth arrest genes p21 and GADD45 during replicative senescence of normal human fibroblasts. *Cancer Res,* 66**,** 8356-60.
JANG, C.-W., CHEN, C.-H., CHEN, C.-C., CHEN, J.-Y., SU, Y.-H. & CHEN, R.-H. 2002. TGF-[beta] induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol,* 4**,** 51-58.
JANMEY, P. A. & MILLER, R. T. 2011. Mechanisms of mechanical signaling in development and disease. *J Cell Sci,* 124**,** 9-18.
JENSEN, P. J. & LAVKER, R. M. 1999. Urokinase is a positive regulator of epidermal proliferation in vivo. *J Invest Dermatol,* 112**,** 240-4.
JEON, K. J., PARK, S. H., SHIN, J. W., KANG, Y. G., HYUN, J. S., OH, M. J., KIM, S. Y. & SHIN, J. W. 2014. Combined effects of flow-induced shear stress and micropatterned surface morphology on neuronal differentiation of human mesenchymal stem cells. *J Biosci Bioeng,* 117**,** 242-7.
JIANHUA, W., TOSHIO, I., NAOTO, O., TAKAYASU, I., TAKASHI, M., BAOLIN, L. & MASAO, Y. 2009. Influence of surface wettability on competitive protein adsorption and initial attachment of osteoblasts. *Biomedical Materials,* 4**,** 045002.
JONES, D. L. & WAGERS, A. J. 2008. No place like home: anatomy and function of the stem cell niche. *Nat Rev Mol Cell Biol,* 9**,** 11-21.
JORGENSEN, P. & TYERS, M. 2004. How cells coordinate growth and division. *Curr Biol,* 14**,** R1014-27.
JORISSEN, R. N., WALKER, F., POULIOT, N., GARRETT, T. P. J., WARD, C. W. & BURGESS, A. W. 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. *Experimental Cell Research,* 284**,** 31-53.
KABIR, T. D., LEIGH, R. J., TASENA, H., MELLONE, M., COLETTA, R. D., PARKINSON, E. K., PRIME, S. S., THOMAS, G. J., PATERSON, I. C., ZHOU, D., MCCALL, J., SPEIGHT, P. M. & LAMBERT, D. W. 2016. A miR-335/COX-2/PTEN axis regulates the secretory phenotype of senescent cancer-associated fibroblasts. *Aging (Albany NY),* 8**,** 1608-35.
KALLURI, R. & NEILSON, E. G. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest,* 112**,** 1776-84.
KALLURI, R. & ZEISBERG, M. 2006. Fibroblasts in cancer. *Nat Rev Cancer,* 6**,** 392-401.
KANE, C. J., HEBDA, P. A., MANSBRIDGE, J. N. & HANAWALT, P. C. 1991. Direct evidence for spatial and temporal regulation of transforming growth factor beta 1 expression during cutaneous wound healing. *J Cell Physiol,* 148**,** 157-73.

KANEDA, H., ARAO, T., MATSUMOTO, K., DE VELASCO, M. A., TAMURA, D., AOMATSU, K., KUDO, K., SAKAI, K., NAGAI, T., FUJITA, Y., TANAKA, K., YANAGIHARA, K., YAMADA, Y., OKAMOTO, I., NAKAGAWA, K. & NISHIO, K. 2011. Activin A inhibits vascular endothelial cell growth and suppresses tumour angiogenesis in gastric cancer. *Br J Cancer,* 105**,** 1210-1217.

KANG, J. S., LIU, C. & DERYNCK, R. 2009. New regulatory mechanisms of TGF-beta receptor function. *Trends Cell Biol,* 19**,** 385-94.

KANG, T., PARK, C. & LEE, B.-J. 2016. Investigation of biomimetic shear stress on cellular uptake and mechanism of polystyrene nanoparticles in various cancer cell lines. *Archives of Pharmacal Research,* 39**,** 1663-1670.

KAWABE, J.-I., OKUMURA, S., LEE, M.-C., SADOSHIMA, J. & ISHIKAWA, Y. 2004. Translocation of caveolin regulates stretch-induced ERK activity in vascular smooth muscle cells. *American Journal of Physiology - Heart and Circulatory Physiology,* 286**,** H1845-H1852.

KAWAKAMI, S., FUJII, Y. & WINTERS, S. J. 2001. Follistatin production by skin fibroblasts and its regulation by dexamethasone. *Molecular and Cellular Endocrinology,* 172**,** 157-167.

KELLER, M. A., TURCHYN, A. V. & RALSER, M. 2014. Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean. *Mol Syst Biol,* 10**,** 725.

KELLERMANN, M. G., SOBRAL, L. M., DA SILVA, S. D., ZECCHIN, K. G., GRANER, E., LOPES, M. A., KOWALSKI, L. P. & COLETTA, R. D. 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 Oncol,* 44**,** 509-17.

KHORASANI, M. T., MIRZADEH, H. & IRANI, S. 2008. Plasma surface modification of poly (l-lactic acid) and poly (lactic-co-glycolic acid) films for improvement of nerve cells adhesion. *Radiation Physics and Chemistry,* 77**,** 280-287.

KIM, J. & MA, T. 2012. Bioreactor strategy in bone tissue engineering: pre-culture and osteogenic differentiation under two flow configurations. *Tissue Eng Part A,* 18**,** 2354-64.

KINGSLEY, D. M. 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev,* 8**,** 133-46.

KIRKHAM, M. & PARTON, R. G. 2005. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta,* 1746**,** 349-63.

KLEIN, E. A., YIN, L., KOTHAPALLI, D., CASTAGNINO, P., BYFIELD, F. J., XU, T., LEVENTAL, I., HAWTHORNE, E., JANMEY, P. A. & ASSOIAN, R. K. 2009. Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. *Curr Biol,* 19**,** 1511-8.

KLINGBERG, F., CHOW, M. L., KOEHLER, A., BOO, S., BUSCEMI, L., QUINN, T. M., COSTELL, M., ALMAN, B. A., GENOT, E. & HINZ, B. 2014. Prestress in the extracellular matrix sensitizes latent TGF-beta1 for activation. *J Cell Biol,* 207**,** 283-97.

KOHAN, M., MURO, A. F., WHITE, E. S. & BERKMAN, N. 2010. EDA-containing cellular fibronectin induces fibroblast differentiation through binding to α4β7 integrin receptor and MAPK/Erk 1/2-dependent signaling. *The FASEB Journal,* 24**,** 4503-4512.

KOHN J , C., ZHOU D , W., BORDELEAU, F., ZHOU A , L., MASON B , N., MITCHELL M , J., KING M , R. & REINHART-KING C , A. 2015. Cooperative Effects of Matrix Stiffness and Fluid Shear Stress on Endothelial Cell Behavior. *Biophys J,* 108**,** 471-8.

KOJIMA, I., NOBUSAWA, R., ZHANG, Y. Q., SEKINE, N., MINE, T. & SHIBATA, H. 1995. Attenuation of glycogenolytic action of activin A in intact rat liver. *Am J Physiol,* 269**,** E846-51.

KONERDING, M. A., MALKUSCH, W., KLAPTHOR, B., VAN ACKERN, C., FAIT, E., HILL, S. A., PARKINS, C., CHAPLIN, D. J., PRESTA, M. & DENEKAMP, J. 1999. Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. *Br J Cancer,* 80**,** 724-32.

KOPECKI, Z., LUCHETTI, M. M., ADAMS, D. H., STRUDWICK, X., MANTAMADIOTIS, T., STOPPACCIARO, A., GABRIELLI, A., RAMSAY, R. G. & COWIN, A. J. 2007. Collagen loss and impaired wound healing is associated with c-Myb deficiency. *J Pathol,* 211**,** 351-61.

KOTTGEN, M., BUCHHOLZ, B., GARCIA-GONZALEZ, M. A., KOTSIS, F., FU, X., DOERKEN, M., BOEHLKE, C., STEFFL, D., TAUBER, R., WEGIERSKI, T., NITSCHKE, R., SUZUKI, M., KRAMER-ZUCKER, A.,

GERMINO, G. G., WATNICK, T., PRENEN, J., NILIUS, B., KUEHN, E. W. & WALZ, G. 2008. TRPP2 and TRPV4 form a polymodal sensory channel complex. *J Cell Biol,* 182**,** 437-47.

KRTOLICA, A., PARRINELLO, S., LOCKETT, S., DESPREZ, P.-Y. & CAMPISI, J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: A link between cancer and aging. *Proceedings of the National Academy of Sciences,* 98**,** 12072-12077.

KU, D. N., GIDDENS, D. P., ZARINS, C. K. & GLAGOV, S. 1985. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis,* 5**,** 293-302.

KUILMAN, T., MICHALOGLOU, C., VREDEVELD, L. C., DOUMA, S., VAN DOORN, R., DESMET, C. J., AARDEN, L. A., MOOI, W. J. & PEEPER, D. S. 2008. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell,* 133**,** 1019-31.

KUMAR, S., MILLIS, A. J. & BAGLIONI, C. 1992. Expression of interleukin 1-inducible genes and production of interleukin 1 by aging human fibroblasts. *Proc Natl Acad Sci U S A,* 89**,** 4683-7.

KURZ, D. J., DECARY, S., HONG, Y. & ERUSALIMSKY, J. D. 2000. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci,* 113 (Pt 20)**,** 3613-22.

KWAN, M. K., LAI, W. M. & MOW, V. C. 1984. Fundamentals of fluid transport through cartilage in compression. *Ann Biomed Eng,* 12**,** 537-58.

L XIE, C. V.-R., BRITAIN ABEL, CARA WILLOUGHBY, SUNGCHAN JANG, GRZEGORZ SOWA 2011. Caveolin-2 is a negative regulator of anti-proliferative function and signaling of transforming growth factor-β in endothelial cells. *American Journal of Physiology - Cell Physiology,* 301.

LARJAVA, H., KOIVISTO, L., HÄKKINEN, L. & HEINO, J. 2011. Epithelial Integrins with Special Reference to Oral Epithelia. *J Dent Res,* 90**,** 1367-76.

LAZZERINI DENCHI, E., ATTWOOLL, C., PASINI, D. & HELIN, K. 2005. Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol,* 25**,** 2660-72.

LEASK, A., DENTON, C. P. & ABRAHAM, D. J. 2004. Insights into the molecular mechanism of chronic fibrosis: the role of connective tissue growth factor in scleroderma. *J Invest Dermatol,* 122**,** 1-6.

LEE, B. Y., HAN, J. A., IM, J. S., MORRONE, A., JOHUNG, K., GOODWIN, E. C., KLEIJER, W. J., DIMAIO, D. & HWANG, E. S. 2006. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell,* 5**,** 187-95.

LEE, H. G. & EUN, H. C. 1999. Differences between fibroblasts cultured from oral mucosa and normal skin: implication to wound healing. *J Dermatol Sci,* 21**,** 176-82.

LEE, H. J., DIAZ, M. F., PRICE, K. M., OZUNA, J. A., ZHANG, S., SEVICK-MURACA, E. M., HAGAN, J. P. & WENZEL, P. L. 2017. Fluid shear stress activates YAP1 to promote cancer cell motility. *Nat Commun,* 8**,** 14122.

LEGESSE-MILLER, A., RAITMAN, I., HALEY, E. M., LIAO, A., SUN, L. L., WANG, D. J., KRISHNAN, N., LEMONS, J. M., SUH, E. J., JOHNSON, E. L., LUND, B. A. & COLLER, H. A. 2012. Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. *Mol Biol Cell,* 23**,** 3566-81.

LEMONS, J. M., FENG, X. J., BENNETT, B. D., LEGESSE-MILLER, A., JOHNSON, E. L., RAITMAN, I., POLLINA, E. A., RABITZ, H. A., RABINOWITZ, J. D. & COLLER, H. A. 2010. Quiescent fibroblasts exhibit high metabolic activity. *PLoS Biol,* 8**,** e1000514.

LEWANDOWSKA, K., CHOI, H. U., ROSENBERG, L. C., ZARDI, L. & CULP, L. A. 1987. Fibronectin-mediated adhesion of fibroblasts: inhibition by dermatan sulfate proteoglycan and evidence for a cryptic glycosaminoglycan-binding domain. *J Cell Biol,* 105**,** 1443-54.

LI, H., COLLADO, M., VILLASANTE, A., STRATI, K., ORTEGA, S., CANAMERO, M., BLASCO, M. A. & SERRANO, M. 2009. The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature,* 460**,** 1136-9.

LI, J., FARTHING, P. M., IRELAND, G. W. & THORNHILL, M. H. 1996. IL-1 alpha and IL-6 production by oral and skin keratinocytes: similarities and differences in response to cytokine treatment in vitro. *J Oral Pathol Med,* 25**,** 157-62.

LI, S., HUANG, N. F. & HSU, S. 2005. Mechanotransduction in endothelial cell migration. *Journal of Cellular Biochemistry,* 96**,** 1110-1126.

LI, W. P., LIU, P., PILCHER, B. K. & ANDERSON, R. G. 2001. Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria. *Journal of Cell Science,* 114**,** 1397-1408.

LI, Y., NICHOLS, M. A., SHAY, J. W. & XIONG, Y. 1994. Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb. *Cancer Res,* 54**,** 6078-82.

LI, Z., DRANOFF, J. A., CHAN, E. P., UEMURA, M., SEVIGNY, J. & WELLS, R. G. 2007. Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology,* 46**,** 1246-56.

LIM, J. Y., HANSEN, J. C., SIEDLECKI, C. A., RUNT, J. & DONAHUE, H. J. 2005. Human foetal osteoblastic cell response to polymer-demixed nanotopographic interfaces. *Journal of The Royal Society Interface,* 2**,** 97-108.

LIN, A. W., BARRADAS, M., STONE, J. C., VAN AELST, L., SERRANO, M. & LOWE, S. W. 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev,* 12**,** 3008-19.

LIN, M., SUTHERLAND, D. R., HORSFALL, W., TOTTY, N., YEO, E., NAYAR, R., WU, X. F. & SCHUH, A. C. 2002. Cell surface antigen CD109 is a novel member of the alpha(2) macroglobulin/C3, C4, C5 family of thioester-containing proteins. *Blood,* 99**,** 1683-91.

LINVILLE, R. M., BOLAND, N. F., COVARRUBIAS, G., PRICE, G. M. & TIEN, J. 2016. Physical and Chemical Signals That Promote Vascularization of Capillary-Scale Channels. *Cell Mol Bioeng,* 9**,** 73-84.

LIU, C., YAO, J., MERCOLA, D. & ADAMSON, E. 2000. The transcription factor EGR-1 directly transactivates the fibronectin gene and enhances attachment of human glioblastoma cell line U251. *J Biol Chem,* 275**,** 20315-23.

LIU, H., ADLER, A. S., SEGAL, E. & CHANG, H. Y. 2007. A transcriptional program mediating entry into cellular quiescence. *PLoS Genet,* 3**,** e91.

LIU, Q., ZHANG, A., XU, W. & DONG, J. 2011. A new view of the roles of blood flow dynamics and Kupffer cell in intra-hepatic metastasis of hepatocellular carcinoma. *Med Hypotheses,* 77**,** 87-90.

LO, C. M., WANG, H. B., DEMBO, M. & WANG, Y. L. 2000. Cell movement is guided by the rigidity of the substrate. *Biophys J,* 79**,** 144-52.

LODISH H, B. A., ZIPURSKY SL, ET AL 2000. Receptor-Mediated Endocytosis and the Sorting of Internalized Proteins. 4th edition ed. Molecular Cell Biology: New York: W. H. Freeman.

LOPEZ-CASILLAS, F., WRANA, J. L. & MASSAGUE, J. 1993. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell,* 73**,** 1435-44.

LORBER, V. & RAYNS, D. G. 1977. Fine structure of the gap junction in the tunicate heart. *Cell Tissue Res,* 179**,** 169-75.

LUDLOW, H., PHILLIPS, D. J., MYERS, M., MCLACHLAN, R. I., DE KRETSER, D. M., ALLAN, C. A., ANDERSON, R. A., GROOME, N. P., HYVONEN, M., DUNCAN, W. C. & MUTTUKRISHNA, S. 2009. A new 'total' activin B enzyme-linked immunosorbent assay (ELISA): development and validation for human samples. *Clin Endocrinol (Oxf),* 71**,** 867-73.

LV, H., LI, L., SUN, M., ZHANG, Y., CHEN, L., RONG, Y. & LI, Y. 2015. Mechanism of regulation of stem cell differentiation by matrix stiffness. *Stem Cell Res Ther,* 6.

LYGOE, K. A., WALL, I., STEPHENS, P. & LEWIS, M. P. 2007. Role of vitronectin and fibronectin receptors in oral mucosal and dermal myofibroblast differentiation. *Biol Cell,* 99**,** 601-14.

MAESHIMA, A., MISHIMA, K., YAMASHITA, S., NAKASATOMI, M., MIYA, M., SAKURAI, N., SAKAIRI, T., IKEUCHI, H., HIROMURA, K., HASEGAWA, Y., KOJIMA, I. & NOJIMA, Y. 2014a. Follistatin, an activin antagonist, ameliorates renal interstitial fibrosis in a rat model of unilateral ureteral obstruction. *Biomed Res Int,* 2014**,** 376191.

MAESHIMA, A., MISHIMA, K., YAMASHITA, S., NAKASATOMI, M., MIYA, M., SAKURAI, N., SAKAIRI, T., IKEUCHI, H., HIROMURA, K., HASEGAWA, Y., KOJIMA, I. & NOJIMA, Y. 2014b. Follistatin, an Activin Antagonist, Ameliorates Renal Interstitial Fibrosis in a Rat Model of Unilateral Ureteral Obstruction. *BioMed Research International,* 2014**,** 376191.

MAH, W., JIANG, G., OLVER, D., CHEUNG, G., KIM, B., LARJAVA, H. & HAKKINEN, L. 2014. Human gingival fibroblasts display a non-fibrotic phenotype distinct from skin fibroblasts in three-dimensional cultures. *PLoS One,* 9**,** e90715.

MAINIERO, F., MURGIA, C., WARY, K. K., CURATOLA, A. M., PEPE, A., BLUMEMBERG, M., WESTWICK, J. K., DER, C. J. & GIANCOTTI, F. G. 1997. The coupling of alpha6beta4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J,* 16**,** 2365-75.

MAK, K., MANJI, A., GALLANT-BEHM, C., WIEBE, C., HART, D. A., LARJAVA, H. & HAKKINEN, L. 2009. Scarless healing of oral mucosa is characterized by faster resolution of inflammation and control of myofibroblast action compared to skin wounds in the red Duroc pig model. *J Dermatol Sci,* 56**,** 168-80.

MALENKA RC, N. E., HYMAN SE 2009. Chapter 10: Neural and Neuroendocrine Control of the Internal Milieu. *Molecular Neuropharmacology: A Foundation for Clinical Neuroscience (2nd ed.*

MALLER, O., DUFORT, C. C. & WEAVER, V. M. 2013. YAP forces fibroblasts to feel the tension. *Nat Cell Biol,* 15**,** 570-2.

MAMMOTO, A., HUANG, S., MOORE, K., OH, P. & INGBER, D. E. 2004. Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27kip1 pathway and the G1/S transition. *J Biol Chem,* 279**,** 26323-30.

MAMMOTO, T. & INGBER, D. E. 2010. Mechanical control of tissue and organ development. *Development,* 137**,** 1407-20.

MAN, X.-Y., FINNSON, K. W., BARON, M. & PHILIP, A. 2012. CD109, a TGF-β co-receptor, attenuates extracellular matrix production in scleroderma skin fibroblasts. *Arthritis Research & Therapy,* 14**,** R144.

MANI, H., SIDHU, G. S., KUMARI, R., GADDIPATI, J. P., SETH, P. & MAHESHWARI, R. K. 2002. Curcumin differentially regulates TGF-beta1, its receptors and nitric oxide synthase during impaired wound healing. *Biofactors,* 16**,** 29-43.

MANI, S. A., GUO, W., LIAO, M. J., EATON, E. N., AYYANAN, A., ZHOU, A. Y., BROOKS, M., REINHARD, F., ZHANG, C. C., SHIPITSIN, M., CAMPBELL, L. L., POLYAK, K., BRISKEN, C., YANG, J. & WEINBERG, R. A. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell,* 133**,** 704-15.

MARENZANA, M., WILSON-JONES, N., MUDERA, V. & BROWN, R. A. 2006. The origins and regulation of tissue tension: identification of collagen tension-fixation process in vitro. *Exp Cell Res,* 312**,** 423-33.

MAROTO, R., RASO, A., WOOD, T. G., KUROSKY, A., MARTINAC, B. & HAMILL, O. P. 2005. TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nat Cell Biol,* 7**,** 179-85.

MARSH, D., SUCHAK, K., MOUTASIM, K. A., VALLATH, S., HOPPER, C., JERJES, W., UPILE, T., KALAVREZOS, N., VIOLETTE, S. M., WEINREB, P. H., CHESTER, K. A., CHANA, J. S., MARSHALL, J. F., HART, I. R., HACKSHAW, A. K., PIPER, K. & THOMAS, G. J. 2011. Stromal features are predictive of disease mortality in oral cancer patients. *J Pathol,* 223**,** 470-81.

MARSH, M. & MCMAHON, H. T. 1999. The Structural Era of Endocytosis. *Science,* 285**,** 215-220.

MARTIN, P. 1997. Wound Healing--Aiming for Perfect Skin Regeneration. *Science,* 276**,** 75-81.

MARUYAMA, J., NAGURO, I., TAKEDA, K. & ICHIJO, H. 2009. Stress-activated MAP kinase cascades in cellular senescence. *Curr Med Chem,* 16**,** 1229-35.

MASON, A. J., NIALL, H. D. & SEEBURG, P. H. 1986. Structure of two human ovarian inhibins. *Biochem Biophys Res Commun,* 135**,** 957-64.
MASSAGUE, J. 2008. TGFbeta in Cancer. *Cell,* 134**,** 215-30.
MASUDA, M. & FUJIWARA, K. 1993. Morphological responses of single endothelial cells exposed to physiological levels of fluid shear stress. *Front Med Biol Eng,* 5**,** 79-87.
MATHIEU D, L. J.-C., WATTEL F. 2006. *Non-healing wounds. In: Handbook on hyperbaric medicine,* , Netherlands: Springer.
MATOS, L., GOUVEIA, A. M. & ALMEIDA, H. 2015. ER Stress Response in Human Cellular Models of Senescence. *J Gerontol A Biol Sci Med Sci,* 70**,** 924-35.
MAY, W. S., JACOBS, S. & CUATRECASAS, P. 1984. Association of phorbol ester-induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. *Proc Natl Acad Sci U S A,* 81**,** 2016-20.
MAYORAL, R., VALVERDE, A. M., LLORENTE IZQUIERDO, C., GONZALEZ-RODRIGUEZ, A., BOSCA, L. & MARTIN-SANZ, P. 2010. Impairment of transforming growth factor beta signaling in caveolin-1-deficient hepatocytes: role in liver regeneration. *J Biol Chem,* 285**,** 3633-42.
MAZZEI, D., GUZZARDI, M. A., GIUSTI, S. & AHLUWALIA, A. 2010. A low shear stress modular bioreactor for connected cell culture under high flow rates. *Biotechnol Bioeng,* 106**,** 127-37.
MAZZEI, D., VOZZI, F., CISTERNINO, A., VOZZI, G. & AHLUWALIA, A. 2008. A High-Throughput Bioreactor System for Simulating Physiological Environments. *Industrial Electronics, IEEE Transactions on,* 55**,** 3273-3280.
MCBEATH, R., PIRONE, D. M., NELSON, C. M., BHADRIRAJU, K. & CHEN, C. S. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell,* 6**,** 483-95.
MCCARTNEY-FRANCIS, N. L., FRAZIER-JESSEN, M. & WAHL, S. M. 1998. TGF-beta: a balancing act. *Int Rev Immunol,* 16**,** 553-80.
MCCLOY, R. A., ROGERS, S., CALDON, C. E., LORCA, T., CASTRO, A. & BURGESS, A. 2014. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell Cycle,* 13**,** 1400-12.
MCCULLOCH, C. A. & BORDIN, S. 1991. Role of fibroblast subpopulations in periodontal physiology and pathology. *J Periodontal Res,* 26**,** 144-54.
MCCULLOCH, C. A., LEKIC, P. & MCKEE, M. D. 2000. Role of physical forces in regulating the form and function of the periodontal ligament. *Periodontol 2000,* 24**,** 56-72.
MCGRATH, J. A. E., R.A.; POPE, F.M. 2004. *Rook's Textbook of Dermatology* Blackwell Publishing.
MCKARNS, S. C., LETTERIO, J. J. & KAMINSKI, N. E. 2003. Concentration-dependent bifunctional effect of TGF-beta 1 on immunoglobulin production: a role for Smad3 in IgA production in vitro. *Int Immunopharmacol,* 3**,** 1761-74.
MCKEOWN, S. T., BARNES, J. J., HYLAND, P. L., LUNDY, F. T., FRAY, M. J. & IRWIN, C. R. 2007. Matrix metalloproteinase-3 differences in oral and skin fibroblasts. *J Dent Res,* 86**,** 457-62.
MENG, A., WANG, Y., VAN ZANT, G. & ZHOU, D. 2003. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res,* 63**,** 5414-9.
MICHEL, U., ESSELMANN, J. & NIESCHLAG, E. 1993. Expression of follistatin messenger ribonucleic acid in Sertoli cell-enriched cultures: regulation by epidermal growth factor and protein kinase C-dependent pathway but not by follicle-stimulating hormone and protein kinase A-dependent pathway. *Acta Endocrinol (Copenh),* 129**,** 525-31.
MILLER, C. J. & DAVIDSON, L. A. 2013. The interplay between cell signalling and mechanics in developmental processes. *Nat Rev Genet,* 14**,** 733-744.
MILLER, J. 1972. Experiments in Molecular Genetics. *In:* COLD SPRING HARBOR LAB. PRESS, P., NY) (ed.).
MITCHELL, H., CHOUDHURY, A., PAGANO, R. E. & LEOF, E. B. 2004a. Ligand-dependent and -independent transforming growth factor-beta receptor recycling regulated by clathrin-mediated endocytosis and Rab11. *Mol Biol Cell,* 15**,** 4166-78.

MITCHELL, H., CHOUDHURY, A., PAGANO, R. E. & LEOF, E. B. 2004b. Ligand-dependent and -independent Transforming Growth Factor-β Receptor Recycling Regulated by Clathrin-mediated Endocytosis and Rab11. *Mol Biol Cell,* 15**,** 4166-78.

MIURA, S., SATO, K., KATO-NEGISHI, M., TESHIMA, T. & TAKEUCHI, S. 2015. Fluid shear triggers microvilli formation via mechanosensitive activation of TRPV6. *Nat Commun,* 6**,** 8871.

MIYAMOTO, S., TERAMOTO, H., GUTKIND, J. S. & YAMADA, K. M. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol,* 135**,** 1633-42.

MIYASATO, S. K., LOEFFLER, J., SHOHET, R., ZHANG, J., LINDSEY, M. & LE SAUX, C. J. 2011. Caveolin-1 modulates TGF-beta1 signaling in cardiac remodeling. *Matrix Biol,* 30**,** 318-29.

MIYAZONO, K., TEN DIJKE, P. & HELDIN, C. H. 2000. TGF-beta signaling by Smad proteins. *Adv Immunol,* 75**,** 115-57.

MIYOSHI, K., HORIGUCHI, T., TANIMURA, A., HAGITA, H. & NOMA, T. 2015. Gene Signature of Human Oral Mucosa Fibroblasts: Comparison with Dermal Fibroblasts and Induced Pluripotent Stem Cells. *BioMed Research International,* 2015**,** 19.

MONTESANO, R., CARROZZINO, F. & SOULIÉ, P. 2007. Low concentrations of transforming growth factor-beta-1 induce tubulogenesis in cultured mammary epithelial cells. *BMC Developmental Biology,* 7**,** 7.

MOON, J. J., MATSUMOTO, M., PATEL, S., LEE, L., GUAN, J. L. & LI, S. 2005. Role of cell surface heparan sulfate proteoglycans in endothelial cell migration and mechanotransduction. *J Cell Physiol,* 203**,** 166-76.

MORI, Y., CHEN, S. J. & VARGA, J. 2003. Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. *Arthritis Rheum,* 48**,** 1964-78.

MOSES, H. L., YANG, E. Y. & PIETENPOL, J. A. 1990. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell,* 63**,** 245-7.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

MOUSTAKAS, A., LIN, H. Y., HENIS, Y. I., PLAMONDON, J., O'CONNOR-MCCOURT, M. D. & LODISH, H. F. 1993. The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J Biol Chem,* 268**,** 22215-8.

MOUW, J. K., OU, G. & WEAVER, V. M. 2014. Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol,* 15**,** 771-85.

MOW, V. C., HOLMES, M. H. & MICHAEL LAI, W. 1984. Fluid transport and mechanical properties of articular cartilage: A review. *Journal of Biomechanics,* 17**,** 377-394.

MU, Y., SUNDAR, R., THAKUR, N., EKMAN, M., GUDEY, S. K., YAKYMOVYCH, M., HERMANSSON, A., DIMITRIOU, H., BENGOECHEA-ALONSO, M. T., ERICSSON, J., HELDIN, C. H. & LANDSTRÖM, M. 2011. TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer. *Nat Commun,* 2**,** 330-.

MUNDT AJ, R. J., CHUNG TD, ET AL. 2003. Biologic Basis of Radiation Therapy. *In:* IN: KUFE DW, P. R., WEICHSELBAUM RR, ET AL. (ed.) 6th edition. ed. Holland-Frei Cancer Medicine.

MUNRO, J., STEEGHS, K., MORRISON, V., IRELAND, H. & PARKINSON, E. K. 2001. Human fibroblast replicative senescence can occur in the absence of extensive cell division and short telomeres. *Oncogene,* 20**,** 3541-52.

MUNSON, J. M. & SHIEH, A. C. 2014. Interstitial fluid flow in cancer: implications for disease progression and treatment. *Cancer Manag Res,* 6**,** 317-28.

MURRAY, L. J., BRUNO, E., UCHIDA, N., HOFFMAN, R., NAYAR, R., YEO, E. L., SCHUH, A. C. & SUTHERLAND, D. R. 1999. CD109 is expressed on a subpopulation of CD34+ cells enriched in hematopoietic stem and progenitor cells. *Exp Hematol,* 27**,** 1282-94.

NADERI, J., HUNG, M. & PANDEY, S. 2003. Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: Resistance of quiescent cells to oxidative stress. *Apoptosis,* 8**,** 91-100.

NAGARAJ, N. S. & DATTA, P. K. 2010. Targeting the Transforming Growth Factor-β Signaling Pathway in Human Cancer. *Expert opinion on investigational drugs,* 19**,** 77-91.

NAGEL, T., RESNICK, N., ATKINSON, W. J., DEWEY, C. F., JR. & GIMBRONE, M. A., JR. 1994. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest,* 94**,** 885-91.

NAGY, G. 2003. [Role of saliva, salivary glands and epidermal growth factor (EGF) on oral wound healing]. *Fogorv Sz,* 96**,** 17-20.

NAKAMURA, T., TAKIO, K., ETO, Y., SHIBAI, H., TITANI, K. & SUGINO, H. 1990. Activin-binding protein from rat ovary is follistatin. *Science,* 247**,** 836-8.

NAKANISHI, M., ADAMI, G. R., ROBETORYE, R. S., NODA, A., VENABLE, S. F., DIMITROV, D., PEREIRA-SMITH, O. M. & SMITH, J. R. 1995. Exit from G0 and entry into the cell cycle of cells expressing p21Sdi1 antisense RNA. *Proc Natl Acad Sci U S A,* 92**,** 4352-6.

NAKAO, A., AFRAKHTE, M., MOREN, A., NAKAYAMA, T., CHRISTIAN, J. L., HEUCHEL, R., ITOH, S., KAWABATA, M., HELDIN, N. E., HELDIN, C. H. & TEN DIJKE, P. 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature,* 389**,** 631-5.

NAKAYAMA, K. H., SURYA, V. N., GOLE, M., WALKER, T. W., YANG, W., LAI, E. S., OSTROWSKI, M. A., FULLER, G. G., DUNN, A. R. & HUANG, N. F. 2016. Nanoscale Patterning of Extracellular Matrix Alters Endothelial Function under Shear Stress. *Nano Lett,* 16**,** 410-9.

NANCI, A. & BOSSHARDT, D. D. 2006. Structure of periodontal tissues in health and disease. *Periodontol 2000,* 40**,** 11-28.

NAULI, S. M., ALENGHAT, F. J., LUO, Y., WILLIAMS, E., VASSILEV, P., LI, X., ELIA, A. E., LU, W., BROWN, E. M., QUINN, S. J., INGBER, D. E. & ZHOU, J. 2003. Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet,* 33**,** 129-37.

NAULI, S. M., KAWANABE, Y., KAMINSKI, J. J., PEARCE, W. J., INGBER, D. E. & ZHOU, J. 2008. Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. *Circulation,* 117**,** 1161-71.

NEREM, R. M., HARRISON, D. G., TAYLOR, W. R. & ALEXANDER, R. W. 1993. Hemodynamics and vascular endothelial biology. *J Cardiovasc Pharmacol,* 21 Suppl 1**,** S6-10.

NG, C. P., HELM, C. L. & SWARTZ, M. A. 2004. Interstitial flow differentially stimulates blood and lymphatic endothelial cell morphogenesis in vitro. *Microvasc Res,* 68**,** 258-64.

NG, C. P., HINZ, B. & SWARTZ, M. A. 2005. Interstitial fluid flow induces myofibroblast differentiation and collagen alignment in vitro. *Journal of Cell Science,* 118**,** 4731-4739.

NG, C. P. & SWARTZ, M. A. 2003. Fibroblast alignment under interstitial fluid flow using a novel 3-D tissue culture model. *Am J Physiol Heart Circ Physiol,* 284**,** H1771-7.

NOLTE, S. V., XU, W., RENNEKAMPFF, H. O. & RODEMANN, H. P. 2008. Diversity of fibroblasts--a review on implications for skin tissue engineering. *Cells Tissues Organs,* 187**,** 165-76.

NONAKA, S., TANAKA, Y., OKADA, Y., TAKEDA, S., HARADA, A., KANAI, Y., KIDO, M. & HIROKAWA, N. 1998. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell,* 95**,** 829-37.

NORTH, T. E., GOESSLING, W., PEETERS, M., LI, P., CEOL, C., LORD, A. M., WEBER, G. J., HARRIS, J., CUTTING, C. C., HUANG, P., DZIERZAK, E. & ZON, L. I. 2009. Hematopoietic stem cell development is dependent on blood flow. *Cell,* 137**,** 736-48.

NUNEZ, M., MAYO, K. H., STARBUCK, C. & LAUFFENBURGER, D. 1993. pH sensitivity of epidermal growth factor receptor complexes. *J Cell Biochem,* 51**,** 312-21.

O'CONNOR, J. W. & GOMEZ, E. W. 2014. Biomechanics of TGFbeta-induced epithelial-mesenchymal transition: implications for fibrosis and cancer. *Clin Transl Med,* 3**,** 23.

O'BRIEN, F. J., HARLEY, B. A., YANNAS, I. V. & GIBSON, L. J. 2005. The effect of pore size on cell adhesion in collagen-GAG scaffolds. *Biomaterials,* 26**,** 433-441.

OGATA, S., MOROKUMA, J., HAYATA, T., KOLLE, G., NIEHRS, C., UENO, N. & CHO, K. W. 2007. TGF-beta signaling-mediated morphogenesis: modulation of cell adhesion via cadherin endocytosis. *Genes Dev,* 21**,** 1817-31.

OHGA, E., MATSUSE, T., TERAMOTO, S., KATAYAMA, H., NAGASE, T., FUKUCHI, Y. & OUCHI, Y. 1996a. Effects of Activin A on Proliferation and Differentiation of Human Lung Fibroblasts. *Biochemical and Biophysical Research Communications,* 228**,** 391-396.

OHGA, E., MATSUSE, T., TERAMOTO, S., KATAYAMA, H., NAGASE, T., FUKUCHI, Y. & OUCHI, Y. 1996b. Effects of activin A on proliferation and differentiation of human lung fibroblasts. *Biochem Biophys Res Commun,* 228**,** 391-6.

OHTANI, N., ZEBEDEE, Z., HUOT, T. J., STINSON, J. A., SUGIMOTO, M., OHASHI, Y., SHARROCKS, A. D., PETERS, G. & HARA, E. 2001. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature,* 409**,** 1067-70.

ORAGUI, E., NANNAPARAJU, M. & KHAN, W. S. 2011. The Role of Bioreactors in Tissue Engineering for Musculoskeletal Applications. *Open Orthop J,* 5**,** 267-70.

ORIMO, A., GUPTA, P. B., SGROI, D. C., ARENZANA-SEISDEDOS, F., DELAUNAY, T., NAEEM, R., CAREY, V. J., RICHARDSON, A. L. & WEINBERG, R. A. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell,* 121**,** 335-48.

OSHIMORI, N. & FUCHS, E. 2012. The Harmonies Played by TGF-β in Stem Cell Biology. *Cell Stem Cell,* 11**,** 751-64.

OSTMAN, A. 2004. PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev,* 15**,** 275-86.

OTA, F., MAESHIMA, A., YAMASHITA, S., IKEUCHI, H., KANEKO, Y., KUROIWA, T., HIROMURA, K., UEKI, K., KOJIMA, I. & NOJIMA, Y. 2003. Activin A induces cell proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. *Arthritis Rheum,* 48**,** 2442-9.

OTRANTO, M., SARRAZY, V., BONTÉ, F., HINZ, B., GABBIANI, G. & DESMOULIERE, A. 2012. The role of the myofibroblast in tumor stroma remodeling. *Cell Adh Migr,* 6**,** 203-19.

OWENS, G. K., KUMAR, M. S. & WAMHOFF, B. R. 2004. Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. *Physiological Reviews,* 84**,** 767-801.

OYAMA, F., MURATA, Y., SUGANUMA, N., KIMURA, T., TITANI, K. & SEKIGUCHI, K. 1989. Patterns of alternative splicing of fibronectin pre-mRNA in human adult and fetal tissues. *Biochemistry,* 28**,** 1428-34.

PAN, B. T. & JOHNSTONE, R. M. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell,* 33**,** 967-78.

PAN, X., CHEN, Z., HUANG, R., YAO, Y. & MA, G. 2013. Transforming growth factor beta1 induces the expression of collagen type I by DNA methylation in cardiac fibroblasts. *PLoS One,* 8**,** e60335.

PANKOV, R. & YAMADA, K. M. 2002. Fibronectin at a glance. *J Cell Sci,* 115**,** 3861-3.

PANSE, K. D., FELKIN, L. E., LÓPEZ-OLAÑETA, M. M., GÓMEZ-SALINERO, J., VILLALBA, M., MUÑOZ, L., NAKAMURA, K., SHIMANO, M., WALSH, K., BARTON, P. J. R., ROSENTHAL, N. & LARA-PEZZI, E. 2012. Follistatin-Like 3 Mediates Paracrine Fibroblast Activation by Cardiomyocytes. *Journal of Cardiovascular Translational Research,* 5**,** 814-826.

PAPAGEORGIS, P. 2015. TGFβ Signaling in Tumor Initiation, Epithelial-to-Mesenchymal Transition, and Metastasis. *Journal of Oncology,* 2015**,** 587193.

PARDANAUD, L. & EICHMANN, A. 2009. Stem cells: The stress of forming blood cells. *Nature,* 459**,** 1068-9.

PARK, J. Y., YOO, S. J., PATEL, L., LEE, S. H. & LEE, S. H. 2010. Cell morphological response to low shear stress in a two-dimensional culture microsystem with magnitudes comparable to interstitial shear stress. *Biorheology,* 47**,** 165-78.

PARSONAGE, G., FILER, A. D., HAWORTH, O., NASH, G. B., RAINGER, G. E., SALMON, M. & BUCKLEY, C. D. 2005. A stromal address code defined by fibroblasts. *Trends Immunol,* 26**,** 150-6.

PARTON, R. G. & SIMONS, K. 2007. The multiple faces of caveolae. *Nat Rev Mol Cell Biol,* 8**,** 185-94.

PASZEK, M. J., ZAHIR, N., JOHNSON, K. R., LAKINS, J. N., ROZENBERG, G. I., GEFEN, A., REINHART-KING, C. A., MARGULIES, S. S., DEMBO, M., BOETTIGER, D., HAMMER, D. A. & WEAVER, V. M. 2005. Tensional homeostasis and the malignant phenotype. *Cancer Cell,* 8**,** 241-54.

PAVALKO, F. M., CHEN, N. X., TURNER, C. H., BURR, D. B., ATKINSON, S., HSIEH, Y. F., QIU, J. & DUNCAN, R. L. 1998. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. *Am J Physiol,* 275**,** C1591-601.

PEDERSEN, J. A., BOSCHETTI, F. & SWARTZ, M. A. 2007. Effects of extracellular fiber architecture on cell membrane shear stress in a 3D fibrous matrix. *J Biomech,* 40**,** 1484-92.

PELHAM, R. J., JR. & WANG, Y. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A,* 94**,** 13661-5.

PELLEGRIN, S. & MELLOR, H. 2007. Actin stress fibres. *J Cell Sci,* 120**,** 3491-9.

PENDER, N. & MCCULLOCH, C. A. 1991. Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci,* 100 (Pt 1)**,** 187-93.

PIERREUX, C. E., NICOLÁS, F. J. & HILL, C. S. 2000. Transforming Growth Factor β-Independent Shuttling of Smad4 between the Cytoplasm and Nucleus. *Molecular and Cellular Biology,* 20**,** 9041-9054.

PIERSMA, B., BANK, R. A. & BOERSEMA, M. 2015a. Signaling in Fibrosis: TGF-beta, WNT, and YAP/TAZ Converge. *Front Med (Lausanne),* 2**,** 59.

PIERSMA, B., BANK, R. A. & BOERSEMA, M. 2015b. Signaling in Fibrosis: TGF-β, WNT, and YAP/TAZ Converge. *Front Med (Lausanne),* 2.

PLUNKETT, N. & O'BRIEN, F. J. 2011. Bioreactors in tissue engineering. *Technol Health Care,* 19**,** 55-69.

POELMANN, R. E., VAN DER HEIDEN, K., GITTENBERGER-DE GROOT, A. & HIERCK, B. P. 2008. Deciphering the endothelial shear stress sensor. *Circulation,* 117**,** 1124-6.

POLACHECK, W. J., CHAREST, J. L. & KAMM, R. D. 2011. Interstitial flow influences direction of tumor cell migration through competing mechanisms. *Proc Natl Acad Sci U S A,* 108**,** 11115-20.

PONCELET, A. C., SCHNAPER, H. W., TAN, R., LIU, Y. & RUNYAN, C. E. 2007. Cell phenotype-specific down-regulation of Smad3 involves decreased gene activation as well as protein degradation. *J Biol Chem,* 282**,** 15534-40.

POTENTA, S., ZEISBERG, E. & KALLURI, R. 2008. The role of endothelial-to-mesenchymal transition in cancer progression. *Br J Cancer,* 99**,** 1375-9.

PRIME, S. S., CIRILLO, N., HASSONA, Y., LAMBERT, D. W., PATERSON, I. C., MELLONE, M., THOMAS, G. J., JAMES, E. N. & PARKINSON, E. K. 2016. Fibroblast activation and senescence in oral cancer. *J Oral Pathol Med*.

PRITCHARD, W. F., DAVIES, P. F., DERAFSHI, Z., POLACEK, D. C., TSAO, R., DULL, R. O., JONES, S. A. & GIDDENS, D. P. 1995. Effects of wall shear stress and fluid recirculation on the localization of circulating monocytes in a three-dimensional flow model. *J Biomech,* 28**,** 1459-69.

QAZI, H., SHI, Z. D. & TARBELL, J. M. 2011. Fluid shear stress regulates the invasive potential of glioma cells via modulation of migratory activity and matrix metalloproteinase expression. *PLoS One,* 6**,** e20348.

RADISKY, D. C., LEVY, D. D., LITTLEPAGE, L. E., LIU, H., NELSON, C. M., FATA, J. E., LEAKE, D., GODDEN, E. L., ALBERTSON, D. G., NIETO, M. A., WERB, Z. & BISSELL, M. J. 2005. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature,* 436**,** 123-7.

RAGHAVAN, V., RBAIBI, Y., PASTOR-SOLER, N. M., CARATTINO, M. D. & WEISZ, O. A. 2014. Shear stress-dependent regulation of apical endocytosis in renal proximal tubule cells mediated by primary cilia. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 8506-8511.

RAGHAVAN, V. & WEISZ, O. A. 2015. Flow stimulated endocytosis in the proximal tubule. *Current opinion in nephrology and hypertension,* 24**,** 359-365.

RAZANI, B., SCHLEGEL, A. & LISANTI, M. P. 2000. Caveolin proteins in signaling, oncogenic transformation and muscular dystrophy. *Journal of Cell Science,* 113**,** 2103-2109.

RAZANI, B., ZHANG, X. L., BITZER, M., VON GERSDORFF, G., BOTTINGER, E. P. & LISANTI, M. P. 2001. Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *J Biol Chem,* 276**,** 6727-38.
REED, R. K., LIDEN, A. & RUBIN, K. 2010. Edema and fluid dynamics in connective tissue remodelling. *J Mol Cell Cardiol,* 48**,** 518-23.
RESNICK, A. 2011. Chronic fluid flow is an environmental modifier of renal epithelial function. *PLoS One,* 6**,** e27058.
RHO, J. Y., ASHMAN, R. B. & TURNER, C. H. 1993. Young's modulus of trabecular and cortical bone material: ultrasonic and microtensile measurements. *J Biomech,* 26**,** 111-9.
RIEF, M., PASCUAL, J., SARASTE, M. & GAUB, H. E. 1999. Single molecule force spectroscopy of spectrin repeats: low unfolding forces in helix bundles. *J Mol Biol,* 286**,** 553-61.
RIZVI, I., GURKAN, U. A., TASOGLU, S., ALAGIC, N., CELLI, J. P., MENSAH, L. B., MAI, Z., DEMIRCI, U. & HASAN, T. 2013. Flow induces epithelial-mesenchymal transition, cellular heterogeneity and biomarker modulation in 3D ovarian cancer nodules. *Proc Natl Acad Sci U S A,* 110**,** E1974-83.
ROBERTS, V. J. & BARTH, S. L. 1994. Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid- and late-gestation rat embryogenesis. *Endocrinology,* 134**,** 914-23.
RODEMANN, H. P. & MULLER, G. A. 1991. Characterization of human renal fibroblasts in health and disease: II. In vitro growth, differentiation, and collagen synthesis of fibroblasts from kidneys with interstitial fibrosis. *Am J Kidney Dis,* 17**,** 684-6.
ROHR, S. 2011. Cardiac fibroblasts in cell culture systems: myofibroblasts all along? *J Cardiovasc Pharmacol,* 57**,** 389-99.
ROJAS, A., PADIDAM, M., CRESS, D. & GRADY, W. M. 2009. TGF-beta receptor levels regulate the specificity of signaling pathway activation and biological effects of TGF-beta. *Biochim Biophys Acta,* 1793**,** 1165-73.
ROTHBERG, K. G., HEUSER, J. E., DONZELL, W. C., YING, Y. S., GLENNEY, J. R. & ANDERSON, R. G. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell,* 68**,** 673-82.
ROWLAND, B. D. & BERNARDS, R. 2006. Re-evaluating cell-cycle regulation by E2Fs. *Cell,* 127**,** 871-4.
RUGGIERO, P. 2010. Helicobacter pylori and inflammation. *Curr Pharm Des,* 16**,** 4225-36.
RUNYAN, C. E., SCHNAPER, H. W. & PONCELET, A. C. 2005. The role of internalization in transforming growth factor beta1-induced Smad2 association with Smad anchor for receptor activation (SARA) and Smad2-dependent signaling in human mesangial cells. *J Biol Chem,* 280**,** 8300-8.
RUSCH, V., KLIMSTRA, D., VENKATRAMAN, E., PISTERS, P. W., LANGENFELD, J. & DMITROVSKY, E. 1997. Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression. *Clin Cancer Res,* 3**,** 515-22.
RUSSELL, W. E., COFFEY, R. J., JR., OUELLETTE, A. J. & MOSES, H. L. 1988. Type beta transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat. *Proc Natl Acad Sci U S A,* 85**,** 5126-30.
RUTKOWSKI, J. M., BOARDMAN, K. C. & SWARTZ, M. A. 2006. Characterization of lymphangiogenesis in a model of adult skin regeneration. *Am J Physiol Heart Circ Physiol,* 291**,** H1402-10.
RUTKOWSKI, J. M. & SWARTZ, M. A. 2007. A driving force for change: interstitial flow as a morphoregulator. *Trends Cell Biol,* 17**,** 44-50.
SAFI, L. M., LIBERTHSON, R. R. & BHATT, A. 2016. Current Management of Ebstein's Anomaly in the Adult. *Curr Treat Options Cardiovasc Med,* 18**,** 56.
SAKAKURA, H., MII, S., HAGIWARA, S., KATO, T., YAMAMOTO, N., HIBI, H., TAKAHASHI, M. & MURAKUMO, Y. 2016. CD109 is a component of exosome secreted from cultured cells. *Biochem Biophys Res Commun,* 469**,** 816-22.
SAMANI, A. & PLEWES, D. 2004. A method to measure the hyperelastic parameters of ex vivo breast tissue samples. *Phys Med Biol,* 49**,** 4395-405.

SANG, L. & COLLER, H. A. 2009. Fear of commitment: Hes1 protects quiescent fibroblasts from irreversible cellular fates. *Cell Cycle,* 8**,** 2161-2167.
SAUNDERS, R. L. & HAMMER, D. A. 2010. Assembly of Human Umbilical Vein Endothelial Cells on Compliant Hydrogels. *Cell Mol Bioeng,* 3**,** 60-7.
SAWAMOTO, K., WICHTERLE, H., GONZALEZ-PEREZ, O., CHOLFIN, J. A., YAMADA, M., SPASSKY, N., MURCIA, N. S., GARCIA-VERDUGO, J. M., MARIN, O., RUBENSTEIN, J. L., TESSIER-LAVIGNE, M., OKANO, H. & ALVAREZ-BUYLLA, A. 2006. New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science,* 311**,** 629-32.
SCALLAN J, H. V., KORTHUIS RJ. 2010. Pathophysiology of Edema Formation. *In:* (CA), S. R. (ed.) *Capillary Fluid Exchange: Regulation, Functions, and Pathology*.: Morgan & Claypool Life Sciences.
SCHERES, N., LAINE, M. L., DE VRIES, T. J., EVERTS, V. & VAN WINKELHOFF, A. J. 2010. Gingival and periodontal ligament fibroblasts differ in their inflammatory response to viable Porphyromonas gingivalis. *J Periodontal Res,* 45**,** 262-70.
SCHLAEPFER, D. D., JONES, K. C. & HUNTER, T. 1998. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol,* 18**,** 2571-85.
SCHMIERER, B. & HILL, C. S. 2007. TGF[beta]-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol,* 8**,** 970-982.
SCHNEYER, A. L., RZUCIDLO, D. A., SLUSS, P. M. & CROWLEY, W. F., JR. 1994. Characterization of unique binding kinetics of follistatin and activin or inhibin in serum. *Endocrinology,* 135**,** 667-74.
SCHREMENTI, M. E., FERREIRA, A. M., ZENDER, C. & DIPIETRO, L. A. 2008. Site-specific production of TGF-beta in oral mucosal and cutaneous wounds. *Wound Repair Regen,* 16**,** 80-6.
SCHWACHTGEN, J. L., HOUSTON, P., CAMPBELL, C., SUKHATME, V. & BRADDOCK, M. 1998. Fluid shear stress activation of egr-1 transcription in cultured human endothelial and epithelial cells is mediated via the extracellular signal-related kinase 1/2 mitogen-activated protein kinase pathway. *J Clin Invest,* 101**,** 2540-9.
SCHWARZ, M. A. & JULIANO, R. L. 1984. Interaction of fibronectin-coated beads with CHO cells. *Exp Cell Res,* 152**,** 302-12.
SCHWENCKE, C., SCHMEISSER, A., WALTER, C., WACHTER, R., PANNACH, S., WECK, B., BRAUN-DULLAEUS, R. C., KASPER, M. & STRASSER, R. H. 2005. Decreased caveolin-1 in atheroma: Loss of antiproliferative control of vascular smooth muscle cells in atherosclerosis. *Cardiovascular Research,* 68**,** 128-135.
SERINI, G., BOCHATON-PIALLAT, M. L., ROPRAZ, P., GEINOZ, A., BORSI, L., ZARDI, L. & GABBIANI, G. 1998. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol,* 142**,** 873-81.
SERLUCA, F. C., DRUMMOND, I. A. & FISHMAN, M. C. 2002. Endothelial signaling in kidney morphogenesis: a role for hemodynamic forces. *Curr Biol,* 12**,** 492-7.
SHAHIN, K. & DORAN, P. M. 2015. Shear and Compression Bioreactor for Cartilage Synthesis. *Methods Mol Biol,* 1340**,** 221-33.
SHANNON, D. B., MCKEOWN, S. T., LUNDY, F. T. & IRWIN, C. R. 2006. Phenotypic differences between oral and skin fibroblasts in wound contraction and growth factor expression. *Wound Repair Regen,* 14**,** 172-8.
SHARIF-NAEINI, R., FOLGERING, J. H., BICHET, D., DUPRAT, F., LAURITZEN, I., ARHATTE, M., JODAR, M., DEDMAN, A., CHATELAIN, F. C., SCHULTE, U., RETAILLEAU, K., LOUFRANI, L., PATEL, A., SACHS, F., DELMAS, P., PETERS, D. J. & HONORE, E. 2009. Polycystin-1 and -2 dosage regulates pressure sensing. *Cell,* 139**,** 587-96.
SHEN, X. J., ZHANG, H., TANG, G. S., WANG, X. D., ZHENG, R., WANG, Y., ZHU, Y., XUE, X. C. & BI, J. W. 2015. Caveolin-1 is a Modulator of Fibroblast Activation and a Potential Biomarker for Gastric Cancer. *Int J Biol Sci,* 11**,** 370-9.

SHERMAN, V. R., YANG, W. & MEYERS, M. A. 2015. The materials science of collagen. *Journal of the Mechanical Behavior of Biomedical Materials,* 52**,** 22-50.
SHERRATT, M. J., BAX, D. V., CHAUDHRY, S. S., HODSON, N., LU, J. R., SARAVANAPAVAN, P. & KIELTY, C. M. 2005. Substrate chemistry influences the morphology and biological function of adsorbed extracellular matrix assemblies. *Biomaterials,* 26**,** 7192-206.
SHI, Z. D. & TARBELL, J. M. 2011. Fluid flow mechanotransduction in vascular smooth muscle cells and fibroblasts. *Ann Biomed Eng,* 39**,** 1608-19.
SHIEH, A. C. & SWARTZ, M. A. 2011. Regulation of tumor invasion by interstitial fluid flow. *Phys Biol,* 8**,** 015012.
SHIFF, S. J., KOUTSOS, M. I., QIAO, L. & RIGAS, B. 1996. Nonsteroidal Antiinflammatory Drugs Inhibit the Proliferation of Colon Adenocarcinoma Cells: Effects on Cell Cycle and Apoptosis. *Experimental Cell Research,* 222**,** 179-188.
SHIMA, S., MATSUOKA, H., IWAMOTO, T. & SAKAI, H. 1984. Antimicrobial action of epsilon-poly-L-lysine. *J Antibiot (Tokyo),* 37**,** 1449-55.
SHIMANO, M., OUCHI, N., NAKAMURA, K., OSHIMA, Y., HIGUCHI, A., PIMENTEL, D. R., PANSE, K. D., LARA-PEZZI, E., LEE, S. J., SAM, F. & WALSH, K. 2011. Cardiac myocyte-specific ablation of follistatin-like 3 attenuates stress-induced myocardial hypertrophy. *J Biol Chem,* 286**,** 9840-8.
SHIMASAKI, S., KOGA, M., ESCH, F., MERCADO, M., COOKSEY, K., KOBA, A. & LING, N. 1988. Porcine follistatin gene structure supports two forms of mature follistatin produced by alternative splicing. *Biochem Biophys Res Commun,* 152**,** 717-23.
SHIVSHANKAR, P., BRAMPTON, C., MIYASATO, S., KASPER, M., THANNICKAL, V. J. & LE SAUX, C. J. 2012. Caveolin-1 Deficiency Protects from Pulmonary Fibrosis by Modulating Epithelial Cell Senescence in Mice. *American Journal of Respiratory Cell and Molecular Biology,* 47**,** 28-36.
SIDIS, Y., MUKHERJEE, A., KEUTMANN, H., DELBAERE, A., SADATSUKI, M. & SCHNEYER, A. 2006. Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins. *Endocrinology,* 147**,** 3586-97.
SIEWEKE, M. H., THOMPSON, N. L., SPORN, M. B. & BISSELL, M. J. 1990. Mediation of wound-related Rous sarcoma virus tumorigenesis by TGF-beta. *Science,* 248**,** 1656-60.
SIMPKINS, S. A., HANBY, A. M., HOLLIDAY, D. L. & SPEIRS, V. 2012. Clinical and functional significance of loss of caveolin-1 expression in breast cancer-associated fibroblasts. *J Pathol,* 227**,** 490-8.
SINGH, O., GUPTA, S. S., SONI, M., MOSES, S., SHUKLA, S. & MATHUR, R. K. 2011. Collagen dressing versus conventional dressings in burn and chronic wounds: a retrospective study. *J Cutan Aesthet Surg,* 4**,** 12-6.
SINHA, B., KOSTER, D., RUEZ, R., GONNORD, P., BASTIANI, M., ABANKWA, D., STAN, R. V., BUTLER-BROWNE, G., VEDIE, B., JOHANNES, L., MORONE, N., PARTON, R. G., RAPOSO, G., SENS, P., LAMAZE, C. & NASSOY, P. 2011. Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell,* 144**,** 402-13.
SITTERLEY G 2008. Poly-L-Lysine cell attachment protocol *Biofiles,* 3**,** 12.
SKALLI, O., PELTE, M. F., PECLET, M. C., GABBIANI, G., GUGLIOTTA, P., BUSSOLATI, G., RAVAZZOLA, M. & ORCI, L. 1989. Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J Histochem Cytochem,* 37**,** 315-21.
SLOUGH, J., COONEY, L. & BRUECKNER, M. 2008. Monocilia in the embryonic mouse heart suggest a direct role for cilia in cardiac morphogenesis. *Dev Dyn,* 237**,** 2304-14.
SONG, E., OUYANG, N., HORBELT, M., ANTUS, B., WANG, M. & EXTON, M. S. 2000. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol,* 204**,** 19-28.
SORKIN, A. & VON ZASTROW, M. 2009. Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol,* 10**,** 609-22.
SORRELL, J. M. & CAPLAN, A. I. 2004. Fibroblast heterogeneity: more than skin deep. *J Cell Sci,* 117**,** 667-75.

SOTGIA, F., DEL GALDO, F., CASIMIRO, M. C., BONUCCELLI, G., MERCIER, I., WHITAKER-MENEZES, D., DAUMER, K. M., ZHOU, J., WANG, C., KATIYAR, S., XU, H., BOSCO, E., QUONG, A. A., ARONOW, B., WITKIEWICZ, A. K., MINETTI, C., FRANK, P. G., JIMENEZ, S. A., KNUDSEN, E. S., PESTELL, R. G. & LISANTI, M. P. 2009. Caveolin-1-/- null mammary stromal fibroblasts share characteristics with human breast cancer-associated fibroblasts. *Am J Pathol,* 174**,** 746-61.

SPENCER, S. L., CAPPELL, S. D., TSAI, F. C., OVERTON, K. W., WANG, C. L. & MEYER, T. 2013. The proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell,* 155**,** 369-83.

SQUIER, C. A. & KREMER, M. J. 2001. Biology of oral mucosa and esophagus. *J Natl Cancer Inst Monogr***,** 7-15.

STEHR, M., ESTRADA, C. R., KHOURY, J., DANCIU, T. E., SULLIVAN, M. P., PETERS, C. A., SOLOMON, K. R., FREEMAN, M. R. & ADAM, R. M. 2004. Caveolae are negative regulators of transforming growth factor-beta1 signaling in ureteral smooth muscle cells. *J Urol,* 172**,** 2451-5.

STEIN, G. H., DRULLINGER, L. F., SOULARD, A. & DULIC, V. 1999. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol,* 19**,** 2109-17.

STERNLICHT, M. D., LOCHTER, A., SYMPSON, C. J., HUEY, B., ROUGIER, J. P., GRAY, J. W., PINKEL, D., BISSELL, M. J. & WERB, Z. 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell,* 98**,** 137-46.

STEWARD, R., TAMBE, D., HARDIN, C. C., KRISHNAN, R. & FREDBERG, J. J. 2015. Fluid shear, intercellular stress, and endothelial cell alignment. *Am J Physiol Cell Physiol,* 308**,** C657-64.

STEWARD, R. L., CHENG, C.-M., YE, J. D., BELLIN, R. M. & LEDUC, P. R. 2011. Mechanical stretch and shear flow induced reorganization and recruitment of fibronectin in fibroblasts. *Scientific Reports,* 1**,** 147.

SUGIURA, S., SAKAI, Y., NAKAZAWA, K. & KANAMORI, T. 2011. Superior oxygen and glucose supply in perfusion cell cultures compared to static cell cultures demonstrated by simulations using the finite element method. *Biomicrofluidics,* 5**,** 022202.

SULYOK, S., WANKELL, M., ALZHEIMER, C. & WERNER, S. 2004. Activin: an important regulator of wound repair, fibrosis, and neuroprotection. *Mol Cell Endocrinol,* 225**,** 127-32.

SUTHERLAND, D. R., YEO, E., RYAN, A., MILLS, G. B., BAILEY, D. & BAKER, M. A. 1991. Identification of a cell-surface antigen associated with activated T lymphoblasts and activated platelets. *Blood,* 77**,** 84-93.

SWARTZ, M. A. & FLEURY, M. E. 2007. Interstitial Flow and Its Effects in Soft Tissues. *Annual Review of Biomedical Engineering,* 9**,** 229-256.

SZETO, S. G., NARIMATSU, M., LU, M., HE, X., SIDIQI, A. M., TOLOSA, M. F., CHAN, L., DE FREITAS, K., BIALIK, J. F., MAJUMDER, S., BOO, S., HINZ, B., DAN, Q., ADVANI, A., JOHN, R., WRANA, J. L., KAPUS, A. & YUEN, D. A. 2016. YAP/TAZ Are Mechanoregulators of TGF-β-Smad Signaling and Renal Fibrogenesis. *Journal of the American Society of Nephrology*.

SZPADERSKA, A. M., WALSH, C. G., STEINBERG, M. J. & DIPIETRO, L. A. 2005. Distinct patterns of angiogenesis in oral and skin wounds. *J Dent Res,* 84**,** 309-14.

SZPADERSKA, A. M., ZUCKERMAN, J. D. & DIPIETRO, L. A. 2003. Differential injury responses in oral mucosal and cutaneous wounds. *J Dent Res,* 82**,** 621-6.

TAKAHASHI, A., OHTANI, N., YAMAKOSHI, K., IIDA, S., TAHARA, H., NAKAYAMA, K., NAKAYAMA, K. I., IDE, T., SAYA, H. & HARA, E. 2006. Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat Cell Biol,* 8**,** 1291-7.

TAKAHASHI, M., ISHIDA, T., TRAUB, O., CORSON, M. A. & BERK, B. C. 1997. Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res,* 34**,** 212-9.

TALELE, N. P., FRADETTE, J., DAVIES, J. E., KAPUS, A. & HINZ, B. 2015. Expression of alpha-Smooth Muscle Actin Determines the Fate of Mesenchymal Stromal Cells. *Stem Cell Reports,* 4**,** 1016-30.

TAMADA, Y. & IKADA, Y. 1993. Effect of Preadsorbed Proteins on Cell Adhesion to Polymer Surfaces. *Journal of Colloid and Interface Science,* 155**,** 334-339.
TANDARA, A. A. & MUSTOE, T. A. 2004. Oxygen in wound healing--more than a nutrient. *World J Surg,* 28**,** 294-300.
TARBELL, J. M. & PAHAKIS, M. Y. 2006. Mechanotransduction and the glycocalyx. *J Intern Med,* 259**,** 339-50.
TCHAFA, A. M., TA, M., REGINATO, M. J. & SHIEH, A. C. 2015. EMT Transition Alters Interstitial Fluid Flow-Induced Signaling in ERBB2-Positive Breast Cancer Cells. *Mol Cancer Res,* 13**,** 755-64.
THIERY, J. P., ACLOQUE, H., HUANG, R. Y. & NIETO, M. A. 2009. Epithelial-mesenchymal transitions in development and disease. *Cell,* 139**,** 871-90.
THOMPSON, T. B., LERCH, T. F., COOK, R. W., WOODRUFF, T. K. & JARDETZKY, T. S. 2005. The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. *Dev Cell,* 9**,** 535-43.
TOMASEK, J. J., GABBIANI, G., HINZ, B., CHAPONNIER, C. & BROWN, R. A. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol,* 3**,** 349-363.
TOMLINSON, R. E. & SILVA, M. J. 2013. Skeletal Blood Flow in Bone Repair and Maintenance. *Bone Res,* 1**,** 311-22.
TORTORIELLO, D. V., SIDIS, Y., HOLTZMAN, D. A., HOLMES, W. E. & SCHNEYER, A. L. 2001. Human follistatin-related protein: a structural homologue of follistatin with nuclear localization. *Endocrinology,* 142**,** 3426-34.
TOULON, A., BRETON, L., TAYLOR, K. R., TENENHAUS, M., BHAVSAR, D., LANIGAN, C., RUDOLPH, R., JAMESON, J. & HAVRAN, W. L. 2009. A role for human skin-resident T cells in wound healing. *J Exp Med,* 206**,** 743-50.
TOURKINA, E., RICHARD, M., GOOZ, P., BONNER, M., PANNU, J., HARLEY, R., BERNATCHEZ, P. N., SESSA, W. C., SILVER, R. M. & HOFFMAN, S. 2008. Antifibrotic properties of caveolin-1 scaffolding domain in vitro and in vivo. *Am J Physiol Lung Cell Mol Physiol,* 294**,** L843-61.
TOURKINA, E., RICHARD, M., OATES, J., HOFBAUER, A., BONNER, M., GOOZ, P., VISCONTI, R., ZHANG, J., ZNOYKO, S., HATFIELD, C. M., SILVER, R. M. & HOFFMAN, S. 2010. Caveolin-1 regulates leucocyte behaviour in fibrotic lung disease. *Ann Rheum Dis,* 69**,** 1220-6.
TRACKMAN, P. C. & KANTARCI, A. 2004. Connective tissue metabolism and gingival overgrowth. *Crit Rev Oral Biol Med,* 15**,** 165-75.
TZIMA, E., DEL POZO, M. A., SHATTIL, S. J., CHIEN, S. & SCHWARTZ, M. A. 2001. Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. *EMBO J,* 20**,** 4639-47.
TZIMA, E., IRANI-TEHRANI, M., KIOSSES, W. B., DEJANA, E., SCHULTZ, D. A., ENGELHARDT, B., CAO, G., DELISSER, H. & SCHWARTZ, M. A. 2005. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature,* 437**,** 426-31.
VARGA, J. 2002. Scleroderma and Smads: dysfunctional Smad family dynamics culminating in fibrosis. *Arthritis Rheum,* 46**,** 1703-13.
VASSEUR, S., MALICET, C., CALVO, E. L., LABRIE, C., BERTHEZENE, P., DAGORN, J. C. & IOVANNA, J. L. 2003. Gene expression profiling by DNA microarray analysis in mouse embryonic fibroblasts transformed by rasV12 mutated protein and the E1A oncogene. *Molecular Cancer,* 2**,** 1-10.
VAUPEL, P. & MAYER, A. 2007. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev,* 26**,** 225-39.
VEERMAN, E. C., OUDHOFF, M. J. & BRAND, H. S. 2011. [Saliva and wound healing]. *Ned Tijdschr Tandheelkd,* 118**,** 253-6.
VERED, M., ALLON, I., BUCHNER, A. & DAYAN, D. 2009. Stromal myofibroblasts accompany modifications in the epithelial phenotype of tongue dysplastic and malignant lesions. *Cancer Microenviron,* 2**,** 49-57.
VERED, M., DOBRIYAN, A., DAYAN, D., YAHALOM, R., TALMI, Y. P., BEDRIN, L., BARSHACK, I. & TAICHER, S. 2010. Tumor-host histopathologic variables, stromal myofibroblasts and risk

score, are significantly associated with recurrent disease in tongue cancer. *Cancer Sci,* 101**,** 274-80.
VERMA, M., LAM, T. K., HEBERT, E. & DIVI, R. L. 2015. Extracellular vesicles: potential applications in cancer diagnosis, prognosis, and epidemiology. *BMC Clinical Pathology,* 15**,** 6.
VERRECCHIA, F. & MAUVIEL, A. 2002. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Invest Dermatol,* 118**,** 211-5.
VIHANTO, M. M., VINDIS, C., DJONOV, V., CERRETTI, D. P. & HUYNH-DO, U. 2006. Caveolin-1 is required for signaling and membrane targeting of EphB1 receptor tyrosine kinase. *Journal of Cell Science,* 119**,** 2299-2309.
VINCI, B., CAVALLONE, D., VOZZI, G., MAZZEI, D., DOMENICI, C., BRUNETTO, M. & AHLUWALIA, A. 2010. In vitro liver model using microfabricated scaffolds in a modular bioreactor. *Biotechnol J,* 5**,** 232-41.
VINCI, B., DURET, C., KLIEBER, S., GERBAL-CHALOIN, S., SA-CUNHA, A., LAPORTE, S., SUC, B., MAUREL, P., AHLUWALIA, A. & DAUJAT-CHAVANIEU, M. 2011. Modular bioreactor for primary human hepatocyte culture: medium flow stimulates expression and activity of detoxification genes. *Biotechnol J,* 6**,** 554-64.
VO, B. T., MORTON, D., JR., KOMARAGIRI, S., MILLENA, A. C., LEATH, C. & KHAN, S. A. 2013. TGF-beta effects on prostate cancer cell migration and invasion are mediated by PGE2 through activation of PI3K/AKT/mTOR pathway. *Endocrinology,* 154**,** 1768-79.
VOGEL, V. & SHEETZ, M. 2006. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol,* 7**,** 265-75.
VOLKMER, E., DROSSE, I., OTTO, S., STANGELMAYER, A., STENGELE, M., KALLUKALAM, B. C., MUTSCHLER, W. & SCHIEKER, M. 2008. Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. *Tissue Eng Part A,* 14**,** 1331-40.
VOLKMER, E., OTTO, S., POLZER, H., SALLER, M., TRAPPENDREHER, D., ZAGAR, D., HAMISCH, S., ZIEGLER, G., WILHELMI, A., MUTSCHLER, W. & SCHIEKER, M. 2012. Overcoming hypoxia in 3D culture systems for tissue engineering of bone in vitro using an automated, oxygen-triggered feedback loop. *Journal of Materials Science: Materials in Medicine,* 23**,** 2793-2801.
VOZZI, F., HEINRICH, J. M., BADER, A. & AHLUWALIA, A. D. 2009. Connected culture of murine hepatocytes and HUVEC in a multicompartmental bioreactor. *Tissue Eng Part A,* 15**,** 1291-9.
WALL, I. B., BHADAL, N., BROAD, S., WHAWELL, S. A., MUDERA, V. & LEWIS, M. P. 2009. Force generation and protease gene expression in organotypic co-cultures of fibroblasts and keratinocytes. *J Tissue Eng Regen Med,* 3**,** 647-50.
WANG, C., BAKER, B. M., CHEN, C. S. & SCHWARTZ, M. A. 2013. Endothelial cell sensing of flow direction. *Arterioscler Thromb Vasc Biol,* 33.
WANG, G. 2009. Transforming Growth Factor-β-inducible Phosphorylation of. 284**,** 9663-73.
WANG, H.-I. C. A. Y. 2011. *Cell Responses to Surface and Architecture of Tissue Engineering Scaffolds*.
WANG, J., ZOHAR, R. & MCCULLOCH, C. A. 2006a. Multiple roles of alpha-smooth muscle actin in mechanotransduction. *Exp Cell Res,* 312**,** 205-14.
WANG, N., BUTLER, J. P. & INGBER, D. E. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science,* 260**,** 1124-7.
WANG, N., NARUSE, K., STAMENOVIC, D., FREDBERG, J. J., MIJAILOVICH, S. M., TOLIC-NORRELYKKE, I. M., POLTE, T., MANNIX, R. & INGBER, D. E. 2001. Mechanical behavior in living cells consistent with the tensegrity model. *Proc Natl Acad Sci U S A,* 98**,** 7765-70.
WANG, S. & TARBELL, J. M. 2000. Effect of fluid flow on smooth muscle cells in a 3-dimensional collagen gel model. *Arterioscler Thromb Vasc Biol,* 20**,** 2220-5.
WANG, X. M., ZHANG, Y., KIM, H. P., ZHOU, Z., FEGHALI-BOSTWICK, C. A., LIU, F., IFEDIGBO, E., XU, X., OURY, T. D., KAMINSKI, N. & CHOI, A. M. K. 2006b. Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *The Journal of Experimental Medicine,* 203**,** 2895-2906.

WANG, Y., SCHULTE, B. A., LARUE, A. C., OGAWA, M. & ZHOU, D. 2006c. Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood,* 107**,** 358-66.
WARBURTON, D., BELLUSCI, S., DE LANGHE, S., DEL MORAL, P. M., FLEURY, V., MAILLEUX, A., TEFFT, D., UNBEKANDT, M., WANG, K. & SHI, W. 2005. Molecular mechanisms of early lung specification and branching morphogenesis. *Pediatr Res,* 57**,** 26r-37r.
WARREN, B. A., SHUBIK, P., WILSON, R., GARCIA, H. & FELDMAN, R. 1978. The microcirculation in two transplantable melanomas of the hamster. I. In vivo observations in transparent chambers. *Cancer Lett,* 4**,** 109-16.
WASHBURN, N. R., YAMADA, K. M., SIMON, C. G., JR., KENNEDY, S. B. & AMIS, E. J. 2004. High-throughput investigation of osteoblast response to polymer crystallinity: influence of nanometer-scale roughness on proliferation. *Biomaterials,* 25**,** 1215-24.
WATANABE, T., ICHIHARA, M., HASHIMOTO, M., SHIMONO, K., SHIMOYAMA, Y., NAGASAKA, T., MURAKUMO, Y., MURAKAMI, H., SUGIURA, H., IWATA, H., ISHIGURO, N. & TAKAHASHI, M. 2002. Characterization of gene expression induced by RET with MEN2A or MEN2B mutation. *Am J Pathol,* 161**,** 249-56.
WEINBAUM, S., TARBELL, J. M. & DAMIANO, E. R. 2007. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng,* 9**,** 121-67.
WELLER, R. O. 1998. Pathology of cerebrospinal fluid and interstitial fluid of the CNS: significance for Alzheimer disease, prion disorders and multiple sclerosis. *J Neuropathol Exp Neurol,* 57**,** 885-94.
WERB, Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology. *Cell,* 91**,** 439-42.
WEYAND, B., NÖHRE, M., SCHMÄLZLIN, E., STOLZ, M., ISRAELOWITZ, M., GILLE, C., VON SCHROEDER, H. P., REIMERS, K. & VOGT, P. M. 2015. Noninvasive Oxygen Monitoring in Three-Dimensional Tissue Cultures Under Static and Dynamic Culture Conditions. *Biores Open Access,* 4**,** 266-77.
WICHA, M. S., LIOTTA, L. A., VONDERHAAR, B. K. & KIDWELL, W. R. 1980. Effects of inhibition of basement membrane collagen deposition on rat mammary gland development. *Dev Biol,* 80**,** 253-6.
WILLE, J. J., AMBROSI, C. M. & YIN, F. C. 2004. Comparison of the effects of cyclic stretching and compression on endothelial cell morphological responses. *J Biomech Eng,* 126**,** 545-51.
WINER, J. P., JANMEY, P. A., MCCORMICK, M. E. & FUNAKI, M. 2009. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A,* 15**,** 147-54.
WIPFF, P. J., RIFKIN, D. B., MEISTER, J. J. & HINZ, B. 2007. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol,* 179**,** 1311-23.
WONG, J. W., GALLANT-BEHM, C., WIEBE, C., MAK, K., HART, D. A., LARJAVA, H. & HAKKINEN, L. 2009. Wound healing in oral mucosa results in reduced scar formation as compared with skin: evidence from the red Duroc pig model and humans. *Wound Repair Regen,* 17**,** 717-29.
WOZNIAK, M. A. & CHEN, C. S. 2009. Mechanotransduction in development: a growing role for contractility. *Nat Rev Mol Cell Biol,* 10**,** 34-43.
WRIGHTON, K. H., LIN, X. & FENG, X. H. 2009. Phospho-control of TGF-beta superfamily signaling. *Cell Res,* 19**,** 8-20.
WU, D. T., BITZER, M., JU, W., MUNDEL, P. & BÖTTINGER, E. P. 2005. TGF-β Concentration Specifies Differential Signaling Profiles of Growth Arrest/Differentiation and Apoptosis in Podocytes. *Journal of the American Society of Nephrology,* 16**,** 3211-3221.
WU, J., NIU, J., LI, X., WANG, X., GUO, Z. & ZHANG, F. 2014. TGF-beta1 induces senescence of bone marrow mesenchymal stem cells via increase of mitochondrial ROS production. *BMC Dev Biol,* 14**,** 21.
WU, M. Y. & HILL, C. S. 2009. Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell,* 16**,** 329-43.

WYNN, T. A. & RAMALINGAM, T. R. 2012. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med,* 18**,** 1028-40.
XIE, Q., CHEN, J., FENG, H., PENG, S., ADAMS, U., BAI, Y., HUANG, L., LI, J., HUANG, J., MENG, S. & YUAN, Z. 2013. YAP/TEAD-mediated transcription controls cellular senescence. *Cancer Res,* 73**,** 3615-24.
XU, L.-C. & SIEDLECKI, C. A. 2007. Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces. *Biomaterials,* 28**,** 3273-3283.
XUE, M. & JACKSON, C. J. 2015. Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring. *Adv Wound Care (New Rochelle),* 4**,** 119-36.
YAMAGUCHI, N., CHIBA, M. & MITANI, H. 2002. The induction of c-fos mRNA expression by mechanical stress in human periodontal ligament cells. *Arch Oral Biol,* 47**,** 465-71.
YAMASHITA, S., MAESHIMA, A., KOJIMA, I. & NOJIMA, Y. 2004. Activin A is a potent activator of renal interstitial fibroblasts. *J Am Soc Nephrol,* 15**,** 91-101.
YAN, C., GRIMM, W. A., GARNER, W. L., QIN, L., TRAVIS, T., TAN, N. & HAN, Y. P. 2010. Epithelial to mesenchymal transition in human skin wound healing is induced by tumor necrosis factor-alpha through bone morphogenic protein-2. *Am J Pathol,* 176**,** 2247-58.
YANG, E. Y. & MOSES, H. L. 1990. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J Cell Biol,* 111**,** 731-41.
YANG, G., TRUONG, L. D., WHEELER, T. M. & THOMPSON, T. C. 1999. Caveolin-1 Expression in Clinically Confined Human Prostate Cancer. *A Novel Prognostic Marker,* 59**,** 5719-5723.
YANG, Y. C., PIEK, E., ZAVADIL, J., LIANG, D., XIE, D., HEYER, J., PAVLIDIS, P., KUCHERLAPATI, R., ROBERTS, A. B. & BOTTINGER, E. P. 2003. Hierarchical model of gene regulation by transforming growth factor beta. *Proc Natl Acad Sci U S A,* 100**,** 10269-74.
YAO, D., EHRLICH, M., HENIS, Y. I. & LEOF, E. B. 2002. Transforming growth factor-beta receptors interact with AP2 by direct binding to beta2 subunit. *Mol Biol Cell,* 13**,** 4001-12.
YAO, Q., CAO, S., LI, C., MENGESHA, A., KONG, B. & WEI, M. 2011. Micro-RNA-21 regulates TGF-beta-induced myofibroblast differentiation by targeting PDCD4 in tumor-stroma interaction. *Int J Cancer,* 128**,** 1783-92.
YIH, L.-H., PECK, K. & LEE, T.-C. 2002. Changes in gene expression profiles of human fibroblasts in response to sodium arsenite treatment. *Carcinogenesis,* 23**,** 867-876.
YING, S. Y. 1987. Inhibins and activins: chemical properties and biological activity. *Proc Soc Exp Biol Med,* 186**,** 253-64.
YU, Y., XIAO, C. H., TAN, L. D., WANG, Q. S., LI, X. Q. & FENG, Y. M. 2014. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. *Br J Cancer,* 110**,** 724-32.
ZEIN, I., HUTMACHER, D. W., TAN, K. C. & TEOH, S. H. 2002. Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials,* 23**,** 1169-1185.
ZENG, Y., LEE, T. S., YU, P., ROY, P. & LOW, H. T. 2006. Mass transport and shear stress in a microchannel bioreactor: numerical simulation and dynamic similarity. *J Biomech Eng,* 128**,** 185-93.
ZHANG, G. Y., YU, Q., CHENG, T., LIAO, T., NIE, C. L., WANG, A. Y., ZHENG, X., XIE, X. G., ALBERS, A. E. & GAO, W. Y. 2011a. Role of caveolin-1 in the pathogenesis of tissue fibrosis by keloid-derived fibroblasts in vitro. *Br J Dermatol,* 164**,** 623-7.
ZHANG, H., LANDMANN, F., ZAHREDDINE, H., RODRIGUEZ, D., KOCH, M. & LABOUESSE, M. 2011b. A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature,* 471**,** 99-103.
ZHANG, W., JIANG, Y., WANG, Q., MA, X., XIAO, Z., ZUO, W., FANG, X. & CHEN, Y. G. 2009. Single-molecule imaging reveals transforming growth factor-beta-induced type II receptor dimerization. *Proc Natl Acad Sci U S A,* 106**,** 15679-83.

ZHANG, Z., VUORI, K., REED, J. C. & RUOSLAHTI, E. 1995. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A,* 92**,** 6161-5.

ZHAO, X. H., LASCHINGER, C., ARORA, P., SZASZI, K., KAPUS, A. & MCCULLOCH, C. A. 2007. Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J Cell Sci,* 120**,** 1801-9.

ZHOU, B., CHEN, W. L., WANG, Y. Y., LIN, Z. Y., ZHANG, D. M., FAN, S. & LI, J. S. 2014. A role for cancer-associated fibroblasts in inducing the epithelial-to-mesenchymal transition in human tongue squamous cell carcinoma. *J Oral Pathol Med*.

Appendix