The Role of microRNAs in Chronic Lung Allograft Dysfunction

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Abstract

Dysregulation of miRNAs has been implicated in obstructive airway diseases including TGF-β1 mediated Bronchiolitis Obliterans Syndrome (BOS), where TGF-β induced epithelial phenotype plasticity or Epithelial-Mesenchymal Transition (EMT) may also contribute to pathophysiology. This study investigated the role of miRNAs in TGF-β1 induced fibrosis and had two main aims: (1) Identification of key miRNAs crucial in TGF-β1 induced EMT and fibrosis, a key clinical feature of BOS and (2) investigating the role of selective miRNAs (miR-200b, miR-200c and miR-146a) in maintaining epithelial cell morphology during EMT using immortalised human bronchial epithelial cells (BEAS-2B cells) and human primary bronchial epithelial cells (PBECs). Initially, NanoString® nCounter miRNA assay was used to profile miRNAs in control versus TGF-β1 stimulated BEAS-2B cells. MiR-200b and miR-200c were downregulated while miR-146a was upregulated post TGF-β1 treatment compared to control BEAS-2B cells. BEAS-2B cells and PBECs were transfected with miR-200b and miR-200c mimics that maintained the expression of epithelial cell markers and downregulated mesenchymal cell markers in the presence of TGF-β1 at RNA and protein level. The same experiment when replicated in PBECs derived from lung allografts yielded similar results. Next, the effect of miR-200b/c mimics was evaluated in TGF-β1 pre-treated cells. MiR-200b and miR-200c mimics reversed established TGF-β1 driven EMT in BEAS-2B cells. Furthermore, miRNA target studies were performed using computational tools, and a luciferase assay validated ZNF532 and ZEB2 as direct targets of miR-200b and miR-200c. Importantly, *in situ* hybridization revealed miR-200b-3p expression in the healthy lung epithelium. Cells transfected with miR-146a did not show any significant changes in EMT marker expression indicating some specificity to the miR-200b/c data. In conclusion, these investigations showed that miR-200b and miR-200c protect airway epithelial cells from EMT. Use of miR-200b/c mimics may therefore represent a novel therapeutic modulator of EMT associated with BOS.

Dedication

I dedicate this thesis to my parents, Cleo, brother and grandparents

“There are two ways to live a pleasant life, either in someone’s heart or in someone’s prayer.”

-Imam Ali (AS)

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"I think you have to be patient, careful, analytical, thoughtful, prudent, and build step-by-step. I don't think it can be done like mixing a glass of Nescafé."

-**Aga Khan IV**

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List of Abbreviations

PGD	Primary graft dysfunction
BOS	Bronchiolitis obliterans syndrome
AM	Alveolar macrophages
NK	Natural killer cells
DC	Dendritic cells
APC	Antigen presenting cells
PRRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecular patterns
DAMPs	Damage-associated molecular patterns
TLRs	Toll like receptors
MyD88	Myeloid differentiation primary response gene 88
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
TCR	T-cell receptor
FEV	Forced expiratory volume
BAL	Bronchoalveolar lavage
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
COPD	Chronic obstructive pulmonary disease
AMR	Antibody mediated rejection
IL	Interleukin
CTLs	Cytotoxic T lymphocytes
IFNγ	Interferon gamma
TGF	Transforming growth factor
TβR	Transforming growth factor beta receptor
PDGF	Platelet-derived growth factor
ECM	Extracellular matrix
EMT	Epithelial to Mesenchymal transition
ZO-1	Zonula occludens-1
FSP1/S100A4	Fibroblast-specific protein-1/S100 calcium-binding protein A4
MMPs	Matrix metalloproteinases
α-SMA	Alpha smooth muscle actin

ZEB	Zinc finger E-box-binding homeobox
AT2	Type 2 alveolar cells
A549	Adenocarcinoma human alveolar basal epithelial cells
BEAS-2B	Immortalised human bronchial epithelial cells
PBECs	Human primary bronchial epithelial cells
BMPs	Bone morphogenetic proteins
GDFs	Growth differentiation factors
LAP	latency-associated peptide
LTBP	latent TGF-beta binding proteins
MAPK	Mitogen-activated protein kinase
miRNA	microRNA
pri-miRNA	primary microRNA
pre-miRNA	precursor microRNA
dsRBD	Double-stranded RNA-binding domain
DGCR8	Digeorge syndrome critical region gene 8
Ago	Argonaute
GERD	Gastroesophageal reflux disease
MCP1	Monocyte chemotactic protein 1
CDS	Coding sequences
RLE-6TN	Rat alveolar type II epithelial cells
IMR-90	Human fetal lung fibroblasts
EV	Extracellular vesicle
DSA	Donor mismatched HLA
ACR	Acute cellular rejection
SNPs	Single nucleotide polymorphisms
DMSO	Dimethyl sulfoxide
SDS	Sodium Dodecyl Sulphate
HRP	Horseradish peroxidase
BCA	Bicinchoninic Acid
PVDF	Polyvinylidene fluoride
PAGE	Polyacrylamide gel electrophoresis
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

TAMRA	Tetramethylrhodamine
ISH	*In situ* hybridization
DIG	Digoxigenin
BCIP	5-bromo-4-chloro-3'- indolylphosphate
HPRT1	Hypoxanthine Phosphoribosyl transferase 1
Luc2	Luciferase gene
PGK	Phosphoglycerate kinase
MCS	Multiple cloning site
Amp^r	Ampicillin resistance
ZNF532	Zinc Finger Protein 532
LB	Luria broth

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Chapter 1: Introduction

1.1 Lung transplantation history

Early lung transplant attempts were made in animals in the 1940s and 1950s by a Soviet researcher (Cooper, 1969). However, the first human lung transplant was performed in 1963 at the University of Mississippi for a patient suffering from an advanced form of lung carcinoma. However, the patient died on day 18 post-surgery (Hardy, 1999). During the following 20 years, only one out of the total 40 lung transplant patients survived the surgery but died soon after due to sepsis (Detterbeck *et al.*, 1995; de Perrot *et al.*, 2004). Since the first successful isolated lung transplant procedure in 1983 performed by Patterson and colleagues at the Toronto general hospital (Toronto Lung Transplant, 1986; Patterson *et al.*, 1988), the International Society for Heart and Lung Transplantation (ISHLT) has reported data from 51,440 adult lung transplants that occurred until June 2014 (Yusen *et al.*, 2015).

1.2 Lung allograft dysfunction

1.2.1 Primary graft dysfunction

Primary graft dysfunction (PGD) is a type of acute lung injury that begins within the first 72 hours post transplantation, developing from several pathological mechanisms integral to the process of transplantation. With every transplant, there is an initial insult to the allograft due to lack of natural blood supply post organ retrieval followed by ischemia during organ preservation stage and then subsequent reperfusion. All these factors contribute to the development of PGD (also known as ischemia reperfusion injury or primary graft failure)(Lee and Christie, 2009). The clinical hallmarks of PGD are varying degrees of impaired oxygenation and alveolar and interstitial edema (Christie *et al.*, 2005a). In its more severe form there is fibrotic

tissue deposition along the alveoli that resembles pathology seen in adult respiratory distress syndrome (ARDS) or other forms of acute lung injury (Christie *et al.*, 1998; Christie *et al.*, 2005b). There is now evidence suggesting that the severe form of PGD is associated with decline in long term allograft survival and is the leading cause of morbidity and mortality rate (first 30 days post transplantation) of 35-60% (Fiser *et al.*, 2001).

The process of ischaemia-reperfusion injury is pivotal in the pathogenesis of PGD. This is driven by the tissue ischaemia and generation of reactive oxygen species during the reperfusion process. These oxygen species subsequently cause oxidative stress at the cellular level directly causing damage to the epithelium and endothelium of the grafted lung (Ng *et al.*, 2006). This triggers a pro-inflammatory cascade with increased cytokine, chemokine and adhesion molecule expression, that leads to the recruitment of macrophages, recipient lymphocytes, and neutrophils to the sites of injury, further propagating the process of lung injury (Moreno *et al.*, 2007). Ultimately, there is activation of downstream signalling such as lipid peroxidation driven by neutrophils, platelets and complement cascade activation. Histologically, this process causes damage to the alveoli, capillary leak, neutrophil infiltration and formation of hyaline membranes along the alveolar spaces (Matthay and Zemans, 2011).

1.2.2 Acute cellular rejection

The incidence of lung allograft rejection is the highest amongst the commonly transplanted solid organs and is associated with poor long term outcomes despite modern immunosuppressive regimes (Yusen *et al.*, 2015). As many as 55% of lung transplant recipients receive treatment for acute allograft rejection in their first year post transplantation and only 50% of recipients survive 5 years after transplant. Furthermore, recipients that have at least one episode of acute rejection that is

thought to be one of the reasons for subsequent predisposition to bronchiolitis obliterans syndrome (BOS). The high susceptibility of the lung to infection and persistent environmental exposure with innate immunity activation contributes to the high rates of early rejection (Martinu *et al.*, 2009).

1.2.2.1 Innate immune responses

The innate immune system is a highly conserved mechanism of host defence that precedes adaptive immunity. This system provides an immediate response, distinguishing self from nonself by using germline-encoded receptors to recognize patterns distinct to pathogens or injured tissues (Medzhitov and Janeway Jr, 2000a). Innate pathways are of significance in the lung as the extensive alveolar surface area is continuously exposed to a wide array of airborne particles and invading microbes during normal respiration. The innate response resolves the infection or airborne challenge, without causing damage to the delicate alveolar structures necessary for gas exchange (Zhang *et al.*, 2000). The anatomy of the upper and lower airways represents the initial barrier to foreign bodies expelling particles larger than 5 μm by cough reflex. Smaller particles, including bacterial, viral and mycobacterial components gain access to the terminal airways and alveolar spaces. Here they encounter a variety of soluble proteins such as defensins, surfactants, lysozyme, lactoferrin, fibronectin and complements are crucial in maintaining a sterile microenvironment (Ganz, 2003; Wright, 2005; Zaas and Schwartz, 2005; Dunkelberger and Song, 2010). These components present in the fluid of the epithelial lining exert direct microbicidal effects and facilitate phagocytosis thus playing an important role in regulating local inflammation (Zaas and Schwartz, 2005).

Cells important to the innate response include airway and alveolar epithelial cells, resident alveolar macrophages (AMs), natural killer (NK) cells, dendritic cells (DCs),

and neutrophils. AMs phagocytose and eradicate inhaled particles on an ongoing basis and account for the majority of leukocytes in a normal healthy lung. A large particulate or exceptionally virulent pathogen may elicit an immune response wherein the AMs produce proinflammatory cytokines and chemokines to initiate recruitment of neutrophils, DCs, and monocyte-derived macrophages that generates a local inflammatory microenvironment. AMs also act as antigen presenting cells (APCs), transporting foreign antigens to regional lymph nodes, where they are taken up by DCs and presented to naïve lymphocytes, thus invoking T-cell proliferation and promoting adaptive immune responses (Martin and Frevert, 2005). Interestingly, lung itself can act as a tertiary lymphoid organ where local antigen presentation and cell maturation can occur in the absence of extra pulmonary lymphoid tissue, a novel discovery relevant to lung transplantation (Gelman *et al.*, 2009).

1.2.2.1.1 Receptors and ligands of the innate immune system

Commencement of the innate immune response depends on pattern recognition receptors (PRRs) that recognize highly conserved molecular patterns on microorganisms (pathogen-associated molecular patterns [PAMPs]). In addition to recognition of foreign molecular patterns, PRRs can elicit a response to injured self-tissue via recognition of damage-associated molecular patterns (DAMPs). PRRs serve a variety of functions depending on the cell type and location of expression (in bloodstream or intracellularly or on the cell surface) (Trinchieri and Sher, 2007).

The Toll-like receptors (TLRs) are the most extensively studied PRRs of relevance to transplant rejection. TLRs are expressed by a variety of cells significant to pulmonary innate immunity, including AMs, DCs, neutrophils, and epithelial cells of the alveoli and conducting airways. There are 11 well-described TLRs (numbered TLR1 through TLR11); TLR 1, 2, 4, 5 and 6 are located on cell surface while TLR 3, 7, 8, 9, 11 are

intracellularly located (Akira *et al.*, 2001; Kawai and Akira, 2007). Most TLRs associate with co-receptors in a tissue-specific manner in order to detect microbial antigens while some do not require a co-receptor. Myeloid differentiation factor 88 (MyD88) dependent pathway utilizes MyD88, an adaptor protein that is shared by all TLRs except for TLR3. It activates transcription factor NFκ (kappa) β thus promoting DC maturation and proinflammatory cytokine production that in turn directs a Th1 immune response. MyD88-independent signalling pathway operates via another adaptor protein, TRIF that initiates a complex intracellular kinase cascade. Hence, TLR signalling helps in distinguishing healthy self from injured self and microbial nonself and directs downstream adaptive immune reactivity (Medzhitov and Janeway Jr, 2000b; Imler and Hoffmann, 2001).

1.2.2.2 Adaptive immune response

The alloimmune response post-allograft injury is predominantly driven by T cell recognition of foreign major histocompatibility complexes (MHC). The MHC, also referred to as the Human Leukocyte Antigen (HLA) represents a protein complex encoded by a set of very closely linked genes (class and class II) that elicit an immune response by presenting antigenic peptides to T cells. Each MHC molecule is composed of an extracellular peptide-binding cleft, a transmembrane domain, and a cytoplasmic domain. The peptide-binding cleft allows binding to variety of antigenic peptides. MHC class I molecules are expressed on most nucleated cells while class II molecules are expressed constitutively on APCs and can be upregulated under inflammatory conditions. In humans, MHC molecules also referred to as HLA genes are located on the short arm of chromosome 6 and are divided into two classes based on historic distinction. The HLA class I and class II genes include A, B, and C loci and DR, DQ, and DP genes respectively (Abbas *et al.*, 2014).

T and B lymphocytes that constitute the adaptive system have the capacity to recognize large number of peptides, generate a memory response and rapidly produce clones that can carry out the immune response. T cells recognize specific antigenic determinants via the T cell receptor (TCR). T cells also express several signal transduction molecules that participate in antigen responses, such as $CD3^+$, $CD4^+$ (Th- T helper cell) or $CD8^+$ (Tc- cytotoxic T cell), $CD28^+$ and CD40L (costimulatory molecules) and adhesion molecules. $CD4^+$ Th cells help other cells by producing cytokines for $CD8^+$ Tc cell stimulation and activation of other inflammatory cells. $CD8^+$ Tc cells have the ability to kill target cells by direct delivery of cytotoxic granules containing enzymes such as granzyme B, that induce apoptosis (Csencsits and Bishop, 2003).

There are two pathways of alloantigen recognition: direct and indirect (Hornick and Lechler, 1997). Direct allorecognition involves recognition of donor antigen-presenting cells (APCs) displaying MHC class II antigens (and peptides) on their surface by recipient T cells. A large proportion of circulating T cells is able to recognise wide range of allo-MHC molecules directly, and this accounts for the dynamic nature of acute rejection in the early post-transplant period when numerous donor APCs are present. During indirect allorecognition recipient APCs engulf and process donor alloantigens and present donor-derived processed peptides to recipient T cells via self-MHC: donor peptide complexes. In this case, the number of T cells with appropriate TCRs is much smaller and this response is thought to develop later and remain active throughout life of the allograft due to the infiltration of recipient APC in the allograft or the availability of donor antigens in the lymphoid tissue (Snyder and Palmer, 2006). Although the indirect pathway is thought to contribute to the development of chronic rejection, direct recognition of MHC by T-

cells can lead to chronic rejection after the depletion of APCs (Snyder and Palmer, 2006). Class I MHC molecules expressed mainly on hematopoietic cells present antigens to $CD8^+$ T cells. There is evidence that $CD8^+$ T cells: class I MHC direct alloreactivity to the graft contributes to obliterative airway disease of transplanted tracheal allografts. In a rat model, class II MHC was upregulated on the lung epithelium and endothelium post transplantation. The expression of class II molecules on non-hematopoietic cells in an allograft may provide a mechanism of direct allorecognition for $CD4^+$ T cells (Vigneswaran and Garrity, 2010).

In lung transplantation, acute rejection occurs frequently within the first year post transplant and is characterized by infiltration of $CD4^+$ and $CD8^+$ T cells and mononuclear cells in the perivascular and peribronchiolar regions of the graft. Early rejection is augmented by local innate immune activation through tissue injury, infection and an autoimmune response to cryptic self-epitopes exposed during lung injury (Vigneswaran and Garrity, 2010). In addition, some lung transplant recipients also engage a humoral response to the allograft that occurs via indirect allorecognition. This process provides help for B cell memory, antibody class switching, and affinity maturation in the presence of appropriate cytokines and co-stimulatory factors. Consequently, successful lung transplants became possible after the introduction of a T cell activation and proliferation blocker, the calcineurin inhibitor cyclosporine that prevents episodes of acute rejection (Longoria *et al.*, 1999; Colvin and Smith, 2005). Other immunosuppressive therapies include use of antiproliferative agents including azathioprine, mycophenolate, sirolimus, everolimus, and corticosteroids. About 50% of lung transplant centres also utilize induction therapy with polyclonal antibody preparations (rabbit anti-thymocyte globulin) or IL-2 receptor antagonists such as daclizumab or basiliximab (Bhorade and Stern, 2009).

1.2.3 Chronic rejection

Chronic rejection is the leading cause of morbidity and mortality thus limiting the long-term success of lung transplantation (Verleden, 2001). About 50 % of lung transplant recipients are affected by this condition by 5 years after transplantation and the patient survival rate is 53% out of the total diagnosed (Boehler *et al.*, 1998). Chronic lung transplant rejection was first described as obliterative bronchiolitis at Stanford in 1984. Fourteen patients out of the 19 heart–lung transplants performed for end-stage pulmonary vascular disease were long-term survivors. Five of these patients developed a progressive obstructive ventilatory defect with a decline in the forced expiratory volume in 1 second (FEV1).

Chronic airway rejection affects the airways wherein there is deposition of granular tissue and dense connective tissue between the epithelium and the elastic lumen. It also involves infiltration of chronic inflammatory cells leading to development of obliterated lumen (Stehlik *et al.*, 2012). Lately the term “chronic lung allograft dysfunction” (CLAD) has been introduced to include specific forms of allograft dysfunction. CLAD is a descriptive term for chronic lung transplant rejection which includes several phenotypes such as BOS, restrictive allograft syndrome, recurrence of primary disease, azithromycin-responsive allograft dysfunction and other specific causes of decline in lung function (Snell *et al.*, 2013).

1.3 Development of BOS

1.3.1 Overview and classification

Lung transplantation has proved to be successful in carefully selected individuals suffering from a variety of end stage lung diseases (Arcasoy and Kotloff, 1999). Despite the advances in immunosuppression, surgical techniques and management of infections, acute lung allograft rejection and development of the BOS is still a problem (Christie *et al.*, 2005a). Progression of BOS is thought to indicate chronic rejection and allograft injury and is characterized by irreversible airway obstruction due to progressive fibroblast proliferation and ECM deposition in the small airways. Ultimately, this leads to loss of lung function and has been defined as being irreversible (Boehler *et al.*, 1998; Belperio *et al.*, 2009; Borthwick *et al.*, 2010b; Hayes, 2011). Because BOS represents an important problem for all lung transplant centres, early identification and prediction of progressive loss of lung function is a common and important goal (Sohal *et al.*, 2013b; Suwara *et al.*, 2014).

The development and severity of BOS has been characterised by the International Society of Heart and Lung Transplantation (ISHLT) that describes the various stages of BOS with increasing loss of lung function (Hayes, 2011) (Table 1.1)**.** It is postulated that initial acute rejection seeds the development of progressive graft deterioration and chronic rejection. This is characterized by decline in lung function that is termed as the BOS. Thus the development of BOS is indicative of chronic graft injury and is clinically diagnosed by determining the forced expiratory volume (FEV) of the patient measured at least 3 weeks apart post lung transplant (Jackson *et al.*, 2002).

Stages	2003 Classification
BOS 0	FEV_1 > 90% of baseline and FEV_{25-75} >75% of Baseline
BOS 0p	FEV_1 81 - 90% of baseline and/or FEV_{25-75} ≤ 75% of Baseline
BOS 1	FEV_1 66 - 80% of baseline
BOS 2	FEV_1 51 - 65% of baseline
BOS 3	FEV_1 ≤ 50% of baseline

Table 1.1: The 5 stages of BOS. The baseline FEV_1 and FEV_{25-75} were recorded as the average of two highest FEV without the use of bronchodilator 3 weeks post transplant. The progressive stages of BOS correlate to declining airflow obstruction. BOS 0p (potential BOS) gives an indication of early decline of lung function (Kesten *et al.*, 1995).

Baseline values for FEV_1 and forced expiratory flow at 25–75% of forced vital capacity (FEF 25–75%) are defined as the average of the two highest values for each measurement that are obtained at least 3 weeks apart post-transplant without the use of a bronchodilator. In order for the diagnosis of BOS to be made, three or more months are required to have elapsed from the time of transplantation so as to distinguish BOS from acute and other complications of lung transplantation; also taking into account the time required to establish a baseline FEV_1 and a decline in FEV_1 (Cooper *et al.*, 1992). As the cut-off value for FEV_1 at 80% may be sensitive to early decline in lung function due to early disease, BOS grade 0-p was added to the classification system that may suggest 'potential BOS' (Estenne *et al.*, 2002) .

The identification of patient groups may allow the recognition of specific risk factors and/or strategies for treatment and prevention that pertain to a subset of patients with BOS. Patients exhibiting early decline in FEV_1 (according to BOS criteria) may represent a BOS phenotype which is characterised by rapid progression and poor prognosis (Jackson *et al.*, 2002; Burton *et al.*, 2007). Another potential BOS phenotype consists of recipients with significant bronchoalveolar lavage (BAL) neutrophilia with responsiveness to azithromycin therapy. A study showed an increase in BOS-like syndromes with BAL neutrophilia, however prophylactic

administration of azithromycin shortly after transplantation suppressed the development of this syndrome and the recipients no longer met the spirometric criteria for BOS. Patients who meet BOS criteria but fail to respond to azithromycin may represent a phenotype with fibro-obliterative BOS (Gottlieb *et al.*, 2008; Vos *et al.*, 2010).

The development of the pathology thought to underly BOS involves a series of events in which various insults (alloimmune dependent and independent) can lead to a similar histological result (Figure 1.1).

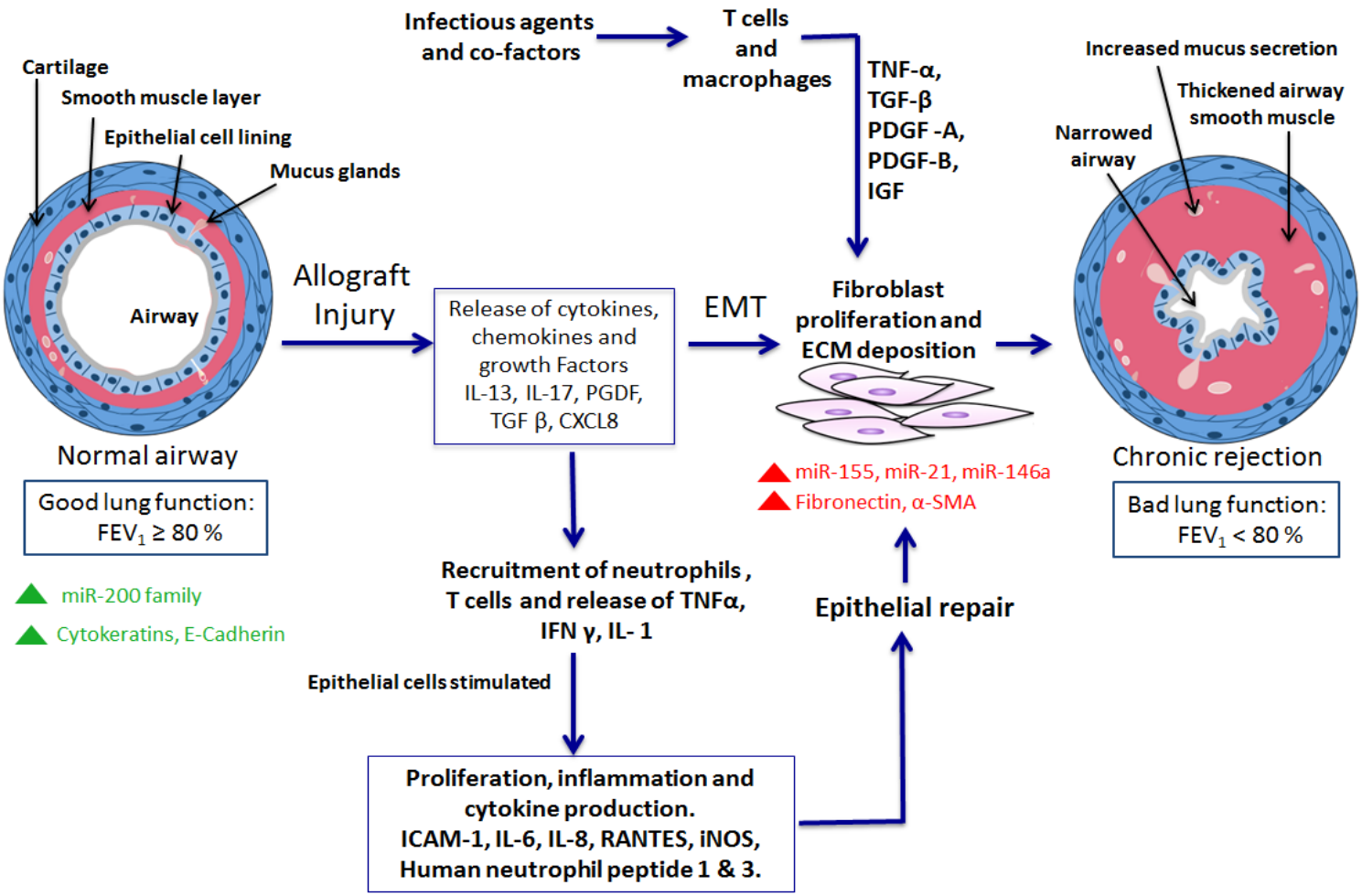

Figure 1.1: Pathogenesis of BOS- Allogeneic injury leads to activation of cytokines, chemokines and growth factors that cause fibroblast proliferation, epithelial mesenchymal transition and finally chronic rejection with the possible involvement of a number of microRNAs (Ladak *et al.*, 2016).

Activation of the innate immune system, via Toll-like receptors, leads to the release of cytokines that are able to activate APCs leading to antigen presentation to T-lymphocytes. This process leads to an increased alloantigen expression by the graft and thus triggers the adaptive immunity. The chemoattractants facilitate recruitment of mononuclear phagocytes and T cells that maintain the levels of cytokine and chemokine at the site of injury. This leads to inflammation and epithelial damage and causes release of profibrotic chemokines such as TGF-β and platelet derived growth factor (PDGF) that promote epithelial repair and vascular remodelling (Neuringer *et al.*, 2008; Song *et al.*, 2008).

1.3.2 Risk factors of BOS

There is evidence that recipients who develop BOS have greater degrees of HLA mismatch (Schulman *et al.*, 1998) and involvement of autoimmune pathways (Burlingham *et al.*, 2007). Additionally, many non-alloimmune mechanisms have also been implicated in BOS pathogenesis such as airway injury due to primary PGD, gastro-oesophageal reflux disease (GERD), various infections, and airway ischaemia due to disruption of the bronchial circulation. These alloimmune-independent factors may promote tissue damage and inflammation that in turn initiate and intensify an alloimmune recipient response (Verleden *et al.*, 2009; Weigt *et al.*, 2010).

1.3.2.1 Alloimmune dependent

In addition to acute rejection there are other alloimmune risk factors associated with the development of BOS in lung transplant recipients. Lymphocytic bronchiolitis, a precursor of BOS, is characterised by submucosal lymphocytic and plasma cell infiltration around the airways and to the smooth muscle layer. Patients who went on to develop BOS in the first year post-transplantation had over twice as many

episodes of LB compared to those without BOS. Thus presence of LB, and especially the severity has been associated with the development of BOS (Yousem, 1993; Husain *et al.*, 1999). In a study involving 221 lung transplant recipients with end stage chronic obstructive pulmonary disease (COPD), single transplant recipients were at higher risk of developing BOS as compared to bilateral transplant recipients (Hadjiliadis *et al.*, 2006).

The nature of development of antibody mediated rejection (AMR) after lung transplantation can manifest itself as BOS. AMR is driven by humoral response/ B-cell immune response as opposed to the classical T-cell mediated rejection or cellular rejection. The presence of HLA incompatibility, due to mismatches between donor and recipient poses a risk factor for BOS. Anti-HLA antibodies have been associated with a worse outcome in all solid-organ transplants. These antibodies pre-existing to the transplant procedure may expose the patient to the risk of acute rejection (Hodge *et al.*, 2009). Studies have shown that development of anti-HLA antibodies post-transplantation is associated with the development of BOS (Song *et al.*, 2008). Binding of these antibodies to the airway epithelium may induce injury and proliferation of the airway epithelial cells.

The development of cell-mediated and humoral response to self-antigens in the lung allograft may play an important role in the inflammation and fibrosis that leads to progressive graft dysfunction. In addition to the alloimmune response to donor HLA, there is increased risk of BOS observed due to non-HLA antibodies such as collagen V and Kα1 tubulin (Goers *et al.*, 2008; Fukami *et al.*, 2009). These epitopes are exposed as a result of ischemia and reperfusion injury or other insults that may damage the respiratory epithelium (Sumpter and Wilkes, 2004) and have been found

in up to 31% of lung transplant recipients with BOS. This type of autoimmune activation seems to be mediated by a Th17 response (Burlingham *et al.*, 2007).

1.3.2.2 Alloimmune independent

Multiple studies have shown that endogenous DAMPs released from ischemic or dying tissues can activate innate pathways via TLR2, TLR4, and the innate PRRs. This response may potentiate alloimmune reactivity and account for the increased risk for BOS in lung allograft recipients with a history of PGD. Interestingly, GERD may directly activate TLRs to provoke downstream inflammation and adaptive processes. For example, aspiration of gastric juice has been shown to cause severe acute lung rejection and increase in innate cytokine levels in rat models (Hartwig *et al.*, 2006). Apart from tissue injury and DAMP release, lung-transplant recipients are also exposed to microbial PAMPs that hold the potential to directly activate TLRs (Hartwig *et al.*, 2006; Appel *et al.*, 2007; Fishman, 2007; Wu *et al.*, 2007).

1.3.2.2.1 Respiratory tract infections

Lower respiratory tract infections have been reported to increase the risk for BOS, including rhinovirus, coronavirus, respiratory syncytial virus, influenza A, parainfluenza, human meta-pneumovirus, and human herpes virus-6 (Kumar *et al.*, 2005; Neurohr *et al.*, 2005). Studies suggest that community-acquired respiratory viral infections increase the risk for BOS (Billings *et al.*, 2002). Infection with Sendai virus, a murine parainfluenza type I-like virus, has shown to increase tracheal fibro-obliteration and alloreactive T cells (Kuo *et al.*, 2006). CMV infection has been well documented in the post-transplant period in patients with both reactivated and donor-derived CMV infection. Smith *et al.* found that CMV mismatch (donor positive/recipient negative) may be a risk factor for developing BOS within 3 years of

transplantation (Khalifah *et al.*, 2004; Weigt *et al.*, 2008; Snyder *et al.*, 2010). The treatment of CMV and the subsequent prevention of BOS remains unclear (Keenan *et al.*, 1991; Glanville *et al.*, 2005).

There is inconsistent data available on literature evaluating role for bacterial infection and colonization in BOS. Positive serostatus for *Chlamydia pneumoniae* in donors and recipients is associated with the development of BOS (Kotsimbos *et al.*, 2005). An interesting study by Weigt and colleagues implicated *Aspergillus* colonization in the lung allograft as a novel BOS risk factor that independently predicted BOS-related morbidity and mortality regardless of acute rejection problem. As *Aspergillus* is recognized through TLR2 and TLR4, this shows that relationship between lung infection and BOS may not be limited to specific pathogens, but rather a broad spectrum of pathogen-associated motifs subsequently initiating an alloimmune common response to injury (Garantziotis and Palmer, 2009; Weigt *et al.*, 2009).

1.3.2.2.2 GERD

GERD is very common post-lung transplant. However, the mechanism by which GERD contributes to BOS remains unclear. The presence of bile acids and pepsin in BAL fluid of lung transplant recipients suggests that aspiration may stimulate airway injury (D'Ovidio *et al.*, 2005). Treatment with proton pump inhibitors has shown to reduce acid reflux but did not affect non-acid reflux, including bile or pepsin. The benefit of surgical correction of GERD is an ongoing area of investigation. In a study, fundoplication has been shown to contribute to increased graft survival together with an improvement of the pulmonary function (Davis *et al.*, 2003). It has also been demonstrated that GERD is associated with an increased number of acute rejection episodes and severity of initial acute rejection (Blondeau *et al.*, 2008). Fundoplication at an early stage decreased the number of late rejections. These studies indicate that

alloimmune-independent mechanisms (in this case GERD) may indeed lead to BOS, and might be to some extent treatable (Hartwig *et al.*, 2004).

In summary, the important findings indicate that BOS develops as a consequence of complex interactions where TLR activation and signalling play a critical role in modulating allograft rejection.

1.3.3 Cytokines and chemokines implicated in BOS

Fibro-obliteration is characterised by the combined action of type 1, type 2 and type 17-alloimmune responses. Acute lung allograft rejection is indicative of Type 1 immune response that initiates cell-mediated immunity. This induces production of IL-2 , IFN- γ and further activates cytotoxic T lymphocytes (CTLs) that mark the onset of inflammation (Toews, 2001). Studies have shown a correlation between high levels of IFN- γ in BAL samples and accelerated acute rejection. It has also been shown that low levels of IL-12 in BAL samples post lung transplant correlate to development of BOS (Deslee *et al.*, 2007; sabel Neuringer*, 2008).

Chronic lung rejection is marked by the activation of humoral and Type 17-alloimmune responses that lead to up regulation of fibro proliferative cytokines IL-6, IL-17, IL-8 and IL-23 (Mangi *et al.*, 2011). Studies have shown that IL-8 (CXCL8), a chemo attractant for recruiting neutrophils at the sight of injury and growth factors such as TGF-β and PDGF contribute to epithelial repair. IL-8 is produced by epithelial cells, endothelial cells, smooth muscle cells and macrophages in response to IL-17. The production of IL-17 is associated with autoimmunity in human diseases and it was also found to be linked with the incidence of BOS post lung transplantation (Wong *et al.*, 2000). Thus the differential expression of these cytokines in lung transplant correlates to the development of BOS (Holbro *et al.*, 2013).

Certain Chemokines are thought to have a critical role in pathogenesis of BOS such as Monocyte chemotactic protein 1 (MCP1). The chronic inflammatory process is persistent during the expression of MCP1, which binds to CCR2. Recent studies have elucidated the potential role of MCP1 in mediating the infiltration of mononuclear phagocytes in the lung allograft ultimately leading to rejection. Experimental studies in mice also suggest the role of CXCL10 in promoting epithelial hyperplasia and inflammatory response that eventually leads to fibro proliferation (Martin, 1999; Deslee *et al.*, 2007).

1.3.4 Role of dysregulated repair in BOS

The risk factors for BOS also contribute to epithelial injury and long term airway obstruction. The integrity of the airway epithelial lining crucial in providing a barrier against microorganisms and other toxic molecules may be disturbed post onset of an infectious or inflammatory-related injury. This may cause the epithelium to attempt to repair itself (Persson *et al.*, 1995; Erjefält and Persson, 1997). In a normal airway epithelium the ciliated cells are terminally differentiated and do not possess the ability to divide further. However, in response to injury they may transdifferentiate into squamous cells in order to maintain the integrity of the epithelium (Wong *et al.*, 2009). The de-differentiated cells then migrate into the wound site while releasing proinflammatory cytokines and growth factors required to restore the ECM (Rock *et al.*, 2010). Once the wound-site is concealed, reepithelialisation can occur by recruitment of progenitor cells. These cells then proliferate and undergo phenotypic differentiation in order to establish integrity and stability of the epithelial layer (Qu *et al.*, 2005).

The normal repair response is similar for different types of injury, but the dysregulated repair response to persistent injury may lead to fibrosis (Horowitz and

Thannickal, 2006), bronchiectasis and airway remodelling (Wilson and Wynn, 2009). A study showed that epithelial cells derived from asthmatic patients when grown in culture and mechanically wounded were unable to close the wound completely suggesting the poor reparative capacity of the airway epithelium (Stevens *et al.*, 2008; Hackett *et al.*, 2009). Exposure to cigarette smoke leads to a repetitive cycles of epithelial injury and abnormal repair leading to COPD (Churg *et al.*, 2008). Thus, it is possible that persistent injury both alloimmune and non-alloimmune post transplantation could lead to development of BOS.

During normal repair there is some degree of epithelial-to-mesenchymal transition (EMT) where epithelial cells at the periphery of the wound de-differentiate, migrate and transiently behave in a manner similar to mesenchymal cells in order to facilitate wound closure (Roberts *et al.*, 2006). Fibroblasts are also important during the wound closure. Activated resident fibroblasts contribute by secreting cytokines that facilitate epithelial airway repair and formation of the ECM (Sacco *et al.*, 2004). In addition, there is infiltration of circulating fibroblasts that may lead to uncontrolled fibrosis. Previous studies have reported the presence of fibroblasts in the BOS lesion, but the source of the fibrotic tissue has not been confirmed. Studies have suggested that fibrosis may be caused by circulating recipient derived fibrocytes, which have been (Andersson-Sjöland *et al.*, 2009) detected in fibrotic foci of BOS lesions (Brocker *et al.*, 2006). Cells isolated from these fibrotic foci exhibit epithelial and mesenchymal characteristics (Ward *et al.*, 2005). Studies have well documented that EMT is one of the pathway leading to fibrosis as a result of injury and dysregulated repair (Iwano *et al.*, 2002).

1.3.5 Treatment for BOS

Prevention and treatment of BOS in order to improve long-term graft survival after transplant has been challenging for physicians. Treatment approaches have targeted the prevention or slowing the onset of BOS (Dudek *et al.*, 2003). However, once BOS is established, it leads to irreversible airway obstruction where re-transplantation is usually required. Administration of macrolides such as azithromycin has shown some success, however most treatments have largely not been successful in lowering the occurrence of BOS (Estenne and Hertz, 2002). Current immunosuppressive treatments include a calcineurin inhibitor, a purine synthesis inhibitor, and corticosteroids. At present, not much is known as to which calcineurin inhibitor at the time of transplantation might reduce the probability of developing BOS. However, several studies have confirmed that substituting tacrolimus for cyclosporine A in patients with acute rejection could possibly help prevent the subsequent development of BOS (Webster *et al.*, 2005).

Treatment after the onset of BOS consists primarily of increasing immunosuppression by changing medications or by employing nonmedicinal immune-modulating therapies; these include polyclonal and monoclonal anti-lymphocyte antibody preparations (Kesten *et al.*, 1996), methotrexate (Dusmet *et al.*, 1996), cyclophosphamide (Verleden *et al.*, 1999) and total lymphoid irradiation. The use of immunosuppressive regime has been effective in treating acute rejection. However, the effectiveness is limited since there is reactivation of disease once the patient is off immunosuppressants. Furthermore, prolonged use of this kind of treatment has adverse effects such as invasive infections and malignancy (Agraharkar *et al.*, 2004).

1.5 Epithelial to mesenchymal transition

EMT is characterised by loss of cell-to-cell contact that is a characteristic feature of epithelial cells, cytoskeleton remodelling which ultimately generates cells with fibroblast like morphology that express mesenchymal markers (Ward *et al.*, 2005). The completion of EMT is characterized by degradation of underlying basement membrane and the subsequent generation of mesenchymal cells, that can migrate away from the region of origin. Various molecular processes are involved in order to initiate EMT. These include activation of transcription factors, expression of specific cell-surface markers, rearrangement of cytoskeletal proteins, production of ECM enzymes and changes in the expression of specific microRNAs (Kalluri and Neilson, 2003). In several studies, these factors are also used as biomarkers to determine the transition of a cell through EMT (Hay, 2005).

TGF-β1 is one of the major inducers of EMT during fibrosis that promotes ECM production and deposition and thereby induces a change in the cell morphology. It has been described as a potent inducer of EMT in epithelial cells in renal proximal cells, alveolar epithelial cells and other cell types (Radisky, 2005; Kalluri and Weinberg, 2009).

1.5.1 Types of EMT

Three distinct biological settings of EMTs exist, each of which carry very different functional roles (Kalluri and Weinberg, 2009).

1.5.1.1 Type 1 EMT: EMT during early development

Type 1 EMT drives important aspects of development from implantation to organ development (Vićovac and Aplin, 1996). The early sign of gastrulation is generation of a primitive streak in the epiblast layer that leads to the formation of the three germ

layers that further form all tissue types of the body (Hay, 1990). The epithelial cells in the epiblast undergo changes driven by expression of specific proteins related to cell migration and differentiation (Thiery and Sleeman, 2006). Once formed, the primitive streak generates the meso-endoderm, which separates to form the mesoderm and the endoderm via epiblast-mesoderm transition (Hay, 1995). The embryonic mesoderm eventually gives rise to primary mesenchyme where the cells exhibit enhanced migratory characteristics (Hay, 2005).

Wnt3 deficient embryos cannot undergo the EMT, this suggests that EMT associated with gastrulation is dependent on Wnt signalling (Liu *et al.*, 1999). The formation of the primitive streak is associated with expression of Wnt8c (Skromne and Stern, 2001). Studies have shown that expression of Wnt8c in embryos leads to generation of multiple primitive streaks. Nodal and Vg1 that belong to the TGF-β superfamily mediate the action of Wnts, and their deficiencies can lead to a defect in mesoderm formation due to absence of EMT (Collignon *et al.*, 1996; Skromne and Stern, 2002). Therefore, EMT is crucial in embryogenesis and organ development (Thiery, 2002).

1.5.1.2 Type 2 EMT: EMT involved in fibrosis

During organ fibrosis, epithelial cell passes through various stages before acquiring mesenchymal properties. This phenotypic conversion requires the molecular reprogramming of epithelium wherein the epithelium in transition loses polarity, tight junctions and adherens junction in order to rearrange their actin stress fibres (Strutz *et al.*, 1995; Okada *et al.*, 1997).

1.5.1.2.1 Loss of epithelial cell markers

As EMT progresses the cells leave the epithelial layer, enter the interstitium of tissue through the underlying basement membrane where they ultimately acquire fibrotic

phenotype and shed epithelial characteristics (Okada *et al.*, 1996). One of the first signature markers lost is E-cadherin that is thought to initiate and promote the EMT pathway. Other markers such as cytokeratins and Zona Occludens-1 (ZO-1) involved in maintaining cell-cell contact and structural integrity are also downregulated (Ward *et al.*, 2005). Formation of tight junction between cells is a unique characteristic of epithelial cells. The loss of barrier junction proteins such as claudin and occludin indicate loss of epithelial cells ability to form tight junctions with one another on the basement membrane that imparts the cobblestone like structure. Therefore, cells become motile and migratory (Hartsock and Nelson, 2008; Furuse, 2010).

1.5.1.2.2 Gain of mesenchymal cell markers

Due to loss of cell adhesion proteins, epithelial cells lose their normal cellular organisation and there is an increased expression of fibroblastic proteins such as vimentin, fibronectin and S100 A4 (Huyard *et al.*, 2014). The most reliable markers that characterises mesenchymal cell properties are α-SMA and fibroblast-specific protein 1 (FSP1 or S100A4 /MTS-1). In addition to these markers vimentin and desmin have also been used to identify cells in lung and other organs that are at the verge of undergoing EMT due to inflammation (Strutz *et al.*, 1995; Kim *et al.*, 2006). Such cells continue to exhibit epithelial cell surface markers while concurrently expressing S100A4 and α-SMA. These cells express an intermediate phase of EMT. This type of behaviour exhibited by epithelial cells under inflammatory stress creates a notion of ‘partial EMT (Strutz *et al.*, 2002).

Inflammatory injury that triggers an EMT leads to release of growth factors, such as TGF-β, PDGF, EGF, and FGF-2. In addition, macrophages and resident fibroblasts release chemokines and matrix metalloproteinases (MMPs), notably MMP-2, MMP-3, and MMP-9 (Koskela von Sydow, 2016). The MMPs comprise of 24 zinc dependent

endopeptidases that are released in response to stimuli such as cytokines, chemokines, inflammatory intermediates and environmental factors. One of the targets of MMPs is collagen IV that constitutes the basement membrane. Thus these enzymes degrade the basement membrane thereby causing damage, differentiation and translocation of epithelial cells thereby promoting EMT (Jugdutt, 2003; Greenlee *et al.*, 2007; Egger *et al.*, 2014). Qin Yu *et al.* and other studies describe the role of MMP-2 and MMP-9 in activation of profibrotic growth factor TGF-β. This growth factor has shown to induce EMT in PBECs and type II lung adenocarcinoma cell line (A549) (Camara and Jarai, 2010; Van Linthout *et al.*, 2014). A study utilised TGF-β1 to induce EMT in alveolar epithelial cells and suggested that the extreme plasticity exhibited by these cells may serve as a source of fibroblasts in lung fibrosis. The downstream mediators of this 'master switch' contribute to activation of series of processes that lead to progressive deterioration of lung function due to airway remodelling (Willis and Borok, 2007; Vancheri *et al.*, 2010).

1.5.1.3 Type 3 EMT: EMT in cancer progression

Excessive epithelial cell proliferation and angiogenesis has been proposed as the critical mechanism for early growth of primary epithelial cancers (Hanahan and Weinberg, 2000) . Many experiments have projected that carcinoma cells acquire a mesenchymal phenotype and express markers such as α-SMA, FSP1 and vimentin. There has been extensive research done on studying the genetic and biochemical mechanisms involved in the acquisition of the invasive phenotype and the subsequent systemic spread of the cancer cell (Thiery, 2002). EMT-derived migratory cancer cells establish secondary colonies at distant sites that resemble the primary tumor from which they arose. In many cases of carcinoma, there is activation of a series of EMT-inducing transcription factors, notably Snail, Slug, zinc finger E-box

binding homeobox 1 (ZEB1) and Twist. Upon activation, each of these factors can act in harmony to heighten the complex EMT (Tse and Kalluri, 2007; Yang and Weinberg, 2008).

1.5.2 Role of EMT in BOS

Recent research in BOS has focused on the prevalence of EMT. Improvements in immunosuppression and other therapies have almost no effect on the reduction in the rate of incidence of BOS and long-term patient survival has marginally improved (Taghavi *et al.*, 2005; Hodge *et al.*, 2009). These observations lead to the investigation of other possible mechanisms responsible for causing BOS. Evidence suggests EMT as a candidate in the pathogenesis of BOS and has been postulated in various models (Ward *et al.*, 2005; Borthwick *et al.*, 2009). Earlier studies have demonstrated that high levels of TGF-β1, a well-known inducer of EMT, is found in the airways of BOS patients. Furthermore, clinical detection of BOS is followed by increasing levels of TGF-β1 that directly correlates to BOS grade. Therefore, TGF-β1 plays a crucial role in the pathogenesis of BOS (Jonigk *et al.*, 2016) .

There is also data available that defines EMT in the allograft lung. A study analysed the expression of mesenchymal cell markers on epithelial cells collected from the bronchial brushings (small and large airways) of BOS^+ transplant recipients. A number of epithelial cells isolated from BOS^+ patients co-expressed markers such as S100A4, EDA-Fn and α-SMA. This study stated progression of BOS from grade 0 to 3 was associated with increase in mesenchymal markers and reduction in epithelial cell markers (Hodge *et al.*, 2009). Another study reported S100A4 and cytokeratin positive fibroblasts in biopsy samples from BOS lesions providing evidence that epithelial cells undergoing EMT are detectable *in vivo* (Ward *et al.*, 2005).

Researchers have attempted to develop model for studying EMT in BOS based on cell lines, primary cells and animals (Hackett and Knight, 2007; Borthwick *et al.*, 2009).

1.5.2.1 Animal models

Murine models were initially developed to study BOS post transplant and they were successfully used to study acute rejection (Belperio *et al.*, 2000) and Idiopathic pulmonary fibrosis (Hertz *et al.*, 1993; Fahrni *et al.*, 1997; Fujita *et al.*, 2001). These models were later modified to study airway obstruction occurring during a later stage post transplantation (Adams *et al.*, 2000). The most commonly used model involves transplanting trachea derived from a donor animal into the subcutaneous pouch of a mismatched allograft recipient. This heterotopic model developed sub epithelial inflammation, necrosis and fibrosis by 21 days, therefore exhibiting symptoms of BOS (Hertz *et al.*, 1993). However, this and other similar models only performed studies on the trachea, whereas BOS is a small airway disease (King *et al.*, 1997; Neuringer *et al.*, 1998). Thus it could be argued whether such models provide a complete picture of BOS pathogenesis.

To counter this problem, murine orthotopic model of transplantation was developed. This involved a more challenging surgery wherein the entire lobe of lung was transplanted (Marck *et al.*, 1985; Okazaki *et al.*, 2007). Although this model was more appropriate to study airway remodeling, the success rate of these models in studying BOS has been variable. Some animals developed symptoms within 10-20 days while others took upto 120 days to develop lesions (Tazelaar and Yousem, 1988; Chung *et al.*, 1999). Several attempts have been made to develop larger animal models. These models utilized terminal bronchi, thus making it more appropriate to study BOS (Ikonen *et al.*, 1998; Maasilta *et al.*, 2000). Although this provides hope in

translational research due to the size and similarities with humans, there may be a high risk of having MHC mismatch alloreactivity (Maasilta *et al.*, 2000). Therefore, to improve BOS research human *in vitro* cell culture models have been developed which are both cost-effective and translatable to humans (Sato *et al.*, 2009).

1.5.2.2 Cell culture models

A number of cell culture models have utilized primary animal cell cultures or primary cells derived from human lungs to study the effect of TGF-β1 on epithelial cells (El-Gamel *et al.*, 1999; Yao *et al.*, 2004; Willis *et al.*, 2005). The immortalized alveolar type II (AT2) cell line, also known as A549 cells, when stimulated with TGF-β1 undergoes EMT and thus is commonly used to study lung fibrosis (Kasai *et al.*, 2005b; Lee *et al.*, 2008). However, authors have raised concerns about using A549 cell line due to its limitations. Firstly, although A549 shares similarity with primary epithelial cells, there are a few differences in EMT between A549 and primary cells. For instance, it is known that 1L-1β induces EMT in epithelial cells. However, treatment with TNF-α or lL-1β didn't induce EMT in A549 cells (Kasai *et al.*, 2005b). Secondly, A549 cell line only represents the morphology of the alveolar regions of the lung (Borthwick *et al.*, 2010b). BOS primarily affects the peripheral airways and causes fibrosis while it manifests as bronchiectasis in the proximal airways at a later stage. Therefore, bronchial epithelial cells have been employed in studies related to fibrosis (Molloy *et al.*, 2008). Transformed bronchial epithelial cell lines such as BEAS-2B, 16HBE4o- and Calu3 stimulated with TGF-β1 have been almost identical pattern of EMT post stimulation with TGF-β (Doerner and Zuraw, 2009b; Buckley *et al.*, 2010; Kamitani *et al.*, 2010).

Primary bronchial epithelial cells acquired from healthy patients have been used as an *in vitro* model (Doerner and Zuraw, 2009a). However, these commercially available cells obtained from healthy adults demonstrate critical functional and biochemical differences when compared to primary epithelial cells in different diseases as well as stable lung transplant recipients (Stecenko *et al.*, 2001). Therefore, although commercially available primary cells may serve as healthy controls, they may not accurately represent the process of EMT in a lung allograft recipient. To overcome these problems it is necessary to establish primary cell culture model directly from the lung epithelium. Furthermore, since BOS is a progressive disease, isolating tissue from different stages of its pathogenesis and comparing to healthy epithelium may be a powerful tool. The drawback of using this *in vitro* transplant fibrosis model is that it only allows collecting airway brushings from the large airways (Romagnoli *et al.*, 1999). Collecting samples from small airways that is the first site of injury poses further technical challenges and is associated with a high risk of developing complications (Oki *et al.*, 2005). To avoid this, a thinner fiberscope is used; but it is costly and not commonly available (Tanaka *et al.*, 1994; Shinagawa *et al.*, 2007).

Since small airway epithelial cells from transplant recipients are not always available, non-immortalized primary cell cultures such as hSAEC (LONZA) are used to conduct experiments (Jyonouchi *et al.*, 1998; Walsh *et al.*, 1999). The drawback of these cultures is that they only represent the ‘healthy’ state and are inapplicable as a model to study transplanted lung. Researchers have successfully collected and cultured the small airway epithelial cells from tobacco smokers to study COPD (Takizawa *et al.*, 2001). This could allow development of an *in vitro* primary small airway epithelial cell model of BOS.

1.5.3 TGF-β1 signalling and its role in EMT

TGF-β1, a multifunctional cytokine, is a member of the TGF-β superfamily of polypeptides that regulates cell proliferation and differentiation, apoptosis and ECM production (Massagué *et al.*, 2000; Derynck and Akhurst, 2007). There are 33 TGF-β related genes that have been identified, including bone morphogenic proteins (BMPs), activins and inhibins, nodal, anti-Mullerian hormone and growth differentiation factors (GDFs) (Vale *et al.*, 1990; Kingsley, 1994; Chen *et al.*, 2012). The individual members of TGF-β family bind to seven type I receptors and 5 type II receptors in different combinations in order to induce downstream signalling cascade. For example, BMP type II receptor pairs with three different BMP type I receptors: BMP-RIA, BMP-RIB and activin type I receptor or ALK2 (Derynck and Feng, 1997).

1.5.3.1 TGF-β isoforms, synthesis and activation

The three TGF-β isoforms (TGF-β 1, 2 & 3) are synthesized as precursor proteins coupled with latency associated proteins (LAPs), which are required for proper folding and dimerization of carboxy-terminal growth-factor domain (mature peptide). (Saharinen *et al.*, 1996). After folding and dimerization, TGF-β dimer undergoes cleavage in the trans-Golgi apparatus creating a ‘large latent complex’ that includes 120-240KDa latent TGF-β binding protein (LTBP).This complex is released by most cultured cells and is composed of EGF-like repeats and eight-cysteine domains. The C-terminal region is covalently bound to TGF-β precursor via LAP and the amino terminal of LTBP is linked to the ECM (Nunes *et al.*, 1996). Activation of TGF-β begins with release of large latent complex from ECM by proteases (Annes *et al.*, 2003). The TGF-β activators found so far are associated with the wound healing process, and include MMPs, thrombospondin-1 and integrin αvβ6 (Frazier, 1991; Munger *et al.*, 1999; Yu and Stamenkovic, 2000).

1.5.3.2 TGF-β receptors and their activation

TGF-β signalling is mediated via three cell surface receptors: TGF-β receptor I (TβRI), II (TβRII) and III (TβRIII). Out of the three receptors, TβRIII (also called betaglycan) is the most abundant binding molecule and is expressed on most cell types and fetal /adult tissues (Cheifetz *et al.*, 1986; Cheifetz *et al.*, 1988). Endoglin (CD105) was also shown to act as type III receptor for TGF-β (Cheifetz *et al.*, 1992). TβRIII binds all three isoforms of TGF-β, but has higher affinity for TGF-β2; while endoglin binds TGF-β1 and -β3 with constant affinity and has only but has weak affinity for TGF-β2 (Yamashita *et al.*, 1994).

TβRI and TβRII are transmembrane serine/theronine kinases that mediate signal transduction. They are organised into an extracellular N-terminal ligand binding domain, a transmembrane region and a C-terminal comprising of serine/threonine kinase (Massagué, 1992). TβRI contains a 20 amino acids highly conserved region in the cytoplasmic part that needs to be phosphorylated for its complete activation (Lyons *et al.*, 1988). TβRII contains a 10bp poly-adenine repeat in the coding region of extracellular domain. Changes or mutations in this region may lead to premature protein terminations resulting in truncated products (Lu *et al.*, 1996). Binding of TGF-β to extracellular domains of TβRI and TβRII forms hetero-tetrameric receptor complexes and induces proper conformation of the intracellular kinase domains. These receptors are subjected to post-translational modifications such as phosphorylation ubiquitylation and sumoylation that in turn regulates the availability and stability of these receptors and also SMAD and non-SMAD pathway activation (Sun and Davies, 1995).

Receptor phosphorylation activates the TGF-β signalling pathway - the ligand binds to TβRII and this is followed by subsequent phosphorylation of a Gly-Ser regulatory

region in TβRI (Miyazawa *et al.*, 2002; Shi and Massagué, 2003). TGF-β1 and TGF-β3 bind to TβRII without involvement of type I receptor, whereas TGF-β2 interacts with the arrangement of both receptors. Next, Ubiquitin-mediated degradation maintains the stability and turnover of receptors. Ubiquitylation occurs via the actions of E1, E2 and E3 ubiquitin ligases. E3 ubiquitin ligases including SMURF1 and SMURF2 regulate stability of TβRI and TGF-β receptor complex. The process wherein the three ligases form a polypeptide attachment is called sumoylation. This process is similar to ubiquitylation and modifies TβRI function by recruitment of phosphorylated SMAD3.

TGF-β1 has been implicated in various lung disorders and changes in its expression contribute to the development of BOS (Broekelmann *et al.*, 1991; Bergmann *et al.*, 1998). Studies have shown that excessive accumulation of TGF-β1 correlates with metastatic phenotype and poor patient outcome (Ito *et al.*, 1995; Shariat *et al.*, 2001). Furthermore, TGF-β1 is the key compound responsible for inducing EMT related fibrosis in lung (Sime and O'Reilly, 2001). It causes the epithelial cells to lose polarity, express MMPs that degrade the basement membrane, induces cytoskeletal rearrangement and increases migration. Prolonged exposure of TGF-β1 and other growth factors ensure that the epithelial cells lose their characteristics and transform into mesenchymal cells (Docherty *et al.*, 2006).

TGF-β receptors are also constitutively internalized via two major endocytic pathways, clathrin-mediated endocytosis and caveolin-mediated endocytosis wherein the latter has shown to be prominent in airway epithelia (Rejman *et al.*, 2004). Clathrin-mediated endocytosis is the most common and well-studied (McLean and Di Guglielmo, 2010). It is utilized by various cell surface receptors such as G protein-coupled receptors, tyrosine kinase receptors and other non-kinase, single

transmembrane receptors (Schmid, 1997). The internalizing receptors are first concentrated on the clathrin-coated pits, which are assembled on the cytoplasmic face of the plasma membrane (Takei and Haucke, 2001). These pit folds into and pinches off from the plasma membrane in a GTPase-dependent manner. After releasing the clathrin coat, the vesicle fuses while the receptors are transported to early endosomes (Hinshaw, 2000). Caveolae mediated endocytosis involves formation of small concave pits comprising of a 21 KDa protein caveolin-1 on the surface of the plasma membrane enriched in glycolipids and cholesterol (Anderson, 1998). This method is highly sensitive to intracellular cholesterol levels and are associated with TβRI/II mediated endocytosis of TGF-β1 (Simons and Toomre, 2000; Razani and Lisanti, 2001). If TGF-β1 endocytosis occurs via clathrin mediated endocytosis it leads to activation of SMAD2 dependent pathway ultimately leading to EMT. However, SMAD7-SMURF2 ubiquitin ligase complex is activated if signalling occurs via caveolar internalisation pathway which leads to degradation of TβRs and thus suppression of TGF-β1 signalling (McLean and Di Guglielmo, 2013). Thus these two pathways function independently and have the ability to act as a master switch to control TGF-β1 activity in cells. Once TGF-β1 signal is internalised, it may then initiate SMAD-dependent or SMAD-independent pathways (Figure 1.2).

1.5.3.3 SMAD dependent TGF-β1 signalling pathways

The SMAD proteins are latent cytoplasmic transcription factors that are directly activated by serine phosphorylation at their related receptors. Based on their function, SMADs are classified into 3 groups: the receptor-regulated SMADs (R-SMADs), SMAD1, SMAD2, SMAD 3, SMAD5 and SMAD8/9; the common SMAD (Co-SMAD), SMAD4, and the inhibitory SMADs (I-SMADs), SMAD6 and SMAD7 (Attisano and Wrana, 2000). R-SMADs and Co-SMAD contain conserved MH1

domain (Mad-homology-1) and MH2 domain (Mad-homology-2; C-terminal) that are connected via a linker segment (Liu *et al.*, 1996). I-SMADs contain only highly conserved MH2 domain and regulate activation of R-SMADs by competing with them and preventing their phosphorylation (Hayashi *et al.*, 1997). SMAD6 is also able to compete with SMAD4 for heteromeric complex formation and inhibits BMP signalling whereas SMAD7 acts as a general inhibitor of TGF-β signalling (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001).

The SMAD pathway is activated directly by the TGF-β cytokines. TβRI recognizes and phosphorylates the SMAD proteins. R-SMAD binding to the type I receptor is mediated by an anchor protein SARA (The SMAD Anchor for Receptor Activation) that recruits non-activated SMADs to the activated TGF-β receptor complex (Tsukazaki *et al.*, 1998). However, TMEPAI, a transmembrane TGF-β inducible protein competes with SMAD anchor for receptor activation for R-SMAD binding thus perturbing SMAD 2/3 recruitment to TβRI. Therefore TMEPAI controls the duration and intensity of TGF-β /SMAD signalling (Watanabe *et al.*, 2010). Receptor mediated phosphorylation of SMAD2 leads to dissociation from SARA (Wu *et al.*, 2001).

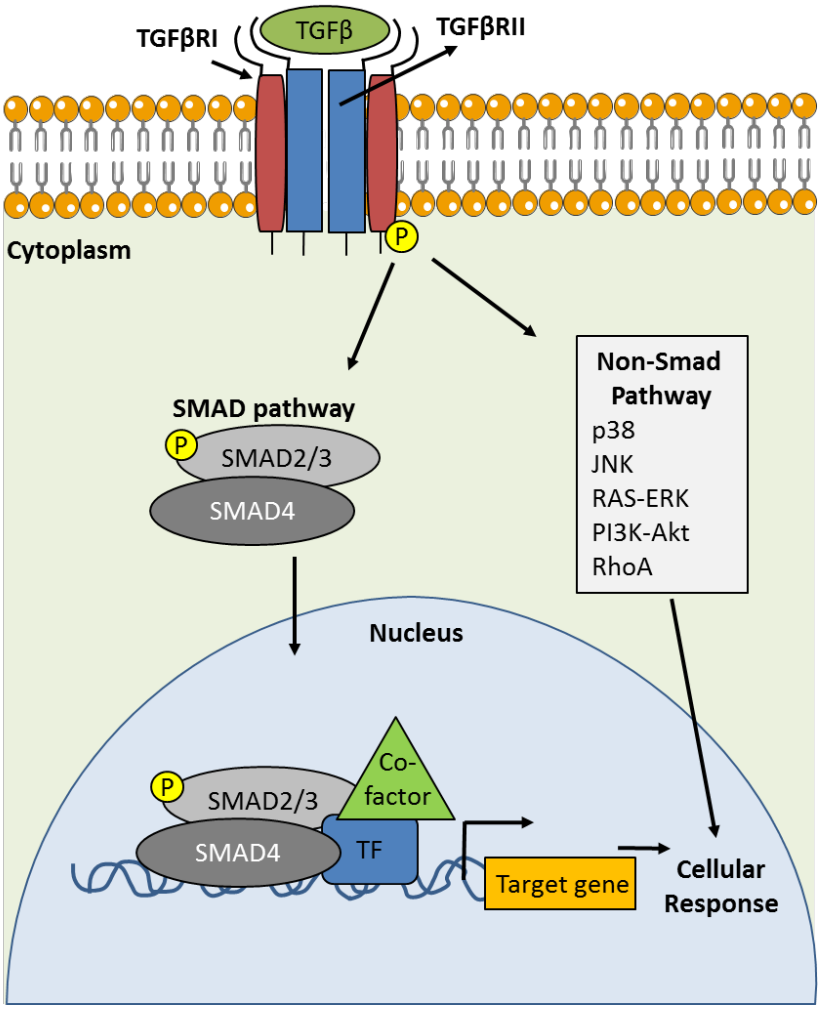

Figure 1.2: Schematic illustration of the TGF-β signalling pathway (TF: transcription factor).

Subsequently, phosphorylated SMAD2/3 forms a complex with SMAD4 and moves to the nucleus. Here, SMURF1 interacts with SMAD2/3, triggers their ubiquitination and inactivation (Zhu *et al.*, 1999). SMURF2 binding to SMAD7 in the nucleus induces transfer and employment to the activated TGF-β receptors, where it causes degradation of receptors and SMAD7 through proteasomal and lysosomal pathways (Kavsak *et al.*, 2000). SMURF1, specific for BMP-SMADs binds to SMAD7 and induces SMAD7 inactivation and translocation into the cytoplasm (Ebisawa *et al.*, 2001).

Several studies revealed that TGF-β proteins stimulate transcription of different genes via interaction of the conserved MH1 domain of SMADs, particularly SMAD1,

SMAD2, SMAD3 and SMAD4 with sequence-specific transcription factors and co-activators such as CBP and p300 *in vitro* and *in vivo* (Pouponnot *et al.*, 1998; Topper *et al.*, 1998). Recent studies indicate miRNA regulation by TGF-β/BMP signalling. R-SMADs have shown to associate with the p68/Drosha/DGCR8 miRNA processing complex thereby regulating miRNA processing in a ligand dependent and sequence specific manner. In this way, SMADs act as sequence specific transcription factors and control diverse signalling pathways (Ross and Hill, 2008; Davis *et al.*, 2010).

The role of SMAD2/3 is crucial to the EMT process. Ectopic expression of SMAD2 and SMAD3 in mouse mammary epithelial cells has shown to elicit EMT (Valcourt *et al.*, 2005). Furthermore, adenovirus infected mouse mammary epithelial cells overexpressing SMAD2, SMAD3 and SMAD4 were also shown to induce EMT, even in the absence of TGF-β1 (Piek *et al.*, 1999). Subsequent inhibition of SMAD2 and SMAD3 blocked TGF-β1 induced EMT (Valcourt *et al.*, 2005). SMAD3 has been shown to play a central role in EMT, as TGF-β1 stimulated renal epithelial cells deficient in SMAD3 fail to undergo EMT (Sato *et al.*, 2003). Furthermore, TGF-β1/SMAD3 is a major pathway that regulates myofibroblast differentiation in lung (Gu *et al.*, 2007).

1.5.3.2 SMAD independent TGF-β1 signalling pathways

In addition to SMAD-mediated signalling, TGF-β activates other signalling cascades such as ERK, JNK and p38 MAPK kinase pathways. Activation with slow kinetics may result from SMAD-dependent responses, while the rapid activation (5–15 min) suggests SMAD-independent responses (Massagué, 2000). Studies using SMAD4-deficient cells support the possibility that MAPK pathway activation is independent of SMADs (Engel *et al.*, 1999). In addition, mutated TGFBRI defective in SMAD activation can activate p38-MAPK signalling in response to TGF-β stimulation (Yu *et*

al., 2002). TGF-β induced activation of ERK and JNK pathways can lead to SMAD phosphorylation while activation of Ras/ERK MAPK signalling can induce TGF-β1 expression, thereby amplifying and inducing secondary TGF-β responses (Engel *et al.*, 1999; Funaba *et al.*, 2002). Activation of MAPK pathways by TGF-β may also have direct effects on SMAD-interacting transcription factors. For example, the JNK substrate c-Jun may allow convergence of TGF-β induced SMAD and MAPK pathways (Massagué, 2000; Moustakas *et al.*, 2001). In addition to convergence, these pathways may also counteract each other (Mazars *et al.*, 2001).

1.6 miRNAs: biogenesis and their mechanism of action

1.6.1 Overview

MiRNAs are a class of 20-22 nucleotide noncoding molecules that are produced by two RNase III proteins, Drosha and Dicer. These regulate posttranscriptional gene expression where more than 60% of the human transcriptome is predicted to be under miRNA regulation (Bartel, 2004; Chekulaeva and Filipowicz, 2009). About 35–40% of miRNA binding sites are found in the 3'UTRs, 40–50% in the coding regions and <5% in the 5'UTRs (Zisoulis *et al.*, 2010; Loeb *et al.*, 2012). In RNA silencing, miRNA base pairs with its target mRNAs, whereas AGO proteins recruit factors that induce translational repression, mRNA deadenylation and mRNA degradation (Huntzinger and Izaurralde, 2011). 5´ end of miRNAs that spans from nucleotide position 2 to 7 is crucial for identification of miRNA-binding sites in the 3'UTR of the mRNA. The downstream nucleotides of miRNA also contribute to base pairing with the targets. Greater than 60% of human protein-coding genes comprise of at least one conserved miRNA-binding site, and since numerous non-conserved sites exist, most protein-coding genes may be under the control of miRNAs (Friedman *et al.*,

2009). Thus the biogenesis and function of miRNAs are tightly regulated, and their dysregulation is often associated with human diseases (Lujambio and Lowe, 2012).

In addition to intracellular regulatory functions, miRNAs can be secreted and detected in blood and urine. These secreted miRNAs are associated with Ago2 proteins, lipoprotein complexes, or packaged into microvesicles or exosomes. Circulating miRNAs are very stable and resistant to treatment with ribonucleases, and extreme experimental conditions such as freezing/thawing (Im and Kenny, 2012; Zhang *et al.*, 2015). The biogenesis and function of miRNAs is shown in Figure 3. In summary, the identification of miRNAs and their target and function in health and disease are one of the big challenges in research (Sayed and Abdellatif, 2011).

1.6.2 miRNA nomenclature

miRNAs are allocated a three letter identifier which indicates the organism (example-hsa in Homo sapiens(human)). Typically, ‘pre-miR’ prefix denotes mature miRNA while ‘pre-miR’ refers to a precursor miRNA. A number is assigned depending on the time of miRNA discovery. Furthermore, identical miRNAs are assigned the same number independent of the organism in which they are present in. In addition multiple miRNAs can be evolutionary related, therefore a letter after a number is used to differentiate members of the same family (example hsa-miR-200b and hsa-miR-200c) (Ambros *et al.*, 2003; Griffiths-Jones *et al.*, 2006; Yang and Lai, 2011). A tag is also included in miRNA name indicating which double-stranded RNA the mature sequence comes from (e.g. has-miR-141-5p comes from the 5' arm of the precursor and hsa-miR-200b-3p from the 3' arm of the precursor) (Ha and Kim, 2014).

1.6.3 Biogenesis and processing

1.6.3.1 miRNA transcription and maturation

Early description of the genomic position of miRNAs indicated that miRNAs are derived from non-annotated parts or intergenic regions of the genome, or the intronic regions as a part of the annotated genes in the sense or antisense orientation (Lagos-Quintana *et al.*, 2001; Lee and Ambros, 2001; Bartel and Chen, 2004). miRNAs may be transcribed as a cluster from its primary transcript (pri-miRNA) (Lagos-Quintana *et al.*, 2001) (Lee and Ambros, 2001). These miRNAs are related to one another with similar seed region, for example miR-200 family (Figure 1.3, 1.4). Transcription of miRNA genes is mediated by RNA polymerase II (pol II). It was initially believed that pol III could mediate miRNA transcription because it transcribes most small RNAs, such as tRNAs. However, pri-miRNAs (primary miRNAs) are several kilobases long, containing sections of more than four uracils, which would have resulted in pre-mature transcription termination by pol III (Lee *et al.*, 2002).

Further processing of hairpin-looped pri-miRNA includes involvement of following factors: RNase-III family proteins, double-stranded (ds) binding proteins and the export receptor (Kim, 2005). The stem-loop structure is cleaved by Rnase III Drosha resulting in the generation of precursor miRNA (pre-miRNA) that is approximately 60-70 nucleotide long.

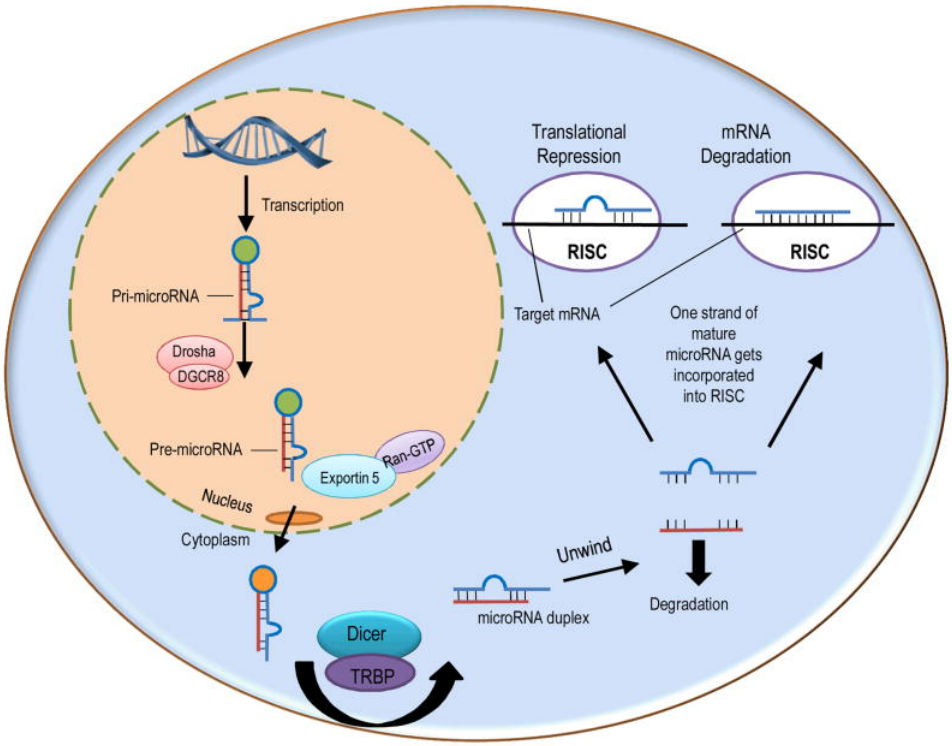

Figure 1.3: Generation and function of miRNA. Pre-miRNA is transcribed in the nucleus and released into the cytoplasm by a nuclear transporter protein exportin-5. In the cytoplasm, pre-miRNA is further processed by dicer to form mature miRNA that forms a RISC complex to regulate its target mRNA (Mas *et al.*, 2013).

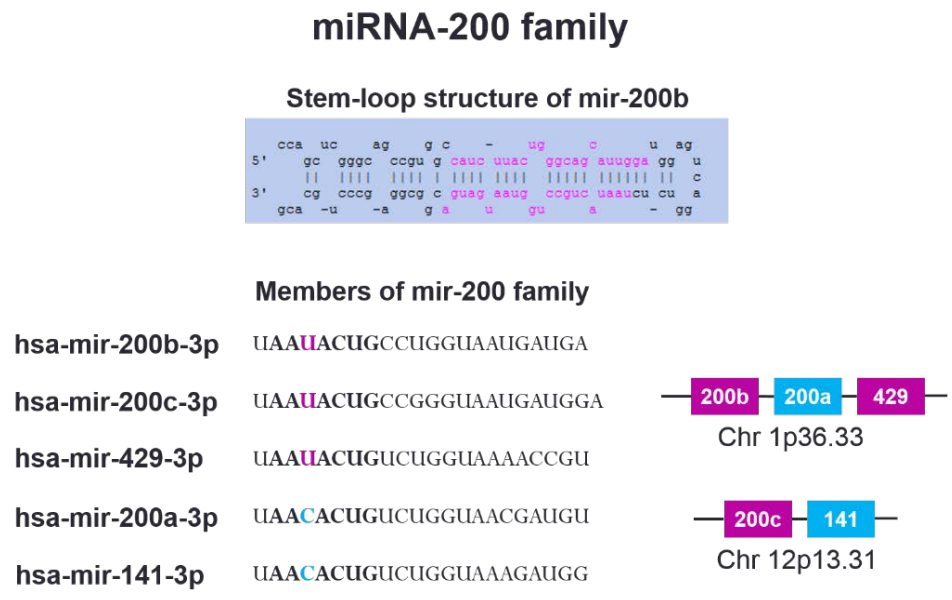

Figure 1.4: Members of miR-200 family have a common seed region although they might be located on different chromosomes. Also the stem-loop structure of miR-200b is a double stranded precursor-miRNA that dissociates to yield two mature miRNAs- miR-200b-5p and miR-200b-3p. The former comes from the 5' arm while the latter comes from the 3' arm of precursor miRNA.

Due to the staggered cutting by RNase III Drosha, there is a 5' phosphate and a 3' overhang (~2nt) (Basyuk *et al.*, 2003). Drosha is a large 160kDa protein that is comprised of two tandem repeats (Han *et al.*, 2004). Following nuclear processing with Drosha, Exportin-5 exports the pre-miRNAs to the cytoplasm. Here pre-miRNAs are subjected to second processing step by dicer (another pol III) that has affinity for the 5' phosphate and the 3' overhang of the pre-miRNA (Bartel, 2004). Dicer makes a double-stranded cut at two helical turns from the base of the pre-miRNA. Until now, two Dicer dependent pathways have been described. The first pathway employs a spliceosome that produces short-hairpin structure for processing by Dicer (Gangaraju and Lin, 2009). The second pathway utilizes unknown nucleases to produce the hair-pin structure which is then processed by Dicer. MicroRNAs derived via the first pathway are termed as miRtrons (Okamura *et al.*, 2007) while those from the second pathway are referred to as endogenous short hairpin derived miRNAs (Babiarz *et al.*, 2008). In the Dicer independent pathway, the pre-miRNA is cleaved by Argonaute 2 (Ago2) proteins that results in the generation of mature miRNA. Therefore, cleavage by Dicer leads to formation of ~22-nucleotide miRNA duplexes (Chendrimada *et al.*, 2005).

1.6.3.2 miRNA assembly in RISC complex and mRNA targeting

Mature miRNAs are incorporated into miRNA-containing ribonucleoprotein complex known as miRNP or miRISC or miRgonaute. This consists of a member of the Argonaute proteins that act as catalysts of the RISC complexes that are located in the cytoplasm and referred to as the P-bodies (Filipowicz *et al.*, 2008). The duplex (miRNA-5p: miRNA-3p) comprises of two strands of miRNA (Ro *et al.*, 2007) and the incorporation of the single-stranded miRNA onto the RISC complex leads to target repression. The RISC identifies target mRNA based on complementarity to the

specific miRNA. Most of miRNAs are associated with RISC complexes, and only less than 3% are present on their own. Thus miRNAs are stable in cells with a long half-life of days to months (Liu *et al.*, 2004; Martinez and Tuschl, 2004).

miRNA target recognition through seed sequence is more productive than to any other region. miRNAs supress gene expression by mRNA cleavage or translational repression (Bartel and Chen, 2004). This mechanism is dependent on the target mRNA. mRNA cleavage is achieved when the incorporated miRNA has complete complementarity with the target mRNA. In animals, partial complementarity between the miRNA and the mRNA sequence at the seed sequence is a prerequisite for translational inhibition (Lemons *et al.*, 2013; Lee and Ajay, 2014). Translational repression might occur at a later stage after initiation of translation. On the other hand, suppression can also be achieved because of product degradation, whilst the rate of translation remains unaltered (Olsen and Ambros, 1999).

1.6.4 MiRNA target identification

Assaying miRNAs can be useful for identification of novel miRNA candidates and for studying miRNA–mRNA and miRNA–protein interactions (Pritchard *et al.*, 2012). Computational tools allow identification of potential mRNA targets by matching the complementarity between the seed region (2-8 bases) of the miRNA and 3' untranslated region of an mRNA.

Various online prediction tools are available to find miRNA-mRNA matches. TargetScan calculates a score after finding a perfect match to the seed region. Based on the match type it also takes into consideration other aspects of seed region such as A-U enrichment (M Witkos *et al.*, 2011). The result screen ranks predicted targets either based on predicted efficacy of targeting or probability of conserved

targeting (Lewis *et al.*, 2003). MiRanda uses a miRSVR score to select the miRNA-mRNA duplexes. It identifies candidate target sites and allocates a miRSVR score to each match. MiRSVR takes into consideration different relevant features such as target site within 3' UTR region, UTR length and AU flanking content (Enright *et al.*, 2003). PicTar algorithm locates all perfect seed (~ 7 seed match) or imperfect seed in 3' UTR and predicts the score for each match. It utilizes sequence alignment to eight vertebrate species to reject false positive results and scores the candidate genes of each species separately to create a combined score for a gene (Krek *et al.*, 2005). DIANA-microT, one of the first online tools to predict targets in human, allows prediction of miRNA binding sites in coding sequences (CDS) and 3' UTR region. The results of predicted target location, binding type and score is linked to Ensembl, miRBase and PubMed and thus is highly reliable (Maragkakis *et al.*, 2009).

Each prediction tool uses a different rule of miRNA targeting and therefore produces a different list of predicted mRNA targets as a result the targets acquired might not be genuine and the definitive targets can be missed. Therefore more than one tool is required for experimental data and only the overlapping results need be considered to conclude if a miRNA-mRNA interaction is reliable (Shkumatava *et al.*, 2009; Thomson *et al.*, 2011).

1.6.5 Regulation of miRNA in lung function and its role in lung injury

Recent findings suggest that most miRNAs are conserved across different species and also most mRNAs are conserved targets of miRNA in mammals. Therefore earlier studies focused on animal models before studying the miRNA diversity in human samples (Grun *et al.*, 2005; Lu *et al.*, 2005; Friedman *et al.*, 2009; Sittka and Schmeck, 2013).

1.6.5.1 miRNA expression in lung epithelial cells

A study reported that let-7d downregulation induced loss of epithelial characteristics and increase in mesenchymal markers such as α-SMA and VIM in A549 cell line, rat alveolar type II epithelial cells (RLE-6TN) and PBECs (Pandit *et al.*, 2010). The role of miR-200 family has been extensively studied in RLE-6TN and overexpression of miR-200b and miR-200c has shown to attenuate TGF-β1 induced EMT (Yang *et al.*, 2012b). Knockout studies also indicated a role for miR-155 in lung development. miR-155 deficiency was correlated with increase in collagen deposition and myofibroblast in bronchioles which is the site of BOS development (Rodriguez *et al.*, 2007). Recently, use of miR-29b mimics inhibited collagen induction in A549 cells, confirming the ability of miR-29b to block EMT (Montgomery *et al.*, 2014b).

Similarly, overexpression of miR-326 mimics in A549 and PBECs caused a significant downregulation in TGF-β1 while miR-326 inhibitors induced TGF-β1 production and promoted increased expression of mesenchymal markers along with decreased expression of epithelial marker, cytokeratin 14 (Das et al., 2014). Thus, miR-326 is capable of maintaining epithelial phenotypes by inhibiting TGF-β1. Post TGF-β1 treatment, miR-424 expression increased in A549 cells, subsequently leading to increase in α-SMA expression. In miR-424 mimic transfected and TGF-β1 treated cells there was further increase in expression of α-SMA and CTGF when compared with the TGF-β1 treated or miR-424 mimics treated A549 cells (Xiao *et al.*, 2015). This suggests that the specific suppression or overexpression of selective miRNAs, may be a viable approach in blocking the excessive EMT process in the fibrotic lungs.

End-stage lung diseases have also shown altered miRNA expression. Due to alterations at gene level, improper recognition and binding to its complementary

targets, the involvement of miRNAs has been indicated in progressive inflammatory disorders (Sonkoly *et al.*, 2008). During the early stages of allograft injury a significant increase in numerous miRNAs occurs due to modulation of several pathways such as cell proliferation and signalling. This activates stress response and mechanisms promoting cell death. In the later phase of repair cells proliferate and release ECM proteins contributing to fibrosis (Lu *et al.*, 2006; Szczepankiewicz *et al.*, 2013). The changes in miRNA expression levels in tissues indicate their role in maintaining the cellular phenotype, tumor suppression and fibrosis (Wang *et al.*, 2009). Loss of miR-34a is noted in various kinds of malignancies including lung cancer. It plays an important role in controlling cell proliferation, cell cycle and senescence. In A549 cells, miR-34a expression leads to cell cycle arrest in G1/G2 phase and transforms cells into large, flat bodies staining positive for senescence-associated proteins (Bader, 2012). Thus the absence of miR34a expression leads to fibroblast proliferation and fibrosis (Pogribny *et al.*, 2010).

Another miRNA potentially associated with lung allograft dysfunction is miR-146a. Increased expression of miR-146a and miR-146b is observed following activation of innate immune response. Perry *et al.* showed changes in miR-146a/146b in PBECs. The study inferred the role of miR-146a in regulating a negative feedback pathway and that its expression was up regulated only during severe inflammation (IL-1β ≥ 0.3ng/ml). Thus use of miR-146a mimics could prove to be a therapeutic approach in down regulating inflammation and chemokine secretion (Perry *et al.*, 2008; Huang *et al.*, 2012). Another study by Chen *et al.* reports the involvement of miR-146a in development and progression of lung cancer. Samples with low expression of miR-146a were associated with metastasis while patients with high expression of miR-146a showed prolonged cancer free survival. It was thus concluded from the study

that miR-146a expression inhibits cell growth and induces apoptosis and is a novel marker of carcinoma (Chen *et al.*, 2013).

MiRNAs are implicated in fibrotic disorders by directly regulating the ECM deposition and profibrotic TGF-β signaling pathway. The profibrotic activity of miR-21, an oncomiR, is proportional to the severity of lung fibrosis found in animal models. Authors showed that increased expression of miR-21 was associated with increase in TGF - β1 (Liu *et al.*, 2010a; Vettori *et al.*, 2012).

1.6.5.2 miRNAs expression in lung fibroblasts

miR-21 transfection of TGF-β1 treated human primary fibroblast cell line, MRC5, induced the increased expression of Fibronectin and α-SMA, coupled with a decrease in SMAD7 expression (Liu *et al.*, 2010b). Thus, miR-21 appears to enhance TGF-β1 signalling in fibroblasts. miR-29 inhibition in TGF-β1 treated human fetal lung fibroblasts (IMR-90) cells increases the expression of ECM associated entities such as collagens and remodelling genes, thus implicating the regulatory role of miR-29 in eliciting fibrotic gene expression in fibroblasts (Cushing *et al.*, 2011). Overexpression of miR-200b and miR-200c attenuated TGF-β1-induced expression of Fibronectin and α-SMA in MRC-5 cell line and in mice derived lung fibroblasts (Yang *et al.*, 2012b).

MiR-145, an inducer of fibroblast differentiation, was found to be upregulated in TGF-β1-treated human lung fibroblasts. Ectopic expression of miR-145 in human lung fibroblasts increased α-SMA expression and promoted the formation of focal and fibrillary adhesions (Yang *et al.*, 2013). On the other hand, miR-326 was found to downregulate TGF-β1 and pro-fibrotic genes expression in NIH/3T3 cells (Das *et al.*, 2014), thus proposing the involvement of an anti-fibrotic miRNA in lung fibroblasts.

Likewise, the antifibrogenic role of miR-26a has been established by two studies (Liang *et al.*, 2014; Montgomery *et al.*, 2014b). In MRC-5 cells, miR-26a eliminated TGF-β1-induced release of collagen, and repressed the expression of fibrotic genes such as collagen IV, α-SMA, and SMAD4. In the second study, miR-26a inhibited TGF-β1-mediated nuclear translocation of pSMAD3 while inhibition of endogenous miR-26a was found to promote proliferation of human lung fibroblasts (Montgomery *et al.*, 2014b). Lastly, Huleihel *et al* found that let-7d overexpression in lung fibroblasts decreased mesenchymal markers expression and delayed wound healing (Huleihel *et al.*, 2014). Thus, the above studies established pro-fibrotic and anti-fibrotic roles of miRNAs in lung fibroblasts.

1.6.6 MiRNA as non-invasive biomarker for allograft rejection

Identification of exclusive miRNAs differentially expressed under various conditions may help in distinguishing outcomes such as early graft dysfunction and others without rejection history. Since miRNAs are relatively stable, they are well preserved in a range of sample types including formalin fixed tissues, urine, serum and blood plasma. Therefore studying the differential expression of miRNA in various samples is relatively easy (Montano, 2011; Zhang *et al.*, 2013). An overview of differential expressed miRNA is given in Table 1.2.

MiRNA profiling has been conducted to investigate their role in different diseased and normal human tissues. A study showed expression of 345 miRNAs in 40 normal human tissues that were universally expressed or exclusively expressed in specific tissues. Human tissue samples were hierarchically clustered based on anatomical position and functions using miRNA expression profile and the predicted miRNA targets were validated. Results suggested that miRNAs and their target genes had coordinated expression patterns in these tissues (Liang *et al.*, 2007). MiRNA

quantification in biofluids has emerged as a promising new approach for disease biomarker detection. It has recently been shown that disease specific exosomes and/or extracellular vesicle (EV) signatures might be useful in differentiating between normal and disease states. EVs include exosomes (<100nm) and microparticles (100–1000 nm) wherein the former are formed and stored in the cell before being released while the latter are generated through a process called ectocytosis (cell membrane shedding) (Julich *et al.*, 2014).

Allograft dysfunction	Differentially expressed miRNAs	Target tissue
Acute injury and inflammation	miR-155, miR-146b, miR-146a, miR-200a, miR-10a, miR-10b,18a	Kidney[44 45]
	miR-326, miR-142-3p miR-10a, miR-31, miR-92a and miR-155, miR-133b	Heart [46 47]
	miR-122, miR-148a, miR-192, miR-194	Liver [48]
	miR-127, miR-146a, miR-181b, miR-24, miR-26a, miR-126,miR-30a/b, miR-135b, miR-346,miR-146a/b	Lung [49 50]
Chronic injury and fibrosis	miR-16, miR-21, miR-155, miR-210, miR-638, miR-192, miR-194, miR-204, miR215/216	Kidney [51 52]
	miR-210, miR-423-5p, miR-320a, miR-22, miR-92b	Heart [53 54]
	miR-29, miR-122, miR-34a	Liver [55 56]
	miR-148b, miR-29b, miR-200, miR-21, miR-146a, miR-150, miR-1, miR-26a	Lung [57 58]

Table 1.2: Differential expression on miRNAs in various tissues during acute and chronic allograft injury (Ladak *et al.*, 2016).

1.6.6.1 miRNAs in lung, liver, kidney and heart transplantation

There are limited miRNA studies that exist in the field of lung transplantation. Therefore, the role of miRNAs in other organ transplants has also been discussed below.

Lung. Pulmonary fibrosis driven by TGF-β reveals the involvement of miRNAs that may help pave a way for effective therapeutics. The development of antibodies to donor mismatched HLA (DSA) has shown association with eight selectively expressed circulating miRNAs (miR-369-5p, miR-144, miR-134, miR-10a, miR-142-5p, miR-195 and miR-155) in lung recipients with BOS as compared to stable lung transplants. Dysregulated expression of TGF-β associated miRNAs - miR-369-5p and miR-144 in lung transplant recipients with BOS suggested their role in fibrosis driven by TGF-β signalling. Furthermore, results acquired from a cohort of DSA^+ BOS^- and DSA^+ BOS^+ lung allograft recipients indicated that the miRNA candidates identified in this study could differentiate lung transplant recipients susceptible to development of DSA and BOS compared to stable lung transplant recipients. In addition to the study described above, mononuclear cell's miRNA profiling from stable lung transplants (LT, n=10), DSA^+ BOS^- LTs (n=10, DSA group) and DSA^+ BOS^+ LTs (n=10, BOS group) revealed that the development of DSA altered the expression of miRNAs affecting TGF-β and other associated signalling pathways that play an integral role in development of BOS (Xu *et al.*, 2015c).

miR-144 is another candidate involved in fibrosis, leading to BOS. The expression of miR-144 was examined in biopsy specimens obtained from lung transplant recipients with and without BOS. BOS^+ patients demonstrated a significant increase in miR-144 expression (4.1 ± 0.8-fold) as compared to the BOS^- patients. Over expression of miR-144 resulted in a significant decrease in (TGF-β)–induced factor homeobox 1 that is a co-repressor of SMADs. Thus miR-144 is an important biomarker of BOS (Xu *et al.*, 2015d). A very recent study elucidated the role of miR-323-3p in BOS. MiR-323-3p was identified to be downregulated in lung epithelium of BOS^+ murine models post transplantation. AntagomiRs of miR-323-3p amplified while mimics

reduce murine lung fibrosis post bleomycin injury. Furthermore, miR-323-3p downregulated CASP3, a component of programmed cell death pathway. This study demonstrated that miR-323-3p limits TGF-β induced fibrotic effects by attenuating apoptotic cell death (Ge *et al.*, 2016).

The development of the bronchiolitis obliterans syndrome is poorly understood. A study hypothesized the involvement of donor HLA to be associated with miRNA dysregulation that predisposes the allograft to BOS. MiR-369-5p and miR-548d downregulation in a DSA^+ (donor HLA) lung transplant group correlated with upregulation of its gene targets LTBP1 and DCN respectively in TGF-β signalling pathways. This showed the importance of miRNAs involved in TGF-β pathway in BOS development (Xu *et al.*, 2015b). It is unclear if there is damage to lung cells during preparation prior to transplant or lung tissue injury post transplantation. Since miRNA profiling yields large number of candidates and parameters such as DSA^+ and/or BOS^+ show variable profile of miRNAs, further investigation is required to define specific marker for lung injury.

Liver. Liver specific miRNA expression (miR-122) in serum is associated with acute injury and inflammation post transplant injury. The extent of rejection has been determined by differential expression of subset of miRNAs at each stage of liver dysfunction. Downregulation of miR-122 and let-7b in liver allograft correlated to acute rejection while expression of miR-142-3p in liver graft suggested alloimmunity during rejection (Wei *et al.*, 2013). A study was devised to identify miRNAs related to hepatocellular carcinoma (HCC) recurrence following orthotopic liver transplantation (OLT). The outcome revealed six miRNA candidates as biomarkers for early prediction of HCC post OLT (Han *et al.*, 2012). Similarly, miR-718 an exclusive biomarker of HCC recurrence post liver transplantation expression was found

significantly low in serum exosomes acquired from patients post liver transplantation with HCC recurrence as compared to with those without HCC recurrence. Furthermore HOXB8 was identified as the target of miR-718 and its increased expression was indicative of HCC progression (Sugimachi *et al.*, 2015).

Kidney. Several studies have identified miRNAs as prognostic marker of kidney fibrosis and ECM deposition post kidney allograft transplantation. Sui *et al* identified and verified 20 differentially expressed miRNA candidates during acute rejection (AR) post transplantation out of which 8 were upregulated and 12 were downregulated. The study indicated that miR-142–5p, miR−155, miR−223, miR−10b, miR−30a-3p and let-7c have a potential role in the pathogenesis of AR (Sui *et al.*, 2008; Harris *et al.*, 2010). Studies have suggested that miRNA expression in urine samples as potential diagnostic and/or prognostic biomarkers of early kidney allograft rejection. MiRNA expression profile was assessed in 19 stable transplant patients and 19 patients before the episode of acute rejection. MiR-210 was elucidated as an important marker for discriminating between stable transplant patients and patients at the risk of rejection. This was the first trial that evaluated the role of miRNAs in urine samples from patients with AR (Anglicheau *et al.*, 2009; Lorenzen *et al.*, 2011). Downregulation of miR-10b correlated to reduced expression of BCL2L11. Overexpressing miR-10b into human renal glomerular endothelial cells prevented endothelial cell apoptosis and release of pro/inflammatory cytokines (IL-6, TNF-alpha, IFN-gamma, and CCL2), whereas use of miR-10b inhibitors had the opposite effects (Hamdorf *et al.*, 2017). Despite several studies in kidney transplantation, there is yet no common marker available for early prognosis of rejection and thus further investigation to find a marker is clinically important.

Heart. Heart transplantation has been recognised as an effective therapy for end stage coronary diseases. However the donor heart undergoes multiple episodes of insults during organ retrieval, preservation and transplantation. This prompts apoptosis of cardiomyocytes leading to myocardial injury following heart transplantation. Wang *et al* studied the expression of cardiomyocyte specific circulating miRNAs during myocardial injury and recovery post heart transplantation. High levels of miR-133b, miR-133a and miR-208a was found in peripheral blood indicated heart injury after transplantation suggesting that circulating miRNAs are promising biomarkers for prediction of early graft dysfunction (Wang *et al.*, 2013). Subsequent studies have helped to clarify the role of selective miRNAs and mRNAs in a model of acute cellular rejection (ACR). MiR-155 expression was significantly upregulated in human and mouse ACR samples while its downregulation reduced inflammation and increased long-term graft survival in the murine ACR model. Thus this study concluded that targeting miR-155 and intermediates of IL-6 pathway may prevent ACR (Van Aelst *et al.*, 2015).

1.6.7 MiRNA-based therapeutics

The regulatory mechanisms of miRNAs are controlled by single nucleotide polymorphisms (SNPs) in the seed region, miRNA editing and other epigenetic modifications (Cai *et al.*, 2009). In general, there are two ways of targeting miRNA in therapeutics: miRNA mimics and miRNA inhibitors/antagonists. miRNA mimics restore a loss of function and the process of incorporating these mimics is commonly called miRNA replacement therapy. This technique involves introduction of double stranded miRNAs that are crucial for maintaining homeostasis. This therapy also activates various endogenous pathways that are required for maintaining a healthy

state and blocks those that may cause disease (Bader *et al.*, 2010; Montgomery *et al.*, 2014a).

Cancerous tissues exhibit dysregulated miRNA expression, however only few can induce phenotypic changes in cancer cells. miR-34a, a master of tumour suppression, represses several gene targets involved in metastasis, oncogenic transcription, apoptosis, cell cycle signalling and cancer cell stemness. Therapeutic activity of miR-34a has been studied in several animal models where it has shown to reduce tumour growth (between 20% and 78%). The application of miR-34a leads to clinical trials wherein using NOV340 technology, MiRNA Therapeutics Inc. pharmacologically formulated NOV340/miR-34a and tested its effect in a model of hepatocellular carcinoma. miR-34a can be efficiently delivered to the liver and cause growth inhibition of human hepatocellular carcinoma cells *in vivo*. MiR-34a replacement therapy has been anticipated to be one of the first miRNA mimics to reach the clinic (Bader, 2012). Let-7 is another emerging target in cancer therapeutics. It targets various oncogenes and key components of developmental pathways thereby preventing angiogenesis and tumor progression. Let-7 replacement therapy is still in the preclinical stage due to lack of an effective delivery system (Barh *et al.*, 2010).

MiRNA antagonists are chemically modified anti-miR that binds to target miRNA and deplete the levels of the specific miRNA. The most clinically advanced miRNA based therapeutic study at present targets hepatitis C virus (HCV). MiR-122 inhibitor (SPC3649) when administered to hepatocytes blocked the replication HCV by targeting the 5’ end of the HCV genome. Clinical trials on chimpanzees showed a long lasting suppression of viral RNA post administrating SPC3649. Thus, miR-122 inhibition provides a way to treat this viral disease in humans (Jopling *et al.*, 2005;

Lanford *et al.*, 2010). Other miRNAs, currently in the preclinical development phase are miR-208/499 and miR-195. MiR-208 is exclusively expressed in the heart and inhibiting its activity induced resistance to fibrosis and cardiomyocyte hypertrophy in mice models. Likewise, miR-499 inhibition slows down myofibroblast proliferation (van Rooij *et al.*, 2007). Overexpressing miR-195 in transgenic mice has shown to cause heart failure and is associated with cardiac remodeling, while downregulating this miRNA reversed the process (You *et al.*, 2014).

1.7 Hypothesis of the project

Dysregulation of miRNAs is implicated in obstructive airway diseases including Bronchiolitis Obliterans Syndrome (BOS), where Epithelial-Mesenchymal Transition (EMT) also contributes to pathophysiology. However, the potential role of miRNAs in the EMT demonstrated in BOS is not fully understood. Manipulating the expression of miRNAs in stressed human airway cells may offer better therapeutic options.

1.8 Specific aims of the study

MiRNAs play an important role in the critical events like inflammation and fibrosis. This study sets out to enhance the current understanding of miRNAs during TGF-β1 induced EMT. The aims of the study were as follows:

1. To investigate the role miRNAs in maintaining bronchial epithelial cell phenotype.

2. Manipulate the expression of key miRNAs in bronchial epithelial cell line and primary human bronchial epithelial cell to modulate EMT.

3. Identify and validate downstream miRNA targets that may have a role in the progression of lung allograft dysfunction.

Chapter 2: Materials and methods

2.1 Risk assessment and ethical approval

The experiments were performed in accordance to Biological Control of Substances Hazardous to Health (BIOCOSH) and Control of Substances Hazardous to Health (COSHH) regulations. The risk assessment was submitted to the University Biological safety committee and tissue culture was carried out in accordance with university regulations for the containment of class II pathogens.

The ethical approval was given by the local research committee for conducting studies on patient samples and tissue sample acquired from lung tissue samples were obtained from donor lung tissue (Ref: 2001/179, The Newcastle and North Tyneside Local Regional Ethics). Informed consent was obtained from donor families and lung transplant recipients (REC 11/NE/0342).

The patient samples (cells) were obtained from patients during routine surveillance bronchoscopy carried out for diagnostic purposes at the Freeman hospital. Anonymised patient details were used for evaluating the varying changes in miRNA expression levels.

2.2 Cell lines and primary cells

2.2.1 Adenocarcinoma human alveolar basal epithelial cell lines (A549)

The A549 cell line (Figure 2.1,A) was isolated in 1972 by D.J. Giard *et al.* from lung carcinoma tissue from a 58-year-old Caucasian male to attempt to establish continuous cell lines from 200 different tumours (Giard *et al.*, 1973). This cell line is representative of the Alveolar Type II pneumocytes of the human lung in culture;

A549 cells exhibit features of an ATII epithelial cell phenotype, form confluent monolayers and are squamous (Corbière *et al.*, 2011; Cooper *et al.*, 2016).

2.3.2 Immortalized human bronchial epithelial cells (BEAS-2B) and human primary bronchial epithelial cells (PBECs)

BEAS-2B (Figure 2.1, B) is a human bronchial epithelial cell line derived from autopsy of non-cancerous individuals transformed by the hybrid adenovirus simian virus 40. The cells are not tumorigenic in immunosuppressed mice, but form colonies in semisolid medium. The adeno 12-SV40 cells contain both the SV40 and adeno T antigens (inhibits apoptosis) and both the viral genomes are operative in these cells. Cells infected with this hybrid have clear and large nuclei (intermediate morphology) (CRL-9609; American Type Culture Collection, Manassas, VA). Squamous differentiation can be observed in response to serum (Reddel *et al.*, 1988; Kinnula *et al.*, 1994).

Human primary bronchial epithelial cells (PBECs) were isolated from healthy individuals by bronchoscopy. Cultured primary cells were expanded upto a maximum of 2 passages as required. PBECs characterised by immunofluorescence prior to conducting experiments exhibited epithelial cell surface marker expression and minimal or no expression of fibrotic markers such as α-SMA and Fibronectin.

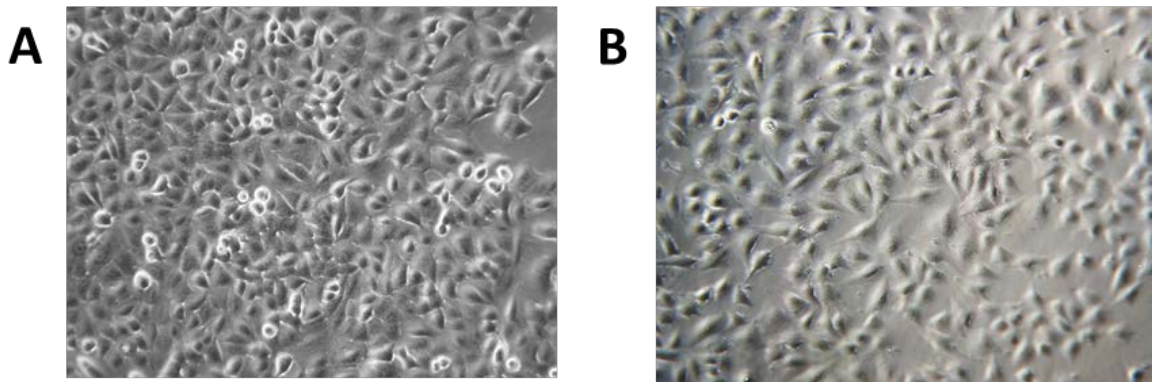

Figure 2.1: Images of cells in culture. Panel A shows A549 human lung adenocarcinoma cell line in culture medium. Panel B shows BEAS-2B cells in culture medium. Images obtained show typical polygonal appearance with epithelial characteristics (×20 magnification).

2.3 Culture media

Mycoplasma screening was carried using MycoAlert PLUS detection kit (Lonza) for the cells prior to experimental use as per manufacturer's protocol (Uphoff and Drexler, 2014).

2.3.1 DMEM media

The A549 cell line was cultured in DMEM (Sigma-Aldrich) medium supplemented with 10% FBS, Penicillin (100 U/ml), Streptomycin (100 U/ml) and L-glutamine (2mM). 15ml of the complete medium was used to maintain cells in a T75 cm^2 culture flask in a humidified incubator at 37°C.

2.3.2 BEBM/BEGM media

The BEAS-2B cell line and PBECs were cultured in BEGM complete medium that was prepared by adding BEGM SingleQuots (Lonza) that included Bovine pituitary extract (2ml), Insulin (0.5ml), Hydrocortisone (0.5ml), Retinoic acid (0.5ml), Transferrin (0.5ml), Triiodothyronine (T3, 0.5ml), Epinephrine (0.5ml), Epidermal growth factor human recombinant (rhEGF, 0.5ml), Penicillin (100U/ml) and Streptomycin (100µl/ml) to BEBM media (Lonza). Complete medium (13ml) was used

to maintain cells in T75 cm^2 culture flask in 5% CO_2 humidified incubator at 37°C. Experiments were conducted in serum free media by incubating cells in BEBM medium only with ITS Liquid Media Supplement (SIGMA-ALDRICH).

Prior to seeding cells, T75 cm^2 culture flask was coated with a mixture of collagen (0.03mg/ml), Fibronectin (0.01mg/ml) and BSA (0.01mg/ml) in BEBM (5ml) for half an hour. This coating allows proper adherence of BEAS-2B cell line by mimicking the basement membrane of the bronchial epithelium.

2.4 Haemocytometry for cell counting

Cell counting was performed using a haemocytometer. Pelleted cells were suspended in 1ml complete media. 10 µl of the cell suspension was then mixed with an equal volume of trypan blue stain that stains cells with a disrupted cell membrane when cells are visualised under a microscope, such that dead cells appear blue. Cells were counted in five squares and the total number was divided by 0.02 (total volume of each square is 0.004 mm^3, therefore for 5 squares 0.004 × 5) and multiplied by 1×10^3 (10^3 cubic mm= $1cm^3$) to obtain total number of cells in 1ml.

2.5 Trypsinization and cell storage

A549, BEAS-2B cells and PBECs grow as a monolayer and adhere to the surface of the flask. Use of Trypsin- EDTA solution (Sigma) dissociates these adherent cells from the surface of the flask. Trypsin is a proteolytic enzyme that breaks the tight bridge of proteins between the cell and the flask surface while EDTA is a calcium chelator that allows trypsin to work efficiently by engaging certain metal ions that might inhibit its activity. The use of trypsin EDTA solution to detach cells is termed trypsinisation.

The initial process involves discarding the cell medium and washing the flask surface with sterile phosphate buffer solution (10ml, 3 times). Trypsin-EDTA solution (6ml) was then added to each flask, which was subsequently incubated at 37°C for 4-5 minutes. To avoid cell damage, complete media was used to neutralize the trypsin activity and the cell solution was subjected to centrifugation at 500 g for 5 minutes. The cell-containing pellet was then suspended in complete media in a new flask or cryopreserved.

Prior to trypsinisation, BEAS-2B and PBECs culture flasks were washed with 5ml HEPES-BSS to eliminate complex medium components such as proteins and calcium that neutralize the trypsin. After trypsinisation, equal volumes of trypsin neutralising solution (Lonza) were added as BEGM complete media is devoid of bovine serum. The harvested cells were then centrifuged at 300 g for 5 minutes to obtain a cell pellet.

Stocks of cryopreserved cells were kept in liquid nitrogen. >90% confluent T75 flasks were split 1 in 2 and (500µl) placed in a cryovial. 100µl dimethyl sulfoxide (DMSO) (Sigma) was added to 900µl FBS, this new solution was added in a 1:1 ratio (500µl) to the cryovial containing the cell suspension. Although DMSO is toxic to cells at room temperature it prevents the formation of ice crystals that can rupture cell membranes during the freezing process. Cryovials were sealed, placed in a Mr Frosty Freezing Container (ThermoFisher Scientific) containing isopropanol at room temperature which was transferred to a -80°C freezer. This procedure allows cells to be frozen gradually, at a rate of -1°C/min. Cells were subsequently transferred into liquid nitrogen for long-term storage. As and when required the frozen cells were quickly thawed at 37°C in a water bath, suspended in culture media and a cell pellet was obtained post centrifugation.

2.6 Protein studies

2.6.1 Immunofluorescence

Cells (5×10^4) were seeded onto a six well chamber slide (ThermoFisher Scientific) and cultured in 250µl of complete media in 5% CO_2 humidified incubator at 37 ^{0}C until the cells adhered to the surface. 5ng/ml TGF-β1 was added to each well and incubated for 72 hours.

Cells were fixed using ice cold methanol for 15 minute that dehydrates cells and allows proteins to denature and precipitate. Chamber slides were then allowed to dry for 15 minutes. To minimize non-specific antibody binding, cells were incubated with 100µl of 5% w/v BSA solution for 1 hour at 4°C. Cells were stained with primary antibody specific for the antigens (overnight) (Table 2.1). After rinsing cells with PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse or anti-rabbit (1:100 dilution; ThermoFisher Scientific, Catlog: R37120 and R37116) made up in 1 % BSA solution and incubated overnight at 4°C. Washing was then carried out three times with PBS solution and 100µl of DAPI solution (1 µl DAPI in 3 ml PBS solution) was added to each well and incubated for 30 minutes at room temperature. The solution was then discarded and chamber superstructure removed from the slide. Fluoromount (Sigma) was used to mount cover slips and kept covered in dark at 4°C until further visualization.

The visualization of immunofluorescence was done using a Leica TCS SP2 UV laser scanning microscope. It is able to detect emission spectra from DAPI (blue, excitation 360 nm and emission 450-550 nm) and FITC (green, excitation 488 nm and emission 510-535 nm). Images were analysed by image J software (Version 1.4.3.67) and the area of fluorescence per cell was calculated.

Antibodies	Species	Company	Dilution factor
E-Cadherin (Monoclonal)	Mouse	BD Transduction Laboratories Catlog no: 610181	1:100
Cytokeratin-19 (Monoclonal)	Mouse	Biolegend Catlog no: 628502	1:50
Fibronectin (Polyclonal)	Rabbit	Santa Cruz Biotechnology Inc. Catlog no: sc- 9068	1:100
α-SMA (Monoclonal)	Mouse	Santa Cruz Biotechnology Inc. Catlog no: sc-32251	1:100

Table 2.1: Specific primary antibodies used for the antigens.

2.6.2 Western Blotting

Western blotting is a widely used technique to detect specific proteins in a sample (tissue homogenate or cell lysate). This analytical technique is based on the separation of proteins by gel electrophoresis followed by protein transfer onto a suitable membrane to be accessible and detectable by specific-antibodies. The separation of proteins was based on the molecular size by using a reducing agent and a detergent, Sodium Dodecyl Sulphate (SDS) and β-mercaptoethanol (Sigma-Aldrich) respectively. The former converts the tertiary structure of the proteins into linear polypeptide chains, while the latter maintains the denatured form of the proteins and coats the proteins with negative charge enabling the separation according to the molecular size. The result is that, the protein becomes coated with negative charges, unfolding into a rod, a conformation that maximally separates those mutually repellent negative charges and the mobility depends exclusively on its length. Along with the addition of SDS to denature (i.e., unfold, destroy native structure of) the cellular proteins, a second reagent is added. This reagent contains a soluble thiol (-SH) group to reduce any inter- or intra-molecular disulfide bonds. This

is important because disulfide bonds, which can exist within as well as between polypeptide chains, prevent the polypeptide from fully unfolding.

Separated proteins are transferred to a Polyvinylidene fluoride (PVDF) membrane for detection of proteins. To avoid non-specific binding, the non-protein bound areas of the membrane are blocked before probing with primary protein specific antibody. After incubation, a secondary antibody conjugated with an enzyme, fluorophore or isotope is added. Horseradish peroxidase (HRP) is one of the most common and safe conjugates. The detection of this enzyme, which correlates with the abundance of the examined protein, is determined indirectly by the addition of a peroxide-luminol based reagent. The peroxidase enzyme catalyses the oxidation of the luminol, resulting in the emission of the light. The emitted light can be captured through exposure to X-ray photographic film (Burnette, 1981).

2.6.2.1 Cell lysate preparation

Stimulated and untreated cells were pelleted post trypsinisation. Cells were lysed using lysis buffer consisting of cell lytic solution (Sigma) supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablets (Roche, USA). To ensure complete cell lysis and protein extraction, samples were sonicated using an MSE Soniprep 150 sonication. The samples were centrifuged at 15000 g for 15 minutes to pellet cells debris and either used immediately or stored at -80°C.

2.6.2.2 Determination of protein concentration

The protein concentration of each sample was estimated colorimetrically using a bicinchoninic Acid (BCA) protein assay kit (Pierce, USA) in accordance with the manufacturer's instructions. In this assay BCA reagent detects the protein sample when Cu^{2+} ions are reduced to Cu^{1+} in alkaline condition. The addition of protein rich

sample allows the colour change thereby exhibiting strong absorbance at 562 nm. The extent of the colour on sample is proportional to the amount of the protein presence. The concentration of the unknown protein was evaluated from a bovine serum albumin standard curve, which was run in parallel with unknown samples. To each well 10 µl of varying concentration of BSA (2 mg/ml, 1 mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml) and 200 µl of working solution was added. Similarly 10 µl of the sample and 200 µl of working solution were added to determine the unknown protein concentration. The working solution was prepared prior to use by mixing reagent A (BCA in alkaline buffer) with reagent B (4% w/v cupric sulphate) in a ratio of 50:1. The plate was incubated at 37°C for 30 minutes before recording the absorbance at 562 nm. A linear regression analysis of the standard curve was calculated and the unknown protein concentration was determined by interpolation (Figure 2.2).

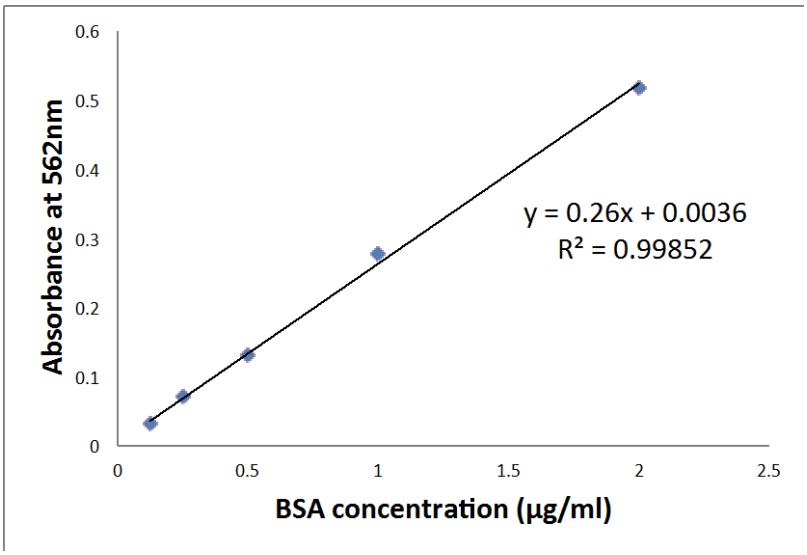

Figure 2.2: Representative standard curve of protein concentration

2.6.2.3 SDS-PAGE Gel Electrophoresis and protein transferring

SDS-PAGE gels consist of a stacking gel on the top of a resolving gel. The amount of acrylamide in the resolving gel determines the percentage of that gel and its pore size. In this project, 10% v/v gel was used to separate large proteins and 12% was

used for smaller proteins (60-40 kDa molecular weight). 5 ml resolving gel solution and 3 ml stacking gel solution was prepared to cast one gel and added to glass and alumina plates (Table 2.2).

The resolving gel polymerised within 30 minutes. After polymerization, stacking gel was added on the top and comb was inserted. The prepared gel was immersed into electrophoretic buffer (10% SDS, Tris Base, Glycine at pH 8.3) and loading sample was prepared by mixing 25 µg equivalent of purified cell lysate (Refer to 2.6.1) and loading dye (containing β-mercaptoethanol) and then subjecting the mix to heating (100°C for 5 minutes) prior to loading on the gel. Parallel to the sample, PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific) was also loaded as a guide for molecular weight. Gel run setting was a maximum of 180V at room temperature for 30-60 minutes (based on gel porosity).

Gels	Composition
10% Resolving Gel **(For 2 slabs-10ml volume)**	4 ml - water 3.3 ml - 30% Acrylamide 2.5ml - 1.5M Tris 0.1ml - 10% SDS 0.1ml - 10% APS 5 µl - TEMED
5 % Stacking Gel **(For 2 slabs – 6ml volume)**	4.1ml - water 1.0ml - 30% Acrylamide 750µl - 1.0M Tris (pH 6.8) 60 µl - 10% SDS 60 µl - 10% APS 6 µl - TEMED

Table 2.2: Gel preparation reagents for SDS-PAGE

TEMED (ThermoFisher Scientific)-Tetramethylethylenediamine; APS (Sigma-Aldrich)- Ammonium persulphate

To ensure presence of purified proteins, gels were stained with Coomassie stain consisting of 10% v/v acetic acid, 10% v/v Isopropanol and 0.1% w/v Coomassie blue powder. After 20 minutes incubation at room temperature on the rocker, the gels were destained in three sequential steps using three different solutions. The stained gels were mixed with destain 1 consisting of 25% v/v propanol with 10% v/v acetic acid for 10 minutes before the addition of destain 2. After the removal of destain 1, destain 2 consisting of 10% v/v propanol and 10% v/v acetic acid was added to the gel and washed many times until the clear bands were obtained. The gels were stored in destain buffer 3 containing 10%v/v acetic acid until further use.

After protein separation, the proteins were transferred to Amersham Hybond LFP 0.2 PVDF (GE healthcare LifeSciences) membrane. This membrane was equilibrated in absolute methanol for ten seconds followed by washing with distilled water twice for 5 minutes each. The gels and transfer electroblotting cassette were soaked in transfer buffer before being assembled. Protein transfer was performed either overnight at 30 V or for 2 hours.

2.6.2.4 Immunoblotting

After transfer, the PVDF membrane was washed once with PBS containing 0.1 % v/v tween 20 (TPBS) for 5 minutes to remove the transfer buffer before blocking. To block the non-specific binding, the membrane was blocked with 5% w/v milk for at room temperature for 1 hour before probing with primary antibody. Primary antibody was diluted in the blocking buffer at a concentration recommended by the manufacture and added to the membrane. This was incubated overnight at 4^0 C with continuous shaking on a rocker. After incubation with primary antibodies, the membrane was washed three times with 30 ml 0.1 % TPBS for 5 minutes each. Membranes were incubated with HRP-conjugated specific secondary antibody

(Santa Cruz Biotechnology; Catlog: sc2005 and sc2004) in 5% w/v milk for 1 hour at room temperature with continuous shaking. After three washes with 30 ml TPBS, peroxidase activity was detected by using 2 ml SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific) for 1 minute. The detection of bound antibodies was dependent on the oxidation of the luminol in the substrate by HRP conjugated to the secondary antibody and consequent emission of light. The resulting bands were visualized by exposure of the membrane to a film using developer and fixer (Tentenal, Germany). The exposure time varied according to the expression of the desired protein, starting from 1 minute. As a control, membrane was stripped at room temperature for 30 minutes using stripping buffer consisting of; 1.5 % w/v glycine, 0.1 % w/v SDS and 1 % v/v Tween 20 followed by washing with 30 ml TPBS and reprobed for GAPDH using the same protocol. Initial attempts were made to optimize minimum protein concentration required to give desirable results (Figure 2.3).

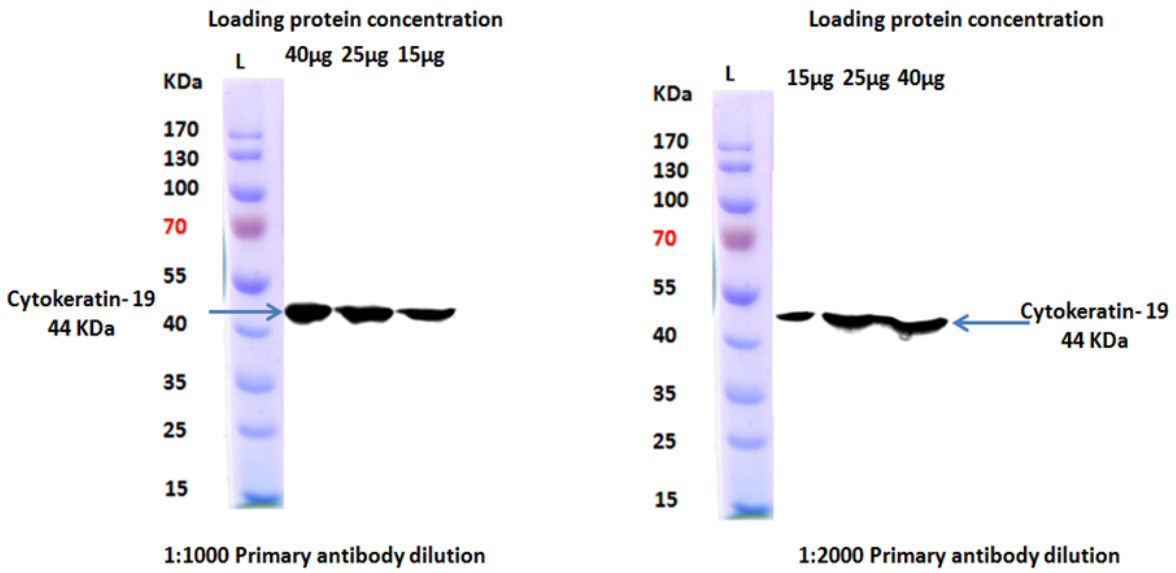

Figure 2.3: Western blot optimization. 25µg equivalent protein and 1:2000 diluted primary antibody was optimum to yield good results. Further western blot experiments were conducted using this optimised concentration and dilution factor for all the markers.

2.7 Molecular biology

2.7.1 Procedure of RNA isolation

2.7.1.1 MRNA isolation using Qiagen RNeasy mini kit

The Trizol/RNeasy hybrid RNA isolating protocol involved the use of phase separation technique as well as RNeasy mini kit (Qiagen). The pelleted cells were homogenized using TRI reagent (Sigma) and the RNA supernatant was collected in an eppendorf post phenol-chloroform phase separation

An equal volume of 100% v/v ethanol was added to this aqueous solution and the mixture was vortexed. 700 µl of the sample was then loaded into RNeasy spin columns (Qiagen) seated in collection tubes and centrifuged for 15 seconds at 8000 g at room temperature. The flow through was discarded and the step was repeated for the remaining sample. 500 µl of RPE buffer was added to the spin columns and centrifuged for 15 seconds at 8000 g to wash the spin column membrane. The flow through was discarded and the step was repeated, but the centrifugation was for 2 minutes to ensure that there was no ethanol contamination during RNA elution step. The spin column was placed in a new collection tube and centrifuged at full speed for 1 minute.This step ensured that any residual RPE buffer was eliminated. The spin column was then transferred in a new collection tube, 50 µl of RNase free water was added to spin column membrane and centrifuged for 1 min at 8000 g to elute the RNA. The OD was measured and sample was stored at -80°C until further use (Figure 2.4, A).

2.7.1.2 miRNA isolation using MiRVana Paris kit (Applied Biosystems)

The pelleted cells were re-suspended in 300 µl of ice-cold Cell Disruption Buffer, followed by mixing with 300 µl of 2x Denaturing solution at room temperature. The guanidinium thiocyanate in the denaturing solution would prevent RNA degradation. Thereafter, 600 µl Acid-Phenol:Chloroform was added and the eppendorf was vortexed for 30-60 secs. The eppendorf was subjected to centrifugation for 5 minutes at 12000 g at room temperature to separate the mixture into aqueous and organic phases. After centrifugation, the upper aqueous phase was carefully pipetted and transferred into a fresh tube. 1.25 volumes of 100% ethanol to the aqueous phase mixed thoroughly and applied to a filter cartridge provided in the kit. The cartridge containing tube was centrifuged at 12000 g for 1 minute and the flow through was discarded. After this, the filter was subjected to washes using 700 µl miRNA Wash Solution 1 (one time) and 500 µl Wash Solution 2/3 (2 times). After each wash the flow through was discarded and the filter was placed back in the collection tube. In the last step after the washes, the filter was placed in a new collection eppendorf, 50 µl preheated nuclease free water was applied carefully to the centre of the filter and let to rest for 1 minute before subjecting to centrifugation for 1 minute at 12000 g. The eluate was collected, kept on ice and RNA concentration and quality was determined using Nanodrop. RNA was then stored at -80°C until further use (Figure 2.4, B).

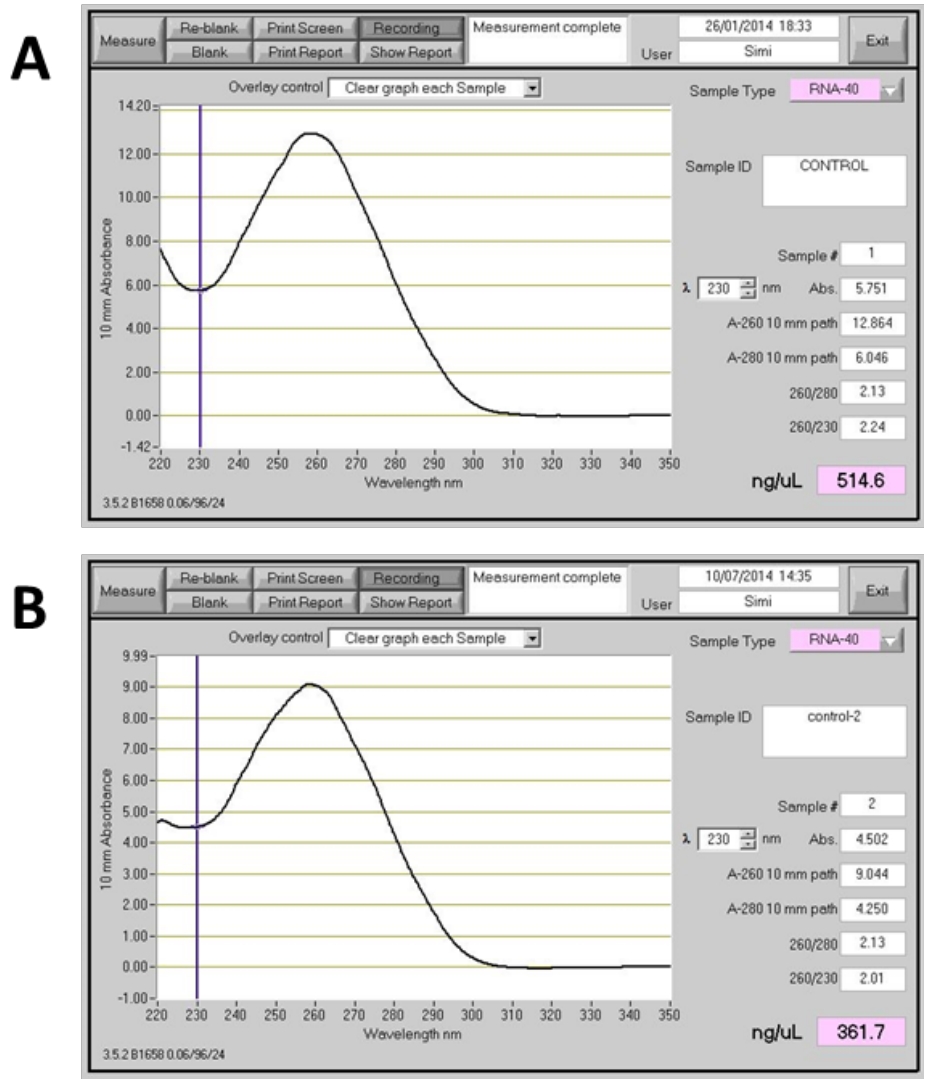

Figure 2.4: Spectrophotometric analysis of RNA samples (Nanodrop). Panel A and B are images taken from spectrophotometer for mRNA and miRNA (in total RNA) isolated respectively. RNA quality is shown by 260/280 and 260/230 ratios. Ratios between 2.0 and 1.8 confirm the good quality and purity of RNA extracted.

2.7.2 Complimentary DNA strand synthesis

2.7.2.1 cDNA synthesis using Tetro cDNA synthesis kit (for mRNA)

Complementary DNA was generated from RNA template using Tetro cDNA synthesis kit (Bioline). The first strand cDNA generated was suitable for RT-PCR with gene specific primers (Figure 2.5). The tube prepared as per table 2.3 was transferred to a T100™ Thermal Cycler (Bio-Rad;45°C - 30 minutes, 85°C - 5 minutes, 4°C - 5 minutes).

Components	Tetro cDNA synthesis
RNA	5 µg equivalent
5X RT buffer	4 µl
10mM dNTP mix	1 µl
Oligo (dT)18 Primer Mix	1 µl
RNase Inhibitor (10 u/µL)	1 µl
Reverse Transcriptase (200 u/µL)	1 µl
RNase free water	Variable
	20 µl total volume

Table 2.3: Contents of first strand Tetro cDNA synthesis reaction.

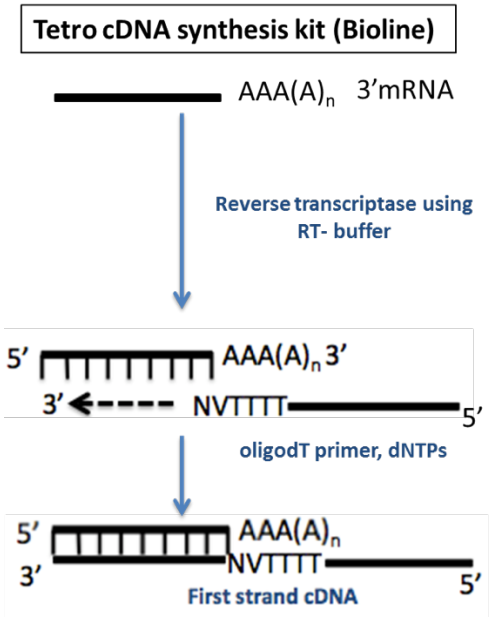

Figure 2.5: First strand cDNA synthesis. First Complementary DNA strand to mRNA was synthesized using Tetro cDNA synthesis kit (Bioline) that utilizes RT buffer.

2.7.2.2 cDNA synthesis using TaqMan miRNA reverse transcription kit (ThermoFisher Scientific)

miRNA cDNA synthesis involved a two-step process. First, a miRNA specific stem–loop RT primer is hybridised to a single stranded miRNA and then reverse transcribed with a MultiScribe reverse transcriptase enzyme. RNA was diluted to the required concentration and added to 7 µl of RT master mix (containing RT buffer, dNTP, Rnase inhibitor, RT enzyme and water). This was subjected to centrifugation at 2000 g for 2 minutes (Table 2.4). Thereafter, RT primer for specific miRNA was added and the eppendorf was incubated on ice for 5 minutes before transferring to the thermocycler (16°C - 30 minutes, 42°C - 30 minutes, 85°C - 5 minutes).

Components	**TaqMan miRNA RT**
RNA	5 µl (100ng/ 20 µl reaction)
10X RT buffer	1.5 µl
dNTP mix w/dTTp (100M total)	0.15 µl
RNase inhibitor (20U/µL)	4.16 µl
MultiScribe™ RT enzyme (50U/µL)	0.19 µl
RNase free water	1 µl
Centrifuge: 2000rpm/2 minutes	
RT primer (miRNA specific)	3 µl
Incubate on ice: 5 minutes	
Volume	15 µl

Table 2.4: Protocol for cDNA synthesis using TaqMan miRNA reverse transcription kit.

2.7.3 Quantitative real time PCR

2.7.3.1 Validation of housekeeping genes and primers

For normalising data in microRNA experiments, an endogenous reference miRNA was chosen from a panel of six different housekeeping genes (5.8S, U54, RNU49, RNT6B, RNU19 and U6; Figure 2.6). The data shown in is a representative of an experiment performed in triplicates. The C_t value (cycle threshold) for each gene gave an estimate of the target cDNA in the samples that is constant across different time points for housekeeping genes. The C_t values for all the housekeeping genes were plotted (BEAS-2B control and TGF-β1 treated cells) to look for any difference between the C_t values across the three time points. There was no statistical significant change between the C_t values across the three time points (for all genes). Although changes in expression of five different housekeeping was studied across various time points (untreated, 8 hrs and 24 hrs TGF-β1 treated BEAS-2B cells) U6 was chosen as an endogenous reference gene for quantification of miRNA using qRT-PCR. This is because RNU6 has been used as an endogenous control for various studies and has shown to be constant across different cell types including human bronchial epithelial cells (Wong *et al.*, 2007; Mujahid *et al.*, 2013; Sohal *et al.*, 2013a; Solleti *et al.*, 2017).

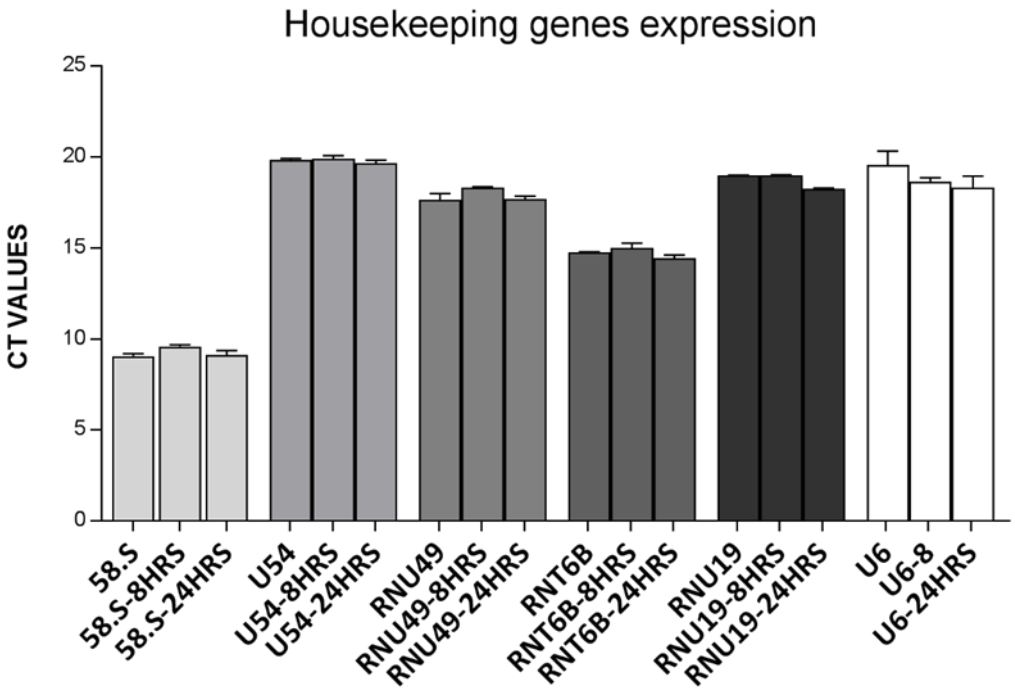

Figure 2.6: Validation of housekeeping genes. The Ct values for each of the six different housekeeping genes 5.8S, U54, RNU49, RNT6B, RNU19 and U6 was obtained by performing qRT-PCR of treated and untreated BEAS-2B cells. The expression level of each gene was then compared across treated and untreated time points using prism 6 software. This data is representative of one experiment done in triplicate.

The primers for the target mRNA and miRNA were validated for their efficiency before proceeding with RT- PCR experiments (Table 2.5). To check for the efficiency and reliability of the primers used, the template cDNA of known concentration was serially diluted (1:10, 1:100, 1:1000 and 1:10000). The resultant C_t values were used to plot a standard curve to calculate q-PCR efficiencies. The standard curve was constructed by plotting the log of dilution factor against the C_t value obtained for each dilution. Each set of dilution was performed in triplicates to check for pipetting error. Using the slope (y) derived from standard curve, percentage amplification efficiency (E) was calculated using the formula $E = [(10^{-1/slope})-1] \times 100\%$. For the assay to be robust and reproducible, the efficiency should lie between 85- 105%. The efficiency

values for miR-200b, miR-146a, miR-34a, miR-21 and RNU6 primer ranged between 93%-110% (Figure 2.7).

Description	Vendor	Product code
miR-34a-5p	Applied Biosystems	4427975
miR-146a-5p	Applied Biosystems	4427975
miR-21-3p	Applied Biosystems	4427975
miR-200b-3p	Applied Biosystems	4427975
RNU6	Applied Biosystems	4427975

Table 2.5: Details of primers used in qRT-PCR

2.7.3.2 TaqMan probe assay

TaqMan based chemistry involves use of sequence specific probes (SensiFast Probe assay, Bioline) carrying a fluorophore FAM at the 5' end of probe (tetrachlorofluorescein) and quencher TAMRA (tetramethylrhodamine) at the 3' end. During the annealing and extension phase of PCR, the probe is cleaved by the 5' to 3' exonuclease activity of Taq DNA polymerase that results in separation of the fluorophore and quencher dyes thus resulting in detectable fluorescence (Figure 2.8).

For mRNA and miRNA studies 10 µl SensiFAST™ Probe Hi-ROX mix (Bioline) was mixed with 2 µl cDNA prepared previously, 1 µl TaqMan primer-probe (Applied Biosystems) and 7 µl of Rnase free water was loaded in each well on a 96 well plate. The thermal cycling conditions were as described in table 2.6.

ΔΔCT based method was used to calculate the gene expression. The comparative ΔΔCT method first determined the ΔCT and the standard deviation of each sample. Following this, ΔΔCT was calculated after calculating the standard error for each sample. The final step involved determining the comparative fold change ($2^{-\Delta\Delta CT}$) of

the gene in sample to the normalised sample. The formulae and calculations are as follows:

$$\Delta\Delta CT = \Delta CT(1) - \Delta CT(2)$$

ΔCT(1) = CT of gene of interest - CT of housekeeping gene.

ΔCT(2) = CT of gene of interest control- CT of housekeeping gene control

The data analysis was carried out using excel spreadsheet.

A

PCR cycling conditions for mRNA			
Cycles	Temperature	Time	Notes
1	95^0C	5 mins	Enzyme activation
40	95^0C	10 secs	Denaturation
	60^0C	20 secs	Annealing and extension

B

PCR cycling conditions for miRNA			
Cycles	Temperature	Time	Notes
1	95^0C	10 mins	Enzyme activation
40	95^0C	15 secs	Denaturation
	60^0C	1 min	Annealing and extension

Table 2.6: Real time PCR cycling conditions for mRNA and miRNA studies

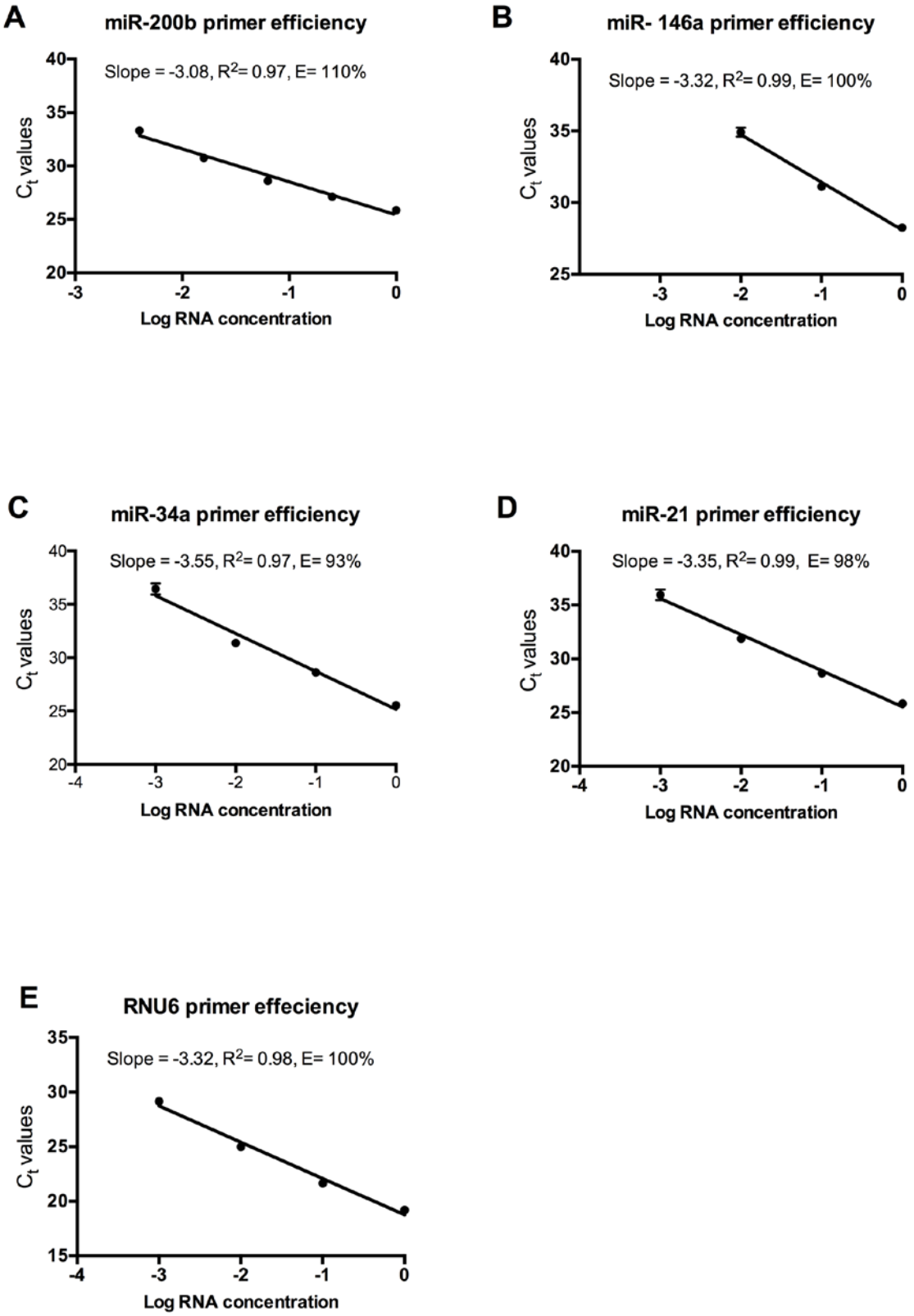

Figure 2.7: RT-PCR miRNA primer efficiencies. Panel A to E shows efficiency of miR-200b, miR-146a, miR-34a, miR-21 and RNU6 primer. The reaction was run on Applied Bio systems (StepOnePlus) for 40 cycles. Efficiency (E) value was calculated from linear regression and x scale was converted to log scale. This data is representative of one experiment done in triplicate.

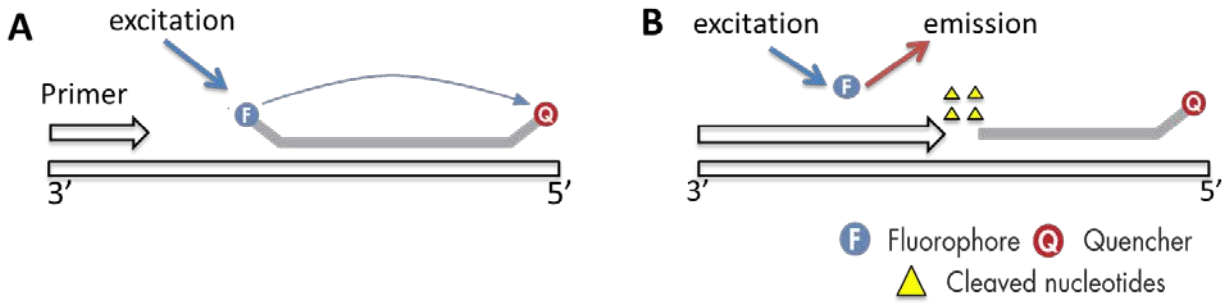

Figure 2.8: TaqMan probes in quantitative RT-PCR. A. The PCR primer and TaqMan probe anneal to target sequence during annealing step of PCR. The proximity between the reporter molecule and quencher keeps the fluorescence low. **B**. During the extension phase, Taq DNA polymerase extends the primer. Upon reaching the probe, the 5' to 3' exonuclease activity of the enzyme cleaves the fluorescent reporter (fluorophore) from the quencher and the fluorescent signal is then measured.

2.8 miRNA transfection

2.8.1 General principle

Cationic lipids such as Lipofectamine transfection regents (ThermoFisher Scientific) assist in delivering DNA and RNA (including siRNA and miRNA) into cells. The structure of a cationic lipid comprises of a positively charged group and one/two hydrocarbon chains. The charged head group directs the interaction between the lipid and the phosphate backbone of the nucleic acid. Positively charged liposomes mediate interaction between the nucleic acid and the cell membrane. This allows for the fusion of a liposome-nucleic acid transfection complex with the cell membrane (that is negatively charged). The complex enters into the cells through endocytosis. Therefore cationic lipids assist in delivering DNA/RNA into the cells mediating DNA or RNA –cellular interactions (Chesnoy and Huang, 2000; Hirko *et al.*, 2003; Liu *et al.*, 2003).

Transfection strategies are classified into two types- stable and transient transfection. During stable transfection, nucleic acid integration persists in the cells long-term and is passed to subsequent generations after cell division (Felgner *et al.*, 1987). When cells are transiently transfected, the transgene is introduced into the nucleus of the cell, but does not integrate into the chromosome. While transfected DNA is translocated into the nucleus for transcription, transfected siRNA/miRNA are found in the cytosol, where they bind to the mRNA to silence the target gene (also known as RNA Interference). Transiently transfected gene can be analysed within 24–96 hours after introduction. This process is most efficient when supercoiled plasmid DNA is used, although siRNAs miRNAs and mRNAs can also be transfected (Kim and Eberwine, 2010).

2.8.2 Optimising transfection parameters

It is crucial to maximize transfection efficiency while minimizing cell toxicity level during transfection. miRIDIAN microRNA Mimic Transfection Control (cel-miR-67) labelled with Dy547 was used to optimize transfection conditions, where Dy547 labelling allowed monitoring delivery into cells. The miRIDIAN microRNA transfection reagents were suspended in RNase-free water (stock concentration = 20 µM) and aliquots were stored at -20°C to limit freeze thawing (Table 2.7). BEAS-2B cells were counted and re-suspended at a density of 40000 cells per well (seeding density: 50000 cells/cm^2) in an 8 well chamber slide (0.8cm^2/well) containing Opti-MEM (ThermoFisher scientific) without antibiotic (e.g. Streptomycin, penicillin). The volume of transfection reagent, miRNA concentration and the length of exposure of cells to transfection reagent/miRNA complexes was optimised (Figure 2.9, n=2).

microRNA transfection controls				
	Transfection control with Dy547	Transfection control (negative)	miRIDIAN microRNA Mimic	miRIDIAN microRNA Inhibitor
ID	cel-miR-67	No homology to human genome	Homology to human genome	Homology to human genome
Accession No	MIMAT0000039	N/A	MIMAT0000318	MIMAT0000039
Molecular weight	18379.0 g/mol	N/A	14084 g/mol	18379 g/mol
Catalog item	CP-004500-01-05 (Dharmacon,GE LifeSciences)	1027280 (Qiagen)	C-300582-07-0005 (Dharmacon, GE LifeSciences)	IN-001005-01-05 (Dharmacon, GE LifeSciences)

Table 2.7: Summary of control transfection reagents

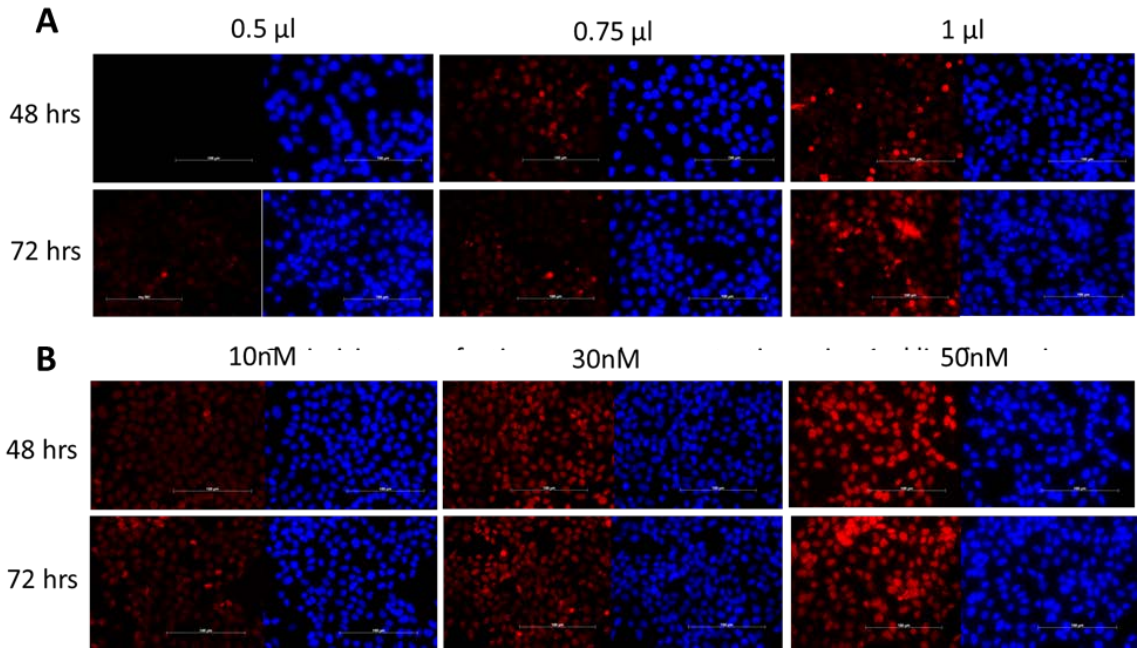

Figure 2.9: Optimizing transfection conditions. BEAS-2B cells were seeded on a 8 well chamber slide and transfected with 30nM transfection control for 48 and 72 hrs using 0.5, 0.75 and 1 µl transfection reagent (A). Transfection control concentration (10, 30 and 50nM) was also optimized using 1 µl transfection reagent at 48 hrs and 72 hrs time point (B). This data is representative of two independent set of experiments, each done in triplicate.

The volume of transfection reagent was optimised by transfecting cells with 30nM transfection control for 48 and 72 hrs using 0.5, 0.75 and 1 µl transfection reagent prepared in Opti-MEM per well. The transfection control concentration was optimised by incubating cells with 10nM, 30nM and 50nM transfection control for 48 and 72 hrs while keeping Lipofectamine volume constant (1 µl). Cells were fixed with methanol and nuclei were stained using DAPI. Slides were visualised under a fluorescence microscope to assess the presence of transfection control.

2.8.3 miRNA-200b Mimic and inhibitor optimisation

BEAS-2B cells were transfected with 10nM, 30nM and 50nM miR-200b mimic (miRIDIAN microRNA Mimic, Dharmacon) and 10nM, 30nM, 50nM, 70nM and 90nM miR-200b inhibitor (miRIDIAN microRNA Hairpin Inhibitor, Dharmacon) for 24 hrs. RNA was isolated followed by cDNA synthesis and q-RT PCR was performed to evaluate changes in miR-200b mimic and inhibitor expression, E-Cadherin expression and fibronectin expression. Phenotypic changes in cell morphology were also recorded by capturing images at 48 hrs post-transfection (Figure 2.10 & 2.11, n=2).

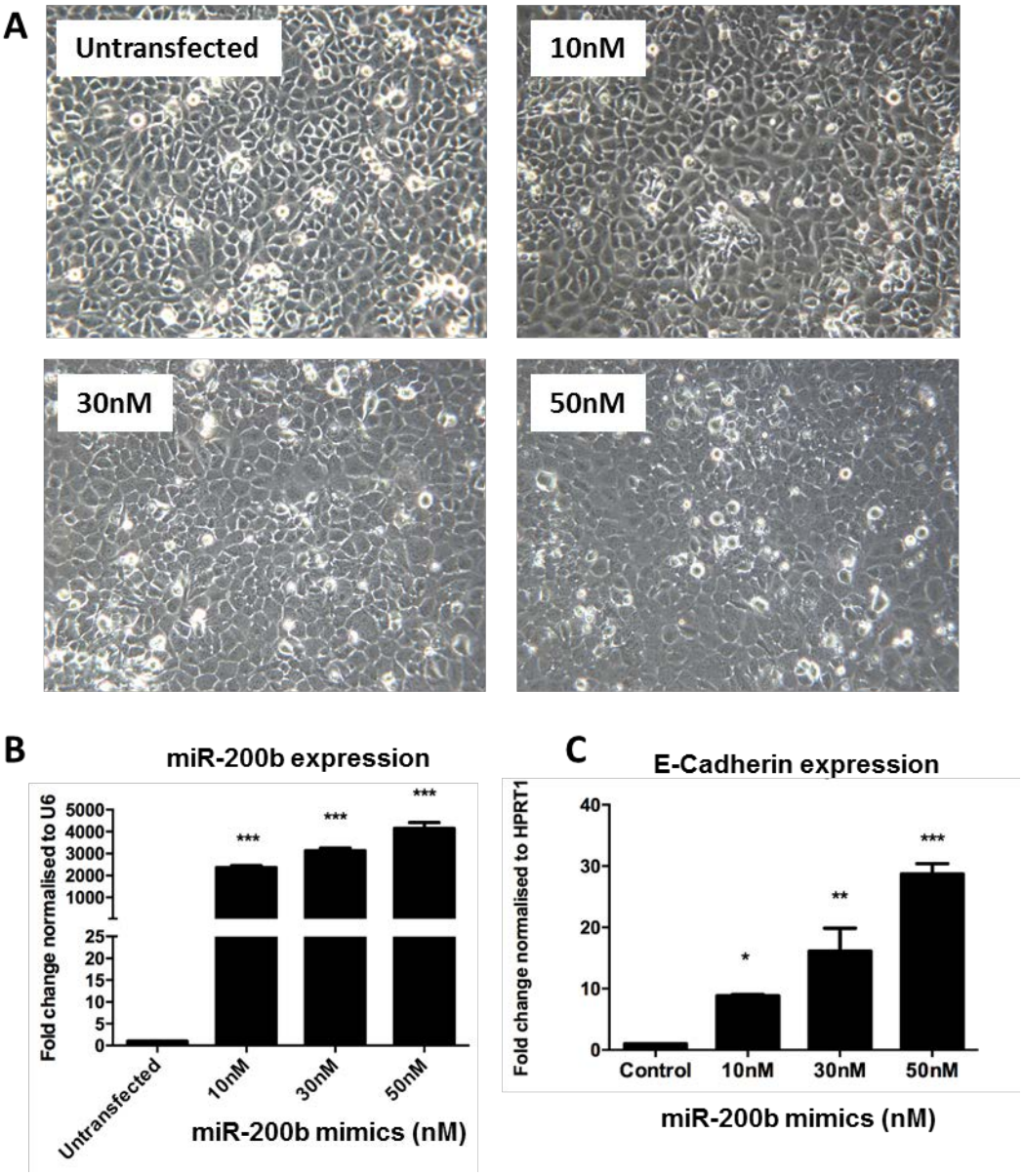

Figure 2.10: miR-200b mimic concentration optimization in BEAS-2B cells. BEAS-2B cells were transfected with 10nM, 30nM and 50nM miR-200b mimics for 24 hrs. RNA was isolated followed by cDNA synthesis and q-RT PCR was performed. Panel A shows phenotypic changes post transfection with varying concentration of miR-200b mimics. miR-200b gene expression (B) and corresponding E-Cadherin expression (C) profile was evaluated and plotted. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001)] compared to the control. The data is representative of two independent set of experiments, each done in triplicate.

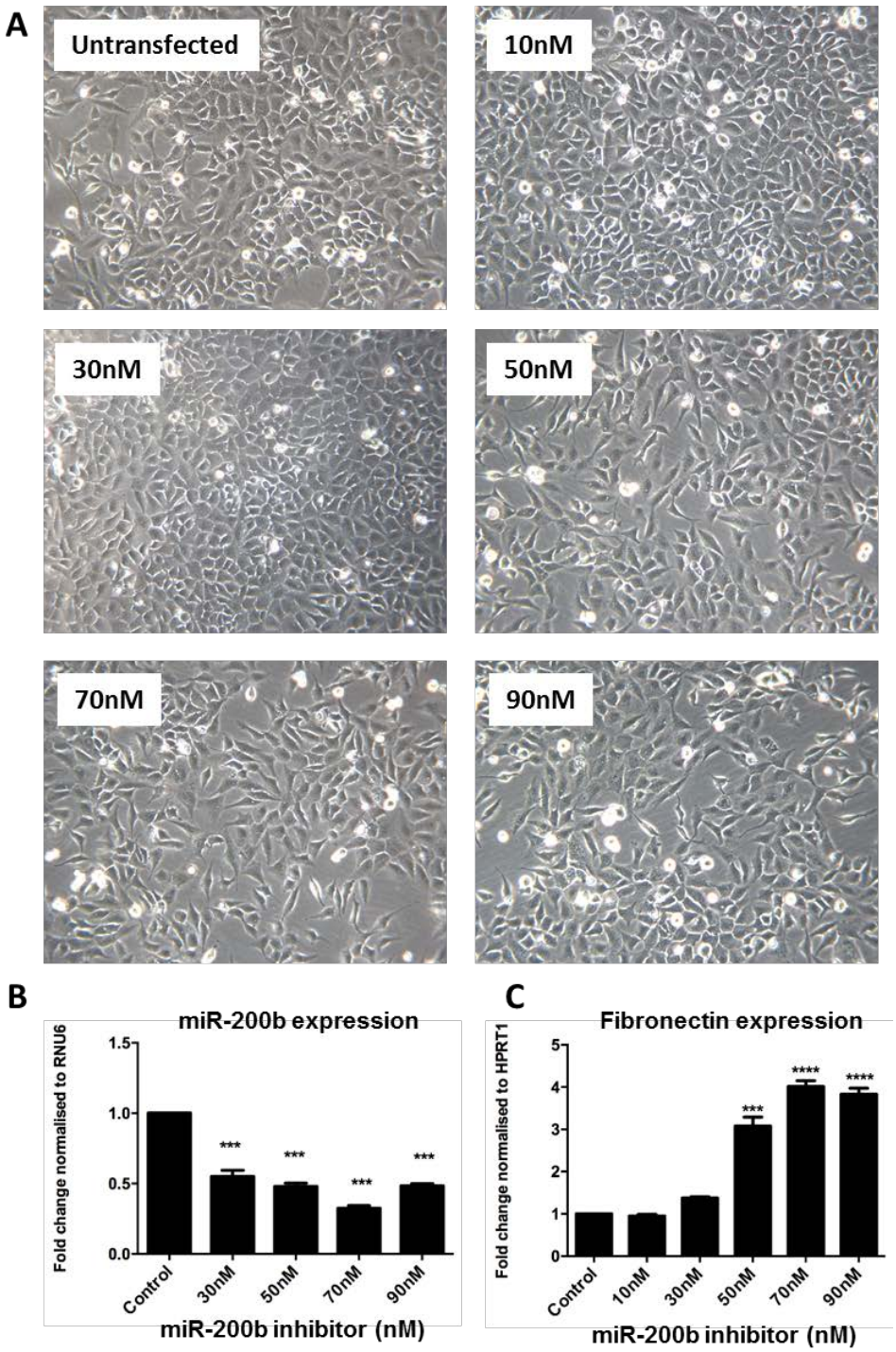

Figure 2.11: miR-200b inhibitor concentration optimization in BEAS-2B cells. BEAS-2B cells were transfected with 10nM, 30nM,50nM, 70nM and 90nM miR-200b inhibitors for 24 hrs. RNA was isolated followed by cDNA synthesis and q-RT PCR was performed. Panel A shows phenotypic changes post transfection with varying concentration of miR-200b inhibitors. miR-200b gene expression (B) and corresponding fibronectin expression (C) profile was evaluated and plotted. The data was analysed by one way ANOVA followed by Bonferroni test (***=p≤0.001) compared to the control. The data is representative of two independent set of experiments, each done in triplicate.

2.8.4 TGF-β1 treatment and miRNA transient transfection

Cells were cultured to reach 60% confluency and then transfected with 30nM miRNA using Lipofectamine transfection reagents in Opti-MEM medium as described in Table 4.2. A 30nM nonspecific miRNA (Qiagen, NSmiRNA) was used as a negative control for the transfection experiments. Cells were transfected for 24 hrs followed by treatment with 5ng/ml TGF-β1 for 48 hours.

Procedure details		
Components	8 well chamber slide	6 well
Adherent cells	$4x10^4$ cells	$5x10^5$ cells
Opti-MEM medium	32 µl	144 µl
Lipofectamine® RNAiMax	1 µl	6 µl
Opti-MEM medium	32 µl	145.5 µl
miRNA mimic/inhibitor (30nM concentration/well)	3 µl Stock miRNA conc: 1µM	4.5 µl Stock miRNA conc: 10 µM
Incubate for 5 minutes at room temperature		
miRNA-lipid complexes/well	66 µl	300 µl
Total volume/well	100 µl	1500 µl

Table 2.8: Summary of transfection protocol (8 well-chamber slides & 6-well plate)

2.9 Statistical analysis

For statistical analysis of data, Prism 6.0 (Graph Pad software, San Diego USA) was used. Comparison between two groups was performed by unpaired Student's t-test. Comparison between more than two groups was performed using one way analysis of variance (ANOVA) or two way ANOVA followed by Bonferroni test as a post hoc test considering the significance at $p \leq 0.05$. In this study, * refers to $p \leq 0.05$, ** refers to $P \leq 0.01$, *** refers to $p \leq 0.001$ and **** refers to $p \leq 0.0001$. Densitometric analysis of western blotting data was performed using Alpha Imager software of Alpha Imager gel documentation system (Alpha innotech, USA).

Chapter 3: The effect of TGF-β1 stimulation in human lung epithelial cells

3.1 Introduction

EMT is a process by which epithelial cells lose their lineage specific characteristics and assume a mesenchymal phenotype. Although EMT is a vital part of foetal development, uncontrolled re-activation of this cellular process in adulthood may lead to tissue fibrosis/remodelling (Borthwick *et al.*, 2009). Pathological findings in lung fibrosis are a consequence of disturbances in physiological processes such as proliferation and apoptosis of fibroblasts, and accumulation and breakdown of ECM. Several possible origins of ECM producing mesenchymal cells have been previously described that include lung fibroblasts (resident and systemic) and differentiation of other circulating fibrocytes or monocytes (Bucala *et al.*, 1994; Hashimoto *et al.*, 2004; Postlethwaite *et al.*, 2004). The bronchial epithelium has been studied as a potential source of fibroblasts and myofibroblasts during the remodelling of airways. This bronchial remodelling is one of the main features of diseases including BOS (Pain *et al.*, 2014).

The crucial effector cell in pulmonary fibrosis is the myofibroblast, a differentiated fibroblast with contractile properties similar to smooth muscle cells. Myofibroblasts are characterised by the presence of α-SMA and minimal expression of epithelial cell characteristics such as E-Cadherin and cytokeratins. In addition to this, recent studies have implicated a cross-talk between damaged epithelial cells and lung myofibroblasts. This interplay imparts support to the process of pulmonary fibrosis, in which altered lung mesenchymal cells coupled with epithelial cell injury result in the accumulation of ECM and remodelling of the lung airways (Horowitz and Thannickal, 2006; Wynn, 2011). Studies have also shown that EMT derived myofibroblasts may

be the primary source of excess scar tissue obstructing the small airways as observed in BOS (Felton *et al.*, 2011).

One of the most common stimulators of EMT evaluated by researchers is excess of TGF-β1. This multifunctional cytokine is involved in cell cycling, apoptosis and cellular differentiation. In excess TGF-β has been shown to induce EMT in various tissues (Willis and Borok, 2007). Subsequently, *in vitro* experiments have shown that primary bronchial epithelial cells undergo EMT when stimulated with TGF-β1 (Borthwick *et al.*, 2009). Previous studies have also reported abnormally elevated levels of TGF-β1 in BOS patients and have proposed that elevated TGF-β1 aggravates BOS symptoms. Therefore, these findings support *in vitro* observation that increased levels of TGF-β1 leads to EMT (Ward *et al.*, 2005). Hence, understanding of ECM components and stimulators of ECM remodelling in pulmonary fibrosis is crucial for presenting novel therapeutic strategies against BOS.

In context, miRNAs have gained significant attention for their role as post-transcriptional regulators of gene expression (Foshay and Gallicano, 2007). In addition to transcription factors such as ZEB1 (Eger *et al.*, 2005) and ZEB2 (Comijn *et al.*, 2001), miR-205, miR-146a and the miR-200 family have emerged as new epithelial markers and repressors of EMT (Gregory *et al.*, 2008). In contrast, miR-21 is upregulated in myofibroblasts during lung fibrosis and promotes TGF-β signalling. Therefore, miRNAs may be attractive candidates for preclinical studies as anti-fibrotic treatment for fibrosis (Yamada *et al.*, 2013).

3.2 Specific aims

1. Optimizing TGF-β1 concentration required to induce EMT in lung epithelial cells.

2. Examining the expression of EMT markers in TGF-β1 treated A549 cells, BEAS-2B cells and PBECs at the protein level.

3. Evaluating the expression profile of selective miRNA candidates in TGF-β1 treated A549 cell line, BEAS-2B and PBECs (for various time points).

4. miRNA profiling using NanoString® nCounter miRNA expression assay kit to identify expression of novel miRNAs in TGF-β1 treated and control BEAS-2B cells.

3.3 Specific materials and methods

3.3.1 Cell viability and proliferation assay

BEAS-2B cells were cultured and grown in BEGM complete media, but for stress inducing studies, cells were incubated in resting media supplemented with ITS Liquid Media Supplement (Sigma Aldrich) to eliminate any chance of interference by media components present in complete medium and to synchronise cells in a non-dividing phase. A preliminary experiment was conducted to test BEAS-2B cells viability in resting media. Percentage cell viability at 12 hours and 36 hours was assessed in untreated control cells and 5ng/ml TGF-β1 treated cells (Figure 3.1, n=1).

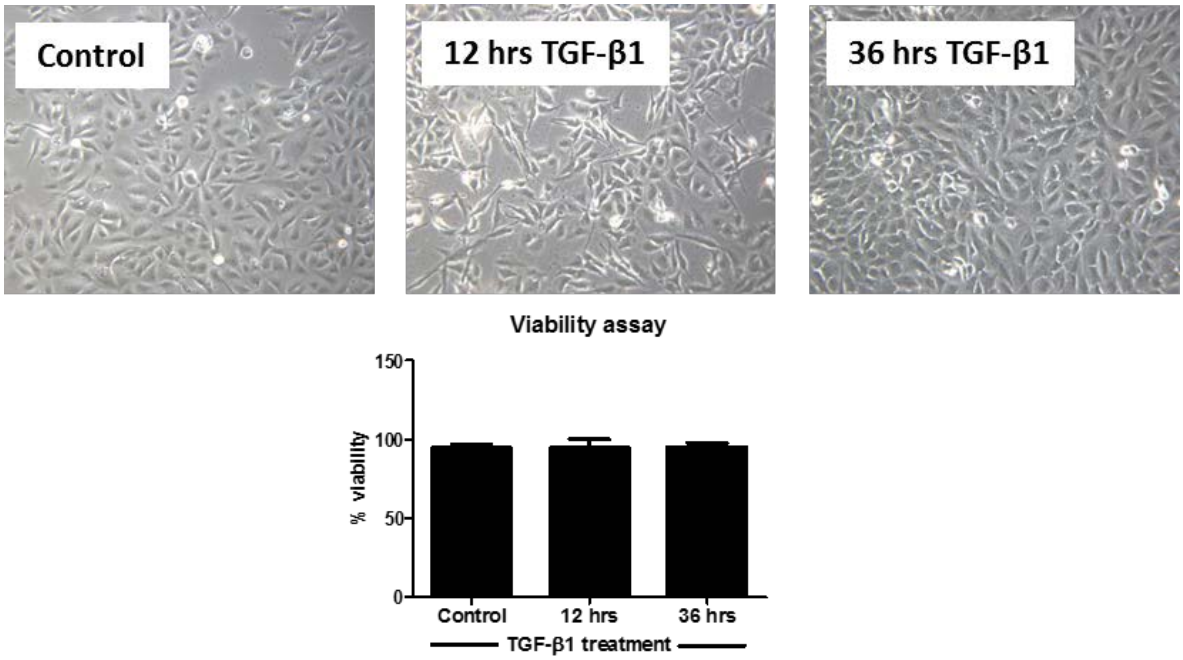

Figure 3.1: Percentage viability was assessed at 12 hours and 36 hours in control and 5ng/ml TGF-β1 treated cells. Data is representative of one experiment performed in triplicate.

A proliferation assay was conducted for duration of 4 days (96 hours). This study was performed to study the effect of 5ng/ml TGF-β1 on the cell number in incomplete media (BEBM supplemented with ITS). A549 and BEAS-2B cells were cultured (10,000 cells) in a 96 well plate at the start of the experiment in serum free media and the total cell number per well were counted every 24 hrs. Trypan blue staining was done to exclude dead cells that are permeable and can take up the dye. This study was performed to study the effect of TGF-β1 on the cell number in the absence of media components (Figure 3.2, n=1).

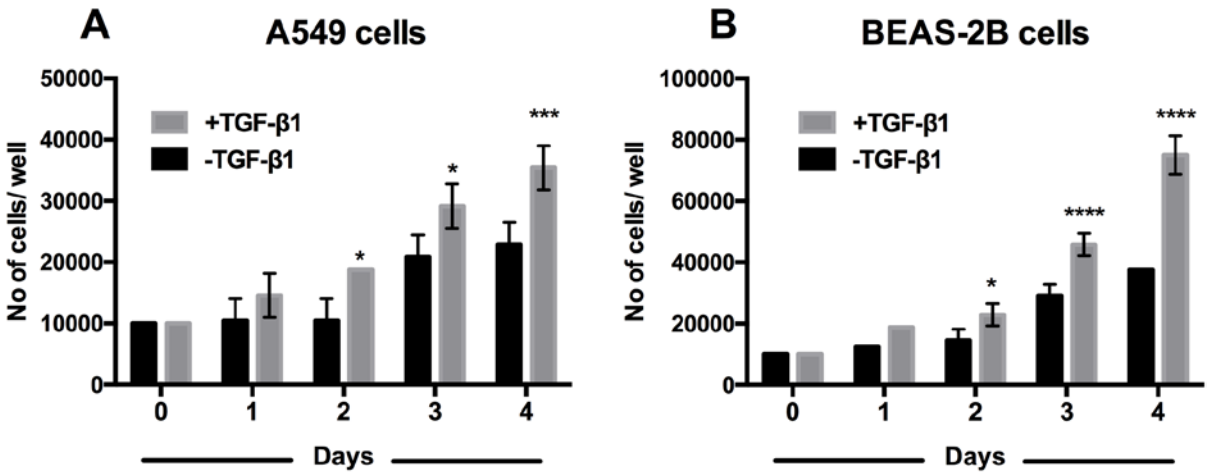

Figure 3.2: TGF-β (5ng/ml) positively regulates proliferation of A549 and BEAS-2B cells. A proliferation assay was conducted for 4 days (96 hours) for A549 and BEAS-2B cells in serum free media and resting media supplemented with ITS respectively in the presence and absence of TGF-β1. The data was analysed by two way ANOVA followed by Bonferroni test [(*=p≤0.05) (***=p≤0.001) (****=p≤0.001)] compared to TGF-β1 treated cells. The data is representative of one experiment done in triplicate.

3.3.2 NanoString technologies nCounter assay

3.3.2.1 Principle and overview

NanoString® nCounter technology is an alternative to traditional techniques such as q-RT-PCR and alternative approaches such as Affymetrix. This platform enables profiling individual miRNA/mRNA in a highly multiplexed reaction by assigning a unique fluorescently labelled probe to each entity. A computerised optical camera counts the probes that are bound to the target molecules.

Since miRNAs are short sequences of about 20-22 nt, the initial step involves miRNA ligation to unique tags for downstream detection. A bridge sequence partially complements to the miRNA and partially to the miRtag sequence assists in ligating the two. Thereafter excess of tag are washed away and bridge is enzymatically removed in a single tube reaction. NanoString nCounter technology relies on the use and detection of colour coded probe pair. Reporter probe carries the signal on the 5’

end and capture probe is attached with biotin at the 3' end. The coloured tag bound to biotin acts as a barcode that comprises of four colours in six positions and is indicative of a specific gene it refers to. After hybridisation, excess probes are washed away and the capture probes enable immobilisation of the complex on the nCounter cartridge surface for data collection. Thereafter the cartridge is placed in the nCounter digital analyser unit. The copies for each unique coded miRNA are counted. NanoString doesn't require any pre-amplification step and therefore there is no possibility of any amplification bias (Figure 3.3).

3.3.2.2 Data analysis

NanoString miRNA assay were performed using 100ng total RNA isolated from BEAS-2B cells for various time points (control, 1 hr, 4 hrs and 24 hrs post 5ng/ml TGF-β1 treatment). Hybridization reactions were conducted according to manufacturer's protocol followed with reading by NanoString nCounter analyser. The data was imported and normalized using nSolver software (provided by NanoString).

Raw data obtained was first normalized against negative control that helps confirm specificity of the ligation reaction and provide a means to estimate background hybridization counts. Background threshold is defined as the mean of all the negative probes. Once the background threshold is determined, it is possible to determine the true counts.

Second, positive control normalization allows assessment of sample preparation conditions such as annealing and ligation with their unique tags in the reaction tubes with their miRNAs. Data was normalized to internal positive controls that are independent of the sample and thus help in eliminating variability unrelated to the sample. Further normalization steps were also carried out as follows:

- Normalization to the internal mRNA controls: The miRNA code-set comprises of five highly expressed mRNA sequences namely ACTB, B2M, GAPDH, RPL19 and RPL0. These controls are used to confirm and normalize successful hybridization and variations in the sample input respectively.

- Normalization to highest expressed miRNAs in an assayed sample: Calculating the mean count of top 100 miRNAs with the highest counts generates a normalization factor. Thereafter the normalization factor is multiplied to the counts in each column.

The data was then exported from the nSolver software and further analysed and visualised using R studio software. R studio helps in reducing the variability between similar groups (such as control 1 and control 2, 1 hr-set 1 and 1 hr-set 2). Correlation plots (Figure 3.4), Volcano plots and heat maps can be generated using R-studio which gives a better understanding of the expression pattern of miRNAs across various time points.

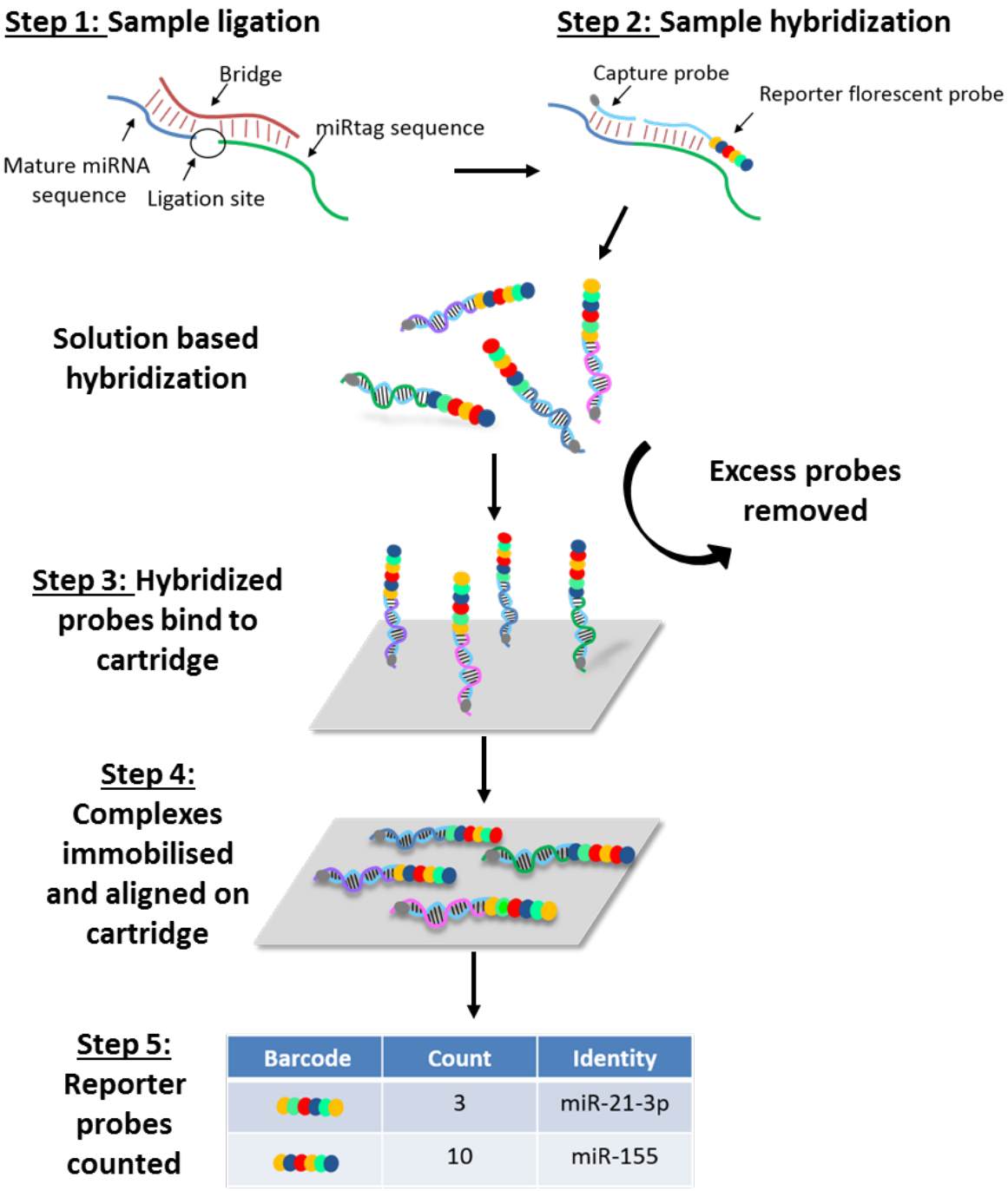

Figure 3.3: Schematic representation of NanoString® nCounter miRNA assay.

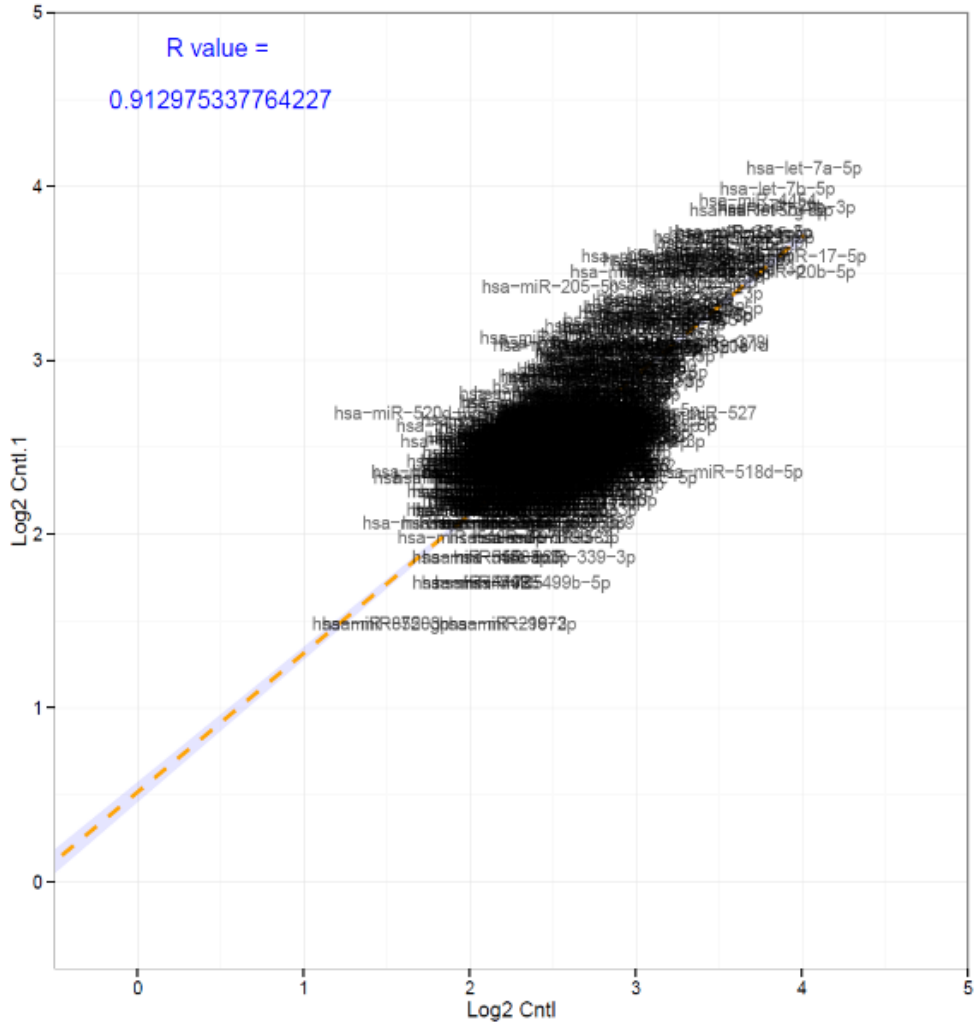

Figure 3.4: Scatterplot of log control ratio. The plot depicts a strong positive association (r value= 0.91) between control 1 and control 2 data sets. This suggests that the expression of miRNAs in control 1 and control 2 data sets are similar and there is acceptable variability of gene expression across the two control wells used in the NanoString nCounter assay.

3.4 Results

3.4.1 TGF-β1 induces EMT in a concentration dependent manner in A549 cells

To determine the optimum concentration required for TGF-β1 to initiate EMT in cell culture, A549 cells were stimulated with varying concentration of TGF-β1 (0.5ng/ml, 1ng/ml, 2ng/ml, 5ng/ml and 10ng/ml) for 72 hrs. Treatment with 2ng/ml ($p \leq 0.05$), 5ng/ml ($p \leq 0.001$) and 10ng/ml ($p \leq 0.01$) TGF-β1 significantly reduced the expression

of E-cadherin at RNA level. 5ng/ml of TGF-β1 stimulation showed maximum reduction in E-Cadherin marker (Figure 3.5, n=2). The expression was normalised using endogenous control gene HPRT1 and compared to the untreated control. Subsequently, phenotypic changes in cell morphology were examined (Figure 3.6). A549 cells acquired mesenchymal like phenotype and displayed reduced cell-cell contact and an elongated morphology when treated with 5ng/ml TGF-β1 for 72 hours while untreated cells retained a characteristic epithelial, “cobblestone/pebble” morphology. Thus, 5ng/ml of TGF-β1 was used in further experiments to induce EMT. These results suggest that lung epithelial cells demonstrate loss of epithelial cell markers and transition into a mesenchymal like phenotype upon TGF-β1 treatment leading to deposition of matrix proteins.

3.4.2 TGF-β1 induces EMT in A549, BEAS-2B cells and PBECs

Changes at protein expression were examined using immunofluorescence and western blotting to further comprehend changes in EMT markers. A549 is a cancer cell line and may not reflect the miRNA profile exhibited by normal alveolar epithelial cells. Moreover studies have indicated abnormal miRNA expression in normal A549 cell line (Kasai *et al.*, 2005a). Also since BOS is an airway disease, it is crucial to assess the relevance of observations by replicating the study using bronchial epithelial cells. Therefore, although initial study was performed using A549 cell line, further studies involved use of only BEAS-2B cells and PBECs.

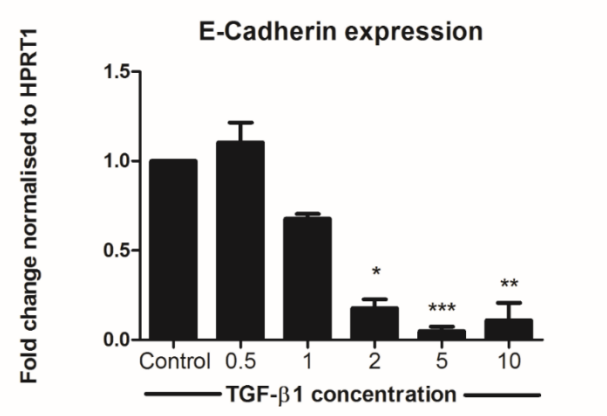

Figure 3.5: E-cadherin expression changes in TGF-β1 stimulated A549 cell line. A549 cells were incubated with upto 10ng/ml of TGF-β1 in serum free media for 72 hours. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (***=p≤0.001)] compared to control. The data is representative of two independent set of experiments, each done in triplicate.

Figure 3.6: TGF-β1 treatment induces morphological changes in the A549 cell line. A549 cells were incubated with varying concentration of TGF-β1 in serum free media for 72 hours. Untreated cells (control) displayed pebble shaped morphology while 5ng/ml TGF-β1 stimulated cells showed reduced cell-cell contact and acquired fibrotic phenotype. Images were captured using a bright field microscope (X20).

3.4.2.1 Immunofluorescence

Cell viability and proliferation study was performed prior to conducting experiments at RNA and protein level. No change in viability between control and BEAS-2B treated cells was observed upto 36 hrs in resting or incomplete media (Figure 3.1). Furthermore, proliferation assay in TGF-β1 treated A549 (A) and BEAS-2B (B) cells

suggested a significant increase in cell number at 48 hrs ($p \leq 0.05$, $p \leq 0.05$) 72 hrs ($p \leq 0.05$, $p \leq 0.0001$) and 96 hrs ($p \leq 0.001$, $p \leq 0.0001$) time point as compared to their respective untreated controls (Figure 3.2).

A significant reduction in epithelial cell markers E-Cadherin and cytokeratin 19 was observed at 72 hrs after TGF-β1 treatment in A549 cells (both $p \leq 0.0001$; Figure 3.7, n=3), BEAS-2B cells ($p \leq 0.001$ and $p \leq 0.01$; Figure 3.8, n=3) and PBECs (both $p \leq 0.001$; Figure 3.9, n=3). Subsequently, there was significant increase in the expression of mesenchymal markers fibronectin and α-SMA post TGF-β1 treatment in A549 cells (both $p \leq 0.0001$), BEAS-2B cells ($p \leq 0.001$ and $p \leq 0.01$) and PBECs (both $p \leq 0.001$).

3.4.2.2 Western blotting

Western Blot was performed to examine and confirm the protein expression pattern obtained from immunofluorescence results. Results showed an increase in the mesenchymal cell markers Fibronectin and α-SMA while a reduction in the expression of E-Cadherin and cytokeratin 19 post TGF-β1 treatment when compared to the untreated A549 and BEAS-2B cells respectively. The PVDF membranes were stripped and reprobed for housekeeping gene, GAPDH. Densitometric analysis was performed using Alpha Imager gel documentation system and a ratio of relative expression of target protein and relative expression of GAPDH was plotted (Alpha innotech, USA) (Figure 3.10). This confirmed that TGF-β1 induced increased expression of mesenchymal cell surface markers while reducing epithelial markers at protein level.

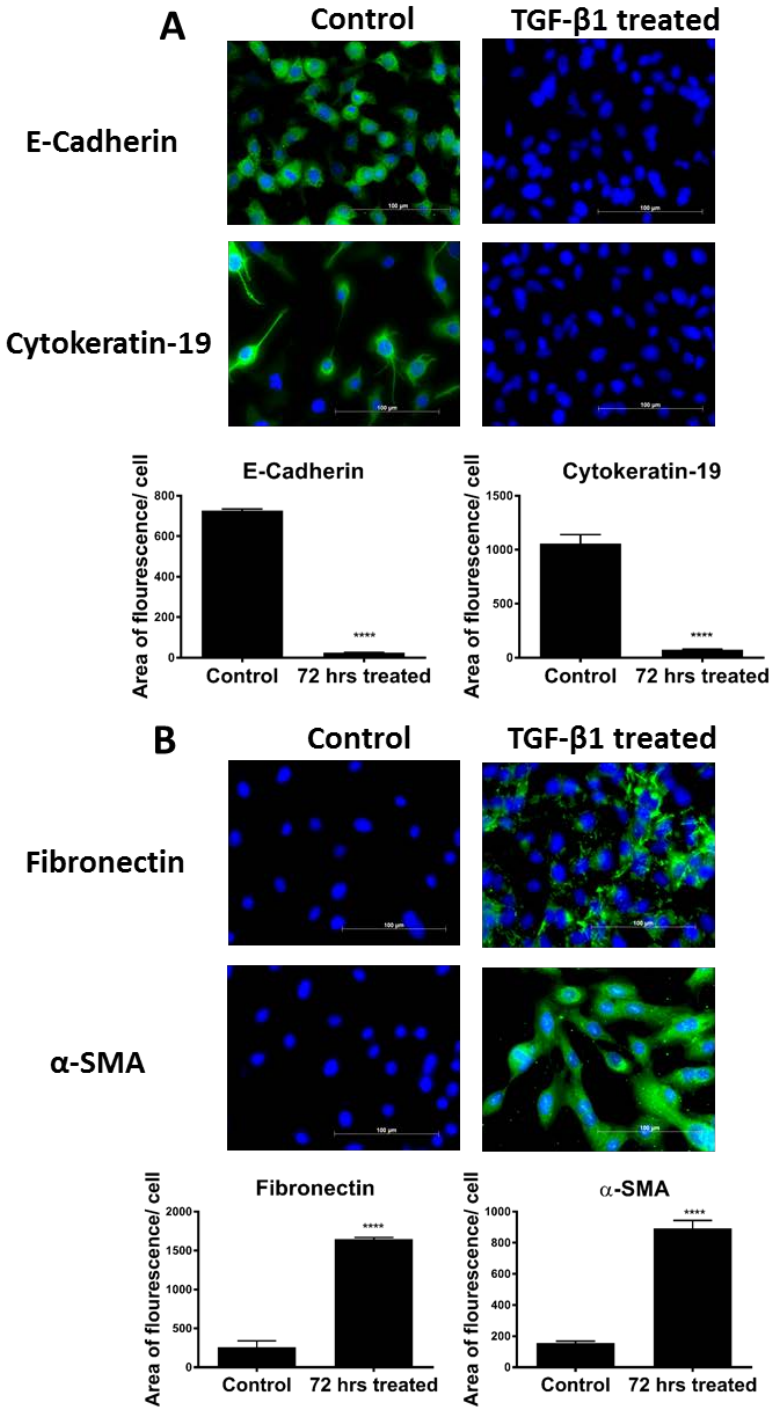

Figure 3.7: Immunofluorescence of TGF-β1 treated A549 cells. Serum starved cells were treated with 5ng/ml of TGF-β1 for 72 hours in 8 well chamber slides followed by methanol fixing. Slides were incubated with primary and secondary antibody for 24 hrs each following which DAPI staining was done. Mounting medium was used and cover slips were placed onto the chamber slides. The cells were visualised and pictures were captured under a Zeiss microscope at 40X magnification .The area of florescence per cell was calculated using Image J software and graphs were made in Prism 6. The data was analysed by unpaired t-test (****=p≤0.0001). The statistical data is representative of three set of independent experiments, each done in triplicate. Scale bar, 100µm

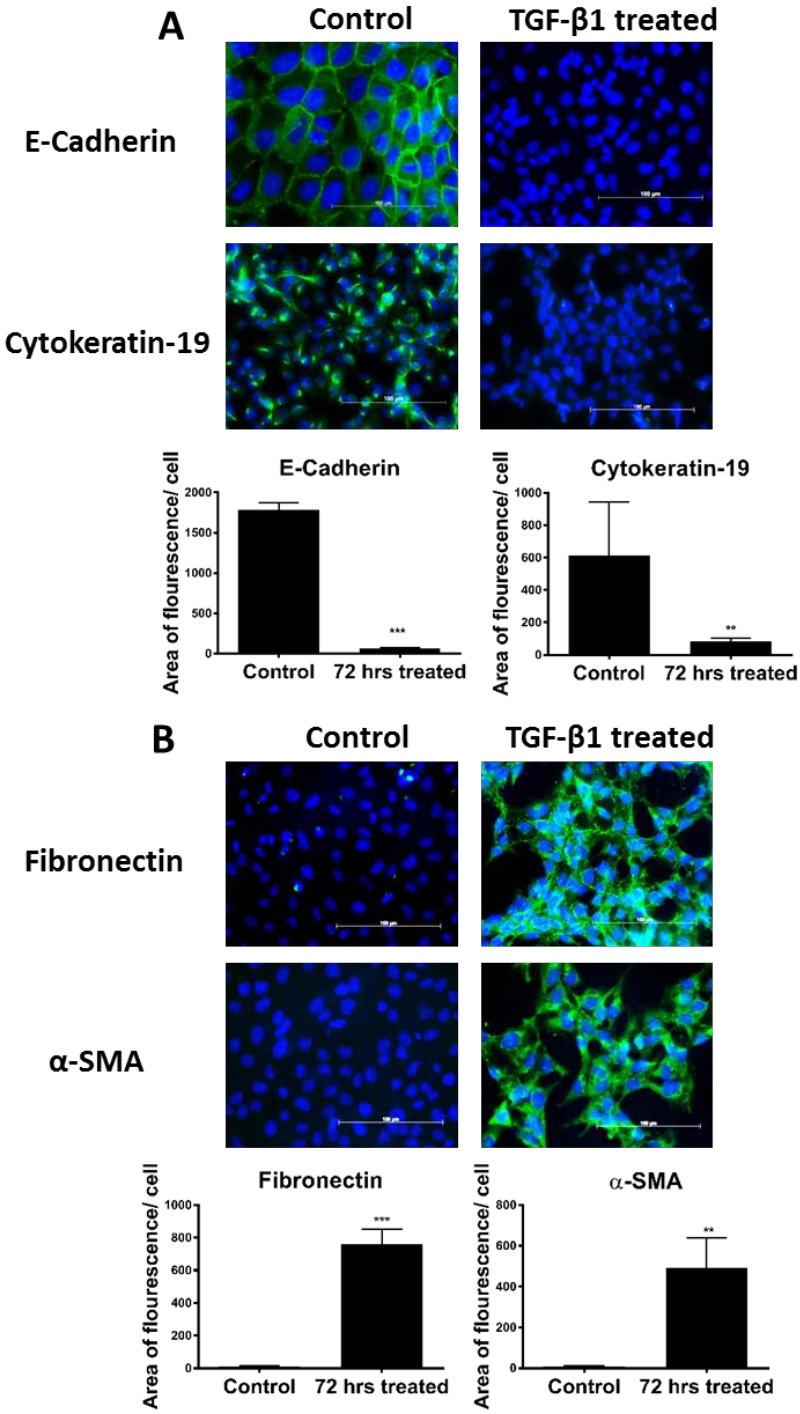

Figure 3.8: Immunofluorescence of TGF-β1 treated BEAS-2B cells. Serum starved cells were treated with 5ng/ml of TGF-β1 for 72 hours in 8 well chamber slides followed by methanol fixing. Slides were incubated with primary and secondary antibody for 24 hrs each following which DAPI staining was done. Mounting medium was used and cover slips were placed onto the chamber slides. The cells were visualised and pictures were captured under a Zeiss microscope at 40X magnification. The area of florescence per cell was calculated using Image J software and graphs were made in Prism 6. The data was analysed by unpaired t-test [(**=p≤0.01) (***=p≤0.001)]. The statistical data is representative of three set of independent experiments, each done in triplicate. Scale bar, 100μm

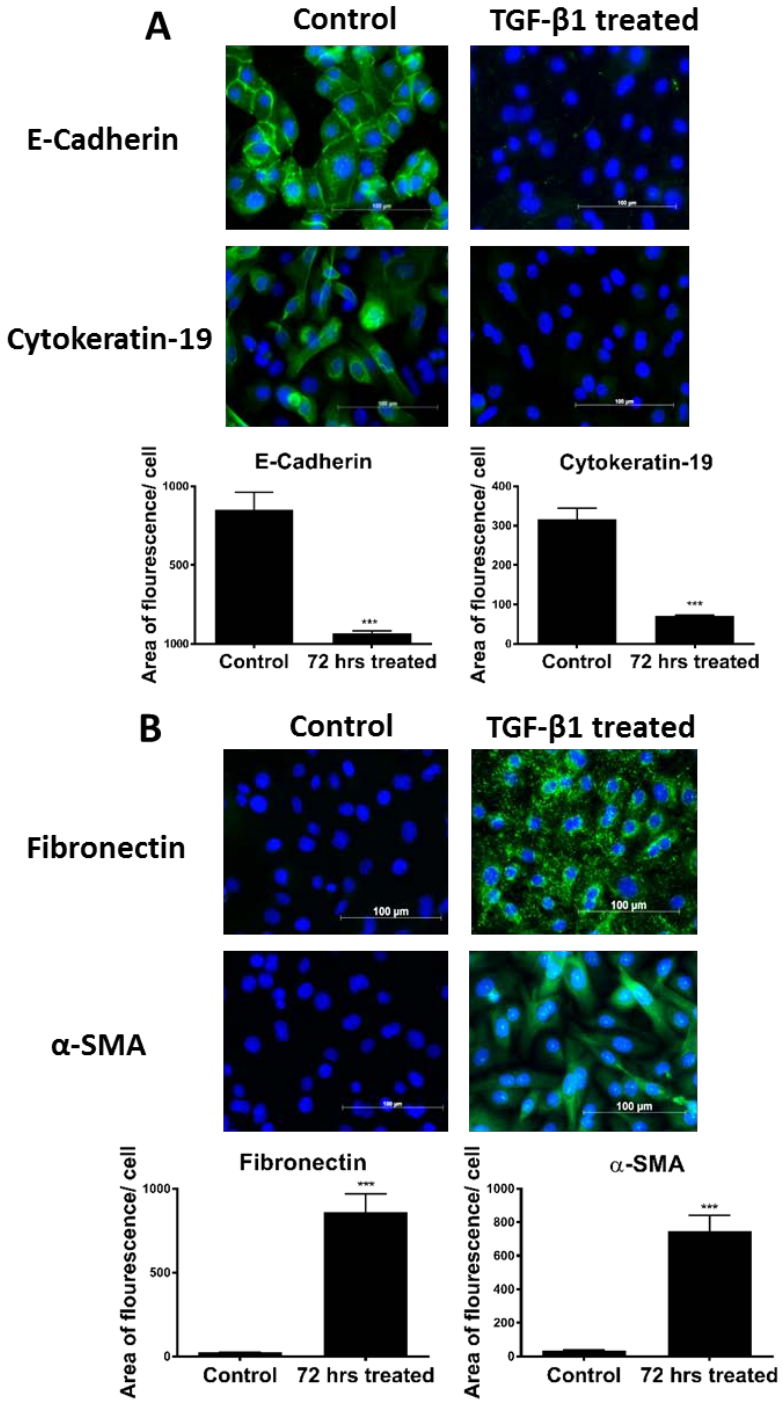

Figure 3.9: Immunofluorescence of TGF-β1 treated PBECs. Serum starved cells were treated with 5ng/ml of TGF-β1 for 72 hours in 8 well chamber slides followed by methanol fixing. Slides were incubated with primary and secondary antibody for 24 hrs each following which DAPI staining was done. Mounting medium was used and cover slips were placed onto the chamber slides. The cells were visualised and pictures were captured under a Zeiss microscope at 40X magnification. The area of florescence per cell was calculated using Image J software and graphs were made in Prism 6. The data was analysed by unpaired t-test (***=p≤0.001). The statistical data is representative of three set of independent experiments, each done in triplicate. Scale bar, 100µm

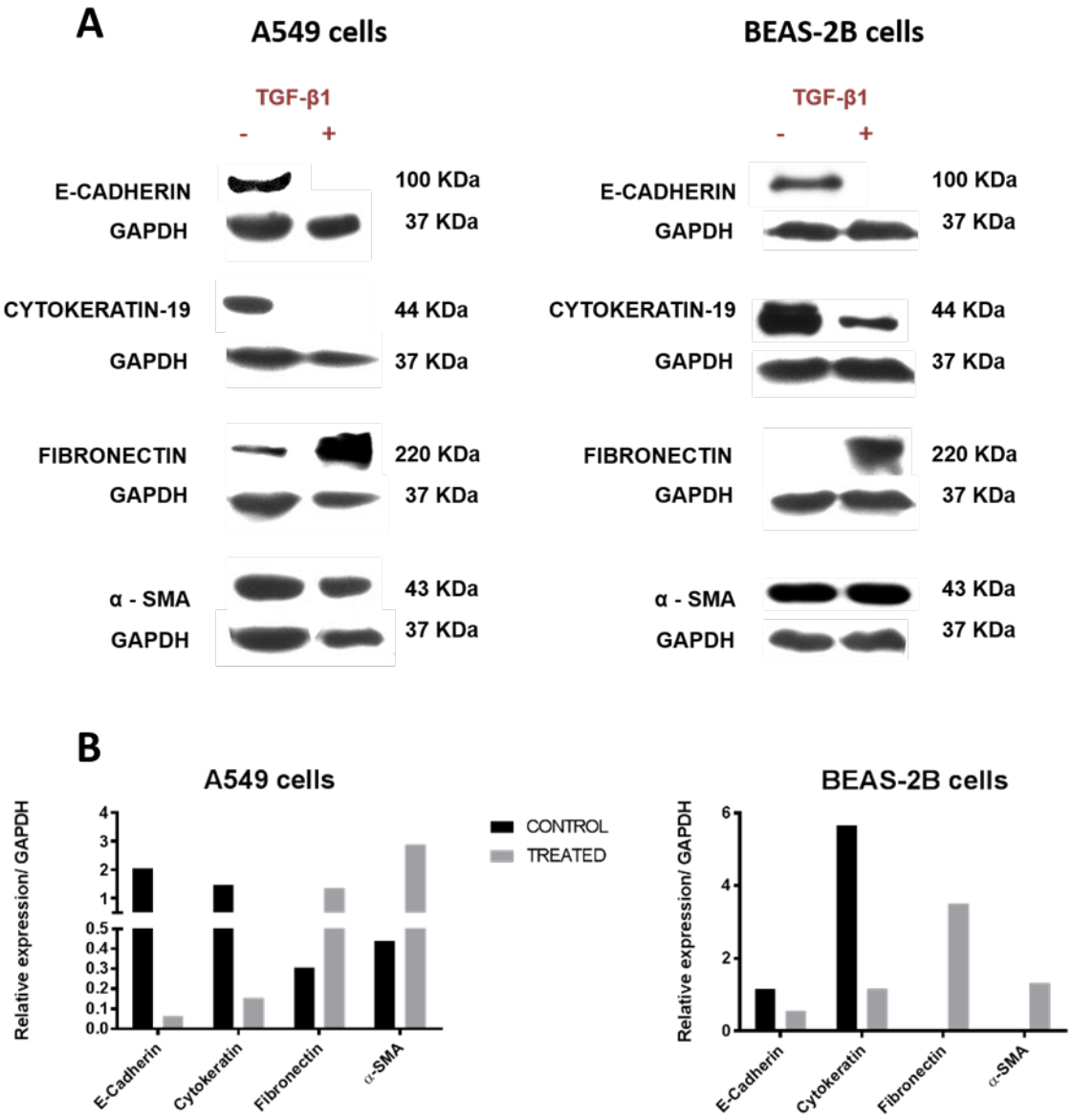

Figure 3.10: Western Blot and densitometry analysis for A549 and BEAS-2B cells. Serum starved cells were treated with 5ng/ml of TGF-β1 for 72 hours followed by cell lysis and western blot. The membrane was probed with various antibodies. The same membrane was stripped and reprobed for GAPDH (A; endogenous control). Densitometric analysis was performed using Alpha Imager software and plotted using Prism 6 (B).

3.4.3 miRNA expression profile in TGF-β1 stimulated versus untreated A549 and PBECS

Expression levels of selected miRNAs- miR-146a, miR-34a, miR-21 and miR-200b involved in fibrosis and inflammation were analysed initially in TGF-β1 stimulated

A549 cells and then in treated PBECs. An initial study was performed using A549 cells at 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs and 24 hrs post TGF-β1 treatment in order to reveal time points suggesting significant changes in miRNA expression (n=2; Figure 3.11). All miRNA experiments were normalized to an endogenous control U6 and compared to untreated samples.

Further study was performed using PBECs at 1 hr, 2 hrs and 24 hrs post TGF-β1 stimulation. miR-21 expression (a marker of fibrosis) significantly peaked at 1 hr ($p \leq 0.05$) but was downregulated at 2 hrs and 24 hrs (both $p \leq 0.001$) post treatment. While there was no significant change observed in the expression profile of miR-34a, miR-200b that is otherwise well expressed in healthy epithelial cells was downregulated significantly at 1 hr ($p \leq 0.05$), 2hrs ($p \leq 0.001$) and 24 hrs ($p \leq 0.05$) post treatment. This correlated well with changes in EMT marker expression studied previously at RNA and protein level since reduction in miR-200b expression has been found to be associated with fibrosis (Yang *et al.*, 2012a). Lastly, the expression of miR-146a involved in inflammation showed an increased trend of expression and was significantly upregulated at 2 hrs and 24 hrs post TGF-β1 stimulation (n=1; Figure 3.12).

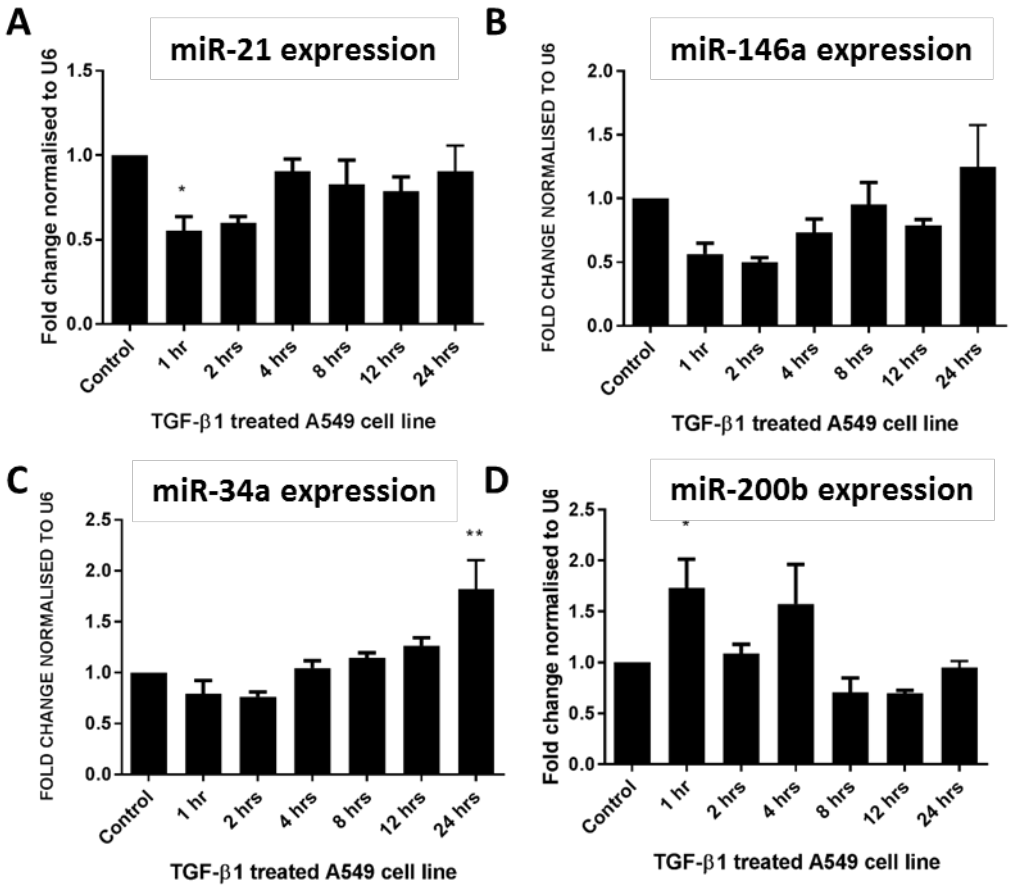

Figure 3.11: Studying miRNA expression in response to TGF-β1 stimuation at various time points in A549 cell line. The graphs show analysis of (A) miR-21, (B) miR-146a, (C) miR-34a and (D) miR-200b expression at 1hrs, 2hrs,4hrs,8hrs,12hrs and 24hrs post TGF-β1 treatment for two independent experiments normalised to U6 and expression levels compared to untreated control. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01)] compared to the control. The data is representative of two independent set of experiments, each done in triplicate.

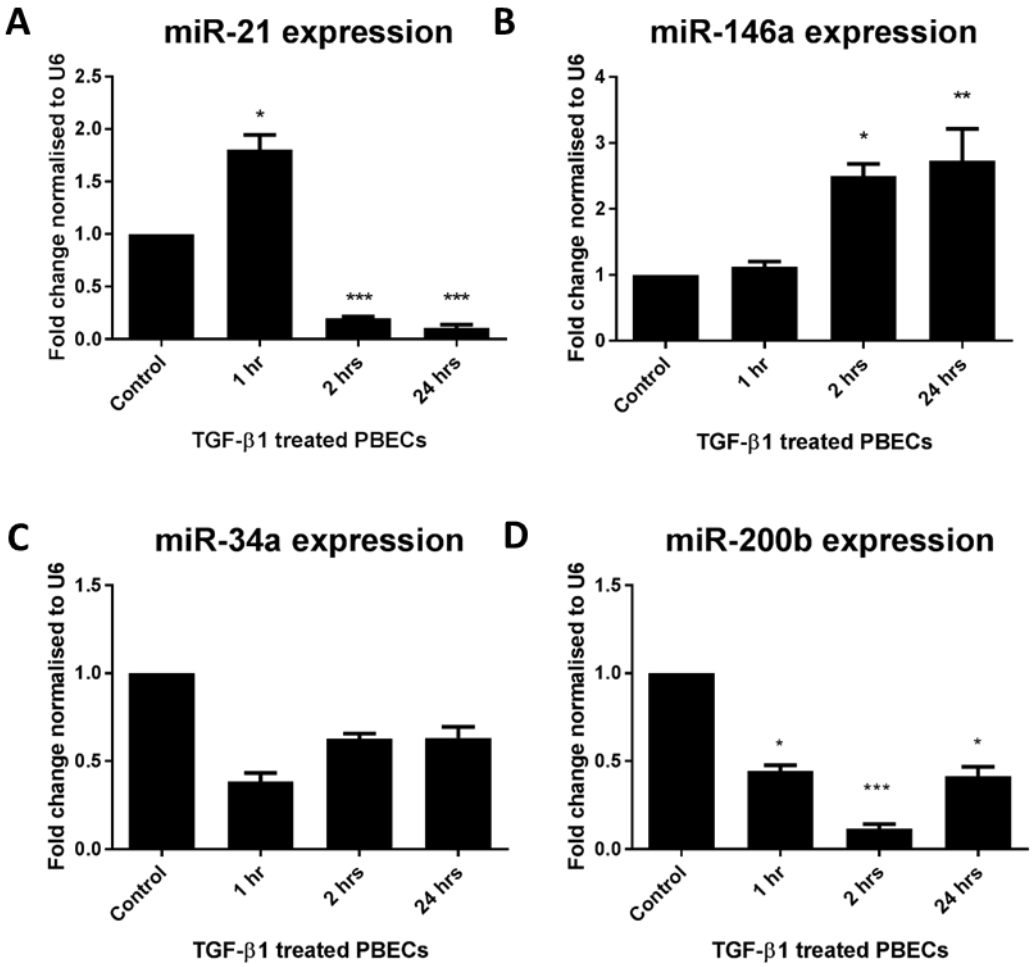

Figure 3.12: miRNA profiling in PBECs following TGF-β1 stimuation . The graphs show analysis of miR-21, miR-146a, miR-34a and miR-200b expression at 1hrs, 2hrs and 24hrs post TGF-β1 treatment normalised to U6 and expression levels compared to untreated control. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001)] compared to the control. The data is representative of one experiment done in triplicate.

3.4.4 miR-200b expression profile in TGF-β1 stimulated versus untreated in BEAS-2B cells

Since A549 cells display aberrant expression of miRNAs that are normally expressed in low levels in healthy epithelium, further studies were performed using BEAS-2B cell line that resemble cells of the airway epithelium (Jeong *et al.*, 2011; He *et al.*, 2012). The expression of miR-200b was also studied in control (untreated cells) and TGF-β1 stimulated BEAS-2B cells (n=3, Figure 3.13). BEAS-2B cell line

morphologically mimics the bronchial epithelium of the respiratory tract and represented a second best option after PBECs that were not available at all times. MiR-200b was significantly downregulated at all-time points post treatment (p≤0.001). Similar trend of miR-200b was noted in PBECs (section 3.4.3) suggesting a role of miR-200b in TGF-β1 induced EMT in bronchial epithelial cells.

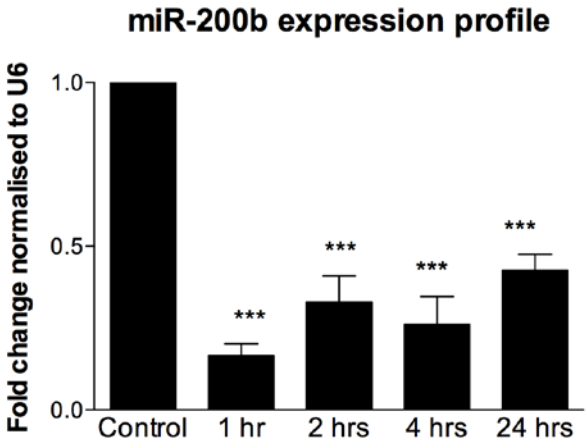

Figure 3.13: TGF-β1 induced downregulation of miR-200b in BEAS-2B cells. Graph shows analysis of miR-200b at 1 hr, 2 hrs 4 hrs and 24 hrs post treatment with 5ng/ml TGF-β1 normalized to U6 and expression levels compared to untreated control. The data was analysed by one way ANOVA followed by Bonferroni test (***=p≤0.001) compared to the control. The data is representative of three independent set of experiments, each done in triplicate.

3.4.5 MiRNA profiling using NanoString® nCounter miRNA expression assay in BEAS-2B cells and miRNA target identification.

To investigate the role of miRNAs in BOS, miRNA profiling was performed using Nanostring technology (n=2). Hierarchical clustering (Figure 3.14A) of 130 most differently expressed miRNAs (control versus various time points) was determined using R (version 3.1.3) and a heatmap was generated (using R; heatmap function). The differential expression of miR-200 family (miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-141-3p and miR-429) between the control and TGF-β1 treated samples was found interesting due to their key role in regulating EMT (Figure 3.14B). MiR-200b-3p

and miR-200c-3p were downregulated at all-time points post TGF-β1 treatment. Expression profile of miR-200b was previously studied in TGF-β1 treated BEAS-2B cells at various time points (Figure 3.13). NanoString authenticated the expression trend of this miRNA candidate. The expression profile was further validated using q-RT-PCR (n=2, Figure 3.14C) that also suggested a significant decrease at 4 hrs ($p<0.05$) and 24 hrs ($p<0.001$) in response to TGF-β1 treatment (n=2). Results suggested a significant decrease in miR-200b-3p expression upon TGF-β1 treatment, which was also associated with loss in epithelial cell markers.

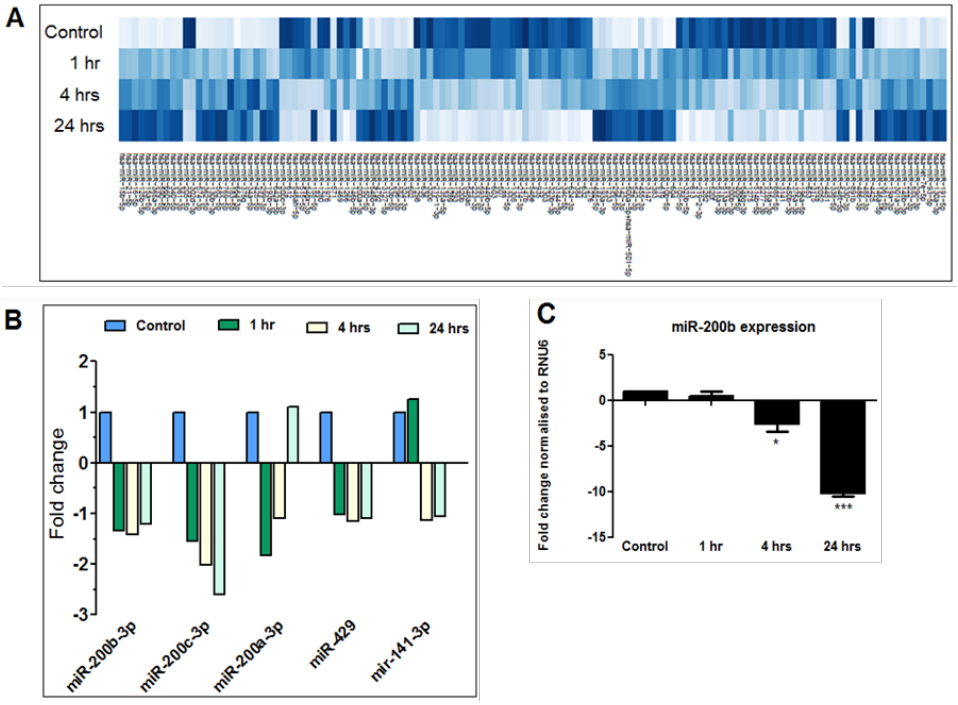

Figure 3.14: miRNA profiling using Nanostring nCounter assay. BEAS-2B cells were treated with 5ng/ml TGF-β1 for 1 hr, 4 and 24 hrs and total RNA was isolated. Each sample was then assayed for expression of miRNA using Nanostring nCounter assay. A heatmap (A) was generated demonstrating differential expression of miRNAs in all samples where an increased intensity of blue signifies increased expression while (B) is expression profile of miR-200 family. For NanoString data, two independent set of experiments were performed, each done once. The expression profile of miR-200b was validated by q-RT-PCR and data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (***=p≤0.001)] compared to the control. This data is representative of two independent experiment, each done in triplicate (C).

3.5 Discussion

This project was an attempt to challenge cells via TGF-β1 stimulation and study the expression levels of selective miRNAs thought to be involved in early and late lung allograft injury. This was done with a view to elucidating candidates that may be biomarkers for early prediction of graft rejection and which may represent novel targets of therapeutic modulation.

An optimum concentration of TGF-β1 (5ng/ml) was determined to stimulate A549 cells and BEAS-2B cells. Published studies also suggest that 5ng/ml of TGF-β1 induces morphological changes in BEAS-2B cells (Doerner and Zuraw, 2009a). Viability and proliferation studies were conducted to ensure there was no significant cell death in control and test sample as cells were incubated in serum free medium throughout the treatment. This allowed studying the effect of TGF-β1 alone without the confounding effects associated with active cell proliferation driven by the components of complete medium.

To confirm the pro-fibrotic effect of TGF-β1 in airway epithelial cells, immunofluorescence and western blot studies were performed using A549 cells, BEAS-2B cells and PBECs. An increase in mesenchymal markers α-SMA and fibronectin and significant reduction in E-cadherin and cytokeratin-19 clearly indicated TGF-β1 induced EMT. This is supported by studies that have demonstrated that alveolar epithelial cells and bronchial epithelial cells undergo EMT as evidenced by loss of epithelial cell markers cytokeratins and ZO-1 and upregulation of mesenchymal markers α-SMA, vimentin and type 1 collagen (Willis *et al.*, 2005; Doerner and Zuraw, 2009a). Post lung transplantation, epithelial cell damage and EMT occurs because of initial graft injury due to alloimmune and non-alloimmune factors. In response to injury and inflammation, epithelial cell repair activates several

downstream fibrotic markers that lead to deposition of extracellular matrix that eventually obstructs the small airways leading to BOS (Martin, 1999; Meloni *et al.*, 2004; Deslee *et al.*, 2007). TGF-β1 signalling controls various cellular processes that triggers downstream activation of Smad proteins that regulate the transcription of various genes. Smad signalling allows TGF-β1 induced protein expression leading to upregulation of α-SMA, collagen and vimentin and reduction in epithelial cell markers, E-Cadherin and ZO-1(Xie *et al.*, 2004).

MiRNA studies were conducted by stimulating A549 cells for 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs and 24 hrs with 5ng/ml TGF-β1. Recent studies have shown rapid changes in significantly important miRNA candidates as early as 24 hrs to 72 hrs post transplantation. The literature has shown that changes in selective circulating miRNA had strong co-relation to early myocardial injury after heart transplantation (Nazarov *et al.*, 2013; Wang *et al.*, 2013). A similar study investigated the role of selective miRNAs in early ischemia and further confirmed the results in a neuroblastoma cell line subjected to ischaemic insult (Dhiraj *et al.*, 2013). These studies suggest the importance of investigating miRNA expression profile for the earlier time points.

Studies have indicated that the A549 cell line exhibits aberrant expression of miRNA involved in cell cycle progression and proliferation. MiR-200b, miR-21 (downregulates tumor suppressor genes), miR-182, miR-375 expression were found to be overexpressed in lung adenocarcinoma specimens when compared to samples from healthy individuals. However, tumor suppressor miRNAs- miR-145, miR-126 and miR-486 were expressed in low levels in tumor samples (Yu *et al.*, 2010; Boeri *et al.*, 2011; Peng *et al.*, 2013). This variability restricts studying the expression of miRNAs in the A549 cell line. However, studies have showed that the normal human bronchial epithelial cells express miR-200b, miR-200c, miR-34a and other miRNAs

involved in the regulation of the cell cycle, maintaining epithelial framework and cell proliferation while miR-155 that has a role in inflammation is expressed in very low levels (Martinez-Anton *et al.*, 2013; Guz *et al.*, 2014). Therefore, further miRNA studies were conducted using cell line (BEAS-2B) and cells (PBECs) derived from human bronchial epithelial cells.

Since miRNA-200b expression was significantly downregulated in TGF-β1 stimulated A549 cell line, its expression profile was further investigated in PBECs and BEAS-2B cells. The results indicated significant downregulation in miR-200b expression in PBECs that could be correlated to changes in EMT marker expression studied previously. MiR-200b has been reported as maintaining the epithelial framework and is only expressed in cells exhibiting epithelial markers and has also been shown to reverse EMT (Park *et al.*, 2008; Lim *et al.*, 2013). In BEAS-2B cells, miR-200b expression significantly reduced with increasing TGF-β1 stimulation time as observed in PBECs. These result were consistent with data from a study performed in kidney proximal tubular epithelial cells (Xiong *et al.*, 2012a).

NanoString has not only been used to validate results of other platforms such as RNA sequencing (Sabo *et al.*, 2014) but also is the favoured technique over microarray and NGS (Chatterjee *et al.*, 2015). A higher number of miRNAs have been detected with high stringency and technical replicates are not required as excellent reproducibility has been previously demonstrated when using NanoString nCounter platform (Balko *et al.*, 2012). Therefore, in my study TGF-β1 stimulated BEAS-2B cells were subjected to miRNA screening using Nanostring nCounter assay. The results showed the differential expression of the miR-200 family between the control and TGF-β1 treated samples occurring during EMT. It is of interest that in limited previous literature the miR-200 family has also been described as regulating

EMT in kidney studies and cancer progression (Sun *et al.*, 2012). Thus, these results suggested the need to investigate the potential role of miR-200b in BOS by manipulating its expression in order to study its effect on TGF-β1 treated cells.

Chapter 4: Role of miRNA-200b in repressing TGF-β1 induced EMT in BEAS-2B and primary bronchial epithelial cells

4.1 Introduction

Dysregulated expression of miRNA is increasingly implicated in various airway diseases and may play an important role in fibrosis; however, a role in BOS is not established. MiRNAs are small non coding RNAs wherein the seed region (2-8 bases) of the miRNA predominately targets the 3' untranslated region (3' UTR) of mRNA leading to degradation, or inhibition of translation (Agarwal *et al.*, 2015; Lin and Gregory, 2015). Since a single miRNA has the potential of targeting more than one mRNA target, they have a suggested role in modulating multiple biological pathways. This induces modulating inflammation (Lv *et al.*, 2016), amplifying the inflammatory microenvironment (McDonald *et al.*, 2016) and predisposing epithelial cells to undergo EMT that leads to extracellular matrix (ECM) deposition and fibrosis (Suwara *et al.*, 2014; Jonas and Izaurralde, 2015; Xu *et al.*, 2015a).

TGF-β1 is a potent inducer of EMT and the SMAD signalling pathway components governs its downstream effects. Once activated the SMAD complex translocates into the nucleus and activates genes involved in fibrosis. MiR-200b plays an important role in regulating EMT in renal proximal cells by preventing renal fibrosis. Experiments confirmed the effect of miR-200b on TGF-β1 induced EMT by transfecting HK2 cells with miR-200b mimics prior to exposing cells to TGF-β1 treatment. Results suggested restoration of TGF-β1 induced downregulation of E-Cadherin levels when miR-200b mimics were added to cell culture. Furthermore, miR-200b also suppressed the expression of fibronectin suggesting a potential reversal of EMT mediated fibrosis (Martinez-Anton *et al.*, 2013; Tang *et al.*, 2013; Saikumar *et al.*, 2014). In 2008, pioneering work achieved by Gregory *et al*

demonstrated miR-200b as a key candidate in regulating EMT by targeting ZEB1 and ZEB2 transcription factors in an immortalised model of distal tubular cells (Gregory *et al.*, 2011). Subsequently miR-200b was shown to prevent TGF-β1 induced ECM production in *in vitro* models of rat tubular epithelial cells (Xiong *et al.*, 2012a). These findings suggest that miR-200b is an important candidate involved in fibrosis but its role in preventing airway/lung injury and molecular mechanisms involved remain unclear.

4.2 Specific aims

1. Determine whether manipulating the concentration of miR-200b (using mimics and inhibitors) leads to a change in expression of EMT markers in BEAS-2B cells and PBECs.

2. Examine the expression of specific predicted miR-200b target genes in BEAS-2B cells, PBECs and patient cells derived post lung transplant.

3. Study the expression of miR-200b target genes that are transcription factors involved in TGF-β signalling.

4. Validate the miR-200b direct targets- ZEB2 and ZNF532 using the pmiRGLO miRNA luciferase assay system.

5. Perform *in-situ* detection of miR-200b in paraffin embedded, human lung sections.

4.3 Specific materials and methods

4.3.1 In-situ hybridisation

4.3.1.1 Overview

In situ hybridization (ISH) is one of the most common methods for visualizing gene expression and localization in cells and specific tissue. The principle behind ISH is

the specific annealing of a labelled nucleic acid probe to its complementary sequences in fixed tissue, followed by visualisation of the localised probe. This technique can be used to detect mRNA, small RNAs such as miRNAs and DNA molecules (Jørgensen *et al.*, 2010).

The miRCURY LNA™ microRNA ISH Optimization kit (FFPE) (Exiqon, Denmark) allows for detection of microRNA in FFPE tissue sections using a non-mammalian hapten digoxigenin (DIG) labelling. During the protocol miRNAs are unmasked using Proteinase-K treatment that allows the entry of double-DIG labelled probes (mimic/inhibitor; Exiqon) to hybridise to the complementary miRNA sequence present in the tissue. The digoxygenins are recognised by DIG specific (Anti-DIG) antibody coupled with the enzyme alkaline phosphatase (AP). Upon introduction of AP substrate, the enzyme converts the soluble substrate 4-nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'- indolylphosphate (BCIP) into a water and alcohol insoluble dark-blue NBT-BCIP precipitates. Finally, a nuclear counter stain is used to allow better histological resolution (Figure 4.1).

The ISH protocol was optimised. Protocol parameters such as hybridisation temperature and Proteinase-K treatment (duration of incubation and concentration) were initially optimised. Using the optimised parameters, a range of probe concentrations were tested in order to achieve a strong specific ISH signal for the positive control miRNA and no/minimum signal for the scrambled negative control miRNA. Finally, detection of the miRNA of interest was carried out at the optimised concentration (Kloosterman *et al.*, 2006).

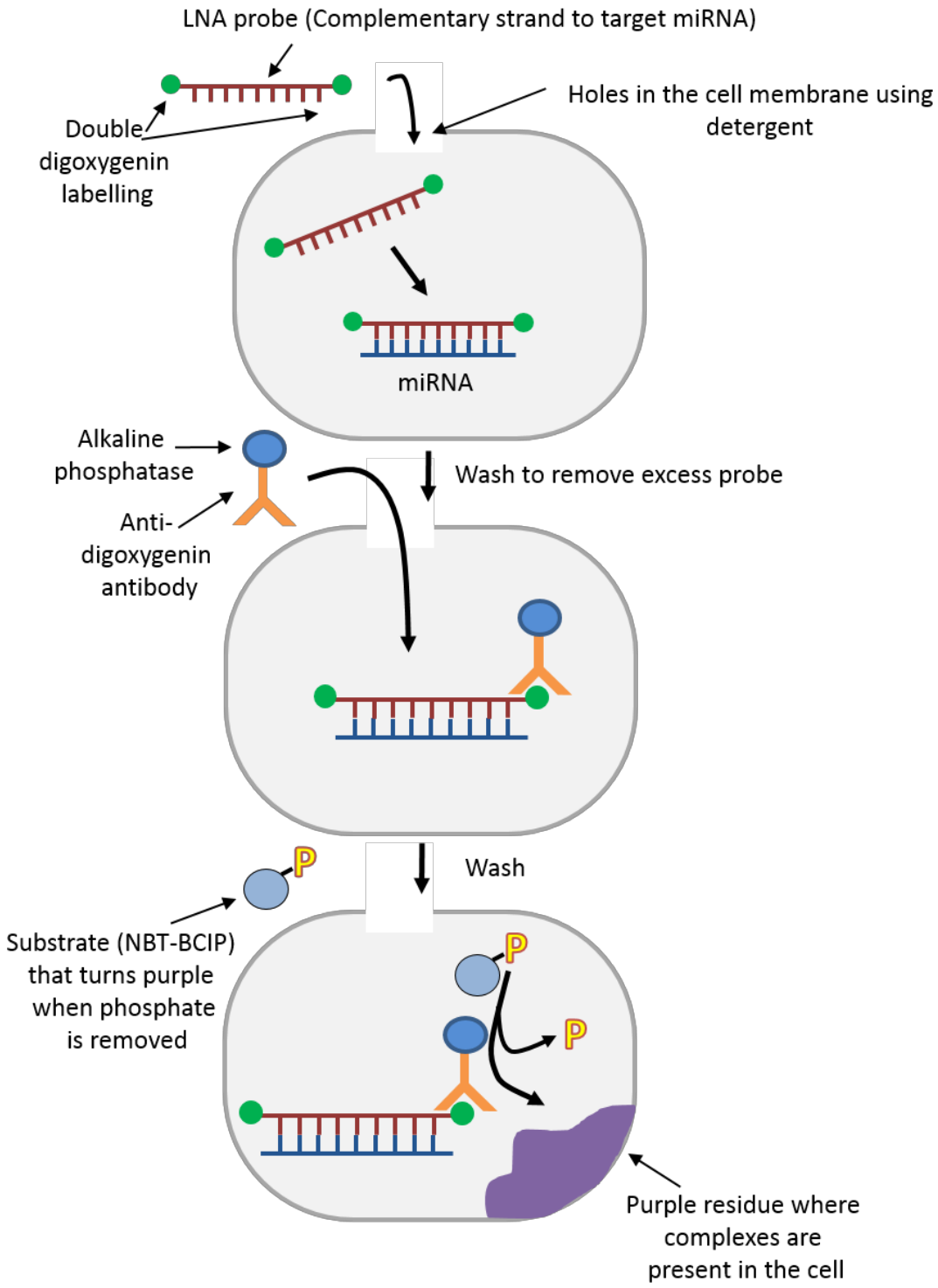

Figure 4.1: Schematic diagram of *in situ* hybridisation (ISH) technique for miRNA detection using double-DIG-labelled LNA™ probes.

4.3.1.2 Hybridization and visualization

A double DIG labelled (5' and 3') miRCURY LNA miRNA detection probe (Exiqon, Denmark) was used to detect the endogenous expression of miR-200b. Deparaffinised lung sections were treated with 15µg/ml Proteinase-K buffer for 10 minutes at 37°C. The sections were hybridised at 50°C (30°C lower than the probe Tm) for 18 hrs in hybridization mix containing 40nM miR-200b DIG-labelled probes or 40nM of scrambled miRNA probes or 40nM of positive control miRNA probes (miR-126). Sections were washed with decreasing standard saline citrate buffer concentration (5xSSC, 1xSSC and 0.2xSSC) at hybridization temperature (for 5 minutes each). For immunostaining, sections were incubated with 1: 800 anti-DIG reagents (Roche) for 1 hr at room temperature followed by incubation with AP substrate (Roche) for 1.5 hrs at 30°C. Nuclear Fast Red (Vector Laboratories) was applied for 1 minute and nuclei were counterstained. Sections were mounted using Eukitt (Sigma) mounting medium.

4.3.2 *TGF-β TaqMan Array, Human TGF-β-Pathway*

4.3.2.1 Overview

The panel of TGF-β TaqMan Array comprises of target genes encoding members of TGF-β superfamily ligands. This includes TGF-β family, BMPs, GDFs, AMH, activin, inhibin and Nodal. The panel also includes gene coding for TGF-β receptors, SMAD family that are central to the TGF-β signal transduction pathway (Figure 4.2). The TGF-β pathway array plate consists of 92 assays to TGF-β associated genes and 4 assays to endogenous control genes (ThermoFisher Scientific).

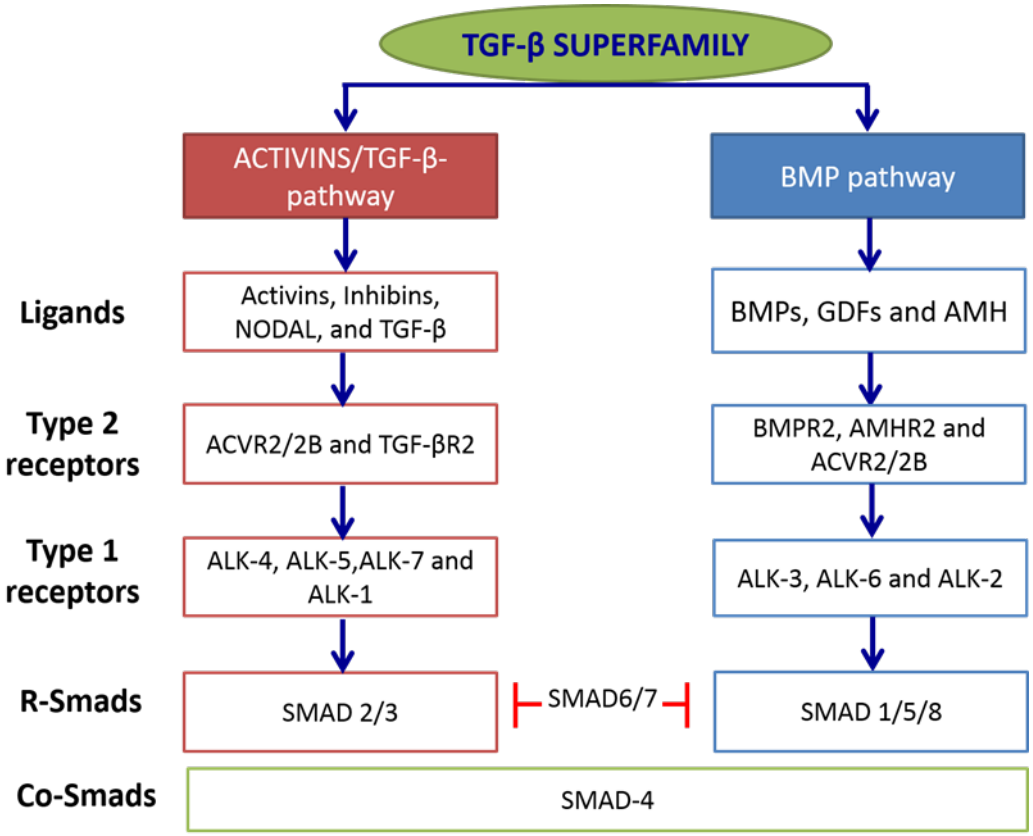

Figure 4.2: TGF-β superfamily signalling pathway. The superfamily is comprised of 30 members including Activins, Inhibins, Nodals, Bone Morphogenetic proteins (BMPs) and Growth differentiation Factors (GDFs). TGF-β superfamily ligands signal via cell-surface serine/threonine kinase receptors to the intracellular SMAD proteins, which in turn translocate to the nucleus to regulate gene expression. In addition to this cascade, SMAD-independent pathways are also activated in a cell specific manner to transduce TGF-β signals.

4.3.2.2 Protocol and data analysis

BEAS-2B cells were cultured in four T75 flasks and grown until they were 60-70% confluent. Two flasks were transfected with 30nM miR-200b mimics for 24 hrs out of which one flask was further subjected to treatment with 5ng/ml TGF-β1 for 48 hrs. The cells in the third flask were treated with only TGF-β1 for 48 hrs. And the last flask consisted of control/untreated cells. Thereafter total RNA was isolated using miRVana Paris kit (Applied Biosystems) and stored at -80°C until further use.

The TaqMan® Array Plate was subjected to centrifugation at 300g for 1 min before loading the TaQman mastermix and cDNA. 50ng of cDNA and SensiFAST™ Probe Hi-ROX mastermix (Bioline) per 10µl reaction was loaded per well and the fast thermal cycling conditions were specified prior to running the plate on StepOnePlus system (Table 4.1).

Hold	Hold	PCR (40 cycles)	
		Melt	Anneal/Extend
50^0C	95^0C	95^0C	60^0C
2 mins	20 secs	3 secs	30 secs

Table 4.1: Cycling conditions for TaqMan array assay.

After completion of the run, C_t values acquired from the StepOnePlus sytem was uploaded on RT^2 profiler software (Qiagen) available online. This software allowed normalising the expression of genes to the endogenous houskeeping genes such as HPRT1 and 18s. The expression of each gene in miR-200b transfected, TGF-β treated and miR-200b transfected followed by TGF-β treatment was also compared to the untreated control and fold change was evaluated. A heat map of differently expressed genes ($p<0.05$) was also plotted using this online software (Figure 4.3).

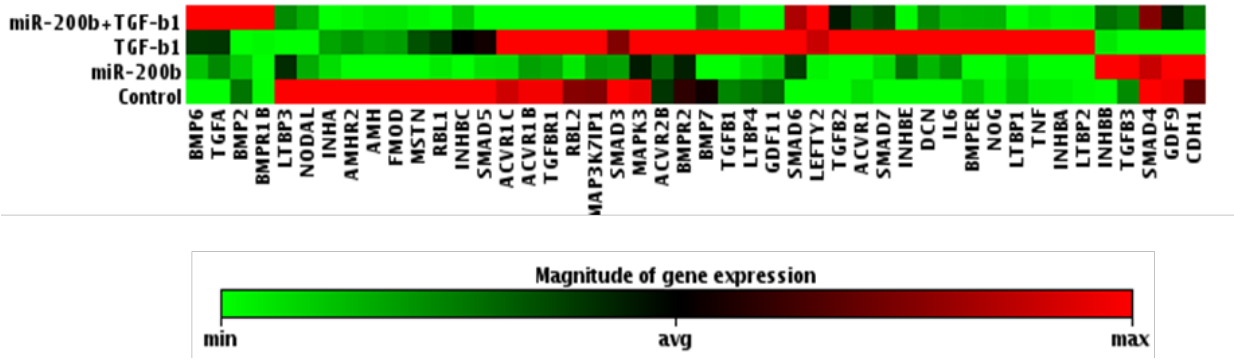

Figure 4.3: Clustering of genes regulated by TGF-β. A heat map of significantly expressed genes was plotted using RT2 profiler software (Qiagen). TGF-β pathway array was used to study the differentially expressed genes in control, miR-200b transfected, TGF-β treated and miR-200b transfected and TGF-β treated BEAS-2B cells. Expression levels were normalized to the housekeeping gene HPRT1.

4.3.3 Reporter gene assay

4.3.3.1 Overview -pmiRGLO dual luciferase miRNA expression vector

The pmiRGLO Vector allows quantitative evaluation of miRNA activity by the insertion of miRNA binding regions/target sites downstream of 3´ region of the firefly luciferase gene (luc2). Reduced firefly luciferase expression indicates the binding of endogenous or introduced miRNAs to the cloned miRNA target sequence. This vector system is based on the dual-luciferase technology comprising of luc2 gene that is the primary reporter to monitor mRNA regulation. It also includes Renilla luciferase (hRluc-neo) that acts as a control reporter for normalization and selection using the Dual-Luciferase Assay. In addition, the vector contains the following features (Coré; Aldred *et al.*, 2011):

1. Non-viral universal human phosphoglycerate kinase (PGK) promoter: This promoter present upstream of the luc2 gene provides low translational expression that leads to desired repose such as a reduction in signal.

2. Multiple cloning site (MCS): This is located downstream of the luc2 gene and contains unique restriction sites that occur only once within a given plasmid such as

XhoI and SacI. Hence, cutting the plasmid with one of the restriction enzymes may serve to insert the miRNA target sequence.

3. SV40 late poly(A) signal sequence positioned downstream of luc2 gene is responsible for transcription termination and mRNA polyadenylation.

4. Ampicillin (Amp^r) gene allows for selecting bacterial colonies that contain the plasmid.

Bacterial transformation was carried out using pmiRGLO miRNA expression vector (Promega, San Luis Obispo, CA, USA). Thereafter, plasmid DNA was isolated, lineralised and purified. On the other hand, primers were designed for ZEB2 and ZNF532 in order to generate inserts (3'UTR region of ZEB2 and ZNF532 containing binding sites for miR-200b) using DNA isolated from BEAS-2B cells. The inserts were then purified. The purified inserts were then incorporated into the pmiRGLO vector using infusion cloning and sent for sequencing to verify successful cloning. Thereafter, luciferase assay was performed after co-transfectiong BEAS-2B cells and PBECs with the vector containing the insert and miRNA-200b/200c mimic (Figure 4.4).

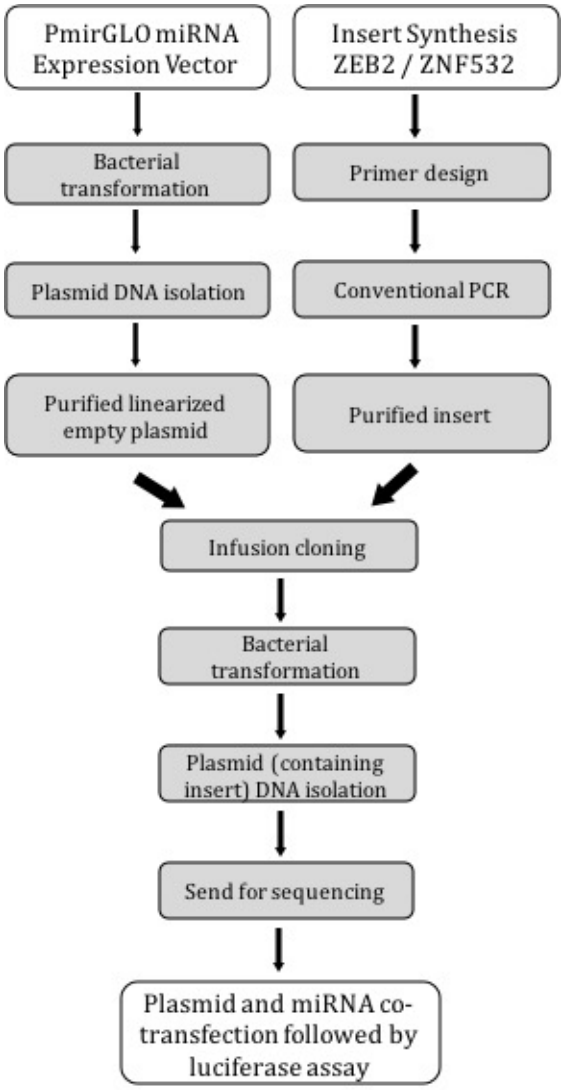

Figure 4.4: Flowchart illustrating the steps involved in generating miRNA expression vector system with ZEB2/ZNF532 3' UTR insert.

4.3.3.2 Bacterial transformation, plasmid DNA isolation and plasmid restriction enzyme digestion and purification.

DH5α bacteria (50µl) were transformed using 10ng of pmiRGLO vector. A negative control containing only bacteria and 2.5µl of pUC19 (100pg/µl) as positive control was also included. The eppendorfs were incubated on ice for 30 minutes, subjected to heat shock for 30 seconds at 42˚C and then incubated on ice for 2 minutes. Bacteria containing the plasmid were incubated for 1 hour at 37˚C in 950µl of LB (Lennox) broth growth medium (Sigma-Aldrich). 100µl of this starter culture was

added to Ampicillin (100µg/ml) containing agar plates and grown overnight in an incubator at 37˚C. A single colony was picked from the agar plate and bacterial cultures were prepared in 10ml Luria broth (LB) and ampicillin (100µg/ml). A colony inoculated in LB broth without ampicillin was considered as a positive control. A negative control consisted of LB broth only. The bacteria were cultured overnight at 37˚C in a shaker incubator.

The Qiagen plasmid midi kit was used to isolate pmiRGLO plasmid DNA from the DH5α bacteria. 50 ml of overnight bacterial growth culture was centrifuged for 20 minutes at 4000 g at 4˚C. The pellets were suspended in 2ml of Buffer P1 to facilitate lysis. Thereafter, 2 ml Buffer P2 was added and the lysate was thoroughly mixed by inverting the falcon tubes and incubating them for 5 minutes at room temperature. 2 ml of Chilled Buffer P3 was added, mixed as previously described and incubated on ice for 15 minutes. The falcon tubes were then subjected to centrifugation at 18000 g for 30 minutes at 4˚C. Clear lysate supernatant containing the plasmid DNA was collected and loaded onto the equilibrated QIAGEN-tip 100 in order to allow it to enter the resin by gravity flow. Following this the Qiagen tip were washed with 10 ml of Buffer QC 2 times and plasmid DNA was eluted with 500 µl of pre-heated Tris-EDTA solution. The quantity and quality of the isolated plasmid was measured using NanoDrop® 1000 spectrophotometer.

pmiRGLO plasmid DNA (5µg) isolated was subjected to restriction enzyme digestion by using 10µl *Xho1* enzyme (10u/µl, Promega), 10µl acetylated BSA (10µg/µl, Promega), 10µl 10X Buffer D (Promega) and RNase free water in a total 100µl reaction mix. The digestion mix was incubated overnight at 37˚C to facilitate complete digestion. The digested plasmid was run on 0.8% w/v agarose gel alongside undigested plasmid DNA and 1kb ladder. Ethidium bromide (0.5µg/ml) in

agarose gel allowed visualisation of DNA when exposed to ultraviolet light (Figure 4.5B).

After the bands were separated, the purified plasmid band (7350bp) was excised from the gel and QIAGEN QIAquick gel extraction kit was used to purify the plasmid DNA. The gel slice was weighed and 3 volumes Buffer QG was added to 1 volume gel and incubated at 50˚C for 20 minutes and vortexed every 3 minutes to help dissolve the gel quickly. After the gel dissolved, 1 gel volume equivalent isopropanol was added and the sample was applied to the QIAquick column for 1 min and the flow through was discarded. The columns were washed with 500 µl Buffer QG and 750 µl Buffer PE. Thereafter, the column was placed into a clean 1.5 ml eppendorf and 50 µl preheated water was applied to the centre of the column membrane and then centrifuged for 1 min. The samples were subjected to speed vacuum at room temperature for 30 minutes to yield 10 µl final volume plasmid DNA as the previously purified DNA concentration was too low. NanoDrop profile was irrelevant as the absorption peak moved from 260nm to 230nm for the linearised DNA extracted from the gel as compared to the undigested DNA possibly due to guanidinium thiocyanate present in buffer QG. There this data wasn’t included (Figure 4.5).

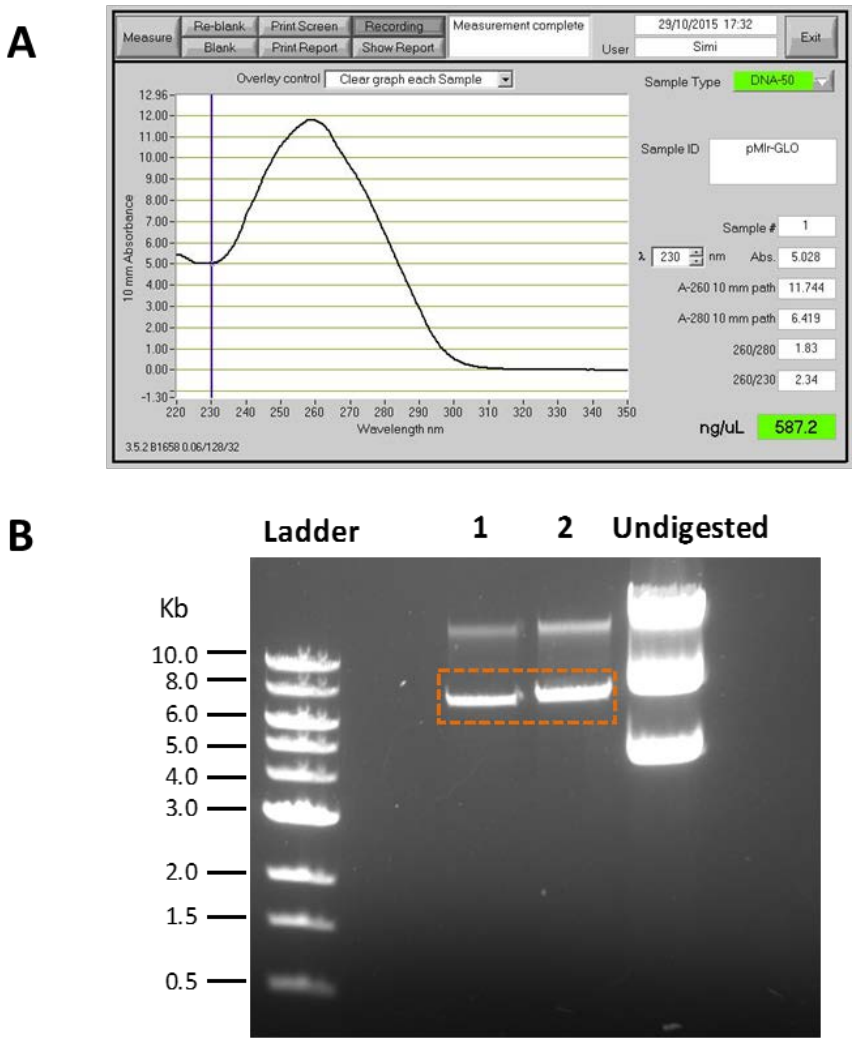

Figure 4.5: pmiRGLO miRNA expression vector isolation, digestion with restriction enzyme Xho-1 and purification. Panel A shows quantity/quality of pmiRGLO plasmid DNA isolated. Isolated pmiRGLO DNA was subjected to restriction enzyme digestion using *Xho1* and agarose gel electrophoresis (0.8% w/v) was performed (2ng & 5ng plasmid, B). The region corresponding to the molecular weight of the plasmid (7350bp) was excised from the gel and purified. Purified plasmid obtained was subjected to SpeedVac in order to concentrate the sample. The concentration was then checked using a Nanodrop (C).

4.3.3.3 Primer design and amplification of ZEB2 and ZNF532 3' UTR region

Online computational tools allowed identifying miR-200b binding sites in the 3' UTR containing (2-8 bases) of ZEB2 and ZNF532 mRNAs. The Ensembl platform was used to scan 3'UTR region of ZEB2 and ZNF532 to find potential binding regions for miR-200b. The sequence containing the binding region was inserted in Primer3 online tool that provided the primer sequences required to amplify the regions of

interest. The size of the 3'UTR regions containing miRNA binding sites were 271 bases for ZEB2 and 215 bases for ZNF532. The primers were then converted into In-Fusion® primers (Forward infusion primer: GCTCGCTAGCCTCGACCTTCCTTCACCTCGTCGTA; reverse infusion primer: CGACTCTAGACTCGAGGAACTGCCCCTGTTACTAAG) and ZEB2 (Forward infusion primer: GCTCGCTAGCCTCGAAGGCAGCAGTTCCTTAGTTT; reverse infusion primer: CGACTCTAGACTCGATGCCCAAATGATCAACGTCA) that added 15 base overhangs complementary to the plasmid DNA sequence in order to provide directional insertion of the amplified 3'UTR into the vector (Figure 4.6). The infusion primers for conventional PCR were synthesised by ThermoFisher scientific.

For Genomic DNA isolation, a T25cm^2 flask was trypsinised and centrifuged to obtain a pellet of BEAS-2B cells. Thereafter, Promega Wizard® SV genomic DNA purification kit was used according to the manufacturer's protocol. A range of annealing temperatures was used for ZEB2 and ZNF532 primers in order to determine the optimum temperature that yields a single band for the PCR product. The range included the following temperatures 40, 45, 50, 55 and 60˚C. Each PCR reaction contained reagents and volumes as outlined in Table 4.2. In addition, the cycling conditions used to amplify the 3'UTR is listed in Table 4.3. Since only single bands were obtained at all temperatures, 20µl reactions (55 & 60˚C) were pooled together used for infusion cloning (Figure 4.7).

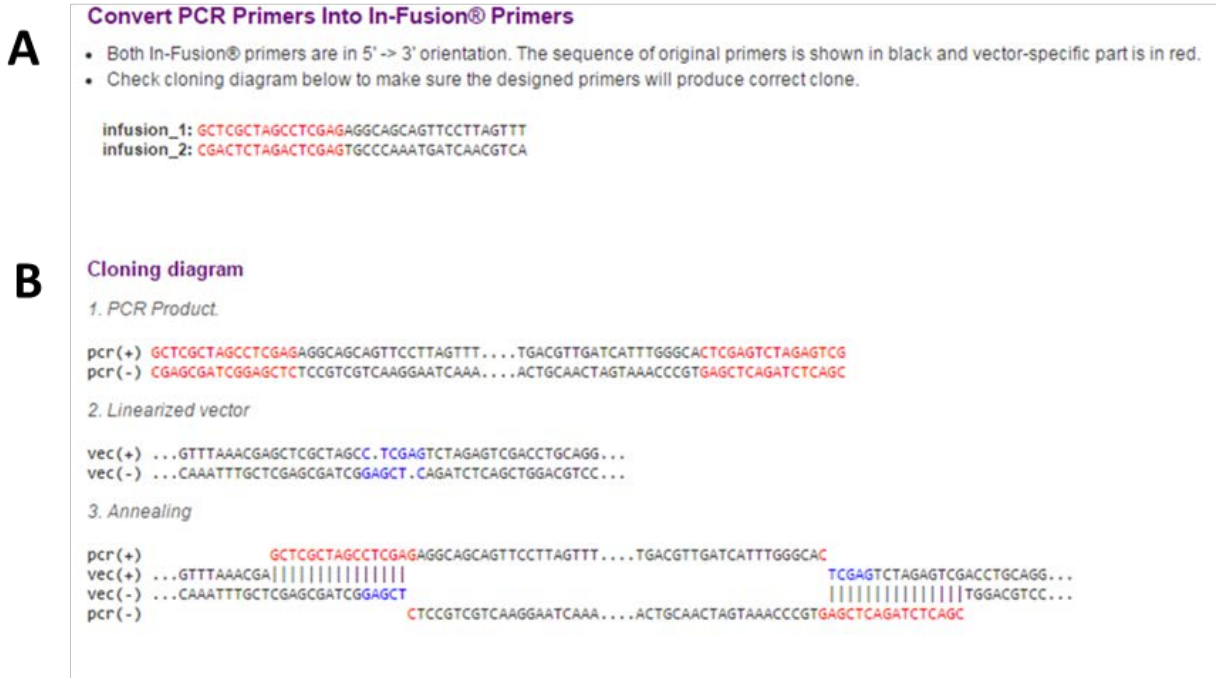

Figure 4.6: Forward and reverse In-Fusion primer sequence for ZEB2. The sequence in black is the conventional primer. The 20 nucleotides coloured in red at the 5'end of the primer are the bases complementary to the vector. These were added as part of the conversion to In-Fusion primers (A). Panel B, the sequence in blue indicates the restriction enzyme site for *Xho-1*, the dashed lines between PCR product and vector in "Step 3. Annealing" indicates the complementarity between the single strand 5' end hangover from the vector (due to 3' to 5' exonuclease activity in In-Fusion cloning's infusion mix) and the 5' hangover on the PCR product.

Name of Reagent	Volume of Reagent/µl
5X Buffer (ThermoFisher)	4
Forward Primer 10mM=[Stock] (ThermoFisher Scientific) 0.5mM=[Final]	1
Reverse primer 10mM=[Stock] (ThermoFisher Scientific) 0.5mM=[Final]	1
Phire Hot Start II DNA Polymerase (ThermoFisher)	0.4
Genomic DNA (39ng/µl)	2.6
dNTPs (ThermoFisher) 200µM each [Final]	1.6
RNase free water (Sigma)	9.4 (adjust to 20µl total volume)

Table 4.2: PCR reaction reagents and volumes for amplification of ZEB2 and 3'UTR insert

Temperature/˚C	Time/seconds
98	30
98 (Denaturation phase)	5
40/45/50/55/60 (Annealing phase)	5
72 (Extension phase)	20
72	60
4	HOLD

Table 4.3: PCR reaction reagents for ThermoFisher Phire® hot start II DNA polymerase.

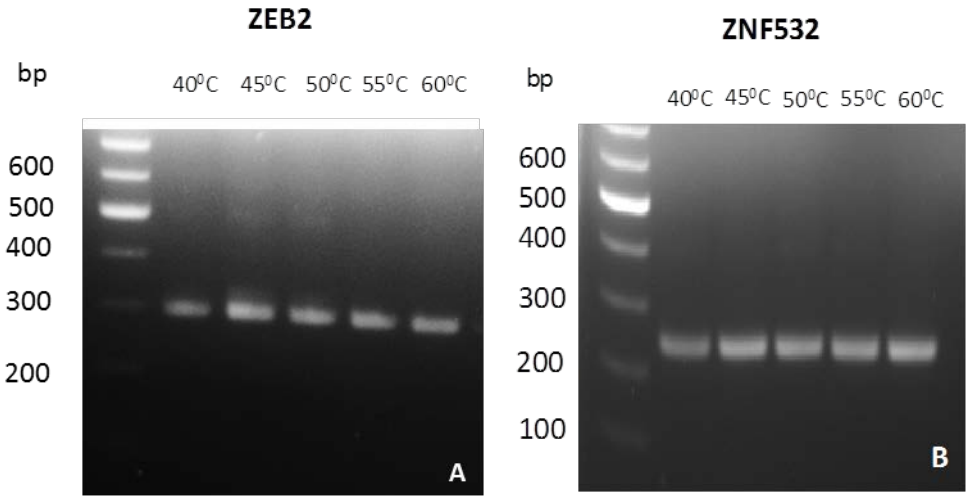

Figure 4.7: 2% w/v agarose gels showing PCR reactions at different annealing temperatures. Gel electrophoresis for the amplified ZEB2 (271 bp) and ZNF532 (215 bp) insert was performed at different temperature (40- 60˚C). A single product band was obtained at all annealing temperatures indicating the primers were highly specific.

4.3.3.4 Infusion cloning and bacterial transformation

The Infusion system (Clontech) is based on the principle of Ligation Independent Cloning method (LIC) that makes use of annealing single-stranded complementary overhangs on the target vector and a PCR-generated insert of 15-bases. The linearised vector and the PCR product (insert) when incubated with In-Fusion HD Enzyme Premix leads to generation of single stranded overhangs by using T4 DNA polymerase and dNTP in the reaction mix. The 3' exonuclease activity of the enzyme begins to chew-back the linearized destination vector and the PCR product from 3' to 5' at the site of the first occurrence of this nucleotide leading to the generation of 5'

overhangs complementary to the termini of the linearized vector (Aslanidis and de Jong, 1990; Betts and Farmer, 2014).

The ClonTech In-Fusion cloning kit was used to generate the recombinant vector containing the amplified 3'UTR insert of either ZEB2 or ZNF532. A range of insert to vector molar ratios were used to obtain optimal cloning (Table 4.4).The vector and insert were incubated with the 2 µl of infusion enzyme mix and Rnase free water (Total volume -10 µl). The reaction was incubated for 15 min at 50°C and then placed on ice and stored at -20˚C until further use.

Reaction	Volume/µl
ZEB2 1:4 (Insert: Vector ratio)	
Vector (27ng/µl)	7.40
Insert (80ng/µl)	0.40
RNase free water	0.20
ZEB2 1:5 (Insert: Vector ratio)	
Vector (47ng/µl)	4.20
Insert (80ng/µl)	0.50
RNase free water	3.30
ZNF532 1:3 (Insert: Vector ratio)	
Vector (47ng/µl)	4.20
Insert (95ng/µl)	0.20
RNase free water	3.60
ZNF532 1:4 (Insert: Vector ratio)	
Vector (47ng/µl)	4.20
Insert (95ng/µl)	0.25
RNase free water	3.55
ZNF532 1:5 (Insert: Vector ratio)	
Vector (27ng/µl)	7.40
Insert (95ng/µl)	0.30
RNase free water	0.30
Negative Control (Linearized vector only)	
Vector (47ng/µl)	4.20
Insert	-
RNase free water	3.80

Table 4.4: In-Fusion cloning reaction mixtures for the most successful attempts. To each reaction 2µl of Infusion primer was added.

Bacterial transformation was carried out using 1.25µl of infusion mixture and stellar competent cells. It was also ensured that more than 5ng of vector DNA was used per 50µl of stellar competent cells. 100µl of transformed cells were spread on ampicillin (100µg/ml) selective agar plates and single colony was picked up and grown

overnight in 10ml of LB broth and ampicillin (100µg/ml) in a shaker incubator (37˚C at 225rpm). The recombinant plasmid DNA was isolated from the bacteria and sent to Source Bioscience (UK) for sequencing to confirm successful cloning (Figure 4.8).

4.3.3.5 Luciferase reporter gene assay

BEAS-2B cells were seeded (35000 cells) in 96 well plates and transfected with 500ng of pmiRGLO vector containing the 3'UTR of ZEB2 (Figure 4.9A) or ZNF532 (Figure 4.9B) or empty plasmid and co-transfected with 30nM miR-200b mimics or NSmiRNA using Lipofectamine 2000 (Invitrogen). Luciferase activity (firefly and renilla luciferase) was measured at 24hrs post-transfection using a Dual luciferase reporter assay system (Promega).

Yellow highlighted region: overhang added to primer to make infusion primers (region complementary to plasmid)

Grey highlighted region: miRNA binding site

Results post sequencing:

For pmirGLO+ZEB2

NNNNNNNNNNNNNNNNTCNAGTTGTTTAACGAGCTCGCTAGCCTCGAAGGCAGCAGTTCTTAGTTTACATA
TGTTTGTGCAATTATTTTCTGTACTTTTTTGTTCATTAATTTTGTCAGTATTACACCAAACTGTTTTTGCAACAAA
AAAATTTTTTTTGCATTCATTTAATTTTAGGTCAAATAACATTTTATTTATGTGGCTCATTTTATATTTCCTAATTT
TATTTATTTCATACTGTAGTGTACAGTATTATAGTTCTTCAATATATAGATATATTTTAGTAAAAAAGGAACATG
ACGTTGATCATTTGGGCATCGAGTCTAGAGTCGACCTGCAGGCATGCAAGCTGATCCGGCTGCTAACAAAGCC
CGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCGGCCGCTTCG
AGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATT
TGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTG
CATTCATTTTATGTTTCAGGTTCAGGGGGAGATGTGNNANN

For pmirGLO+ZNF532

NNNNNNNNNNNNNNNNNGTTGTTTNNGAGCTCGCTAGCCTCGACCTTCCTTCACCTCGTCGTATATATCCT
CGATAAGTATTAAAACAGTATTTGAGTTTAAAAGAGTTTGTATATATTTAAATGAATAACTTTTTATACTCTTTG
TTACATGTTTGTATCAGTATTTAGTGGAAAACCATTTGAGTTGTTTTGGGTTAGAATTTTTCTTTTTGTACTGTTT
CTTTAAAACAGAGTTCTTAGTAACAGGGGCAGTTCCTCGAGTCTAGAGTCGACCTGCAGGCATGCAAGCTGAT
CCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC
CTTGGGGCGGCCGCTTCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCA
GTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACA
AGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGATGTGGGAGG

Figure 4.8: Sequencing data post in-fusion cloning. PmiRGLO plasmid samples cloned with ZEB2 and ZNF532 3'UTR region were sent for sequencing to verify successful cloning. Sequencing data post cloning indicates presence of primer overhangs (yellow highlighted region) and miRNA binding site (grey highlighted region).

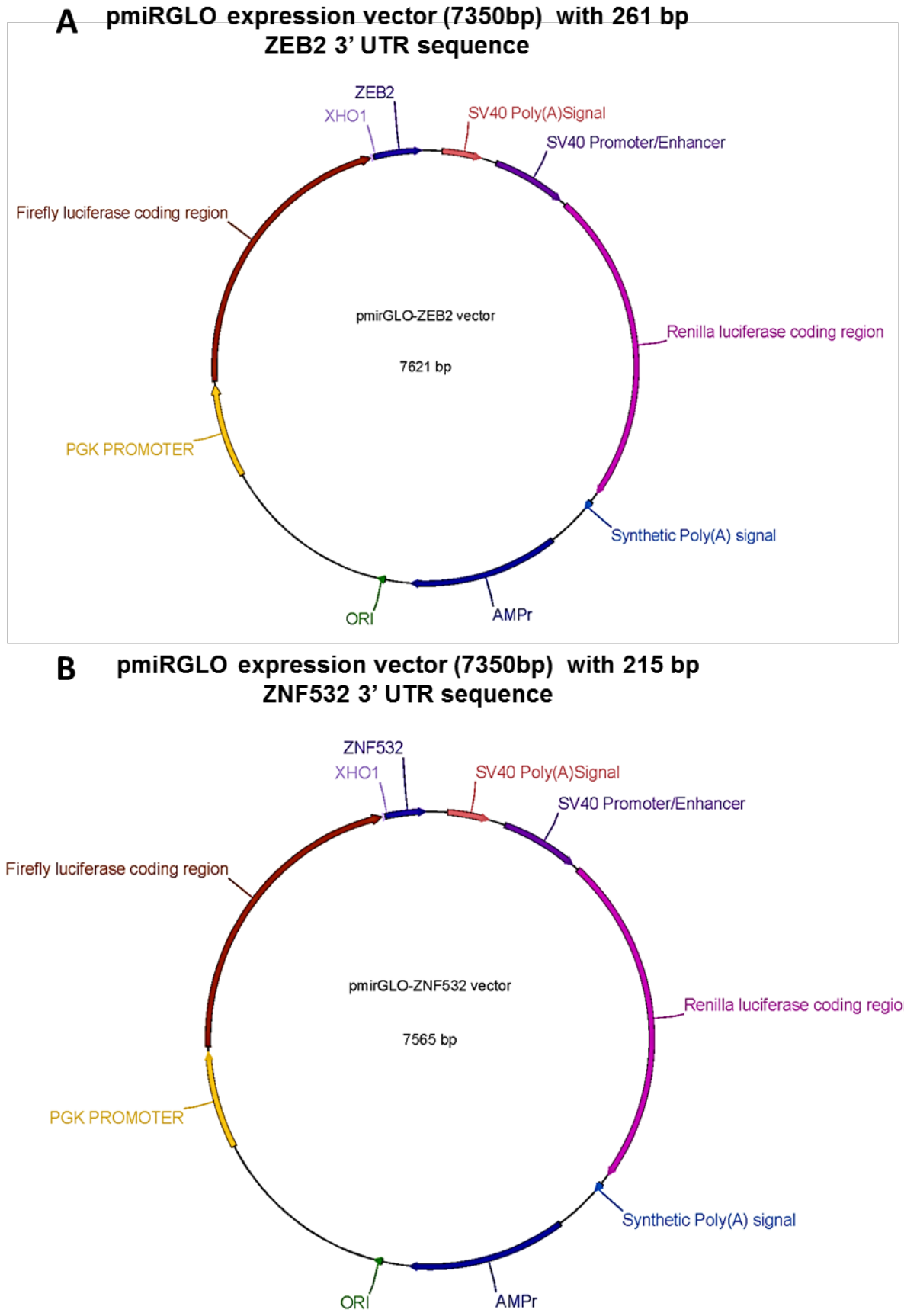

Figure 4.9: pmiRGLO vectors with ZEB2 and ZNF532 3' UTR insert as a result of infusion cloning

4.4 Results

4.4.1 BEAS-2B and PBECs maintain epithelial cell characteristics when transfected with miRNA-200b

To examine the changes in cell morphology post miR-200b transfection and TGF-β1 treatment, BEAS-2B cells and PBECs were first transfected with 30nM miR-200b mimics for 24 hrs followed by treatment with 5ng/ml TGF-β1 for 48 hrs. BEAS-2B cells under control conditions (Figure 4.10A) exhibited a typical epithelial like "cobblestone" morphological phenotype, whereas upon transfection with NSmiRNA (AllStars Negative Control siRNA, Qiagen) followed by TGF-β1 treatment cells acquired an elongated spindle shaped like phenotype (Figure 4.10B) indicating morphological change due to EMT. Cells transfected with miR-200b mimics followed by stimulation with TGF-β1 maintained their epithelial framework and cell-cell contact. They appeared more cobbled shape (Figure 4.10C).

Human PBECs were isolated from the bronchial epithelium of healthy patients. The bronchial epithelium plays an important role in the development of BOS since it is not only the target but also the mediator of BOS through response to injury (Forrest *et al.*, 2005). Columnar PBECs displayed typical epithelial cell morphology but possess a limited regeneration capacity as they acquire a less well-differentiated phenotype (fibroblasts) and become unsuitable for use as primary cells (Brodlie *et al.*, 2010). PBECs were therefore cultured only until passage 2. The morphology of PBECs under normal conditions (Figure 4.10D) and in miR-200b transfected and TGF-β1 treated sample (Figure 4.10F) were identical. However cells pre-incubated with NSmiRNA and treated with TGF-β1 displayed an elongated fibrotic morphology similar to that of mesenchymal cells (Figure 4.10E).

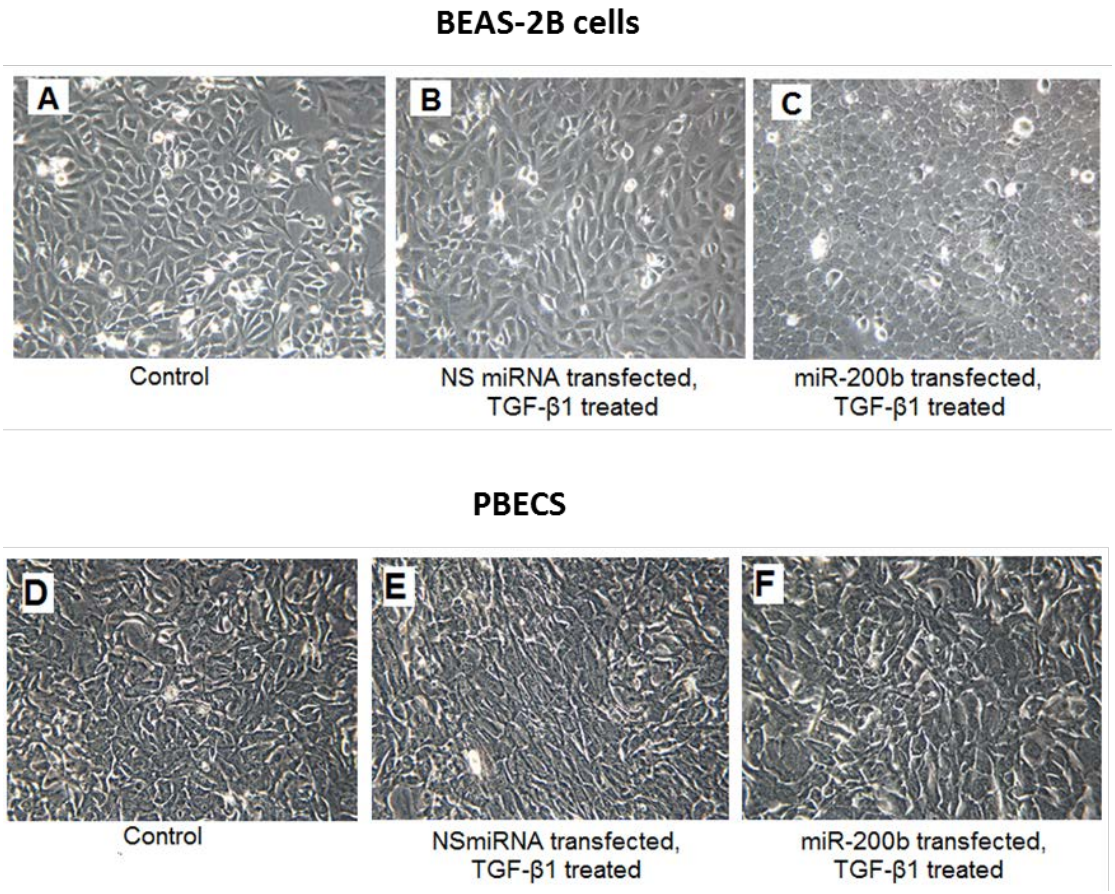

Figure 4.10: BEAS-2B cells and PBECs transfected with miR-200b and treated with TGF-β1 maintained epithelial characteristics. BEAS-2B cells and PBECs treated with TGF-β1 acquired a fibrotic phenotype compared with control (A, D) while cells transfected with 30nM miR-200b and treated with TGF-β1 (C) exhibited cobblestone-like morphology with increased cell-cell contact in BEAS-2B cells indicating a presentation of epithelial characteristics. PBECs transfected with miR-200b and treated with TGF-β1 phenotypically resembled the control cell morphology (F) (n=3) consistent with preservation of epithelial characteristics in TGF-β1 challenged cells transfected with miR-200b.

4.4.2 Ectopic expression of miR-200b followed by TGF-β1 treatment maintained E-Cadherin and reduced fibronectin expression at the RNA level

Transfection efficiency of miRNAs in BEAS-2B cells was assessed and a significant increase in fluorescence was found at 48 hrs and 72 hrs post transfection (Figure 2.9). In addition, miR-200b mimic concentration was optimised by transfecting BEAS-2B cells with varying concentration of mimics for 48 hrs. There was significant

increase in miR-200b expression post-transfecting cells with 10nM, 30nM and 50nM ($p \leq 0.001$) as compared to the non-transfected control. E-Cadherin expression was also significantly upregulated at all time points (10nM- $p \leq 0.05$; 30nM- $p \leq 0.01$; 50nM- $p \leq 0.001$) when compared to non-transfected cells (Figure 2.10). The optimum concentration of miRNA mimic that induced significant changes at RNA level and phenotypically was 30nM. Further experiments were performed to study the effect of miR-200b mimics in the presence and absence of TGF-β1 at the RNA level using BEAS-2B cells, PBECs and cells.

BEAS-2B cells (n=3), PBECs (n=2) and cells from transplant brushings (n=3) were transfected with miR-200b for 24 hrs followed by treatment with TGF-β1 for 48 hrs. Total RNA was isolated and expression level of E-Cadherin and fibronectin was studied using q-RT-PCR. Results suggested a significant restoration of E-Cadherin levels in miR-200b transfected and TGF-β1 treated BEAS-2B ($p<0.01$, Figure 4.11A) and PBECs ($p<0.05$, Figure 4.11B) when experiments were normalised to endogenous control HPRT1 and compared to NSmiRNA + TGF-β1 treated sample. Furthermore, there was an increase in E-Cadherin expression in miR-200b transfected BEAS-2B cells (**=$p \leq 0.01$) and PBECs (**=$p \leq 0.01$) compared to NSmiRNA transfected cells. One- way ANOVA followed by Bonferroni test was performed for evaluating significance difference in the expression levels post qRT-PCR. On the other hand, there was a significant downregulation of fibronectin in miR-200b transfected and TGF-β1 treated BEAS-2B cells ($p<0.01$) and PBECs ($p<0.0001$) when compared to NSmiRNA + TGF-β1 treated cells. The study was replicated in cells acquired from patients post lung transplant (n=3). Results showed a similar trend of significant fibronectin downregulation ($p<0.001$, Figure 4.11C) as seen in BEAS-2B and PBECs. There was no significant change in E-Cadherin

expression in miR-200b +TGF-β1 treated cells as compared to NSmiRNA +TGF-β1 treated cells.

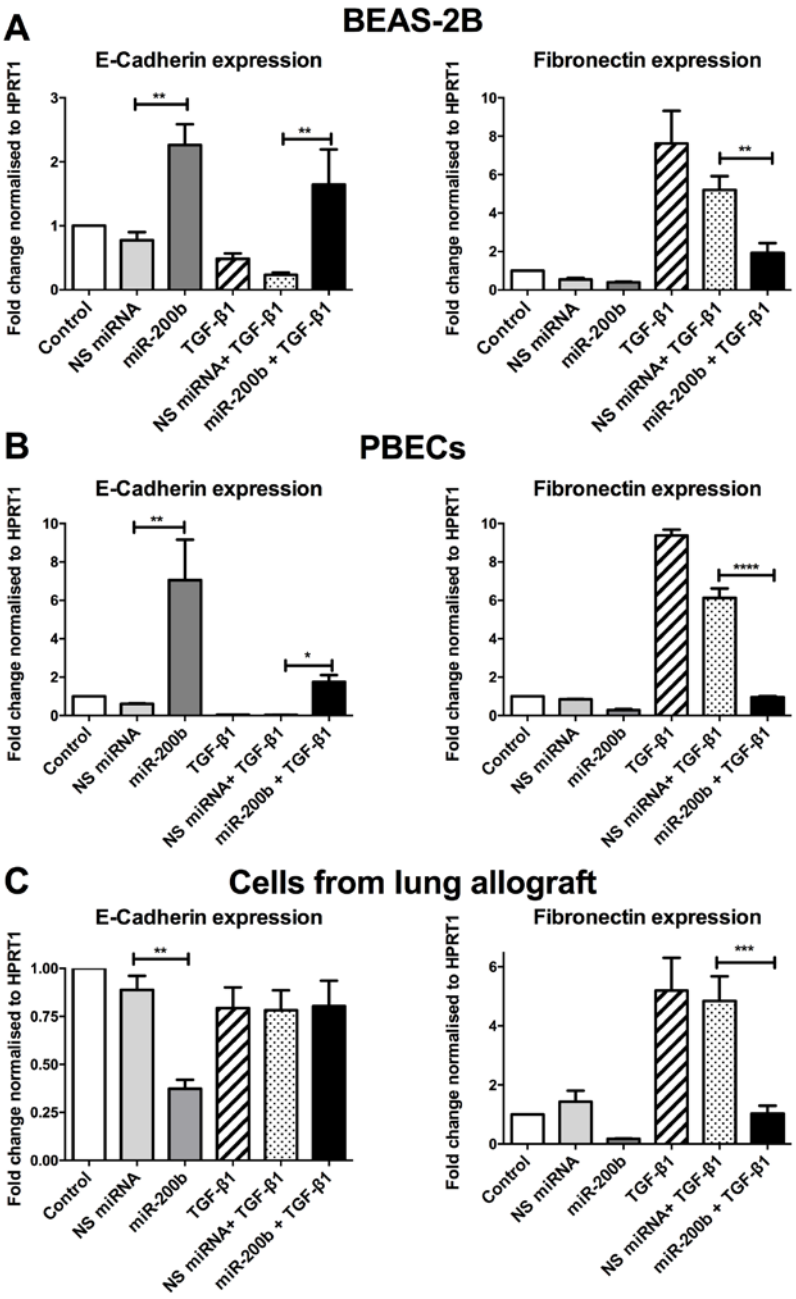

Figure 4.11: miR-200b mimics maintained epithelial markers while reducing fibronectin levels in the presence of TGF-β1. BEAS-2B cells (A), PBECs (B) and PBECs from lung allograft (C) were transfected with 30nM miR-200b for 24 hrs followed by TGF-β1 (5ng/ml) treatment for 48 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001) (****=p≤0.0001)]. The data is representative of three (A, C) or two (B) independent set of experiments, each done in triplicate.

4.4.3 MiR-200b mimics lead to reduction in extracellular matrix proteins in TGF-β1 pre-treated BEAS-2B cells

Changes in protein expression were examined using immunofluorescence (Figure 4.12-1) and western blot (Figure 4.12-2). In BEAS-2B cells, results were consistent with changes in mRNA expression. There was loss of E-Cadherin and cytokeratin-19 and an increase in α-SMA and fibronectin expression in NSmiRNA +TGF-β1 treated BEAS-2B cells (After evaluating fibronectin expression, PVDF membranes were washed and reprobed with α-SMA antibody. The PVDF membrane was subjected to second cycle of stripping and reprobing with GAPDH antibody). However, miR-200b transfection restored E-Cadherin ($p \leq 0.01$) and cytokeratin ($p \leq 0.05$) levels and supressed the expression of α-SMA ($p \leq 0.001$) and fibronectin ($p \leq 0.001$) in NSmiRNA +TGF-β1 treated BEAS-2B cells. This trend of protein expression was confirmed by performing western blot.

Similar expression of EMT markers was observed when experiment was replicated in PBECs (Figure 4.13, n=1).

4.4.4 Overexpression of MiR-200b post TGF-β1 treatment reverses EMT in treated BEAS-2B cells

The effect of overexpressing miR-200b post TGF-β1 treatment is also crucial to understand the potential effect of modulating fibrosis through modulation of miR-200b. MiR-200b overexpression for 24 hrs post TGF-β1 treatment was able to restore E-Cadherin levels (Figure 4.14A) and downregulate fibronectin (Figure 4.14B) in (for both, $p \leq 0.0001$) NSmiRNA + TGF-β1 treated (48 hrs) BEAS-2B cells when expression was normalised to endogenous control HPRT1 (n=3). Therefore, miR-

200b was able to reverse EMT in cells that had already acquired a fibrotic phenotype implicating its potential role in future therapeutic strategies in reversing EMT.

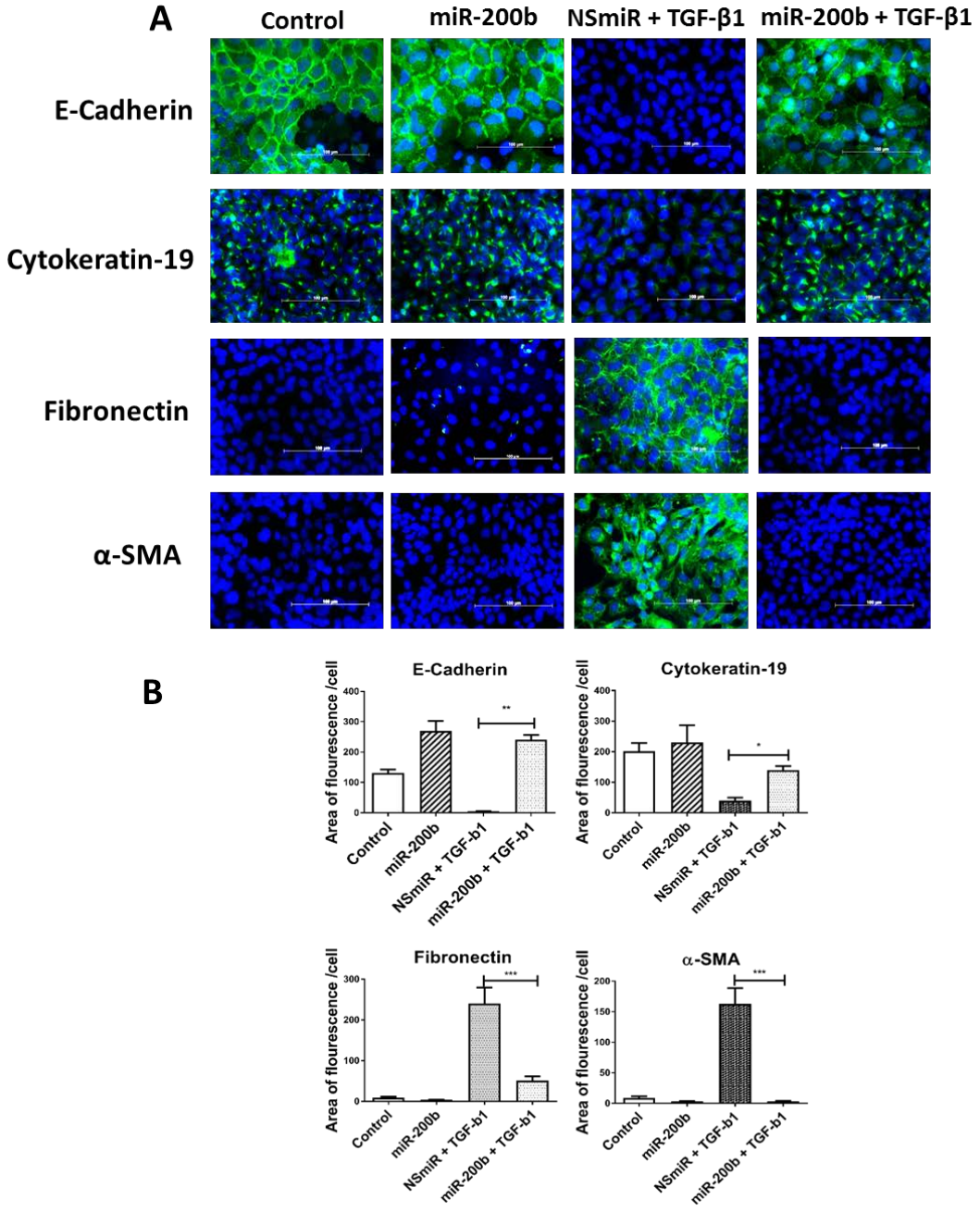

Figure 4.12-1: Overexpression of miR-200b blocked TGF-β1 induced epithelial to mesenchymal transition *in vitro*. BEAS-2B cells were transfected with NSmiRNA (negative control) and 30nM miR-200b mimics. Post-transfection cells were treated with/without 5ng/ml TGF-β1 for 48 hrs. Pictures were captured using Zeiss Axioimager microscope and the two channels DAPI and FITC were merged (A). Bar graphs depict the quantification of immunofluorescence for each of the EMT markers in terms of area of fluorescence per cell (B). The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001)]. The data is representative of three independent set of experiments. Scale bar, 100µm.

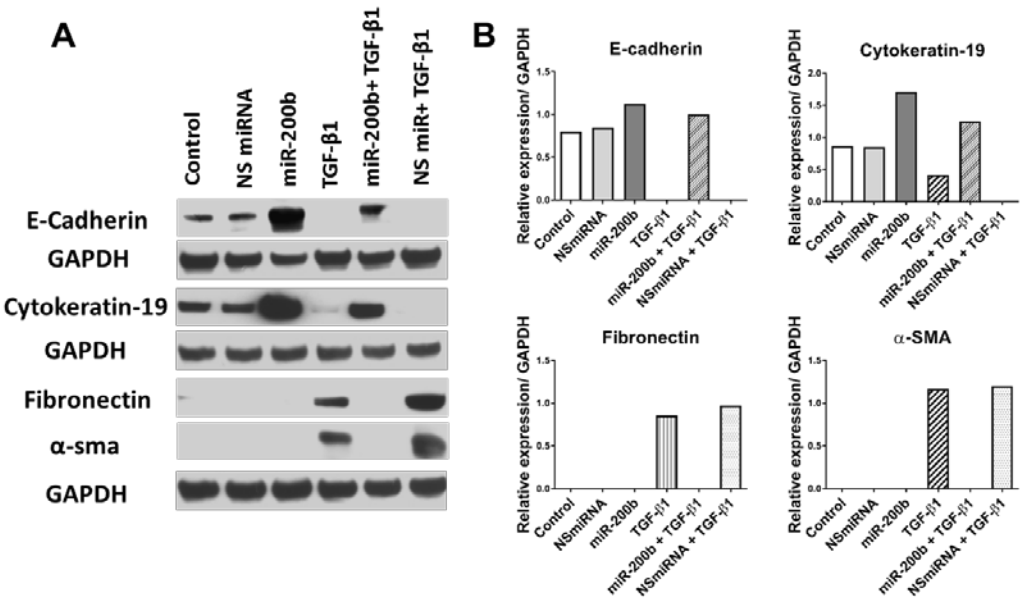

Figure 4.12-2: miR-200b suppresses the expression of fibrotic markers in TGF-β1 treated BEAS-2B cells at the protein level. MiR-200b transfected BEAS-2B cells (24 hrs) were subjected to treatment with 5ng/ml of TGF-β1 for 48hrs. Total protein lysate was harvested and protein production was determined using western blot studies (15µg protein per well). Relative expression of each EMT marker was normalised to the housekeeping gene GAPDH. The data is representative of three independent set of experiments.

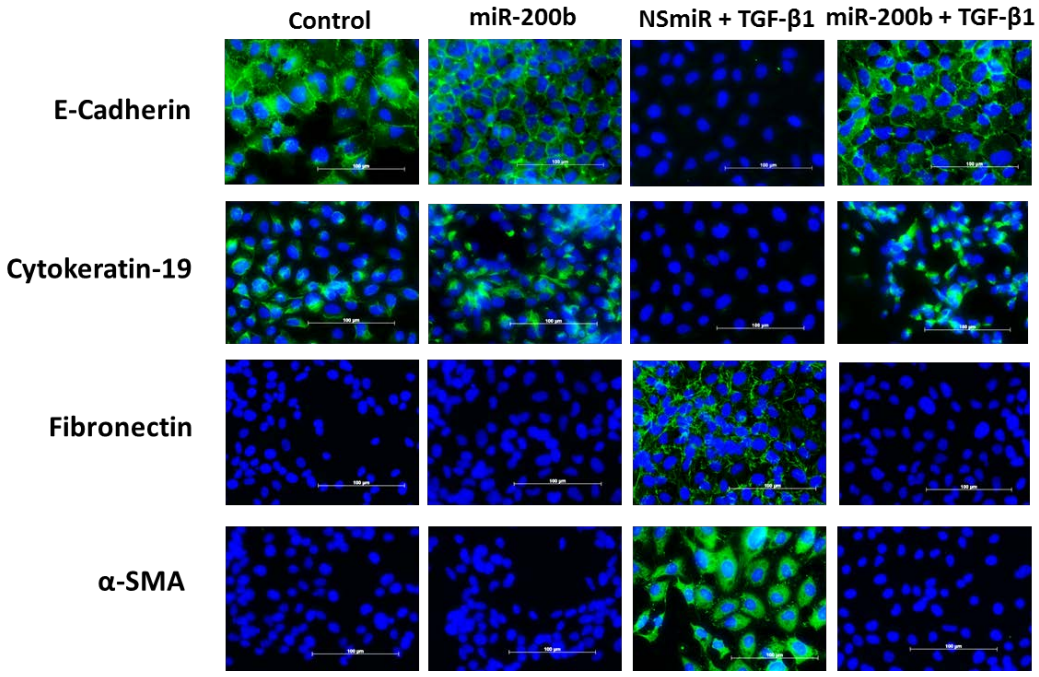

Figure 4.13: Overexpression of miR-200b blocked TGF-β1 induced epithelial to mesenchymal transition in PBECs. PBECs were transfected with NSmiRNA (negative control) and 30nM miR-200b mimics. Post-transfection cells were treated with/without 5ng/ml TGF-β1 for 48 hrs. Pictures were captured using Zeiss Axioimager microscope and the two channels DAPI and FITC were merged. Scale bar, 100µm. The data is representative of one set of experiment.

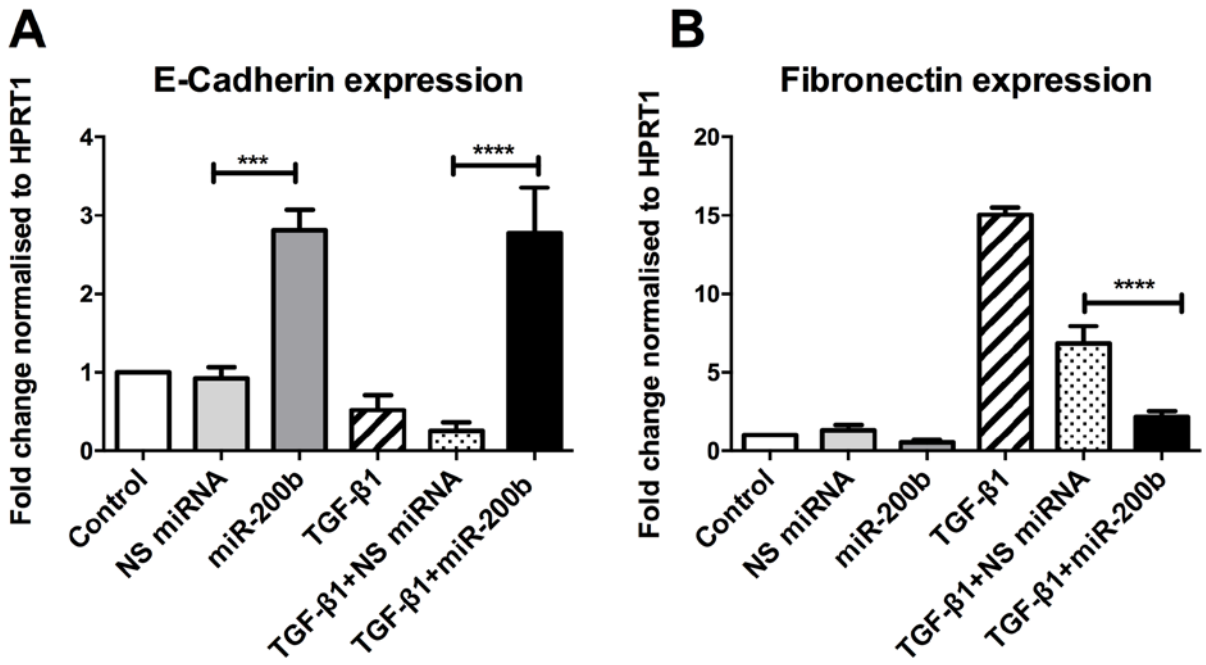

Figure 4.14: miR-200b reversed EMT in BEAS-2B cells treated with TGF-β1. BEAS-2B cells (n=3) were treated with TGF-β1 (5ng/ml) for 48 hrs followed by transfection with 30nM miR-200b for 24 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data was analysed by one way ANOVA followed by Bonferroni test [(***=p≤0.001) (****=p≤0.0001)]. The data is representative of three independent set of experiments, each done in triplicate.

4.4.5 miR-200b supresses expression of target genes involved in TGF-β1 signalling

To identify miR-200b targets, four different software algorithms were employed to find the conserved target site throughout the human transcriptome. Computational tools allowed identification of 7 common genes as potential targets of miR-200b by matching the complementarity between the seed region (2-8 bases) of the miRNA and 3' untranslated region of an mRNA using TargetScan, MiRanda, DIANA-Micro-T and PicTar (Figure 4.15A). Out of the 7 targets the expression of 4 selective miRNA target genes was assessed.

miR-200b significantly reduced the expression of ZNF532 in the presence of TGF-β1 as compared to NSmiRNA + TGF-β1 treated cells in BEAS-2B cells (p≤0.0001), and

PBECs ($p \leq 0.05$). In PBECs from lung allograft, although there was no significant reduction in ZNF532 in miR-200b transfected and TGF-β1 treated cells when compared to NSmiRNA + TGF-β1 treated cells, the trend of expression was similar to that observed in BEAS-2B cells and PBECs.

While a significant reduction in ZEB2 was only observed in BEAS-2B cells ($p \leq 0.0001$). PBECs and PBECs from lung allograft showed a significant reduction in RHOA ($p \leq 0.05$) and SMURF2 ($p \leq 0.0001$) respectively. In PBECs and PBECs from lung allograft, the expression of ZEB2 was only detectable in TGF-β1 treated cells and so the data couldn't be plotted (Figure 4.15B, n=3).

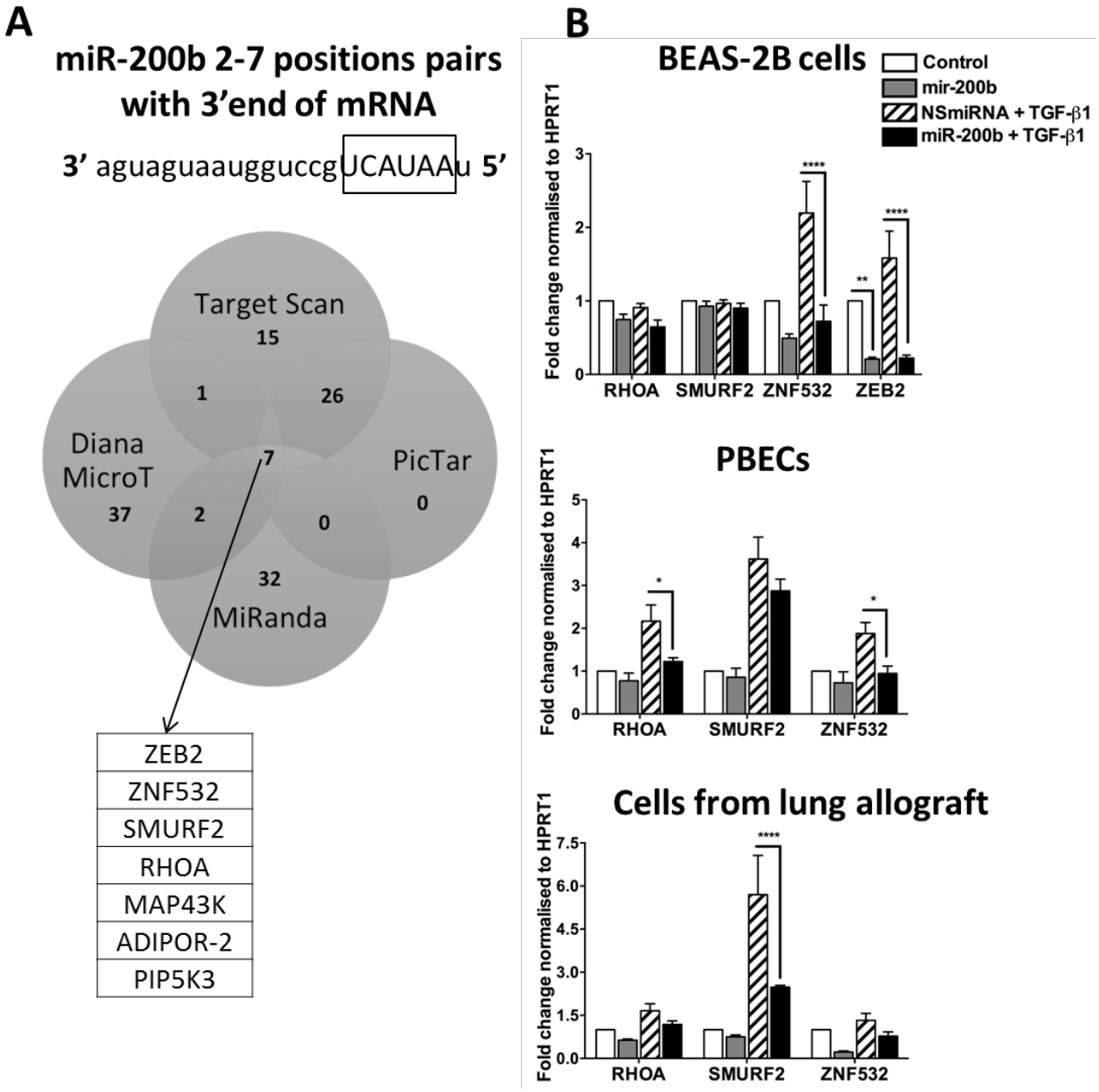

Figure 4.15: miR-200b targets were predicted and analysed using computational tools and qRT-PCR. Venn diagram shows the intersection analysis of miR-200b target genes in TargetScan, PicTar, Diana-Micro T and MiRanda. Seven common mRNA targets were predicted using the online prediction tools (A). BEAS-2B cells, PBECs and cells from transplant brushings were transfected with 30nM miR-200b before being exposed to 5ng/ml TGF-β1 for 48 hrs. mRNA expression of target genes RHOA, SMURF2, ZNF532 and ZEB2 was quantified using q-RT-PCR (B). The data (B) was analysed by two way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (****=p≤0.0001)]. The data (B) is representative of three independent set of experiments, each done in triplicate.

4.4.6 miR-200b modulates the expression of specific transcription factors involved in mediating TGF-β induced fibrosis

A TGF-β array was performed to study other differentially expressed genes in miR-200b transfected and TGF-β treated cells. Target genes were categorised according to the pathway that activates them- activins/inhibins receptor mediated pathway, BMP receptor mediated pathway and TGF-β receptor mediated pathway (Figure 4.16, n=1). These results demonstrated the need to use online computational tools in order look up which mRNA candidates (out of all differentially expressed genes in the results) had binding sites for miR-200b or are direct targets of miR-200b.

4.4.7 miR-200b directly targets ZEB2 and ZNF532 that are integral members of the TGF-β signalling pathway

The likelihood of a direct targeting mechanism of miR-200b to ZEB2 and ZNF532 3'UTR was studied (n=2). The luciferase reporter plasmids containing binding regions (ZEB2 and ZNF532 3'UTR regions) to miR-200b were co-transfected with 30nM miR-200b mimics. There was 62 % reduction in luciferase activity ($p \leq 0.01$) in cells co-transfected with the mimic and plasmid containing ZEB2 gene as compared to cells co-transfected with NSmiRNA and plasmid containing ZEB2 gene. MiR-200b mimics reduced luciferase activity by 54% in cells transfected with plasmid containing ZNF532 gene as compared to cells co-transfected with NSmiRNA and plasmid containing ZNF532 gene (Figure 4.17A). Similar results were obtained when experiments were conducted using PBECs (Figure 4.17B). This confirms that miR-200b directly targets ZEB2 and ZNF532 mRNA.

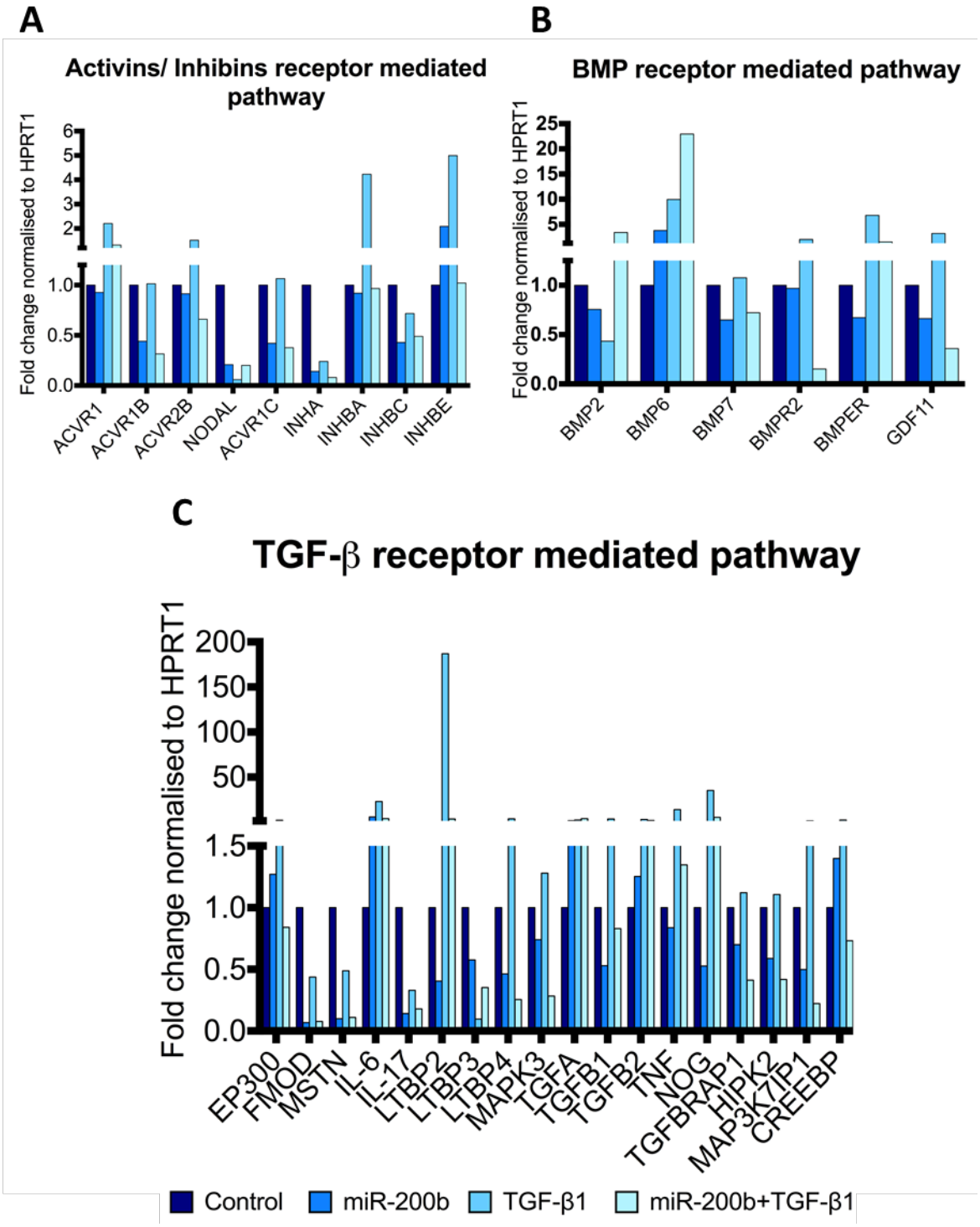

Figure 4.16: Expression profiling of genes regulated by TGF-β. BEAS-2B cells were transfected with 30nM miR-200b for 24 hrs followed by TGF-β1 (5ng/ml) treatment for 24 hrs. Total RNA was harvested and TGF-β pathway array was used. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT)}$ in comparison to the untreated control cells.

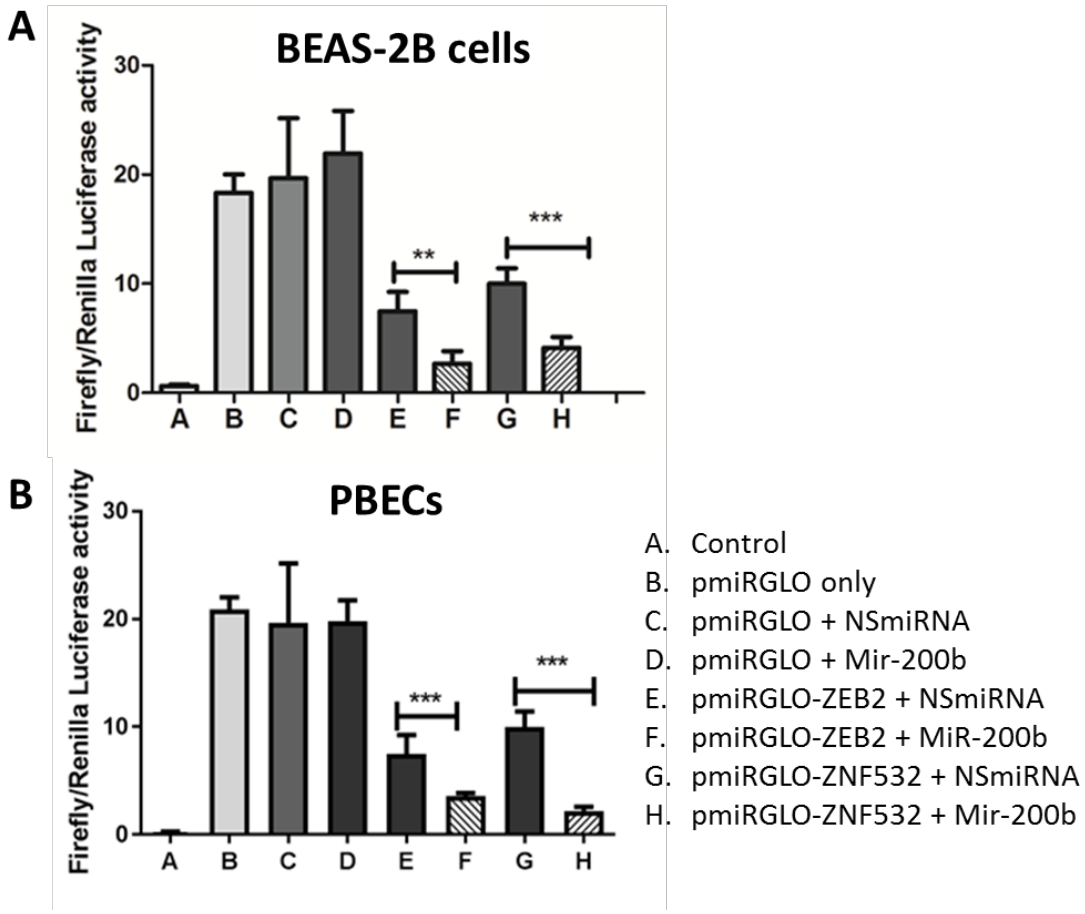

Figure 4.17: miR-200b binds to the 3'UTR region of ZEB2 and ZNF532 gene. 3'UTR region of ZEB2 and ZNF532 containing miR-200b binding site was cloned into the 3'UTR region of luciferase firefly gene in pmiRGLO expression vector. The cloned plasmid was co-transfected with 30nM miR-200b mimics or non-specific miRNA in BEAS-2B cells (A) and PBECs (B). Firefly luciferase activity was measured at 24 hrs post transfection and normalised to renilla luciferase activity (n=2). miR-200b significantly reduced the luciferase activity in cells transfected with plasmid containing 3' UTR of ZEB2 and ZNF532. The data was analysed by one way ANOVA followed by Bonferroni test [(**=p≤0.01) (***=p≤0.001)]. The data is representative of two independent set of experiments, each done in quadruplicate.

4.4.8 In-situ detection of miR-200b in normal paraffin embedded lung sections

The study was designed to examine the expression of miR-200b in tissue derived from normal donor lung tissue that was subsequently used for transplantation. A strong positive staining for miR-200b was observed in the bronchial epithelium region of lung tissue (Figure 4.18A) while the negative control (scrambled miRNA) showed

no staining (Figure 4.18C). There was strong staining (deep purple) when sections were incubated with the positive control miRNA-126 (Figure 4.18B).

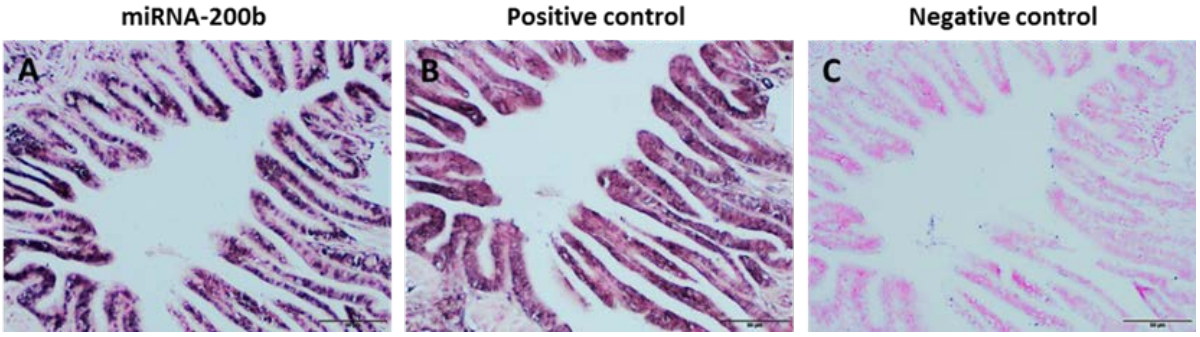

Figure 4.18: Localization of miRNA-200b in EVLP sections by in-situ hybridization (FFPE). Strong expression of miR-200b was observed in bronchial epithelium region (A). Sections were positive for miR-126 (deep purple staining; B) and no staining was observed for sections probed with scrambled miRNAs (C). Images were captured at 20X magnification; scale bar 50µm (n=3).

4.4.9 Effect of miR-200b inhibition in TGF-β1 treated BEAS-2B cells

The miRNA-200b inhibitor concentration was optimised before manipulating its expression in TGF-β1 treated and untreated cells (Section 2.8.3). A significant decrease in miR-200b ($p \leq 0.001$) was observed when cells were transfected with miR-200b inhibitor. Therefore, 50nM miR-200b inhibitor was selected as an optimum concentration after monitoring the changes in BEAS-2B cell phenotype and changes in fibronectin expression at RNA level (Figure 2.11).

BEAS-2B cells were transfected with 30nM miR-200b hairpin inhibitor or NSmiRNA for 24 hrs followed by 48 hrs of TGF-β1 treatment (n=3). The expression of E-cadherin and Fibronectin was studied using q-RT-PCR. E-cadherin expression was inhibited in miR-200b inhibitor transfected and TGF-β1 treated cells, however the change was not significant when compared to NSmiRNA + TGF-β1 treated cells (Figure 4.19). There was no significant reduction in E-Cadherin expression in cells

transfected with miR-200b inhibitor when expression was compared to NSmiRNA transfected cells. miR-200b inhibitor transfection did not induce an increase in fibronectin expression when expression was compared to NSmiRNA transfected cells. Furthermore, there was no change in Fibronectin expression in miR-200b transfected and TGF-β1 treated cells when expression was compared to NSmiRNA + TGF-β1 treated cells.

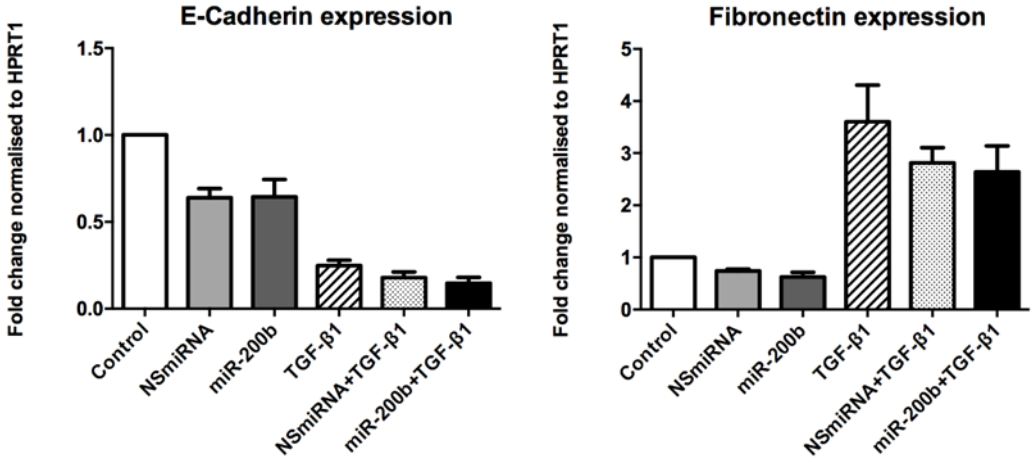

Figure 4.19: miR-200b inhibition had no effect on EMT marker expression with or without TGF-β1. BEAS-2B cells were transfected with 50nM miR-200b inhibitors for 24 hrs followed by TGF-β1 (5ng/ml) treatment for 48 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data is representative of three independent set of experiments, each done in triplicate.

4.5 Discussion

The initial miRNA study elucidated the potential role of miR-200b in the development of airway fibrosis. miRNA mimic transfection studies were conducted to confirm the involvement and importance of these exclusive candidates. Thereafter, online computational tool analysis revealed ZEB2 and ZNF532 as potential direct targets of miR-200b. In order to confirm the direct targeting, a luciferase assay using pmiRGLO miRNA expression vector was performed (Figure 4.20).

BOS occurs because of progressive loss of lung function. Airway EMT that involves a complex series of events is a potential major leading cause of lung allograft fibrosis. However, the role of miRNAs in EMT and development of BOS is not well established (Yang *et al.*, 2012a). A recent study elucidated the role of circulatory miRNA as a potential biomarkers of BOS. A selected group of miRNAs were significantly upregulated in BOS + patients prior to clinical BOS diagnosis with miR-21, miR-103 and miR-191 involved (Budding *et al.*, 2016). In another study, *in situ* hybridisation revealed that miR-34a and miR-21 were upregulated in explanted human BOS lungs (Di Carlo *et al.*, 2016). These studies listed miRNA candidates upregulated during BOS, but failed to recognise miRNAs that could prevent TGF-β induced EMT, which may play a central role in BOS pathogenesis (Hodge *et al.*, 2009). In my study the effectiveness of miR-200b to prevent TGF-β1 induced EMT in BEAS-2B cells and patient derived PBECs was demonstrated. My aim was to detect and quantify changes in EMT in bronchial epithelial cells as previously shown by Hodge S *et al* (Hodge *et al.*, 2009) in miR-200b transfected cells pre-treated with TGF-β1. I also replicated this study in cells from transplant brushings. It was of interest that the work in transplant brushings showed no significant change in E-cadherin expression although a significant decrease in fibronectin was noted in miR-200b transfected and TGF-β1 treated cells. This could be due the fact that following transplant, cells show increased expression of IL-8, MMP9, MMP2 and IL-6 as compared to cells acquired from healthy individuals. Therefore, the expression profile of markers in these cells may differ to those normally expressed in PBECs acquired from normal epithelium (Kalluri and Neilson, 2003). TGF-β1 induced changes in cells were reversible following miR-200b manipulation. I also showed that, miR-200b was able to restore the loss of the epithelial cell marker, E-Cadherin while significantly reducing the expression of fibronectin in BEAS-2B cells.

Online computational tools revealed 7 potential miRNA targets out of which, four mRNA genes namely RHOA, SMURF2, ZNF532 and ZEB2 were studied that belong to the TGF-β1 signalling pathway. These targets have an exact match to positions 2 to 7 of the mature 3' arm of miR-200b and thus interact with high specificity. Recent studies have reported that miR-200b downregulates zinc finger proteins. ZEB2 and ZNF532 are E-Box– binding proteins that are involved in repressing E-cadherin transcription and hence revoke E-cadherin–mediated intercellular adhesiveness. These actions of zinc finger proteins may make them important candidates in the early stage of EMT (Comijn *et al.*, 2001; Dinney *et al.*, 2011).

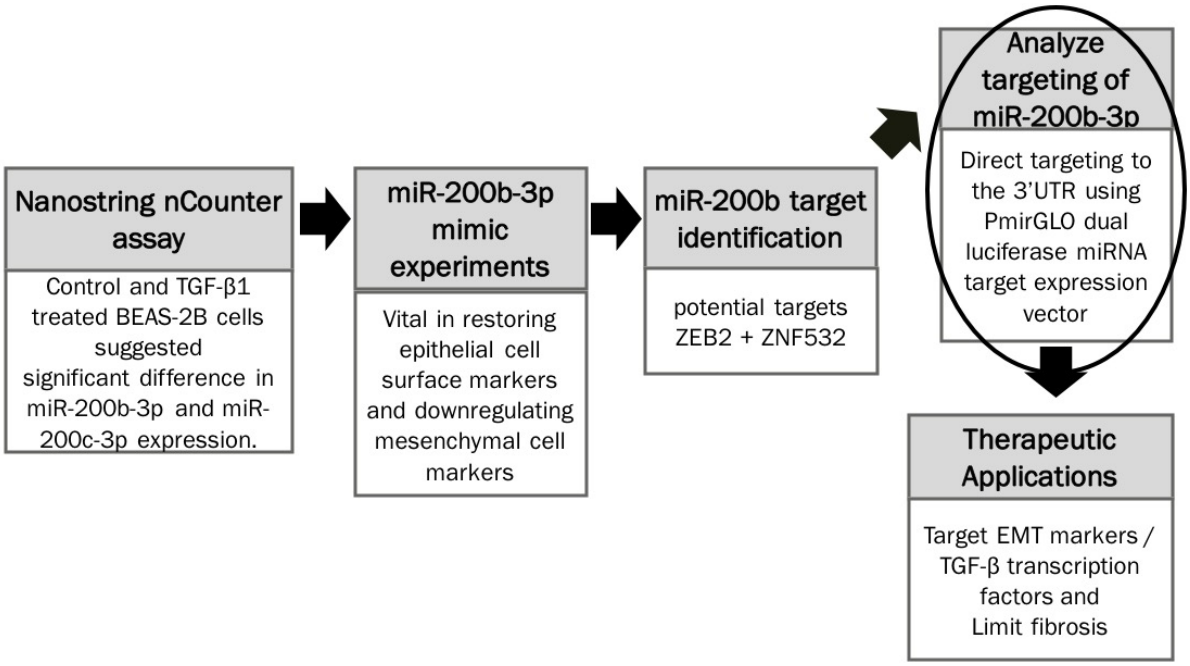

Figure 4.20: Overview of miR-200b study and importance of investigating miR-200b targeting. The initial miRNA profiling revealed that expression miR-200b and miR-200c-3p was significantly downregulated post TGF-β1 treatment (1hr, 4 hrs and 24 hrs). Thereafter miR-200b mimic transfection in the presence and absence of TGF-β1 was studied in BEAS-2B cells. miR-200b targets were identified using online computational tools. After performing studies at RNA level, ZEB2 and ZNF532 were found to be potential direct targets of miR-200b. To confirm the direct targeting, PmiRGLO miRNA expression vector and luciferase assay was used.

Several reports have also established that ZEB1 and ZEB2 are key transcriptional regulators of TGF-β mediated suppression of E-cadherin (Shirakihara *et al.*, 2007). In

my study, mature miRNA-200b was able to directly target transcription factors ZEB2 and ZNF532 3'UTR region upon transfection in BEAS-2B cells with and without TGF-β1 stimulation, representing the first such data in airway epithelial cells. In PBECs, although the expression of ZEB2 was undetermined using qRT-PCR, the luciferase reporter assay was performed and the results were consistent with data from my BEAS-2B study. Hence specific targeting of ECM markers and transcription factors to limit fibrosis and unwanted EMT may be a potential therapeutic strategy in airway epithelial cells and BOS post lung transplantation (Sato *et al.*, 2003; Wight and Potter-Perigo, 2011).

In addition to the candidate gene approaches, a TGF-β array was used to search for possible genes and gene networks that are differently regulated in miR-200b transfected and TGF-β1 treated cells as opposed to TGF-β1 treated cells only. It was recognised that the expression of several transcription factors such as EP300, CREEBP and other proteins belonging to the TGF-β superfamily were downregulated when BEAS-2B cells were transfected with miR-200b and treated with TGF-β1 as compared to TGF-β1 treated only (Attisano and Wrana, 2002; Mauviel, 2005; Ghosh and Varga, 2007). Thus, it could be concluded that miR-200b has the potential of inhibiting the activity of other signalling molecules even in the presence of TGF-β1, a finding which might provide novel therapeutic opportunities.

Next, I used *in situ* hybridization on paraffin embedded donor lung tissue sections. A strong staining for miR-200b in these sections was restricted to the healthy bronchial epithelium. These findings suggest miR-200b may be a key homeostatic system in the epithelium, and overall our study indicates that miR-200b may modify the development of EMT, which is known to be associated with BOS.

To study the effect of miR-200b inhibition in the presence of TGF-β1, miR-200b inhibitors were used. BEAS-2B cells transfected with these inhibitors in the presence and absence of TGF-β1 did not show significant changes in EMT marker expression at the mRNA level. This could be due to the fact that the inhibitor only suppresses the expression of miR-200b but not the other members of the miR-200 family that shares the same seed region. An inhibitor crossreactivity amongst members of the miRNA family that share extensive sequence identity has been previously reported (Robertson *et al.*, 2010). Therefore, use of microRNA family inhibitors (Exiqon) could be the best way of studying the impact of miRNA inhibition on EMT marker expression as they use a pool of inhibitors to target a group of related miRNAs at a low dosage rather than a single inhibitor (Rottiers *et al.*, 2013).

To conclude, the results in this chapter suggest the potential therapeutic relevance of miR-200b in inhibiting EMT even in the presence of TGF-β1 and that this involves targeting the transcription factors ZEB2 and ZNF532. TGF-β pathway array analysis revealed other potential candidates that could be direct targets of miR-200b, but this needs further investigation. Finally, *in-situ* hybridisation showed that miR-200b is mainly localised in the epithelium, which is the primary target of injury post-lung transplantation and which is a biologically plausible location for a miRNA implicated in EMT.

Chapter 5: Involvement of miR-200c and miR-146a in bronchiolitis obliterans syndrome

5.1 Introduction

Most miR-200 studies have been carried out in the field of EMT (Park *et al.*, 2008) and in cardiovascular diseases (Magenta *et al.*, 2017). In contrast, the role of the miR-200 family in BOS is still poorly investigated. Previously, a study demonstrated a decrease in miR-200a and miR-200c levels in lungs of patients with IPF. Furthermore, introduction of mimics diminished fibrosis in bleomycin induced lung fibrosis mice models. Overall, the data suggested that restoring miR-200 levels in alveolar epithelial cells might represent a novel therapeutic approach for treatment of IPF (Yang *et al.*, 2012a). My data identified miR-200b regulation in epithelial cells stimulated with TGF-β1. It was therefore crucial to study the involvement of other members of the miR-200 family, for instance miR-200c.

A role for miR-146a has been indicated in innate immunity, inflammatory diseases (Ichii *et al.*, 2012; Feng *et al.*, 2017) and TGF-β1 induced liver fibrosis (He *et al.*, 2012). *In vitro* screening of differentially expressed miRNA candidates in asthma progression revealed that miR-146a was downregulated during asthma (Garbacki *et al.*, 2011). Overexpression of miR-146a reduced the production of various pro-inflammatory cytokines and chemokines (Perry *et al.*, 2008). Increasing evidence suggests that miR-146a limits the intensity and duration of inflammation via a feedback control mechanism by reducing levels of genes such as COX2 that are involved in inflammation and pain (Sato *et al.*, 2010). In my study, the expression of miR-146a was significantly upregulated in a dose dependent manner post TGF-β1 treatment in BEAS-2B cells. Further experiments were conducted to study the effect of ectopic expression of miR-146a in TGF-β1 treated BEAS-2B cells.

5.2 Aims

1. Determine whether transfecting cells (BEAS-2B cells and PBECs) with miR-200c mimics (pre and post TGF-β1 stimulation) lead to a change in expression of EMT markers.

2. Study the expression of miR-200c target genes that are involved in TGF-β signalling.

3. Validate the miR-200c direct targets- ZEB2 and ZNF532 using the pmiRGLO miRNA luciferase assay system.

4. Study the effect of using miR-146a mimics in BEAS-2B cells treated and untreated with TGF-β1.

5.3 Results

5.3.1 miR-200c mimics restored TGF-β1 induced downregulation of E-Cadherin and reduced fibronectin at the RNA level

miRNA profiling (NanoString) data suggested a significant decrease in miR-200c-3p expression in TGF-β1 treated BEAS-2B cells at 1 hr, 4 hrs and 24 hrs, as compared to the untreated control (Figure 5.1A; n=2). This was in accordance with the expression profile previously demonstrated for miR-200b-3p (Chapter 3; Figure 3.14). To validate the expression profile of miR-200c, qRT-PCR was performed. The results suggested a significant decrease in miR-200c expression at 4 hrs ($p<0.001$) and 24 hrs ($p<0.001$) in TGF-β1 treated BEAS-2B cells when compared to the control/ untreated cells (Figure 5.1B; n=3).

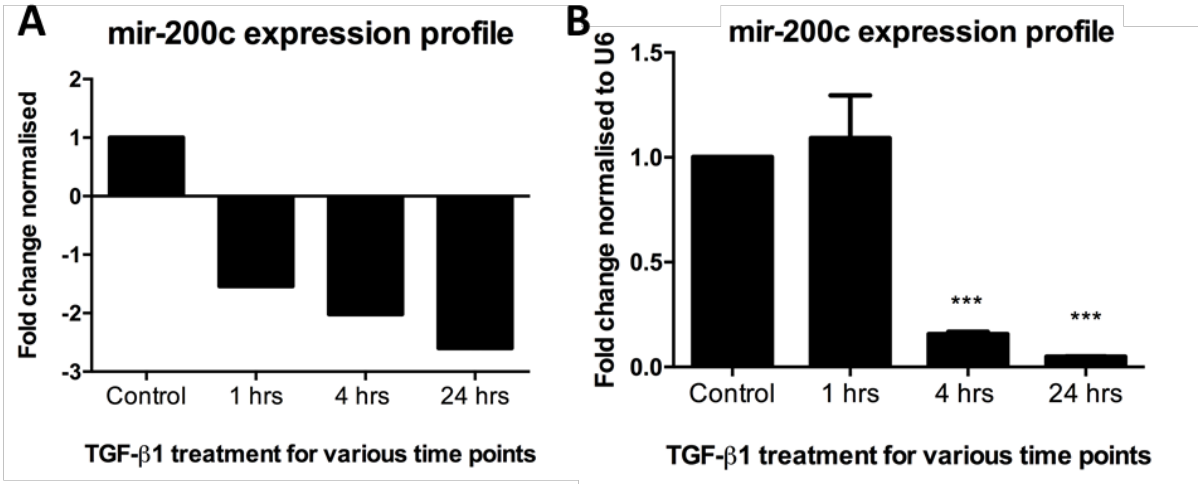

Figure 5.1: MiR-200c expression at various time points in BEAS-2B cells. BEAS-2B cells were treated with TGF-β1 for various time points. Panel A shows graph obtained post NanoString analysis. Panel B shows analysis of miR-200c at 1 hr, 2 hrs 4 hrs and 24 hrs post treatment with TGF-β1 (using q-RT PCR) normalized to U6 and expression levels compared to untreated control (n=3). The data was analysed by one way ANOVA followed by Bonferroni test (***=p≤0.001) compared to the control. The data (B) is representative of three independent set of experiments, each done in triplicate.

To evaluate the effect of miR-200c mimics, BEAS-2B (n=3) and PBECs (n=3) were transfected with miR-200c for 24 hrs followed by treatment with TGF-β1 for 48 hrs. Total RNA was isolated and expression level of E-Cadherin and fibronectin was and downregulated the expression of fibronectin in TGF-β1 treated BEAS-2B cells ($p<0.05$, $p<0.0001$) and PBECs ($p<0.01$, $p<0.0001$). There was also significant increase in E-cadherin expression in BEAS-2B cells and PBECs following miR-200c mimic transfection ($p<0.0001$, $p<0.01$). The experiments were normalised to endogenous control HPRT1 and compared to TGF-β1 cells (Figure 5.2).

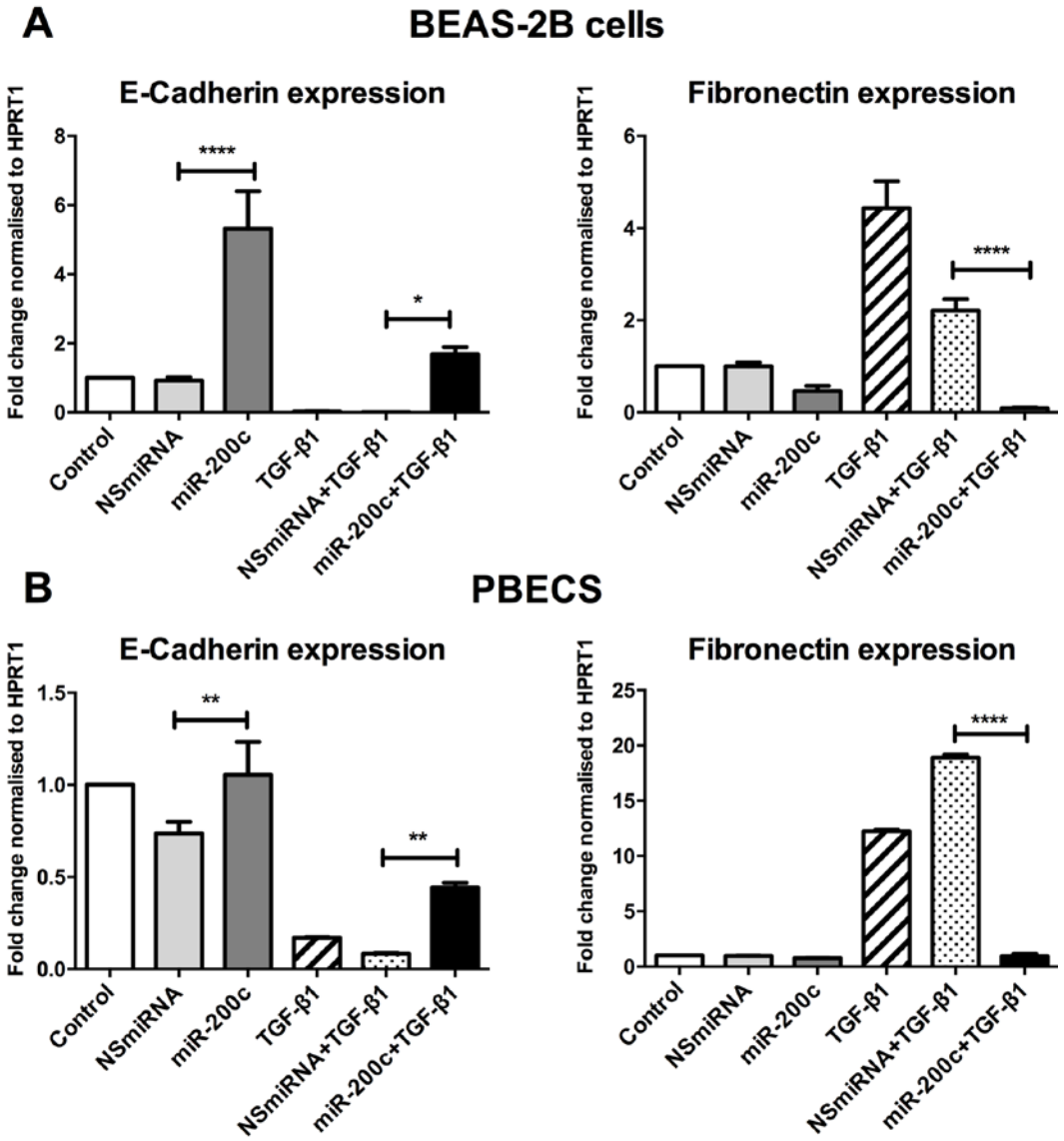

Figure 5.2: miR-200c mimics restored TGF-β1 induced downregulation of E-Cadherin and reduced fibronectin at the RNA level. BEAS-2B cells (A, n=3) and PBECs (B, n=3) were transfected with 30nM miR-200c for 24 hrs followed by TGF-β1 (5ng/ml) treatment for 48 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (****=p≤0.0001)]. The data is representative of three independent set of experiments, each done in triplicate.

5.3.2 miR-200c reduces expression of fibrotic markers at protein level.

Changes in protein expression post miR-200c transfection were examined using immunofluorescence. The results were consistent with changes in mRNA expression

in BEAS-2B cells. The expression of E-Cadherin and Cytokeratin-19 decreased while expression of α-SMA and fibronectin increased in NSmiRNA +TGF-β1 treated BEAS-2B cells. However, transfecting BEAS-2B cells with miR-200c mimics lead to restoration of E-Cadherin and Cytokeratin-19 expression while the expression of α-SMA and fibronectin diminished post TGF-β1 treatment (Figure 5.3). Thus, the results confirmed that miR-200c (of the miR-200 family) like miR-200b restored epithelial cell markers while significantly reducing fibrotic marker expression even in the presence of TGF-β1.

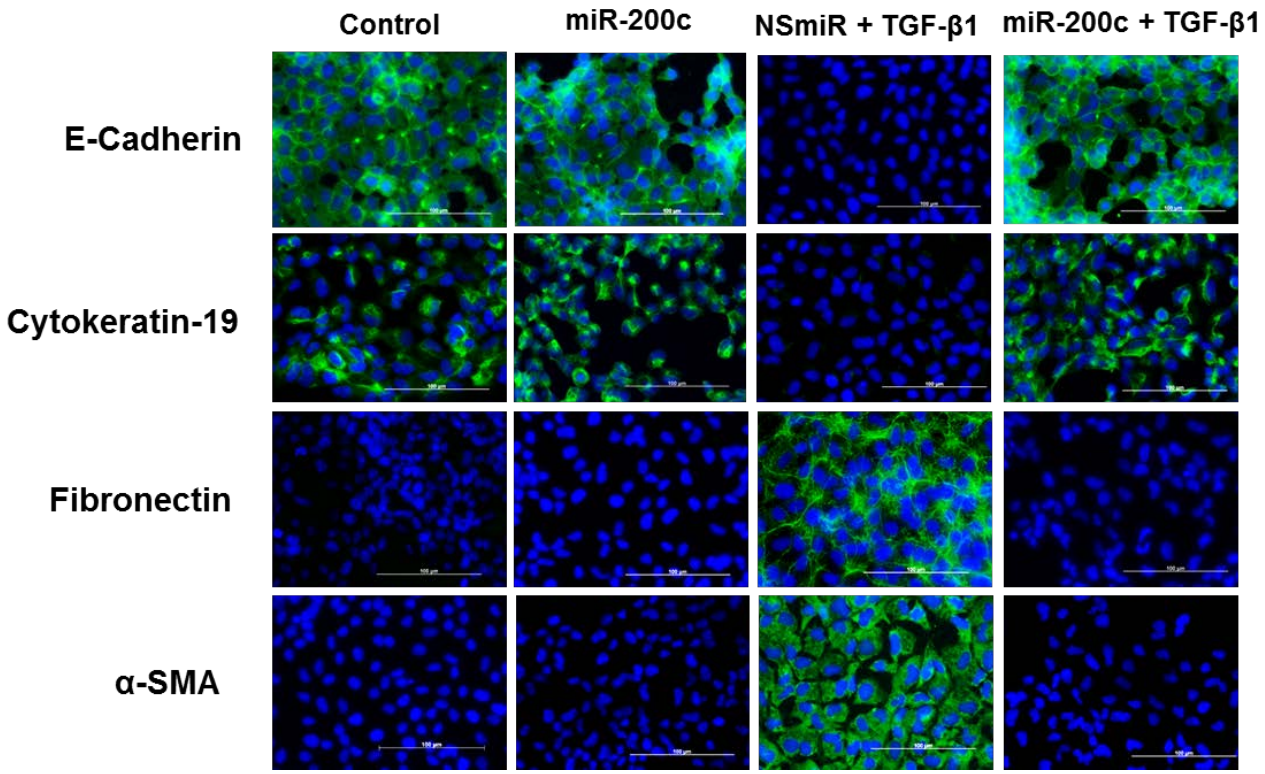

Figure 5.3: miR-200c mimic transfection reduced expression of fibrotic markers at protein level. BEAS-2B cells were transfected with NSmiRNA (negative control) and 30nM miR-200c mimics. Post-transfection cells were treated with/without 5ng/ml TGF-β1 for 48 hrs (n=3). Pictures were captured using Zeiss Axioimager microscope and the two channels DAPI and FITC were merged (40X). Scale bar, 100µm

5.3.3 miR-200c reverses EMT in TGF-β1 treated BEAS-2B cells

To investigate whether miR-200c could restore epithelial cell markers after the onset of fibrosis, BEAS-2B cells were treated with TGF-β1 followed by transfection with miR-200c. MiR-200c mimic transfection (24 hrs) restored E-Cadherin levels and downregulated fibronectin (both $p<0.0001$) in TGF-β1 treated (48 hrs) BEAS-2B cells when expression was normalised to endogenous control HPRT1. Furthermore, there was significant increase in E-Cadherin expression ($p<0.0001$) in miR-200c mimic transfected cells as compared to NSmiRNA transfected cells (Figure 5.4). Therefore, miR-200c was able to reverse EMT in cells that had already acquired fibrotic characteristics.

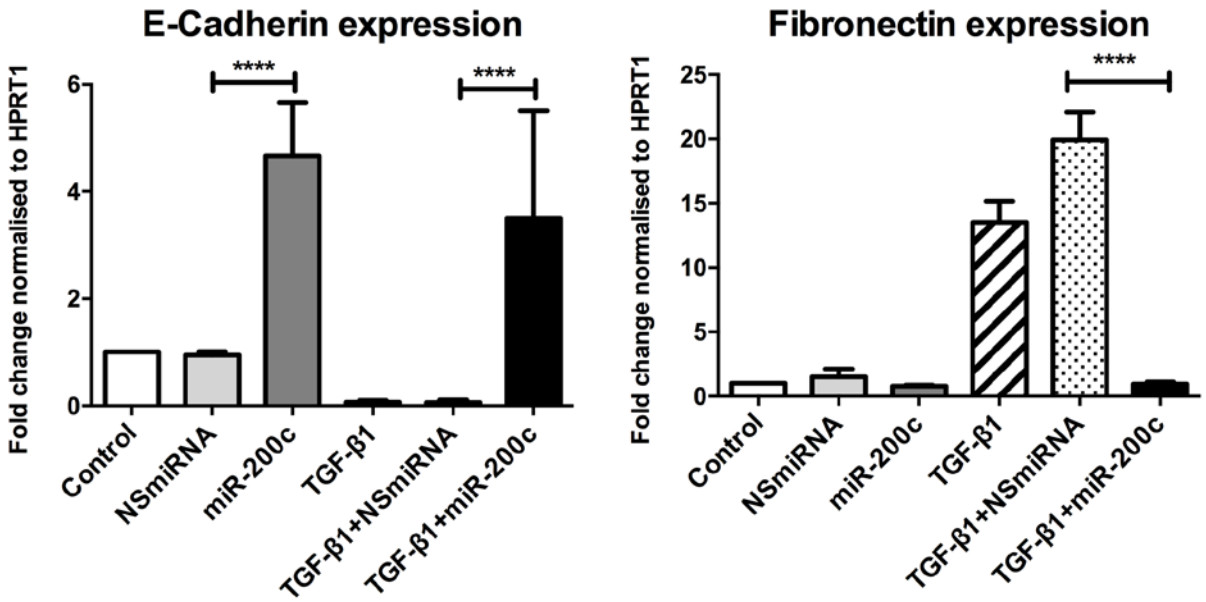

Figure 5.4: MiR-200c reverses EMT in TGF-β1 treated BEAS-2B cells at mRNA level. BEAS-2B cells were treated with TGF-β1 (5ng/ml) for 48 hrs followed by transfection with 30nM miR-200c for 24 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data was analysed by one way ANOVA followed by Bonferroni test (****=$p\leq0.0001$). The data is representative of three independent set of experiments, each done in triplicate.

5.3.4 miR-200c supresses expression of selective target genes involved in TGF-β1 signalling

MiR-200b, miR-200c, and miR-429 (AAUACU) share the same binding sequence region and therefore they would bind to the same mRNA targets. I previously studied the expression of RHOA, SMURF2, ZNF532 and ZEB2 in TGF-β1 treated cells that were transfected with miR-200b mimics. The gene expression was then compared to cells treated with TGF-β1 only. In order to examine whether miR-200c has similar effect as previously demonstrated with miR-200b mimics, the experiment was replicated using miR-200c mimics in BEAS-2B cells and PBECs.

miR-200c significantly reduced the expression of ZNF532 and ZEB2 alone ($p \leq 0.01$, $p \leq 0.05$) and in the presence of TGF-β1 ($p \leq 0.05$, $p \leq 0.001$) as compared to untreated cells and NSmiRNA+TGF-β1 treated BEAS-2B cells respectively (Figure 5.5A). In PBECs, a significant reduction in SMURF2 ($p \leq 0.001$) and RHOA ($p \leq 0.001$) was observed (Figure 5.5B). There was significant reduction in ZNF532 expression in miR-200c transfected cells ($p \leq 0.01$) and miR-200c+TGF-β1 treated cells ($p \leq 0.001$) as compared to the control cells and NSmiRNA+ TGF-β1 treated cells respectively. Furthermore, the expression of ZEB2 was only detectable in TGF-β1 treated PBECs and therefore data for the same could not be plotted. The results suggest that it is likely that miR-200c enhances E-Cadherin expression by directly reducing the expression of its target genes involved in TGF-β signalling pathway.

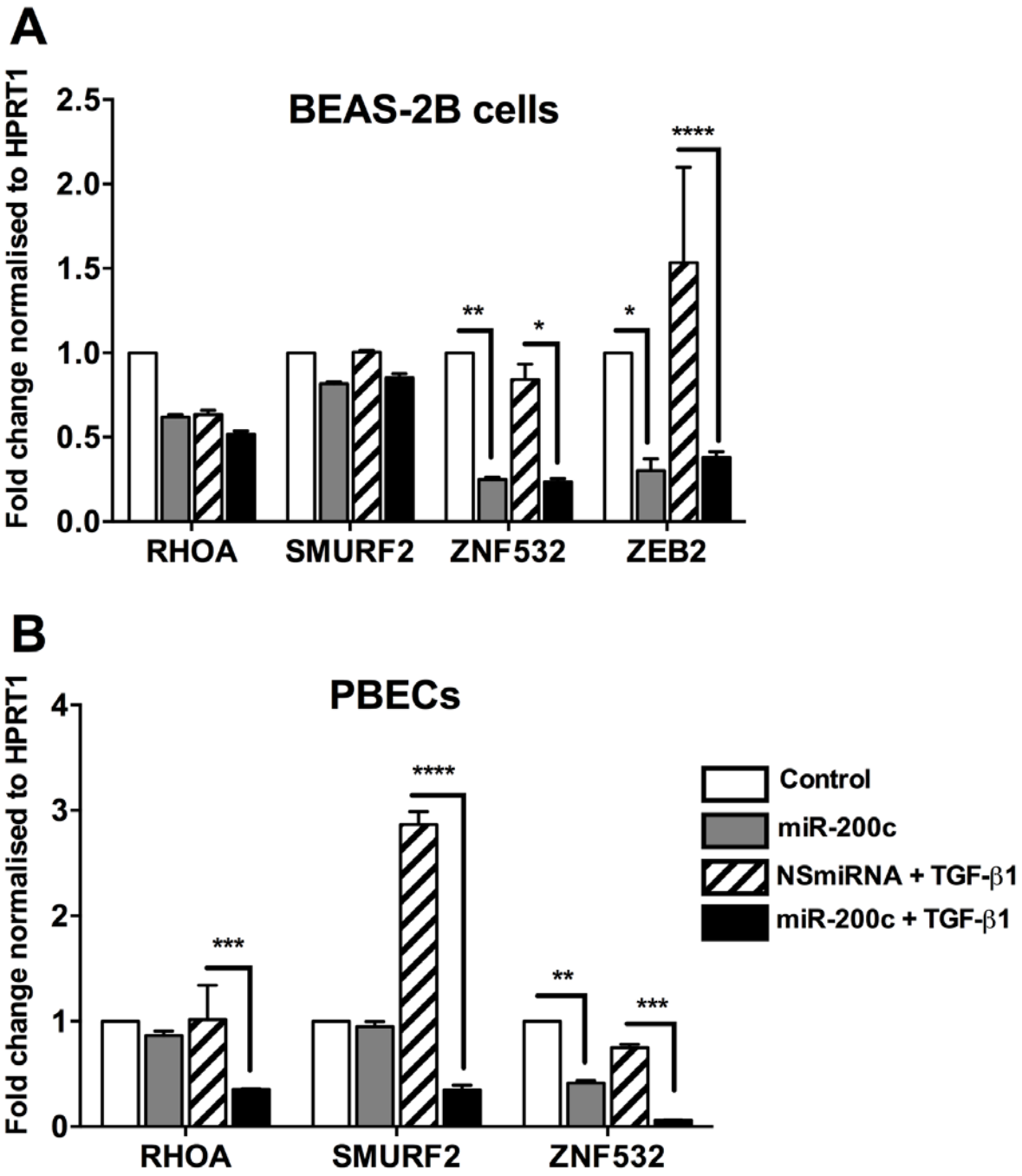

Figure 5.5: miR-200c supresses expression of selective target genes involved in TGF-β1 signalling. BEAS-2B cells (A) and PBECs (B) were transfected with 30nM miR-200c before being exposed to 5ng/ml TGF-β1 for 48 hrs. MRNA expression of target genes RHOA, SMURF2, ZNF532 and ZEB2 was quantified using q-RT-PCR. The data was analysed by two way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001) (****=p≤0.0001)]. The data is representative of three independent set of experiments, each done in triplicate.

5.3.5 miR-200c directly targets transcription factors ZEB2 and ZNF532 thereby reducing their expression.

To test whether miR-200c directly targets ZEB2 and ZNF532, BEAS-2B cells and PBECs were co-transfected with luciferase reporter plasmids containing binding regions for ZEB2 and ZNF532 3'UTR regions and 30nM miR-200c mimics. There was significant reduction in luciferase activity in miR-200c mimic and plasmid transfected BEAS-2B cells (both ZEB2 and ZNF532 p≤0.001) and PBECs (both ZEB2 and ZNF532 p≤0.001) as compared to cells transfected with NSmiRNA and plasmid (containing ZEB2 and ZNF532 region). The reduction in luciferase activity demonstrated that miR-200c binds to specific sites in the 3'UTR of ZEB2 and ZNF532 thereby significantly reducing downstream protein synthesis (Figure 5.6, n=2).

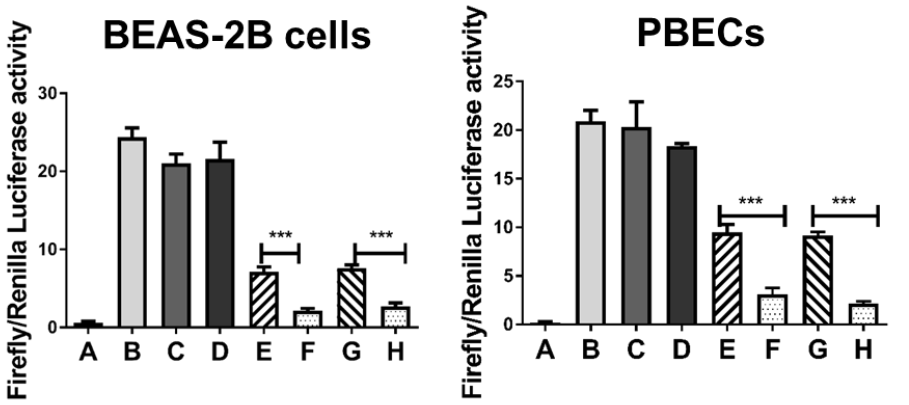

Figure 5.6: miR-200c directly targets transcription factors ZEB2 and ZNF532 thereby reducing their expression. 3'UTR region of ZEB2 and ZNF532 containing miR-200b binding site was cloned into the 3'UTR region of luciferase firefly gene in pmiRGLO expression vector. BEAS-2B cells (A, n=3) and PBECs (B, n=3) were co-transfected with 30nM miR-200c and the cloned plasmid containing ZEB2/ZNF532 3'UTR region. Firefly luciferase activity was measured at 24 hrs post transfection and normalised to Renilla luciferase activity. The data was analysed by one way ANOVA followed by Bonferroni test (***=p≤0.001). The data is representative of two independent set of experiments, each done in quadruplicate.

5.3.6 No effect of miR-146a overexpression in BEAS-2B cells treated and untreated with TGF-β1.

NanoString nCounter miRNA assay (n=2) revealed that miR-146a that is involved in inflammation was significantly upregulated at all time points post TGF-β1 treatment (Figure 5.7A). This was validated by qRT-PCR (n=3) that also suggested a significant increase in miR-146a expression at 1 hr ($p \leq 0.05$), 4 hrs ($p \leq 0.01$) and 24 hrs ($p \leq 0.001$) post TGF-β1 stimulation (Figure 5.7B).

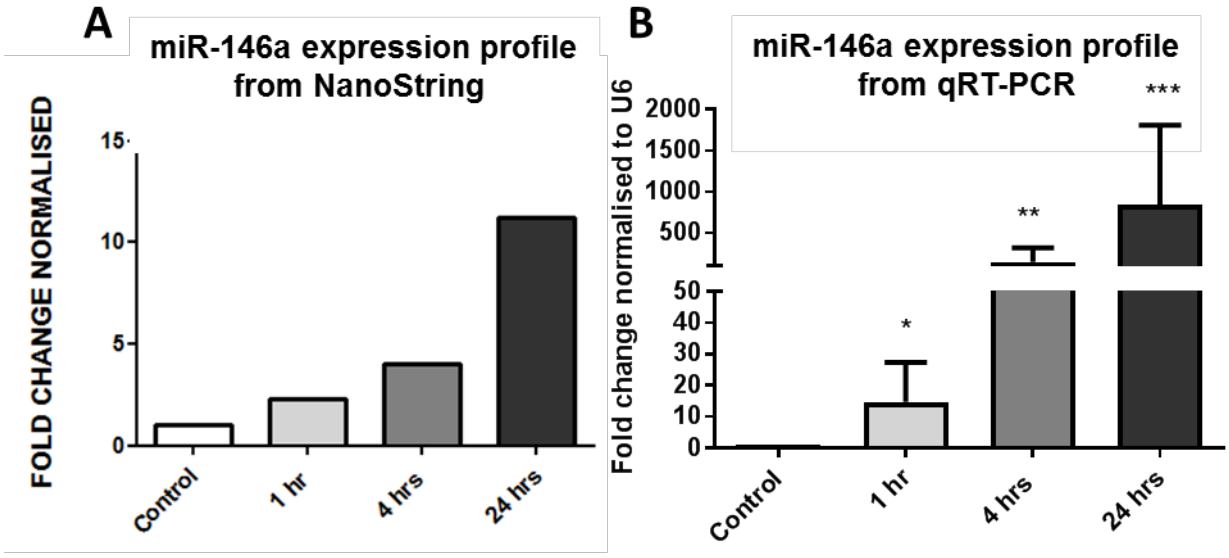

Figure 5.7: miR-146a expression at various time points in BEAS-2B cells. BEAS-2B cells were treated with TGF-β1 for various time points. Panel A (n=2) shows graph obtained post NanoString analysis. Panel B (n=3) shows expression of miR-146a at 1 hr, 2 hrs 4 hrs and 24 hrs post treatment with TGF-β1 (using q-RT PCR) normalized to U6 and expression levels compared to untreated control. The data was analysed by one way ANOVA followed by Bonferroni test [(*=p≤0.05) (**=p≤0.01) (***=p≤0.001)] compared to the control. The data is representative of three (B) independent set of experiments, each done in triplicate.

miR-146a mimic concentration was optimised (n=2) by transfecting BEAS-2B cells with varying concentration of the mimic, isolating RNA and performing q-RT-PCR. MiR-146a expression level was significantly upregulated at 10nM, 30nM, 50nM, 70nM and 90nM post transfection with miR-146a mimics (all p≤0.0001; Figure 5.8B). Furthermore, no morphological changes in cells were observed when cells were

transfected with 10nM, 30nM and 50nM miR-146a mimics. Using concentration higher than 50nM resulted in increased cell death (Figure 5.8A). Therefore, for further experiments 10nM of miR-146a mimics was used.

To study the effect of miR-146a in TGF-β1 induced EMT, BEAS-2B cells were transfected with 10nM miR-146a mimics in the presence and absence of TGF-β1. Thereafter, changes in expression of EMT markers were evaluated at the RNA level. There was no significant reduction in E-Cadherin and Fibronectin expression in miR-146a mimics transfected cells and miR-146a mimics transfected and TGF-β1 treated cells as compared to NSmiRNA and NSmiRNA+ TGF-β1 treated cells respectively (Figure 5.9, n=3). Therefore, manipulating the expression of miR-146a had no effect on EMT marker expression.

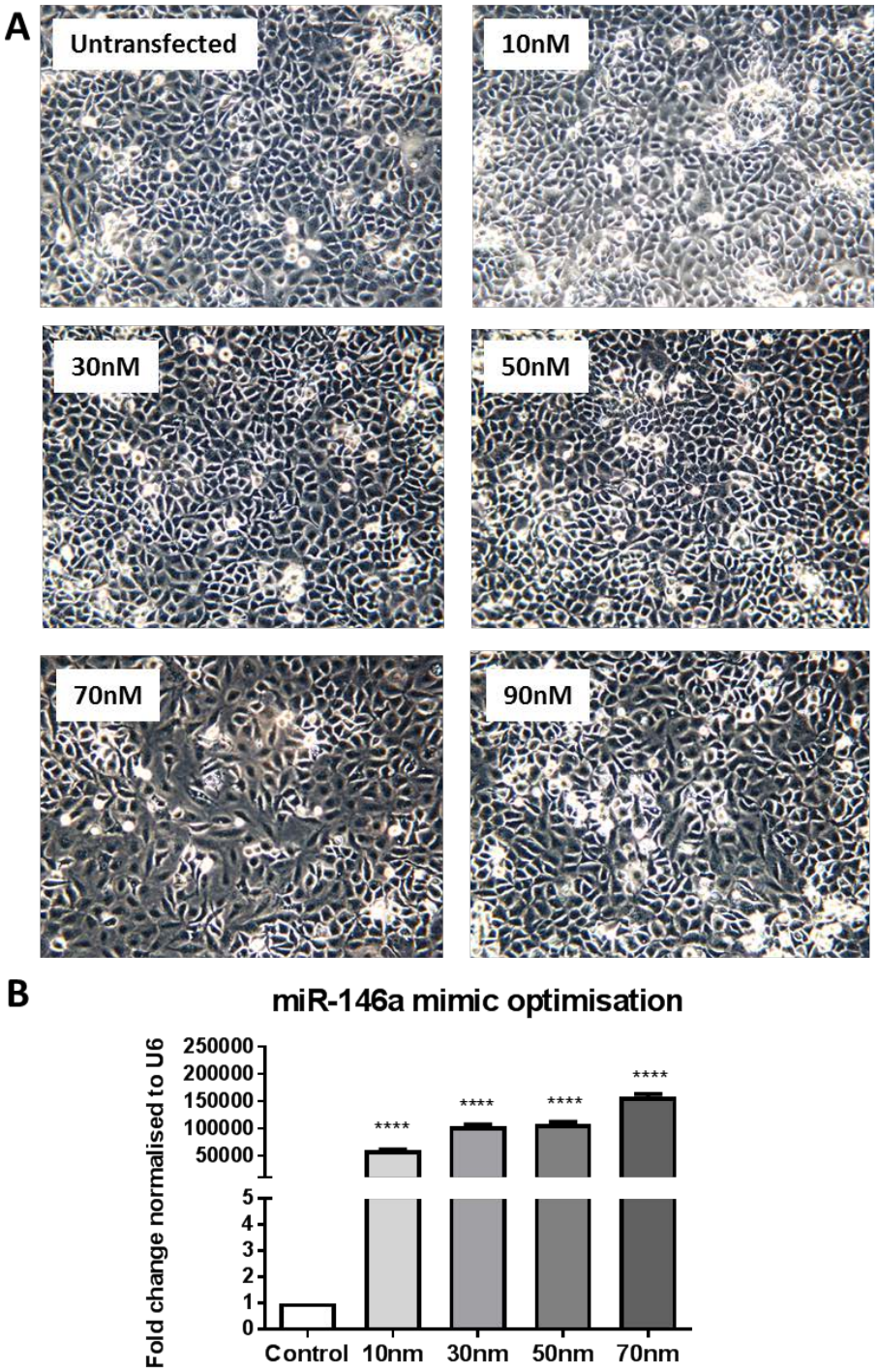

Figure 5.8: miR-146a concentration optimization in BEAS-2B cells. BEAS-2B cells were transfected with 10nM, 30nM, 50nM, 70nM and 90nM miR-146a mimic for 24 hrs. RNA was isolated followed by cDNA synthesis and q-RT PCR was performed. Panel A shows phenotypic changes post transfection with varying concentration of miR-146a mimics. miR-146a gene expression (B) profile was evaluated and plotted. Excel was used to analyse the data and graphs were plotted using prism 6 software. The data was analysed by one way ANOVA followed by Bonferroni test (****=p≤0.0001) compared to the control. The data (B) is representative of two independent set of experiments, each done in triplicate.

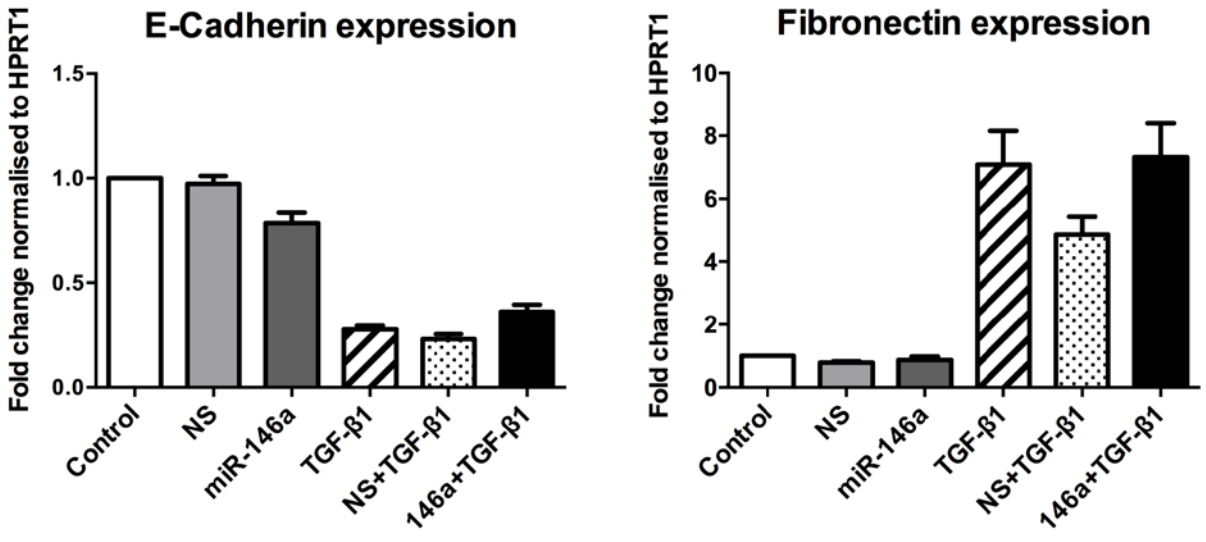

Figure 5.9: miR-146a mimic transfection post TGF-β1 stimulation in BEAS-2B cells had no effect on EMT markers and TGF-β signalling molecules. BEAS-2B cells (n=3) were transfected with 10nM miR-146a for 24 hrs followed by TGF-β1 (5ng/ml) treatment for 48 hrs. Total RNA was harvested and q-RT-PCR was performed. Expression levels were normalized to the housekeeping gene HPRT1 and calculated as fold change ($2^{-\Delta\Delta CT}$) in comparison to the untreated control cells. The data is representative of three independent set of experiments, each done in triplicate.

5.4 Discussion

Studies have previously linked the miR-200 family with epithelial phenotype and their expression is significantly downregulated during EMT (Korpal *et al.*, 2008; Gregory *et al.*, 2011). It has also been suggested that miRNA's from the same family may target the same process co-operatively thereby obtaining a more effective regulation (Barnes *et al.*, 2007). In animals, miRNAs function by base pairing (seed region) with the complementary sequence in the 3'UTR of the target mRNA. Based on the similarity of the seed region nucleotides, miR-200b and miR-200c are predicted to interact with the same targets. Therefore, following my work implicating miR-200 in EMT, it was logical to study another candidate from the miR-200 family to examine whether transfecting BEAS-2B cells and PBECs with miR-200c mimics had the same

effect as observed previously with miR-200b mimics. In this study, the effect of ectopic expression of miR-200c in TGF-β1 treated cells was examined. Like miR-200b, miR-200c was not only able to maintain the expression of epithelial cell surface markers in the presence of TGF-β1 but also was able to restore the expression of epithelial cell marker- E-cadherin when BEAS-2B cells were pre-treated with TGF-β1. This was observed by performing studies at the RNA level and protein level and by examining changes in epithelial cell morphology. Previous study has shown that administration of miR-200c diminishes bleomycin-induced pulmonary fibrosis in IPF lungs (Yang *et al.*, 2012a). Consistent with that study, my results indicate that miR-200c plays a role in maintaining epithelial phenotype through inhibiting EMT and has a potential to reverse EMT.

Reports have also shown that miR-200 family regulates EMT by targeting EMT accelerators such as Zinc finger E-box-binding homeobox (Lamouille *et al.*, 2014). My results demonstrated that ectopic expression of miR-200c hindered EMT progression in TGF-β1 treated BEAS-2B cells and PBECs by keeping ZEB2 and ZNF532 levels low. This result was confirmed by luciferase assay performed after co-transfecting cells with miR-200c mimics and pmiRGLO vector containing ZEB2 or ZNF532 3' UTR region. In accordance with downregulation of ZEB2 and ZNF532, an increase in the level of E-cadherin and decrease in fibronectin was observed, indicative of their influence on E-cadherin and fibronectin transcription. Taken together these data indicate that miR-200c can prevent EMT by targeting transcription factors ZEB2 and ZNF532 and thus may provide an important avenue for therapeutic targeting during fibrosis.

Another miRNA candidate that has shown potential involvement in inflammation and fibrosis is miR-146a. Previous study has shown that miR-146a targets SMAD4 and

TRAF6 that are important mediators of TGF-β signalling (Min *et al.*, 2017). In my study, I attempted to evaluate the effect of transfecting TGF-β1 treated BEAS-2B cells with miR-146a mimics. No significant change in EMT marker expression was observed in miR-146a transfected and TGF-β1 treated cells when compared to TGF-β1 treated cells. Previous studies have shown the responsiveness of miR-146a to IL-1β, a cytokine that induces inflammation (Li *et al.*, 2012). Furthermore, miR-146a expression has been found to be upregulated in human dermal fibroblasts cells in response to TGF-β1 stimulation and the expression of its target SMAD4 was studied (Liu *et al.*, 2012). Therefore, instead of examining the changes in EMT marker expression post miR-146a overexpression, studying the expression of its potential target genes would be a better experimental plan.

My findings of an important role for miR-200b and miR-200c in enforcing the epithelial phenotype are supported by studies across various tissue types. It is noteworthy that miR-200 is enriched in tissues where epithelial cells predominate in humans (Thomson *et al.*, 2004; Baskerville and Bartel, 2005); an architectural location that is ideally suited for a role in epithelial phenotypic homeostasis. Furthermore, miR-200 family members are highly expressed during skin morphogenesis (Yi *et al.*, 2006). In summary, this chapter shows that in a similar way to miR-200b, miR-200c is a key determinant of epithelial cell identity and the expression of transcription factors ZEB2 and ZNF532 is controlled by miR-200b and miR-200c. This suggests that downregulation of these miRNAs may be an essential early step in progression of lung fibrosis, which could represent a novel therapeutic target.

Chapter 6: General discussion

6.1 Summary of aims and outcomes

1. To investigate the role miRNAs in maintaining bronchial epithelial cell phenotype.

A cell culture model was developed to study EMT in lung epithelial cells. Initial studies were performed using A549 cell lines that are derived from the alveolar epithelium. However, since BOS affects the large and the small airways, PBEC cultures that were collected during normal and post-transplant surveillance bronchoscopy and BEAS-2B cells were utilised for further studies. Changes in EMT marker expression using immunofluorescence and western blot studies demonstrated that bronchial epithelial cell line and primary cells are capable of undergoing TGF-β1 induced EMT. Next, the changes in miRNA expression were assessed in normal versus TGF-β1 treated BEAS-2B cells and PBECs. Results from miRNA assays and qPCR showed that miR-200b and miR-200c were significantly downregulated in response to TGF-β1 stimulation, which was also associated with loss in epithelial cell markers.

2. Manipulate the expression of key miRNAs in bronchial epithelial cell line and primary human bronchial epithelial cell to modulate EMT.

Expression of several miRNAs is dysregulated in diseased conditions. However, their role in experimental models of airway EMT is not well characterised. The first section of chapter 4 and chapter 5 aimed to determine whether manipulating the expression of miR-200b, miR-200c and miR-146a *in vitro* led to a change in EMT marker expression in TGF-β1 treated BEAS-2B cells and PBECs. Results showed that miR-200b and miR-200c mimics had similar effects on epithelial cell phenotype in BEAS-2B cells and PBECs. Results demonstrated that miR-200b and miR-200c mimics not

only limited epithelial cells to undergo EMT but also reversed TGF-β1 induced EMT. However, transfecting BEAS-2B cells with miR-146a had no effect on EMT marker expression. This study demonstrated a potential use for miRNA-200b and miR-200c in therapeutics in order to prevent and reverse TGF-β1 induced EMT.

3. Identify and validate downstream miRNA targets that may have a role in the progression of lung allograft dysfunction.

MiRNAs control gene expression by binding to complementary sequences in target mRNAs. Therefore, various miRNA target prediction programs were used to determine the direct targets of miR-200b and miR-200c. Out of the 7 predicted targets ZEB2 and ZNF532 were further studied as RNA studies suggested that miR-200b and miR-200c significantly downregulated these targets even in the presence of TGF-β1. A miRNA expression vector system was used to generate plasmids containing 3'UTR region of ZEB2 and ZNF532 that had binding sites for miR-200b/200c. Co-transfection studies and luciferase assay revealed that the 3' UTR of ZEB2 and ZNF532 are likely targets of miR-200b/200c and that use of mimics blocks their activity thereby preventing TGF-β induced effects. In order to study other potential targets of miR-200b that are transcription factors involved in TGF-β signalling, TGF-β array was used and the results were categorised according to the pathway that is activated. However due to time restriction further work of using online computational tools in order to find miRNA targets could not be carried out. Lastly, in order to show that miR-200b is extensively expressed in the bronchial epithelium, lung sections from normal patients were used to perform *in situ* hybridisation. Results demonstrated that miR-200b was expressed in high levels in the epithelium as compared to the extracellular matrix.

6.2 Implications

Increasing numbers of lung transplants are being successfully carried out globally to prolong and improve the lives of patients suffering from end-stage lung disorders (Christie *et al.*, 2010). However, long-term survival is restricted due to the incidence of BOS and the mechanism leading to this irreversible airway obstruction remains unclear. Emerging evidence suggests that EMT may be the common pathway leading to loss of lung function and fibrosis (Todd *et al.*, 2012). MiRNAs have been implicated in end stage lung diseases and are a major focus for therapeutics in the clinic. Although the role of several miRNAs has been investigated in lung *in vitro* (Sato *et al.*) and animal models (Ji *et al.*, 2015; Gubrij *et al.*, 2016) only a few make it through the phase II clinical trials due to lack of efficacy and off target effects (Rupaimoole and Slack, 2017). This highlights the greater need to investigate the role of miRNAs in lung allograft dysfunction, with an emphasis on their clinical utility. My study attempted to use specific miRNAs in an *in vitro* model of BOS in order to reverse EMT marker expression.

A debatable issue in research into BOS has been the choice of an appropriate model. Animal models, while having the advantage of producing lesions that are histologically similar to BOS, have been repeatedly challenged, as the lesions in the whole-lung transplant model are not consistently reproducible (Mimura *et al.*, 2015). Given this background, studying the changes in EMT marker expression in human bronchial epithelial cell line and primary cells could lead to a better understanding of disease pathogenesis. A number of publications investigating the role of EMT in BOS have been conducted using PBECs (Forrest *et al.*, 2005; Ward *et al.*, 2005; Borthwick *et al.*, 2010a). Although these studies provided critical insight into the subject, one drawback is that it only utilises primary cells and failed to develop a

model using a cell line that would be a much more feasible/reproducible option for understanding the mechanism. Therefore, my initial study aimed to develop an *in vitro* model using A549 cells and BEAS-2B cells to study the phenomenon of EMT and how it may lead to fibrosis and then determine its reproducibility in PBECs. The immunofluorescence results of this study in Chapter 3 add significantly to the understanding gained from previously reported observations in lung epithelial cells (Section 1.5.1.2)(Hackett *et al.*, 2009; Kamitani *et al.*, 2011; Gong *et al.*, 2014). My data demonstrates the ability of BEAS-2B cells and PBECs derived from normal lung to undergo EMT in the presence of TGF-β1.

The expression of miRNAs was then evaluated in BEAS-2B cells that were pre-treated with TGF-β1 and compared to the untreated control. Expression of selective miRNAs was studied followed by miRNA screening using Nanostring nCounter platform. This is the first time that nCounter miRNA assay was used to study the differentially expressed miRNAs in BEAS-2B cells. The data suggested that miR-200b and miR-200c, which were downregulated post TGF-β1 treatment, are suitable candidates for further analysis. Several cancer studies have utilised mimics to study the effect of increasing the concentration of specific miRNA on EMT (Kong *et al.*, 2012; Park *et al.*, 2015; Li *et al.*, 2016; Liu *et al.*, 2017) . A number of reports have highlighted changes in miR-200 family expression as fundamental regulators of EMT (Fatatis, 2012). Furthermore, there is evidence that miR-200 is a determining factor of the epithelial phenotype and that its direct targets are E-cadherin transcriptional repressors (Park *et al.*, 2008). Since E-cadherin expression is known to be lost during EMT, my study aimed at using miR-200b/c mimics to correct the EMT marker expression by increasing the naturally occurring miRNAs that are underexpressed post TGF-β1 treatment. The use of mimics attenuated the expression of fibrotic

markers in TGF-β1 stimulated BEAS-2B cells and this was reproducible in PBECs and cells from lung allografts. Therefore, miR-200b and miR-200c showed potential for reversing a fibrotic phenotype induced in epithelial cells by TGF-β. This study demonstrated that miR-200b and miR-200c were capable of reprogramming TGF-β1 stimulated epithelial cells to an epithelial like state.

Next, the miRNA-target interactions were studied. Online computational tools revealed ZEB2 and ZNF532 as one of the predicted targets of miR-200b/c. The former has been previously studied (Park *et al.*, 2008), however ZNF532 is a novel target thought to participate in TGF-β signalling pathway. In this study, the direct targets were not only identified but also validated using qRT-PCR and miRNA vector system customised with 3' UTR ZEB2/ZNF532 gene containing binding sites for miR-200b/c. MiR-200b/miR-200c attenuated EMT marker expression in TGF-β1 stimulated cells (BEAS-2B cells and PBECs) by directly targeting ZEB2 and ZNF532. Furthermore, miR-200b localisation was also studied. My results demonstrate that miR-200b expression is highest within the normal epithelium as shown in previous cancer studies that demonstrate a reduction in miR-200 with disease progression (Mongroo and Rustgi, 2010; Zaravinos, 2015). Furthermore, a previous study shows that miR-200a localisation in proximal tubular cells of obstructed kidneys after unilateral ureteric obstruction was markedly decreased as compared to normal kidneys (Xiong *et al.*, 2012b). Overall, my results demonstrate the need to study the expression of miR-200b/c in BOS+ lung tissue and that miR-200b/c mimics have future clinical applicability in lung fibrosis.

TGF-β TaqMan array analysis identified significantly downregulated candidates in miR-200b transfected and TGF-β1 treated BEAS-2B cells. However, the candidates couldn't be verified due to time constraints. Future study verifying the targets using

qRT-PCR and miRNA expression vector system will be required to clarify the association between the miRNA and the candidates. Lastly, the role of miR-146a was also studied in BEAS-2B cells. However, since miR-146a is mainly upregulated during inflammation (Aronica *et al.*, 2010), it may not hold much relevance in TGF-β1 induced EMT.

Differential changes observed in epithelial cells might just be the average of all miRNAs expressed by different cells. Although, the epithelium is the initial target in lung fibrotic disorders like BOS, it would be worth investigating the miRNA profile of endothelial cells as they also undergo a transition to acquire fibrotic phenotype under stressful conditions.

6.3 Limitations

The main limitation of the study was the lack of PBECs from BOS+ patients. It would be interesting if the miR-200b/c expression could be manipulated in these cells and see whether that reverses EMT marker expression. Furthermore, due to time restriction the miR-200b expression could not be evaluated in tissue sections acquired from BOS+ patients. This study would allow comparing the expression of miR-200b in normal versus diseased lung epithelium *in vivo*. Lastly, miRNA inhibitors had no significant effect *in vitro* and use of miRNA power inhibitors may offer an efficient inhibition of microRNA activity.

6.4 Future directions

The ability to screen cell-free miRNAs in biofluids has allowed early detection in cases where there is limited tissue availability. It has recently been shown that disease specific exosomes and/or extracellular vesicle (EV) signatures might be useful in differentiating between normal and disease states. One recent study has

demonstrated the use of tissue-specific targeting of recombinant exosomes. Therefore, manipulating the expression of miRNAs within the exosomes may be an effective tool for target-specific therapy (Ladak *et al.*, 2016).

The future work could focus on profiling archived lung transplant Bronchoalveolar lavage (BAL) samples for selected circulating miRNAs in BOS patients. The experimental plan could be as follows:

1. To investigate the prognostic value of selected circulating miRNAs in BOS patients.

My PhD data has shown the importance of bronchial epithelial cell specific miRNAs and how they are differentially expressed in normal versus BOS patients. The next step would be to study the expression of circulating miRNAs from different cell origins. Extracellular vesicles can be isolated from BAL samples acquired from normal individuals and BOS patients and their cellular origin could be determined by flow cytometry. Levels of selective miRNAs between control and BOS group can be classified and correlated with clinical outcome.

2. Identify genes and pathways that are targeted by miRNAs differentially expressed in normal versus BOS patients by using bioinformatics tools.

Differentially expressed miRNAs would be further investigated. miRNA-target analysis would be performed as previously done during my PhD. Furthermore, pathway analysis using Ingenuity online software will allow identifying miRNA targets and their pathway interaction. These targets will then be verified and validated using qRT-PCR and luciferase reporter assays.

3. Co-localize the expression of selective miRNA/s and their target/s in lung tissues acquired from normal and BOS patients.

In situ hybridisation was performed during my PhD to localise miR-200b expression in the lung bronchial epithelium acquired from normal individuals. However, the expression of miR-200b wasn't evaluated in BOS+ lungs. Therefore, in addition to miR-200b the expression of other selective miRNAs and their validated targets will be co-localised in tissue sections acquired from healthy versus BOS patients using *in-situ* hybridization (Exiqon).

4. Determine the expression of selective miRNAs differentially expressed in lung tissues using quantitative methods.

Post *In-situ* hybridization, laser capture microdissection would allow quantification of miRNA expression in normal and BOS+ epithelium isolated from sections.

5. Study the expression profile of selective miRNAs in ex vivo *lung perfusate in transplanted versus non-transplanted lung*.

A final study would involve studying the expression of a panel of selected miRNAs (from previous experiments, including miR-200b/c) in *ex vivo* lung perfusate in transplanted versus non-transplanted lungs. This would allow devising a panel of miRNAs that may allow accurate prediction of graft's susceptibility to developing BOS.

6.5 Conclusion

My study provides proof of concept that miR-200b and miR-200c both protect the airway epithelial cells from EMT and that miR-200b and miR-200c augmentation can reverse established TGF-β driven EMT. Manipulation of miR-200b/200c may therefore represent a novel therapeutic modulator of EMT, which is associated with the devastating pathophysiology of BOS in lung transplant recipients.

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Conference presentations

National

Jun-16 American transplant congress, Boston USA

Apr-16 Lund University Respiratory Network meeting, Ystad Sweden.

Oral presentation

Sep-15 European congress of immunology conference, Vienna Austria

International

Aug-17 British association for lung research conference, Belfast, UK.

Nov-16 Open Lab Book demonstration at NEPG 2016, Newcastle, UK.

Oral presentation

Aug-16 European respiratory society congress, London UK.

Jul-16 British association for lung research conference, Sheffield, UK.

Oral presentation

Dec-15 British thoracic society winter meeting, London UK. Oral presentation

Nov-15 ICM research seminar, Newcastle University. Oral presentation

Sep-15 British association of lung research conference, Bath UK.

May-15 University of Newcastle MicroRNA day. Oral presentation

Apr-15 PrICM poster evening event, Newcastle University. Best poster award

Dec-14 British society of Immunology annual congress, Brighton UK

Oct-14 North-East postgraduate conference, Newcastle UK

Sep-14 ICM research seminar, Newcastle University. Oral presentation

List of publications

- **Ladak, S.S**., Ward, C. and Ali, S., 2016. The potential role of microRNAs in lung allograft rejection. The Journal of Heart and Lung Transplantation, 35(5), pp.550-559.

• (*Manuscript submitted to Scientific Reports*) **Ladak S S**, Powell J, Fisher A, Ward C, Ali S. MiR-200b-3p: a potential therapeutic target for the bronchiolitis obliterans syndrome

• (*Manuscript in progress, pending submission*) Adil Aldhahrani, **Shameem Ladak**, Bernard Verdon , Malcolm Brodlie , Paul Corris, Andrew Fisher, Simi Ali, Jeffery Pearson*, Chris Ward*. Bile acids promote Epithelial Mesenchymal Transition in primary human airway epithelial cells from lung allografts

• (*Manuscript in progress, submitted to The Laryngoscope*) Adil Aldhahrani, Jason Powell, **Shameem Ladak**, Bernard Verdon, Jeffery Pearson, Chris Ward. The potential role of bile acids in acquired laryngotracheal stenosis

List of conference publications

• **Ladak S**, Ward C, Ali S. MiRNA-200b Inhibits Epithelial-Mesenchymal Transition in TGF-β1 Induced Human Bronchial Epithelial Cells. Am J Transplant. 2016;16 (suppl 3).In: American Transplant congress

• **Shameem Sultanali Ladak,** Chris Ward, Simi Ali. MiRNA-200b inhibits epithelial-mesenchymal transition in TGF-β1 induced human bronchial epithelial cells. Eur Respir J 2016; 48: Suppl. 60, 4034

• **S. Ladak**, C. Ward, and S. Ali. MicroRNA-200b represses TGF-β1 induced EMT in BEAS-2B and primary bronchial epithelial cells. In: THORAX, 2015, vol. 70, pp. A68--A68. BRITISH MED ASSOC HOUSE, TAVISTOCK SQUARE, LONDON WC1H 9JR, ENGLAND: BMJ PUBLISHING GROUP.

• **Ladak SS**, Ali S, Ward C. The potential role of miRNA-200b in the development of Bronchiolitis obliterans syndrome. In: British Society for Immunology Annual Congress. 2014, Brighton, UK: Wiley-Blackwell Publishing

Awards and Grants

- Overseas research scholarship (ORS), 2013-2016.
- Post-Submission Academic Scholarship, Newcastle University: £1200 (October-December)
- BALR travel award, Belfast, August 2017: Awarded £100
- Invitation to Celebrating Success event for winning two travel awards to attend the 2016
- British society for immunology travel award, September 2016: £700.
- BLF (British lung foundation)-ERS (European respiratory society) travel fellowship, 2016 : Awarded £750.
- Newcastle University travel award, 2016: Awarded £ 650.
- Biolegend travel award, 2016: £ 300.
- Invitation to Celebrating Success event for winning PGR Innovation Fund 2015 for 'Open Lab Book'. Newcastle University, December 2016.
- BALR (British association for lung research) travel award, September 2015: Awarded £ 100.
- BSI-ECI 2015 travel award, June 2015: Awarded £ 440.
- PrICM poster evening event, Newcastle University, April 2015. Best poster award
- PGR Innovation Fund, Newcastle University, 2015: Awarded £ 4000 for the open lab book.