2.1. TGF-β SUPERFAMILY

The TGF-β superfamily can be subdivided into three families of ligands: bone morphogenetic proteins (BMPs), activin/inhibin/nodal and TGF-β. A schematic classification for different members of TGF-β superfamily is given in Figure 1. The bone morphogenetic proteins (BMPs) form the largest group of ligands and includes BMP2, BMP7, GFD5 (growth and differentiation factor-5), Dpp (*Drosophila* orthologs decapentaplegic) and 60A (Shi and Massague, 2003). The BMPs are known to be involved in a multitude of cellular functions, including embryogenesis, growth, cell differentiation, anterior/posterior axis specification, cellular homeostasis, maintenance and repair of bone and other tissues in the adult (Massague et al., 2000). Nodal and Activin belong to a structurally more diverse group, with essential roles in the initial stages of development. These factors play important roles in osteogenesis and embryogenesis, which are complementary to that of the BMPs (Schier, 2003). They also regulate many hormones of the pituitary, gonads and the hypothalamus, besides serving as nerve cell survival factors. Conversely, the various forms of TGF-β are more actively involved in the late stages of embryogenesis and in the mature organism (Newfeld et al., 1999). The crucial functions of TGF-β include the inhibition of epithelial growth, immune and hematopoietic functions, besides promoting the growth of connective tissues (Letterio and Roberts, 1998; Wahl, 1994).

2.1.1. TGF-β Isoforms

Five TGF-β isoforms have been identified till date, out of which three isoforms (TGF-β1, TGF-β2 and TGF-β3) are present in mammals. All three TGF-β isoforms are encoded from individual genes located on different genomic locations. These TGF-β
isoforms show similar, although not identical, biological functions. A differential expression of the TGF-β isoforms occurs in various tissues. For example, although all 3 isoforms are expressed in the male gonads, TGF-β3 is the major isoform expressed in the mature testis, whereas TGF-β1 is specifically expressed in the Sertoli cell and is considered to be important for spermatogenesis (Memon et al., 2008). TGF-β1 is known as an extremely potent promoter of extracellular matrix accumulation, while the effects of TGF-β2 and TGF-β3 on fibroblast collagen synthesis and degradation have not been extensively studied so far (Coker et al., 1997). Recent insights into the functions of TGF-β1 and TGF-β3 have been gained by loss-of-functions deletions for each of the corresponding genes in mice. Although TGF-β1 knock-out does not yield any gross embryonic abnormality, newborn mice exhibit lethal multifocal inflammation, and the phenotype manifests as early as three weeks after birth (Schmid et al., 1991). Hence, TGF-β1 seems to be an essential regulator of the immune system.

2.2. LATENT TGF-β

A distinct feature of the members of the TGF-β superfamily is that it is secreted as a latent complex (Li et al., 2006b). The word “latency” while referring to a cytokine, describes a condition which allows a molecule to be reversibly maintained in an inactive state until required by the cell. Latency seems to be an efficient mechanism to control the activity of a growth factor. Consistently, most members of the TGF-β superfamily, including the TGF-β isoforms are synthesized as dimeric precursor proproteins, which are cleaved during activation to yield the mature forms. The mature form of TGF-β remains associated with its propeptide through noncovalent interactions and must be released from this latent complex to elicit its biological
activity (Gleizes et al., 1997). This process is known as latent TGF-β activation or TGF-β formation. Along with TGF-β transcriptional regulation, latency appears to be a critical step in controlling of TGF-β activity, as an enhanced expression of TGF-β does not always correlate with the increased levels of active TGF-β (Theodorescu et al., 1991). Thus, latency regulates the bioavailability of TGF-β by preventing the cytokine from eliciting a cellular response until it is converted into the active form. It may limit its diffusion from the secreting cell, thereby modulating the autocrine and paracrine actions of this cytokine family.

2.2.1. Formation of LAP-TGF-β1 Complex

TGF-β1 is encoded as a 392 amino acid polypeptide, which dimerizes through disulfide bonds to form the precursor molecule. In the inactive form, the C-terminal fragment of TGF-β1 remains associated with the latency-associated peptide (LAP, or β1-LAP) at its N-terminal, mainly through non-covalent interactions. β1-LAP dimerization is further stabilized by two interchain disulfide bonds. Although the mature forms of TGF-β1, TGF-β2 and TGF-β3 are highly homologous, the sequences of the corresponding propeptides are more divergent. These differences in LAP:TGF-β interactions may partially contribute in certain functional differences between TGF-β isoforms (Annes et al., 2003).

2.2.2. Disruption of LAP-TGF-β1 Complex

Till date, two enzymatic processes have been reported to yield active TGF-β. The first option involves the treatment of the inactive complex with glycosidases, thus suggesting the role of carbohydrates in the stability of latent TGF-β (Miyazono and
Heldin, 1989). The second alternative utilizes the proteolytic degradation of LAP. It has been reported that serine proteases plasmin and cathepsin D release TGF-β from the latent complex, apparently by a cleavage of N-terminal region from LAP (Li et al., 2006b; Lyons et al., 1990).

The TGF-β:LAP interaction can be disrupted under in vitro conditions by physiochemical treatments that take advantage of the greater resistance of mature TGF-β towards denaturing conditions as compared to LAP. The conditions which could promote LAP disruption, thereby activating latent TGF-β includes heat (80°C for 10 min), detergents, or extreme pH conditions (acidic or basic) (Gleizes et al., 1997). Although it remains unclear whether these conditions are physiologically relevant, some specific examples mimic such an in vitro TGF-β activation. These include the disruption of the LAP:TGF-β complex by osteoclasts during bone resorption, which is linked to the acidification (pH < 3) of the osteoclast pericellular space (Oreffo et al., 1989). The in vitro activation allows an accurate estimation of total TGF-β present in cells or tissues.

2.3. TGF-β SIGNALLING PATHWAY

The TGF-β signalling pathway has been the focus of attention for many researchers over the past decades due to its ability to control a plethora of cellular functions, ranging from animal embryo development to the maintainence of tissue homeostasis. The mechanism of signal transduction from cell membrane to the nucleus can be fundamentally described as a ligand-receptor interaction followed by the phosphorylation of nuclear proteins, thus resulting in the transcriptional regulation of
target genes. However, the multiple players involved in this signalling cascade add to the complexity and our current understanding is based on the extensive research over the past years.

2.3.1. Amplification of the Signalling Cascade

The signalling cascade initiates with the binding of a TGF-β superfamily ligand to a TGF-β type II receptor. Ligand binding induces the assembly of type I and type II receptors into a complex, within which the Type II receptor (a serine/threonine receptor kinase) catalyzes the phosphorylation of the Type I receptor. This phosphorylation is both essential and sufficient for TGF-β signalling. The ligand-receptor interaction further activates downstream signalling by the phosphorylation of a family of receptor substrates (the Smad proteins), which assemble into multisubunit complexes and thus regulates transcription (Massague and Chen, 2000; Massague, 1998).

The receptor substrates which are activated in response to ligand-receptor interaction are the essential regulators of downstream TGF-β signalling (Moustakas et al., 2001; Ten and Hill, 2004). They can be broadly classified into three functional classes: the receptor-regulated Smad (R-Smad), the Co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5, and 8) are directly phosphorylated at their carboxy-termini by ligand-activated TGF-β receptor kinases. While Smad2 and Smad3 act specifically downstream of only TGF-βs, activin and Nodal, other R-Smads such as Smad1, 5 and 8 are activated by BMPs only. Smad4 is not phosphorylated at its carboxy-terminus, but undergoes homotrimerization to form a complex with R-Smads,
which gets translocated into the nucleus with other nuclear cofactors, and regulates the transcription of target genes. Thus, Smad4 is also referred to as the co-Smad. Smad6 and Smad7 belong to the I-Smad (Inhibitory Smad) sub-group, which competes with R-Smads for Co-Smad or receptor interaction and target the receptors for degradation (Watanabe et al., 2010; Xu, 2006; Yan et al., 2009). Hence, Smad6 and Smad7 negatively regulate TGF-β signalling. A schematic representation for all the events involved in the amplification of TGF-β signalling cascade is represented in Figure 2.

The Smads share sequence similarities in the N-terminal and C-terminal regions, known as MH1 (Mad Homology 1) and MH2 domains respectively. The sequence between the MH1 and MH2 domains is quite variable among the Smads and is termed as the linker region (Massague and Wotton, 2000).

At the basal state, R-Smads and Smad4 are mostly present in the cytoplasm, which translocate into the nucleus upon TGF-β stimulation (Macias-Silva et al., 1996; Hoodless et al., 1996). Such a spatio-temporal mechanism allows a conditional access of Smads to their target genes. Further, the amount of Smads accumulated in the nucleus and the duration of Smads occupancy on their target promoters are critical determinants of transcriptional outcomes (Wharton et al., 2004; Nicolas and Hill, 2003; Wilson et al., 1997). Hence, the nucleocytoplasm translocation of Smads is a crucial regulatory mechanism in TGF-β signalling.

2.3.2. Transcriptional control of TGF-β

Although structural similarities are observed between the various isoforms of TGF-β, the promoter sequences of these isoforms show little similarity in sequence, thus
suggesting diverse transcriptional control (Geiser et al., 1993). Consistent with this, subtle differences were observed in the transcriptional control of these isoforms. For example, while the promoters of TGF-β1 and -β3 are strongly stimulated by Sp1, TGF-β2 was reported to be completely unaffected. Significant differences were also observed for the binding affinity for AP-1, where TGF-β1 showed a strong affinity, in contrast to TGF-β2 and -β3 promoters which showed affinity towards AP-2 binding sites (Roberts et al., 1991). Moreover, TGF-β1 promoter also showed affinity towards HIF-1 binding (Sanchez-Elsner et al., 2004) and Egr-1 mediated repression (Dey et al., 1994; Liu et al., 1996), indicating a strong transcriptional control.

TGF-β1 expression is further regulated at multiple levels, including mRNA stability and posttranslational processing. TGF-β1 mRNA can be rapidly (2–6 hours) induced by stimulation of mesangial cells and T lymphocytes through intracellular signalling kinases including PKC or ERK. Interestingly, recent data also suggests the presence of the AU-rich elements (AREs) in TGF-β 3’UTR. Several functionally distinct RNA-binding proteins such as TTP (tristetraprolin), HuR (Hu antigen R), AUF1 (ARE-binding factor 1) and YB-1 (Y-box binding protein 1) bind to these AU-rich elements and modulate the differential mRNA decay of proinflammatory cytokines, such as TGF-β1 (Shen et al., 2008). Both transcriptional and post-transcriptional modes of regulation work in concert to critically maintain the levels of TGF-β in the cell. A schematic representation of the transcriptional and post-transcriptional factors with regulate TGF-β1 is shown in Figure 3.

Another important property of TGF-β is that its effects are bimodal; while TGF-β has a mitogenic effect at low concentration, these effects are abolished at high
concentrations (Rooke, 2001). This bell-shaped concentration response curve seems to be critical for the maintainence of cellular homeostasis. For example, the localized, tightly controlled production of TGF-β occurs as a necessary component of an active inflammatory process, whereas the resulting excess TGF-β may inhibit the same immune and inflammatory pathways. It is not inconceivable that this built-in negative feedback loop, occurring in TGF-β excess, has been incorporated in the process of evolution for the protection of the host during bouts of rampant inflammation (Allison et al., 1998). The various modes of regulation, including transcription, post-transcription and autoregulation are critical and a dysregulation leads to aberrant production of TGF-β, resulting in various diseases.

2.4. SOURCES AND TARGETS OF TGF-β

The pleiotropic effects of TGF-β1 are involved in the regulation of cellular differentiation, survival and function of many immune cells. A plethora of cell types including lymphocytes, natural killer cells, macrophages, mast cells, granulocytes and dendritic cells secrete TGF-β, which exerts both autocrine and paracrine effects to control the state of activation and proliferation of these immune cells (Letterio and Roberts, 1998; Kadin et al., 1993; Patil et al., 2011). A typical characteristic function of TGF-β is that it exhibits a bell-shaped concentration response curve. For example, TGF-β can modulate the expression of adhesion molecules and provide a chemotactic gradient for leukocytes and other cell-types participating in an inflammatory response, while inhibits them after activation. The various structural and immune cell types which secrete TGF-β are represented in Figure 4.
2.4.1. Sources of TGF-β in the Normal Lung

The expression of TGF-β has been well-documented during embryogenesis and the development of the lung. Small amounts of TGF-β mRNA and protein expression, along with type I and II receptors have been reported in the normal adult lung. In mice, TGF-β1 mRNA was found to be localized in the bronchiolar epithelium, Clara cells, mesenchymal cells, vascular endothelium, and alveolar cells and macrophages (Coker et al., 1996). In another study, mRNA and protein of all three isoforms of TGF-β were found to be expressed exclusively in the proximal conducting airways, smooth muscle cells and connective tissue fibroblasts but were absent in alveoli of the distal airways (Pelton et al., 1991). This study also showed that TGF-β protein expression was highest in bronchiolar epithelium cells. An independent study in humans also confirmed that bronchial epithelial cells produce the largest amounts of TGF-β protein, and the signal was found to be more intense at the apical poles of the cells (Magnan et al., 1994). The presence of TGF-β in the normal lung suggests its physiological function in the maintainence of lung homeostasis. These functions include local immunomodulation in the lung, control of normal tissue repair as well as the regulation of cell differentiation and proliferation.

2.4.2. Sources of TGF-β in Lung Diseases

The most common characteristic feature of many lung diseases is an inflammatory phase, initiated by tissue injury and followed by the repair phase (Elssner et al., 2000). Lung tissue injury resulting from an immunologic response leads to the production of TGF-β, which may limit inflammatory reactions or mediate tissue remodeling and repair (Sporn and Roberts, 1992). Several cellular sources of TGF-β appear to be
activated in different forms of lung diseases. These sources may vary in the various phases of the reparative process. While platelets and epithelial cells serve as important reservoirs of TGF-β during the early phases of chronic lung diseases (Coker et al., 1996; Border and Ruoslahti, 1992), alveolar macrophages seems to be the major source during the phase with maximum TGF-β secretion (de Boer et al., 1998; Santana et al., 1995). Macrophages also persist in neonates during respiratory distress syndrome and are associated with the development of chronic lung disease (Ogden et al., 1984). Similar observations were reported in bleomycin-induced mouse model of pulmonary fibrosis (described in detail later), in which TGF-β was found to be localized mostly in the bronchial epithelium cells in the initial phase, whereas it was predominantly present in macrophages of the alveolar interstitium during the time of maximum TGF-β production (Santana et al., 1995; Zhang et al., 1995). Mouse models with radiation-induced fibrosis have been reported to exhibit an increase in TGF-β expression by macrophages during the early phase of pulmonary injury, whereas fibroblast and Type II pneumocytes appear as the predominant sources of TGF-β production at the later phase of fibrosis (Rube et al., 2000).

2.4.3. TGF-β Dependent Processes

2.4.3.1. Epithelial to Mesenchymal Transition

Epithelial-Mesenchymal Transition (EMT) is a biologic process which occurs during development and in the context of different morphogenetic events. During this process, the cells undergo multiple biochemical changes to lose their epithelial characteristics, including polarity and specialized cell-cell contacts, and acquire a mesenchymal cell phenotype (Kalluri and Weinberg, 2009; Lee et al., 2006). This
enhances their migratory capacity, invasiveness, and allows an increase in the production of ECM components. This is accompanied by a degradation of underlying basement membrane, which allows them to move away from the epithelial cell community and integrate into surrounding tissue, even at remote cellular locations. The transition of epithelial cells into a mesenchymal phenotype usually exhibits differentiation plasticity during development and is complemented by the reverse process, known as mesenchymal to epithelial transition (MET) (Mani et al., 2008; Scheel and Weinberg, 2011).

2.4.3.1.1. Initiation and Characterization of EMT

While EMT is an integral process under spatio-temporal regulation during normal embryonic development, it is recapitulated under pathological conditions, most prominently during fibrosis and in invasion and metastasis of carcinomas (Loboda et al., 2011; May et al., 2011; Voulgari and Pintzas, 2009; Willis and Borok, 2007). Hence, EMT is considered as an important step for the onset of tumor progression (Klymkowsky and Savagner, 2009; Singh and Settleman, 2010). A number of distinct molecular processes are essential for the initiation and progression of an EMT response. These include activation of several transcription factors, expression of specific cell-surface markers and cytoskeletal proteins, production of certain ECM-degrading enzymes, along with subtle changes in the expression of specific microRNAs (Willis and Borok, 2007; Katoh and Katoh, 2008). These factors are used as biomarkers to demonstrate the induction and establishment of EMT in a cell (Radisky, 2005). Some of the important markers used for the characterization of in vitro and in vivo models of EMT have been highlighted in Figure 5.
2.4.3.1.2. Role of TGF-β in EMT

TGF-β signalling plays a significant role in the initiation and progression of EMT and has received much attention over many years as a major inducer of EMT during embryogenesis, cancer progression and fibrosis (Miettinen et al., 1994; Roberts et al., 2006; Cufi et al., 2010). In fact, most of the in vitro models of EMT can be induced by the exogeneous addition of TGF-β to cultured epithelial cells (Xu et al., 2009; Zavadil and Bottinger, 2005). Although much less characterized, BMPs have been shown to participate in EMT during development, specifically associated with gastrulation initiation and mesoderm patterning (Kishigami and Mishina, 2005).

2.4.3.1.3. Regulation of EMT

The induction of EMT by TGF-β was first recognized using a cell culture based model (Miettinen et al., 1994). It is also reported that TGF-β1, TGF-β2 and TGF-β3 share the capacity to induce EMT in epithelial cells (Piek et al., 1999; Valcourt et al., 2005). As described previously, TGF-β induced activation of the receptor complex leads to phosphorylation of Smad2 and Smad3, which forms a heterotrimer with Smad4 and translocates into the nucleus. Here, the complex associates and co-operates with transcription factors to bind to DNA, thereby activating or repressing target gene transcription. Moreover, Smad6 and Smad7 could inhibit the activation of the receptor-regulated Smads. Consistent with the pathway, an increased expression of Smad2 or Smad3 with Smad4 was reported to induce EMT, or enhance the process by an activated form of TβRI in NMuMG cells (Piek et al., 1999; Valcourt et al., 2005; Thuault et al., 2008). In contrast, the expression of dominant negative versions of Smad2 or Smad3 blocked TGF-β induced EMT (Valcourt et al., 2005; Tojo et al.,
Smad4 was also found to be indispensable for EMT, as RNAi strategy to knockdown Smad4 or the expression of a dominant negative mutant of Smad4 resulted in preserved E-cadherin expression (Valcourt et al., 2005; Takano et al., 2007; Kaimori et al., 2007). Further, an increase in the expression of Smad7 has been reported to block TGF-β-induced EMT in multiple tissues (Xu et al., 2007; Dooley et al., 2008; Zavadil and Bottinger, 2005).

Following the activation and translocation of the Smad complex, the loss of epithelial cell markers and simultaneous acquisition of mesenchymal features is achieved through a well-coordinated transcription program involving three families of transcription factors, namely, the Snail, ZEB and bHLH families, represented in Figure 6. The expression of these transcription factors is induced in response to TGF-β signalling, either through a Smad-dependent pathway (ie, in the case of Snail proteins) or through the indirect activation of other transcription factors (Nagaishi et al., 2012; Leroy and Mostov, 2007; Come et al., 2006; Yang et al., 2004). Three Snail families of proteins have been identified in vertebrates: Snail1 (initially known as Snail), Snail2 (also known as Slug) and a more recently discovered Snail3. All of these Snail proteins function as transcription repressors. An increase in Snail1 expression has been reported in all EMT processes (Nieto, 2002), and correlates with more invasive tumor types (Batlle et al., 2000). Snail2 is more broadly expressed than Snail1, and some reports suggest that Snail1 and Snail2 cooperate to control the transcription network that regulates EMT (Barrallo-Gimeno and Nieto, 2005). Both genes are transcribed in response to TGF-β during the initiation of EMT. Although TGF-β-mediated induction of Snail1 is mediated by Smad3 expression, an indirect activation of Snail has been
reported and may involve other transcription factors, such as Ets-1, which induces MMP-2 expression (Taki et al., 2006). Further, Sp-1 and Ets-1 are thought to be partially responsible for the upregulation of MMP-9 expression (Jorda et al., 2005).

Two ZEB family transcription factors have been well-characterized in vertebrates: ZEB1 (known as dEF1 or AREB6) and ZEB2 (also known as Smad-interacting protein 1, or SIP1). TGF-β signalling induces the expression of ZEB transcription factors during EMT through an indirect mechanism mediated by Ets-1 (Shirakihara et al., 2007). Following activation, ZEB proteins directly interacts with Smad3 and suppresses the expression of epithelial marker markers, possibly by the recruitment of a co-repressor, CTBP (Postigo et al., 2003). The HLH (Helix-loop-helix) family of proteins is divided into seven categories based upon their tissue distribution, dimerization properties and DNA-binding specificity (Massari and Murre, 2000). Among these classes, the class I proteins (E12 and E47), class II proteins (Twists) and class V proteins (Ids) have been reported to be directly involved in EMT. Ectopic expression of E12 or E47 represses E-cadherin expression, while inducing the expression of vimentin and fibronectin to promote migration (Perez-Moreno et al., 2001). Similarly, a loss of Id protein expression is associated with a decrease in E-cadherin expression (Kondo et al., 2004). Twist class of proteins also show a similar downregulation of E-cadherin expression, thus enhancing migration and invasion (Ansieau et al., 2008).

2.4.3.2. T-cell Differentiation

CD4⁺ T lymphocytes are important mediators of adaptive immune responses and are vital for host defense (Kaiko et al., 2008a). An unprimed, naïve CD4⁺ T cell follows
distinct fates of differentiation that are determined by the pattern of signals received
during the initiation of an immune response. On the basis of the signature cytokines
and transcription factors generated by these differentiated T cells, they are classified
into 4 distinct populations, namely Th1, Th2, Th17 and induced regulatory T (iTreg)
cells (Feili-Hariri et al., 2005; O’Garra et al., 2008; Manel et al., 2008). These T cell
subsets exhibit a predominant role in various immunological disorders. For instance,
while allergic airway inflammation is thought to be mediated by Th2 cells and their
cytokines, it is becoming increasingly clear that Th1, Th17 and Treg cells also play a
role in modulating the process (Zhu and Paul, 2008; Currie et al., 2012). A recent
report also suggests IL-17A as a critical mediator of inflammation, neutrophilia and
pulmonary fibrosis after the exposure of mice to bleomycin or IL-1β, a recently
described initiator of fibrosis (Wilson et al., 2010). Not only does each of these T cell
differentiation pathways show complex feedback and feedforward regulation, but it
also actively involves cross-inhibition of the other T cell lineage fates (Reiner,
2009; Kaiko et al., 2008b).

TGF-β plays a critical role in regulating the T-cell differentiation process, as it may
have both pro-inflammatory (Th17-inducing) and anti-inflammatory (Treg-inducing)
roles, depending on the circumstances (Li and Flavell, 2008; Volpe et al., 2008). A
number of in vitro studies indicate that the levels of TGF-β are extremely critical in
regulating this differentiation process, as a combination of high amounts of IL-6 and
intermediate amounts of TGF-β lead to Th17 induction, whereas high amounts of
TGF-β alone facilitate iTreg differentiation (Mangan et al., 2006; Bettelli et al., 2006).
A schematic representation of crucial roles of TGF-β during T-cell differentiation is
shown in Figure 7. Studies also indicate that besides T cell differentiation, TGF-β acts as a critical regulator of thymic T cell development (Li et al., 2006a), peripheral T cell homeostasis (Jameson, 2005) and tolerance to self antigens (Li et al., 2006b).

2.5. TGF-β IN HUMAN DISEASES
The immunoregulatory functions of TGF-β can induce multiple cellular responses, and therefore have been implicated in the development of several pathogenic conditions including cancer, asthma, vascular disorders and fibrosis.

2.5.1. TGF-β in Cancer
As TGF-β is a potent growth inhibitor of many cell types, mutations in components of the TGF-β signalling pathway offer a selective advantage for the growth of cells. Malignant cells may circumvent the immune-suppressive actions of TGF-β through the inactivation of TGF-β receptors (Levy and Hill, 2006). They may also utilize the immunoregulatory roles of TGF-β to its advantage, which allow the cells to acquire invasion capabilities, producing autocrine mitogens, or releasing prometastatic cytokines (Massague, 2008). Epithelial-mesenchymal transition and myofibroblast mobilization further enhances invasiveness and dissemination during cancer metastasis (Davidson et al., 2012; Massague, 2008).

2.5.2. TGF-β in Asthma
Several reports suggest that the levels of TGF-β are elevated in the lungs of asthmatics (Redington et al., 1997). Airway remodeling plays an important role in the development of asthmatic features and is associated with inflammation, along with the
disruption of repair response to injury (Nihlberg et al., 2006; Yamauchi, 2006). Airway remodeling initiates a complex array of events including epithelial layer dysfunction, subepithelial fibrosis, goblet cell hyperplasia, airway smooth muscle cell (ASMC) hypertrophy and vascular remodeling (Postma and Timens, 2006; Shale and Ionescu, 2004). These events exert a cumulative effect during the progression of pulmonary dysfunction (James et al., 1989). TGF-β is secreted by both inflammatory cells as well as structural cells that regulate the airway remodeling process (Panettieri, Jr., 2003; Xu et al., 2003; Kay et al., 2004). The multiple effects of TGF-β on the same or different cell types, depending on the microenvironment, play a crucial role in airway remodeling.

### 2.5.3. TGF-β in Pulmonary Fibrosis

Pulmonary fibrosis is a fatal disease resulting from a variety of insults to the lung, including autoimmune, drug-induced, infectious, environmental toxins or traumatic injuries. The type of tissue response is dictated by a number of host factors including age, genetic susceptibility and environmental factors (Thannickal et al., 2004; Wynn, 2011). The resulting histopathological changes in the lung are characterized by varying degrees of inflammation and fibrosis. Drug induced lung disorders, such as cellular non-specific interstitial pneumonia (NSIP) are more likely to respond to discontinuation of drug treatment, with or without a short course of steroid therapy. However, this might not work for interstitial pneumonia (UIP), which is characterized by a greater degree of fibrosis (Thannickal et al., 2004; Kim and Meyer, 2008; Maher, 2012). In some cases, histopathological changes accompanying fibrosis occur without an identifiable etiological agent, and are therefore termed as “idiopathic”. The
pathological features of idiopathic UIP are synonymous with the clinical syndrome of idiopathic pulmonary fibrosis (IPF) (Katzenstein and Myers, 1998), which is described as a chronic, progressive form of lung disease with no effective treatment till date. Alterations in the alveolar microenvironment account for the dysregulated repair and aberrant tissue remodeling of the lung, which are characteristic features during the progression of fibrosis in IPF. These alterations includes imbalance in the production of various profibrotic cytokines, chemokines and growth factors, loss of alveolar epithelial cells accompanied by the accumulation of activated fibroblasts/myofibroblasts (Figure 8). These factors contribute towards a reduction in extracellular matrix turnover, aberrant angiogenesis, impaired fibrinolysis and an increase in oxidative stress response, which further promotes the dysregulation of tissue homeostasis (Gunther et al., 2012;Faner et al., 2012;Richeldi, 2012).

2.5.3.1. Prevalence of the Disease

Pulmonary fibrosis is considered as a rare disease. However, it is estimated that patients with this disease comprise about 15% of a pulmonary physician’s practice. A 2006 study reported that about 128,000 people in the United States have pulmonary fibrosis, which accounts for 1 in every 2400 Americans (Coultas and Hughes, 1996). However, other reports indicate that as many as 200,000 Americans may be affected by the disease (about 1 in 1550). Due to lack of prognosis and proper treatment, pulmonary fibrosis results in over 40,000 deaths per year. This means that it kills as many Americans every year as does breast cancer (du Bois, 2012;Cottin, 2012). Although the exact incidence and prevalence of the disease in India is not known, reports suggest a definite increase in the frequency of prognosis in the recent years (Maheshwari et al., 2004).
2.5.3.2. Critical Role of TGF-β as a Marker of Pulmonary Fibrosis

TGF-β has been implicated as a marker of tissue repair and remodeling in several lung diseases. An increase in TGF-β levels is regarded as an early event for the development of chronic lung diseases, and is associated with the severity of lung function abnormalities, lung pathology and reduced survival times (de Boer et al., 1998; Minshall et al., 1997). An increase in TGF-β expression is a crucial factor for the onset and progression of pulmonary fibrosis and has been extensively studied. While increased levels of TGF-β1 mRNA were found to be specific to early inflammatory lesions in the lung biopsy specimens of patients with idiopathic pulmonary fibrosis, TGF-β1 protein was found to be more abundant in fibroblast-loci during the advanced fibrotic changes of the lung (Limper et al., 1991). Further, in patients with sarcoidosis, an increase in TGF-β1 expression was found to be associated with alterations in lung functions, leading to fibrosis (Salez et al., 1998). The pro-fibrotic mediators of TGF-β involved in the direct and indirect remodeling of the lung have been highlighted in Figure 9.

2.6. EXPERIMENTAL MODELS OF PULMONARY FIBROSIS

Animal models have been widely used by researchers to obtain insights into the molecular mechanisms of pulmonary fibrosis. Comparable to chronic diseases which are difficult to model, IPF is even more complicated since the etiology of the disease is unknown till date and no single trigger is able to induce “IPF” in animals. Most mouse models of pulmonary fibrosis developed over the years are able to mimic some, but never all features of human IPF. In particular, the progressive and irreversible nature of the disease is difficult to replicate using in vivo models. The
most common methods used in the development of \textit{in vivo} models of pulmonary fibrosis include the instillation of chemical agents such as bleomycin, silica or asbestos, radiation-induced damage, fluorescein isothiocyanate (FITC) or gene transfer employing fibrogenic cytokines (Christensen et al., 1999; Hattori et al., 2000; Lai et al., 2009; Sabo-Attwood et al., 2005; Banerjee and Henderson, Jr., 2012; Phillips et al., 2011; Brickey et al., 2012). Till now, bleomycin has been used as the standard agent for induction of experimental pulmonary fibrosis in animals and is the best characterized model till date.

2.6.1. Development of Bleomycin-Induced Model of Lung Fibrosis

Bleomycin is a glycopeptide antibiotic produced by the bacterium \textit{Streptomyces verticillus} and most commonly used as a chemotherapeutic antibiotic (Adamson, 1976). Its use in animal models of pulmonary fibrosis began with the observation that fibrosis is a major side-effect of bleomycin in human cancer therapy. Bleomycin is believed to mediate its effects by causing single and double-strand DNA breaks in tumor cells, thereby interrupting the cell cycle. This is accompanied by the chelation of metal ions, which leads to production superoxides and hydroxide free radicals (Claussen and Long, 1999). This over-production of reactive oxygen species initiates to an inflammatory response, further resulting in the activation of fibroblasts, pulmonary toxicity and subsequent fibrosis. The lungs also maintain low levels of bleomycin hydrolase, a bleomycin-inactivating enzyme and therefore are more susceptible to bleomycin-induced tissue injury (Sebti et al., 1989). It is suggested that lung toxicity develops in $\sim 10\%$ of patients receiving bleomycin, and it is clinically associated with a deterioration of lung function parameters, along with other
symptoms such as cough, dyspnea, fever and cyanosis (Canadian Pharmacists Association, 2006).

Bleomycin has been shown to induce pulmonary fibrosis and lung injury in a wide variety of experimental animals including mice, rats, hamsters, rabbits, guinea pigs, dogs, and primates. It is effective over a range of doses via different modes of delivery, including intraperitoneal (ip), intravenous (iv), subcutaneous (sc), intranasal (in) or intratracheal (it). In most studies, bleomycin is given by a single instillation on day 0, with most frequent end-points of the model between day 14 and day 28 (Liu et al., 2010). Recently, a mouse model of lung fibrosis induced by the repetitive lung injury with bleomycin has been reported (Degryse et al., 2010). The lung fibrosis induced in this model was accompanied by lesser inflammation at later doses, and demonstrated a substantial distortion in the lung architecture, marked collagen deposition and enhanced EMT. Most importantly, these features do not resolve spontaneously even when the stimulus is removed. However, a mortality rate of 33.3% was observed in the repetitive-dose bleomycin group as compared to 8.3% in the single-dose group, indicating the experimental difficulties of this model.

2.6.2. Phases of Disease Progression in Lung Fibrosis

Following the administration of bleomycin, disease progression begins with the onset of an acute inflammatory response, which lasts up to 8 days. This is followed by fibrogenic changes resulting in deposition of matrix and distortion of lung structure. This phase extends until 28 days, and is accompanied by all the characteristic histological changes seen in fibrosis. Any drug interventions for treatments during the
first seven days are considered as “preventive”, whereas treatments during the later stages are known as “therapeutic”. The sequence of events which takes place during the progression of bleomycin-induced pulmonary fibrosis are represented in Figure 10.

### 2.6.3. Cell-types Involved in Lung Fibrosis

Both structural and inflammatory cells play a crucial role during the progression of pulmonary fibrosis. Epithelial-mesenchymal transition (EMT) is considered as a source of fibroblasts in the setting of lung injury induced by bleomycin, thus contributing to disease pathogenesis (Tanjore et al., 2009; Wu et al., 2007; Senoo et al., 2010). Activated mesenchymal cells serves as a potent source of monocyte/neutrophil chemotactic activity and induce the recruitment of inflammatory cells to site of injury (Hogaboam et al., 1999; Hogaboam et al., 1998). Thereby, neutrophils and macrophages play crucial modulatory roles in the fibrogenic process. Neutrophils secrete higher concentrations of oxidants, alongwith primary and secondary granule enzymes resulting in epithelial cell injury (Cantin et al., 1987; Obayashi et al., 1997; Cailes et al., 1996). The activation of alveolar macrophages is likely to influence tissue remodeling and fibrogenesis (Martinet et al., 1987; Carre et al., 1991).

### 2.6.4. Cytokines Involved in Lung Fibrosis

Several reports suggest that a network of cytokines is capable of modulating the different phases of lung fibrosis pathogenesis, which involve both inflammation and fibrogenesis. Among these cytokines, IL-1β exerts potent inflammatory properties (Dinarello, 1997) and induces a profibrotic phenotype through induction of PDGF
(Platelet-derived Growth Factor), leading to fibroblast proliferation and synthesis of procollagen type I and type III (Raines et al., 1989; Goldring et al., 1988). Transient overexpression of IL-1β in lung epithelial cells of in vivo lung injury models causes acute inflammation and tissue destruction, followed by the secretion of fibrogenic cytokines, such as TGF-β, resulting in progressive interstitial fibrosis (Kolb et al., 2001; Lappalainen et al., 2005).

The pleiotropic effects of TNF-α stimulates an inflammatory response by acting on a plethora of cells, including mononuclear cells, neutrophils, and endothelial cells. TNF-α triggers fibroblast proliferation and has the ability to degrade the extracellular matrix (Fujita et al., 2001). It is produced by epithelial cells, endothelial cells, activated macrophages and lymphocytes and stimulates cell–cell adhesion as well as early events leading to cytokine and chemokine production (Zhang et al., 1997; Miyazaki et al., 1995). A large number of studies on IPF indicate that this cytokine is present in areas of lung fibrosis. Moreover, the transgenic overexpression of TNF-α in mouse lungs showed proinflammatory and profibrotic actions, and subsequent secretion of TGF-β (Miyazaki et al., 1995; Lundblad et al., 2005). However, some contradictory studies also report that TNF-α may protective against bleomycin-induced lung inflammation (Kuroki et al., 2003; Fujita et al., 2003).

IL-13 has been implicated in the pathogenesis of many diseases which involve tissue fibrosis, including hepatic fibrosis, progressive systemic sclerosis, pulmonary fibrosis, and nodular sclerosing Hodgkin’s disease (Fallon et al., 2000; Chiaramonte et al., 1999; Hancock et al., 1998; Ohshima et al., 2001). Studies with transgenic
overexpression of IL-13 in the murine airway caused subepithelial airway fibrosis, and the fibrotic response seen was similar to remodeled human asthmatic airways (Zhu et al., 1999). It is believed that IL-13 mediates its fibrogenic effects in the lung and other organs by altering TGF-β homeostasis (Lee et al., 2001; Bellini et al., 2012; Xiong et al., 2011). A recent study also indicates a critical role for IL-17A in bleomycin-induced fibrosis, which could be involved in tissue remodeling in cooperation with IL-13. This study also suggests that TGF-β and proinflammatory mediators like IL-1β promote fibrosis by up-regulating the production of IL-17A (Wilson et al., 2010).

TGF-β1 is one of the most important pro-fibrotic mediators in lung fibrosis. As explained earlier, it plays a crucial role in the direct and indirect remodeling of the lungs. Patients with IPF invariably show high expression of TGF-β1 in the lung. Studies show that treatment of lung fibroblasts with bleomycin increases the transcription rate of TGF-β, as well as its protein secretion (Breen et al., 1992). The early fibrogenic changes result from TGF-β1 production by alveolar macrophages (Peng et al., 2011; Juniantito et al., 2012).

### 2.6.5. Key Players of TGF-β Signalling Involved in Lung Fibrosis

The network of cytokines, along with structural changes in the lung architecture results in the characteristic features of fibrosis. The components of TGF-β signalling play a key role in the pathogenesis of the disease. Active TGF-β1 stimulates the transcription of *COLIA1* and *COLIA2*, which are key genes involved in collagen synthesis (Grande et al., 1993; Vindevoghel et al., 1998). This results in excessive
collagen deposition and further promotes the deposition of a connective tissue matrix, which are characteristic features of bleomycin-induced pulmonary fibrosis. Particularly, as these histopathological changes are also observed in human IPF, they are considered as the salient features of this model. The downstream activation of Smad proteins is also critical for the pathogenesis of pulmonary fibrosis. Studies suggest that Smad3 deficient mice have decreased $COL1$ gene expression and lung hydroxyproline content as compared to bleomycin-treated mice (Zhao et al., 2002).

Remodeling of the lung architecture during pulmonary fibrosis involves extensive alterations of lung extracellular matrix (ECM). Matrix metalloproteinases (MMPs) have been extensively reported as key moderators of ECM because of their unique ability to cleave structural proteins, such as elastin and collagens (Nagase and Woessner, 1999; Parks and Shapiro, 2001; Sternlicht and Werb, 2001). The role of MMP-9 (type IV collagenase; also known as Gelatinase B) has been extensively studied in lung pathology. It is present in low quantities in the healthy adult lung but increases in several lung diseases, including IPF (Lemjabbar et al., 1999). Studies indicate that there is a complex interaction between the pro-inflammatory cytokines and dysregulation of fibrogenesis during the progression of pulmonary fibrosis. Several enzymes, including MMPs play a critical role not only in the remodeling of the lung tissue, but also release and activate pro-fibrotic growth factors. Reports suggest that gelatinases such as MMP-9 and MMP-2 may be involved in proteolytic activation of latent TGF-β complexes (Yu and Stamenkovic, 2000). Consistent with these reports, independent studies suggest that MMP-9 levels are elevated in BALF from IPF patients (Suga et al., 2000). It is also reported that transcription factors such
as Ets-1 upregulate MMP-9 expression (Jorda et al., 2005). Moreover, Ets-1 expression is also up-regulated by TGF-β, and it acts in cooperative manner with the transcription factors of the ZEB family, such as ZEB1 and SIP1, leading to EMT (Shirakihara et al., 2007). A schematic representation of key players of TGF-β signalling and the downstream events involved in the pathogenesis of pulmonary fibrosis are represented in Figure 11.

2.7. CURRENT STATUS OF CLINICAL TRIALS INVOLVING TGF-β

The clinical strategies used to inhibit increase in TGF-β expression in lung diseases, such as IPF, have been generally based on either antibody-based neutralization strategies for ligands or receptors, or inhibition using small-molecules. Although some showed clinical success at Phase I trials, subsequent success has not been achieved so far.

2.7.1. Clinical Trials so far

- **CAT-192**: An antibody against TGF-β1, showed no effect on lung function parameters in systemic sclerosis patients (Denton et al., 2007).
- **GC-1008**: A monoclonal antibody against TGF-β, completed Phase I trial for IPF without further progress in phase II trials (Datta et al., 2011).
- **Imatinib**: An oral kinase inhibitor against PDGF-R, which failed in placebo-controlled trials in IPF patients (Daniels et al., 2010; Chhina et al., 2008).
- **Etanercept**: Recombinant soluble TNF-α receptor to antagonize TNF-α, which failed at phase II trials (Raghu et al., 2008; Nathan, 2006).
- **Pirfenidone**: An oral pyridine derivative, showed some success but via unclear mechanisms (Moeller et al., 2008; Hilberg et al., 2012; Luppi et al., 2012; Azuma, 2012; Azuma et al., 2011).

- **SB-431542 and LY2109761**: Act as inhibitors of TGF-β Receptor Kinase, showed moderate success in some recent pre-clinical trials but clinical trial outcomes are awaited (Halder et al., 2005; Zhang et al., 2011).

### 2.7.2. Possible Causes of Failures

An important property of TGF-β1 is its ability to activate its own mRNA expression and thereby increase its own secretion (Van Obberghen-Schilling et al., 1988; Kim et al., 1989; Ventura et al., 2004). This suggests a mode of local autocrine amplification of TGF-β1 in the lungs (Figure 12) which would make systemic neutralization strategies difficult, consistent with the clinical experience so far. Specifically, circulating neutralizing antibodies are unlikely to sufficiently inhibit local concentrations of cytokines, especially when there are strong autocrine loops. Direct suppression of TGF-β synthesis or secretion should be a better strategy but has not been possible to date, although some inhibition has been noted through pirfenidone via unclear mechanisms. Direct inhibition of TGF-β1 by nucleotide-based therapy could provide a possible solution for future therapeutics.

### 2.8. MicroRNAs IN HEALTH AND DISEASE

MicroRNA(s) (miRNAs) belong to a family of 20-25 nucleotide long RNAs that have recently emerged as fundamental regulators of cognate target gene expression (Bartel, 2004; Ebert and Sharp, 2012; Ma et al., 2012). These growing class of non-coding
RNAs are involved in a wide variety of biological processes and have a role in networking and fine-tuning of target gene expression in the cell (Malumbres, 2012; Pfaff et al., 2012; Zhou et al., 2011). These have been widely reported to be evolutionarily conserved and show unique tissue-specific, developmental stage-specific and disease-specific patterns of gene expression (Espinoza-Lewis and Wang, 2012; Avila-Moreno et al., 2011; Weiland et al., 2012).

The biogenesis of microRNAs follows a distinct pathway and is processed through a series of post-transcriptional biogenesis steps (O’Carroll and Schaefer, 2012; Gregory and Shiekhattar, 2005; Volk and Shomron, 2011; Davis and Hata, 2009). The pathway initiates with the transcription of longer precursors, known as primary transcript (pri-miRNA). The pri-miRNA fold on themselves to form a characteristic hairpin RNA structure, which is recognized by the nuclear RNAse-III enzyme Drosha, and its cofactor DGCR8. These proteins form a complex with several other proteins, known as the Microprocessor. The Microprocessor cleaves the pri-miRNA to generate a shorter hairpin, which is about 70 nucleotides in length, and is known as the precursor miRNA (pre-miRNA). Pre-miRNA is then exported out from the nucleus to the cytoplasm via Exportin-5. RNase III endonuclease Dicer further cleaves pre-miRNA in the cytoplasm to generate the final mature miRNA duplex. Based on the thermodynamic stability, one of the strands of this duplex becomes the biologically active miRNA, while the other complementary strand is considered as an inactive strand, known as miRNA* or passenger strand. Further, the miRNA* strand is degraded, whereas the mature miRNA strand is taken up by RNA-induced silencing complex (RISC), in association with Argonaute proteins to form a
microribonucleoprotein (miRNP) complex. The mature miRNA strand then guides this complex to its target gene, where it participates in the post-transcriptional regulation either through mRNA degradation or translation inhibition. A schematic representation for the miRNA biogenesis pathway is depicted in Figure 13.

MicroRNAs can be transcribed either inter- or intragenically. When transcribed from an intergenic location, their expression could be coordinated by other miRNAs in genomic proximity. When the genomic distance between the microRNAs is less than 10 kb, they are generally considered to be a part of a single cluster (Altuvia et al., 2005; Ożsolak et al., 2008). When transcribed from an intragenic location, namely introns of the protein-coding gene, intronic microRNA are often expressed from the same strand as their host-gene (Rodriguez et al., 2004; Liu et al., 2008; Okamura et al., 2008; Kim and Kim, 2007) and at correlated levels (Baskerville and Bartel, 2005). However, some reports also suggest host-gene independent transcription for intronic microRNA (Monteys et al., 2010; Isik et al., 2010).

Over the past years, several reports have demonstrated the pivotal roles exhibited by miRNAs in regulating gene expression. By extensive use of microarray technology and high-throughput sequencing, global miRNA expression profiles have been generated and several differentially regulated microRNAs have been identified in various model systems (Costa et al., 2012; Pritchard et al., 2012). Interestingly, several miRNAs have been identified which seem to regulate various immunological processes (Lodish et al., 2008). Gain- and loss-of-function approaches have also been adopted to define the function(s) of these miRNAs. Several reports suggest the
importance of microRNAs in many human diseases, including cancer, fibrosis, autoimmune and vascular diseases (Alvarez-Garcia and Miska, 2005; Lu et al., 2008). Interestingly, a few reports also suggest the role of microRNAs in modulating TGF-β signalling pathway. A recent report suggests that within the developing liver, members of miR-23b cluster target three Smads (Smad3, Smad4 and Smad5), thereby inhibiting the anti-proliferative response mediated by TGF-β and fostering hepatocyte proliferation (Rogler et al., 2009). This is particularly interesting, as this study demonstrates how a single miRNA cluster targets several Smads concomitantly despite of having a weak effect on their own. This provides experimental evidence that the simultaneous attack of miRNAs on a common set of regulatory proteins can amplify their effect. Recent reports also suggest the roles of miR-106b-25 and miR-17-92 clusters as key modulators of TGF-β signalling in gastrointestinal and other tumors, where they interfere with cell cycle arrest as well as apoptosis, when overexpressed in cancer cells (Petrocca et al., 2008; Dews et al., 2010). These reports suggest that miR-17-92 is a potent inhibitor of TGF-β signalling by functioning both upstream and downstream of pSmad2, which triggers downregulation of multiple key effectors of the TGF-β signalling cascade. However, no report till date has shown a direct effect of microRNA(s) on TGF-β and its importance in a biological context. This is particularly important, as TGF-β binding to its receptor initiates this signalling pathway and the levels of TGF-β can be deregulated in a number of diseases. As discussed earlier, since the levels of TGF-β are critically maintained as the transcriptional and post-transcriptional levels, it is quite possible that microRNA-mediated control of TGF-β signalling provides another layer of regulation for the fine-tuning of gene expression.