Role of Smads in remodelling of the large airways in patients with COPD

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XXII Ciclo, 2008-2010
“Dio non solo gioca a dadi, ma a volte li tira persino dove non possono essere visti”
Stephen Hawking

A mio padre Alessandro e a mio figlio/a
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BACKGROUND

1. Basic features of Smad proteins

1.1 Smad family members

SMADs (small mothers against decapentaplegic) are intracellular proteins that transduce extracellular signals from transforming growth factor beta (TGF-β) ligands to the nucleus where they activate downstream TGF-β gene transcription (Massaguè, 1998; Wrana et al, 2000). The name Smads was coined in reference to its sequence similarity to the Drosophila Medea protein, mothers against decapentaplegic “Mad” and to the Caenorhabditis elegans protein “Sma”. Actually eight Smad proteins are encoded in the human and mouse genome, four in Drosophila and three in C. elegans (Massaguè, 1998) (Figure 1). Only five of the mammalian Smads (R-Smads, namely Smad1, Smad2, Smad3, Smad5 and Smad8) act as substrates for the TGF-β receptors family; indeed Smad1, 5, and 8 act mainly as substrates for the Bone Morphogenetic Protein (BMP) and anti-Muellerian receptors, while Smad2 and 3 are substrates for the TGF-β, activin and nodal receptors. Co-Smad-Smad4 serves as a common partner for all R-Smads while Smad6 and 7 are inhibitory proteins. Functional studies, together with the X-ray crystal structure analyses, showed that these ~500 amino acids proteins consist of two conserved globular domains (MH1 and MH2 domains) coupled by a flexible linker region rich of binding sites for Smurf (Smad ubiquitination-related factors) ubiquitin ligase, of phosphorylation sites for several classes of protein kinases and, in Smad4, a nuclear export signal (NES) involves on nucleus-cytoplasmatic translocation (Massaguè et al, 2005) (Figure 2). The N-terminal Mad-Homology 1 (MH1) domain is conserved in all R-Smads and in Co-Smad but not in inhibitory Smads; it functions as DNA-binding site containing a
β-hairpin structure and is stabilized by a tightly bound zinc atom (Shi et al, 1997; Dennler et al, 1998), furthermore it can interact with other proteins and carries nuclear localization signals (NLSs). The C-terminal MH2 domain is conserved in all Smads and is involved on type I receptor binding, on Smad homo-and hetero-oligomerization and on R-Smad activation/phosphorylation (Dijke et al, 2006). Nearby, in R-Smads, a basic pocket interacts with the phosphorylated region of the activate receptor type I, or with the phosphorylated tail of R-Smad, in case of Smad4; in the other site, a set of contiguous hydrophobic patches, constituting the “Hydrophobic corridor”, are involved in interaction with nucleoporins and cytoplasmatic retention proteins (Ross et al, 2008).
Figure 1: Three subclasses of Smads. The receptor-regulated Smads include two groups of closely related forms; the SSXS receptor phosphorylation sequence (P) is located in the C-terminus or receptor regulated Smads. The Co-Smads include Drosophila Medea and C. elegants Sma-4, which are more similar to Smad4 than to any other family member. Antagonistic Smads lack most of the conserved MH1 domain and their MH2 domain is quite divergent from the MH2 domain if the other family members (Massaguè et al, 2005).
**Figure 2:** Molecular structure of Smads and interaction sites in TGF-β type I receptor cytoplasmatic domain (Massaguè et al, 2005).
1.1.2 Receptor internalisation

Binding of TGF-β to the type I and type II receptors, induces formation of heterotetrameric complexes in the presence of the dimeric ligand (Wrana et al, 2008). The serine/threonine kinase receptor family in the human genome comprises 12 members, 7 type I and 5 type II receptors, all involved in TGF-β signaling; both types of receptors consist of about 500 amino acids, organized sequentially into a N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain. In contrast with BMPs, TGF-β and activin display an high affinity for the type II receptor (TβRII) and do not interact with the isolated type I receptor (TβRI) (Massaguè et al, 1998). Initially TGF-β binds tightly to the ectodomain of the type II receptor, this binding allows the subsequent dimerization with the type I receptor, forming a large ligand-receptor complex in which are involved a dimeric ligand and four receptor molecules. Lin (Lin et al, 2006) suggested that once type II receptors phosphorylate type I receptors, they could interact at the cell surface with the cytoplasmatic form of promyelocytic leukemia protein (cPML) that acts as a bridging factor between a Smad Anchor for Receptor Activation (SARA) and Smad2/3. cPML promotes the transfer of the complex formed into early endosomes, where cPML could dissociate from the complex, allowing Smad2/3 to interact with SARA and to be phosphorylated. SARA is an anchor protein which acts presenting R-Smads to the activated type I receptor and thereby regulating TGF-β transduction signal. It is a multidomain protein that contains an 80 amino acids Smad-Binding Domain (SBD) and a FYVE phospholipid-binding domain that avidly binds to phosphatidyl inositol 3-phosphate on endosomal membranes and targets SARA preferentially to early endosomes (Di Guglielmo et al, 2003). As revealed by the X-ray crystal structure of the Smad2–SBD complex, the SBD of SARA makes contact with the three consecutive hydrophobic patches of the hydrophobic corridor (Wu et al, 2000), blocking
translocation of Smad2/3 into the nucleus and the formation of transcriptional complexes; the activated TGF-β receptor complex therefore undergoes endocytosis via two distinct routes: by internalization via coated vesicles to early endosomes and via caveolae to caveolin-positive vesicles for degradation (Di Guglielmo et al, 2003). However other adaptor proteins were found to cooperate with SARA on Smad signaling, such as FYVE-containing protein, HGS (Mirura et al, 2000) Disabled-2 (Hocevar et al, 2001), Axin (Furuhashi et al, 2001), and the ELF β-spectrin (Tang et al, 2003).

1.1.3 Smad phosphorylation and nuclear translocation

In the basal state, Smads form homoligomers and remain in an inactive conformation through an hydrophobic interaction between the MH1 and MH2 domains. In solution, the unphosphorylated Smad2 MH2 domain is a monomer but, once phosphorylated, both R-Smads and Smad4 form homotrimeric complexes (Lagna et al, 1996; Shi et al, 1997). As revealed by X-ray crystallographic studies, the binding of R-Smad to Smad4 forms oligomers, heterodimers (R-Smad–Smad4) or heterotrimers (two R-Smads molecules plus one Smad4 molecule) which are stabilized by interactions within an extensive protein–protein interface between adjacent MH2 domains (Wu et al, 2001). Phosphorylation can occur in two different sites: in the linker region sites by several kinases, including MAPKs and CDK (Brown et al, 1999; Shi et al, 2004) and mainly on the C-terminal conserved Ser-Val-Ser (Ser-Met-Ser in Smad2) motif of Smad3 by the activated receptor (Massaguè et al, 2005; Ross et al, 2008). Actually the cytoplasmatic region of the type I receptor contains a canonical protein kinase domain preceded by a regulatory region or GS domain to which the inhibitor FKBP12 binds to enforce the inactive basal state. Phosphorylation of the GS domain by the type II receptor creates a repeated pS-X-pS motif that serves as a docking site for both R-Smads (Massaguè et al, 2005); as a
result, R-Smads decrease their affinity for SARA and the activated oligomer complex is translocated into the nucleus where can bind directly to DNA promoters or indirectly binding to transcription factor or co-factors. (Xu et al, 2000; 2002). Recently two studies showed that In the basal state, R-Smads are predominantly localized in the cytoplasm, I-Smads tend to be nuclear, while Smad4 is distributed equally in both, cytoplasm and nucleus; however Smads do not reside statically in the cytoplasm in absence of ligands or in the nucleus upon TGF-β stimulation: there is a spontaneous bi-directional shuttling of the R-Smads/Smad4 complex across the nuclear envelope without TGF-β stimulation (Xu et al, 2002) even if the nuclear import of the oligomer complex is offset by export forces, so they cannot reach an high concentration in the nucleus (Nicolas et al, 2004). However TGF-β-independent signals can involve target sites rich of multiple serine and threonine, as on the linker region as on the N-terminal domain. Indeed the linker regions of R-Smads are subject to phosphorylation by Cam Kinase II (Ca^{++}- and Calmodulin-dependent kinase II) (Wicks et al, 2000), while the N-terminal and the linker region of Smad3 can be phosphorylated by cyclin-dependent kinases (CDKs) (Matsuura et al, 2004) or by G protein-coupled receptor kinase 2 (GRK-2) which phosphorylates Ser197 in Smad2 (Ho et al, 2005).
Figure 3: Molecular mechanism of Smads pathway. 1-2) Upon TGF-β binding, anchor protein SARA captures R-Smads (Smad2/3) for presentation to the activated type I receptor. 3-4) Activated R-Smads dissociate from the receptor/SARA complex and form an oligomeric complex with Co-Smad-Smad4. 5-6-7) The complex R-Smad/Co-Smad translocates into the nucleus where can bind to transcription factors or directly interact with DNA in Smad Binding Elements (SBE). 8) Gene target transcription is activated. 9) I-Smads inhibit signals competing with R-Smads for interacting with type I receptor and preventing phosphorylation (Magno et al, 2008).
Smad activation and transcriptional functions can be regulated in both cytoplasm and nucleus. Several types of mechanisms were evolved to limit their activity (Figure 4): one type includes transcriptional co-repressors, such as the homeodomain protein TGIF. Binding of TGIF prevents the interaction of Smads with nuclear co-activators and interferes with activation of TGF-β-target genes expression. The inhibitory Smads, Smad6 and 7, interfere with Smad-receptors or Smad-Smad interactions; Smad7 inhibits TGF-β/activin and BMP signaling, while Smad6 inhibits more selectively the BMP pathway showing high selectivity for specific amino acid residues of the BMP type I receptors, ALK1, ALK2, ALK3 and ALK6 (Goto et al, 2007). Smad6 competes with Smad4 for interaction with the receptor-activated Smad1, such that inactive Smad1-Smad6 complexes are formed (Hata et al, 1998). In contrast, Smad7 acts as a competitive inhibitor able to bind the activated TGF-β and BMP receptors, competing with R-Smads and preventing or blocking their activation (Nakao et al, 1997), furthermore it is able to binds to all type I receptors via specific lysine residues in its MH2 residues (Mochizuki et al, 2004). Although Smad7 primarily acts at the type I receptor level, it also resides in the nucleus and new evidences suggests that it can bind to DNA and to nuclear complexes inhibiting Smads transcriptional activity (Zhang et al, 2007). In addition, Smad7 is involved in regulation of TGF receptor ubiquitination by smurf ubiquitin ligase (Suzuki et al, 2002). The chaperon protein Hsp90 binds to TβRII and to TβRI and protects them from ubiquitination by Smurf2, contributing positively to TGF-β signaling (Wrighton et al, 2008). Smurf1 and Smurf2 are HECT-domains containing E3 ubiquitin ligases that recognize R-Smads as substrates through a PPXY motif; Smurf2 can bind to Smad7, without immediate ubiquitination and degradation, but favouring export of Smad7 to the cytoplasm. The complex Smurf-Smad7 is able to bind to the TGF-β receptor complex and promotes its ubiquitination,
thereby down-regulating signaling; however the activity of Smad7 is regulated at many levels: is induced by TGF-β, activin and BMP signaling and by ERK (Huo et al, 2007) or inflammatory signals such as interferon-γ, TNF-α and interleukin-1β (Bitzer et al, 2000). Activated R-Smads undergo ubiquitination and subsequent degradation. Increasing evidence indicates that ubiquitin modification is important for regulating components of TGF-β signaling and can occur in both the basal and the activated state: for example, phosphorylated Smad2 is eliminated by the fast action of phosphatases but also by the slower action of ubiquitin-dependent proteosome degradation. The susceptibility to ubiquitination may be controlled by acetylation of the same lysine residues. Following phosphorylation, Smad2 and 3 are translocated into the nucleus where interact with co-activators and/or co-repressors. Smads transcriptional activity is significantly enhanced by recruitment of co-activators and histone acetyltransferases, such as p300, C/EBP-binding protein (CBP) and p300/CAF-associated factor (P/CAF), which are able to facilitate the initiation of transcription (Schmierer et al, 2007). CBP is a co-activator able to transfer the acetyl group from acetyl coenzyme A to the lysine residues in histones, allowing remodelling of chromatin to a more relaxed conformation to allow transcription. Smad2/3 can be acetylated by p300/CBP in a ligand-dependent manner enhancing their DNA-binding activity in mammalian cells (Simmonsson et al, 2006; Tu et al, 2007). This event involves at least three lysine residues, Lys19, Lys20 and Lys39 which are required for efficient acetylation of Smad2. in addition, an acetylation event is required for Smad2 to mediate activin and TGF-β signaling: mutations of the three key lysine residues did not alter the stability of Smad2 or its ability to form a complex with Smad4 on promoter DNA, but prevent nuclear accumulation of Smad2 and subsequent TGF-β and activin responses (Tu et al, 2007). Nuclear Smad7 can be also acetylated by p300 in two N-terminal lysines, which are also the sites of ubiquitin attachment. Acetylation prevented receptor-induced ubiquitination of Smad7 by Smurf,
without affecting the subcellular distribution of Smad7. Another Smads regulatory process is **Sumoylation**. In higher eukaryotes are present three SUMO (Small-Ubiquitin-like Modifier) proteins, SUMO-1, SUMO-2 and SUMO-3, that alter proteins function by creating a composite interaction domain (Song et al, 2004) and regulating protein stability and subcellular localization. Two sumoylation consensus motifs were observed in Smad4, one in MH1 domain and one in the linker region (Song et al, 2004); as consequence of this regulation, however, two opposite effects were observed, an increase and a decrease of transcriptional activity (Long et al, 2004; Chang et al, 2005), probably as result of potential competition between ubiquitination and sumoylation of the same lysine residue in Smad4. Ubiquitination of R-Smad/Smad4 complex can be target of proteosome and as a result, gene expression is reduced. By sumoylation, ubiquitination effects are blocked, the half life of the activated complex is prolonged and gene expression is increased. SUMO modification also represses transcriptional activity of a number of transcription factors, such as Sp3, c-Jun, c-Myb, AP2, nuclear receptors, Elk-1 and the general co-activator p300, regulating their biological role (Long et al, 2004). A negative regulatory mechanism of Smad signaling can also implicate the **linker domain phosphorylation**; a recent report showed that nuclear GSK3β negatively controls TGF-β signaling phosphorylating three distinct Smad3 linker residues and down-regulating its transcriptional activity, as consequence of ubiquitination and proteosomal degradation of Smad3 (Guo et al, 2008). Than Smad3 can be phosphorylated in its MH2 domain by casein kinase 1 γ2, which leads to the specific ubiquitination and degradation of the activated form of Smad3 (Guo et al, 2008b).
Figure 4: TGF-β and BMP signaling. A) TGF-β and activin/nodal pathway; B) BMP pathway; C) Non-Smad signaling pathways downstream of the TGF-β receptors. The nuclear Smad complexes that lead to gene regulation are shown for each pathway (Moustakas et al, 2009).
1.2 Smads and lung diseases

1.2.1 TGF-β signal transduction pathway

The transforming growth factor β appears to be the most important cytokine involved on stimulation and regulation of lung extracellular matrix (ECM) components. Structurally the mammalian TGF-β family is characterized by a specific three-dimensional fold and by a conserved number and spacing of cystein residues in the C-terminus of the mature polypeptide (Derynck et al, 2008). It is possible to observe three prototypic TGF-β isoforms: TGF-β1, TGF-β2 and TGF-β3; each of them is encoded by a distinct gene and is expressed in both a tissue-specific and developmentally-regulated fashion. Specifically, TGF-β1 is expressed in endothelial, haematopoietic and connective tissue cells; both TGF-β1 and TGF-β3 are expressed in structures undergoing morphogenesis and in mesenchymal cells, while TGF-β2 alone is expressed in differentiating and mature epithelium (Taipale et al, 1998; Blobe et al, 2000). TGF-β superfamily members are involved on regulation of different cellular processes, such as cell growth, development, motility, adhesion and apoptosis (Hogan et al, 1996; Mehler et al, 1997) and contribute to tissue patterning and regulation and differentiation of stem cell self-renewal (De Robertis et al, 2004; Watabe et al, 2009). However, the cellular response can be variable and depending on the cell type and stimulation context (see Table 1): for instance, was observed that TGF-β1 stimulates cellular proliferation in vitro even if it is generally considered to be an inhibitor of proliferation and a promoter of cellular hypertrophy and differentiation (Choi et al, 1993); whereas was observed that it causes epithelial cell apoptosis and growth arrest (responses which suppress carcinogenesis) even if it can also induce epithelial-mesenchymal transition (EMT) and can mediate fibroblast activation (responses implicated in promoting carcinogenesis and fibrotic diseases) (Siegel et al, 2003). Members of this family are secreted as latent ligands
by binding to their pro-peptide, or in trapped form by binding to occluding factors. The active form of TGF-β is a dimer stabilized by hydrophobic interactions, which in most cases are further strengthened by an inter-subunit disulfide bridge. As reported previously, TGF-β signal is initiated by interacting with and complexing two membrane serine/threonine kinase receptors, type I and type II receptors. Ligand binding mediates formation of a heterotetrameric complex in which the constitutively type receptor II phosphorylates the glycine-serine rich domain in the juxtamembrane region of the type I receptor resulting in activation of kinase. The most well characterized mechanism whereby TGF-β superfamily members initiate signal transduction is via phosphorylation and activation of the Smads proteins by the type I receptor (Rahimi et al, 2007). However a number of reports also supported the existence of other effector pathways operating downstream of TGF-β receptors: the best characterised of these is the c-Jun terminal kinase (JNK) family in which activation of JNK by TGF-β can be mediated through mitogen-activated protein kinase (MAPK), kinase kinase-1 and MAPK kinase-4 (Hocevar et al, 1999; Mazars et al, 2000). Ozdamar and co-workers described also the pathway that seems to be involved on EMT: TβRII phosphorylates the protein PAR6, which regulates the local degradation of the RHOA small GTPase that controls the assembly of intercellular tight junction in mammalian cells, causing the de-differentiation known as epithelial-mesenchymal transition (Ozdamar et al, 2005). In addition TGF-β is able to elicit EMT via Smad signaling, leading to the transcriptional induction of the major inducers of this differentiation process (Thuault et al, 2008). In a distinct mechanism TβRI phosphorylates both serine and tyrosine residues in the SHCA (SHC1) adaptor; causing the activation of the Ras-Raf-MEK-ERK mitogen-activated protein kinase signaling cascade which can regulate cell proliferation and migration (Lee et al, 2007); as a final example, the tyrosine kinase Src can phosphorylate Tyr284 in the cytoplasmatic domain of the TβRII, leading to GRB2 and
SHC recruitment and to the activation of the p38 MAPK pathway (Galliher et al, 2007).

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Table 1: TGF-β regulatory effects on target cells (Magno et al, 2008).

1.2.2 Cross-talk between MAPKs and Smads signaling pathway for TGF-β

Several studies reported a cross-talk between kinases and members of Smad family and demonstrated that activation of p38 MAP kinase (Kamaraju et al, 2005) and ERK MAP kinase (Hayashida et al, 2003) may augment Smad signaling. Indeed MAPKs are able to bind the consensus sites in the linker region and are responsible of Smad2/3 phosphorylation and/or Smad4 activation (Derynck et al, 2003; Hayashida et al, 2003). In vitro, ERK1 and 2 are able to activate Smad1, 2 and 3 and attenuate the nuclear accumulation and signaling activity of these proteins in response to agonists (Kretzschmar et al, 1999) and chemical inhibitors (Rhyu et al, 2005); several studies demonstrated that the use of ERK and JNK inhibitors inhibited TGF-β1-stimulated activation of Smad2/3 in human airway smooth muscle ASMC cells (Shaoping et al, 2005) and that the ERK inhibitors, PD98059 and UO126, were able to inhibit promoter region and to reduce Smad3 protein expression in both, human alveolar type II epithelial adenocarcinoma A549 cell line and ASMC (Ross et al, 2007). However, the interaction between ERK and Smads could alter signaling in either positive and negative manner in according to the cell type studied: actually, human cells of mesenchymal origin appear to show synergy in ERK-Smad interaction whereas epithelial cells generally appear to show inhibition (Hayashida et al, 2003). Recently Hou suggested for the first time that Smad7 plays an important role in the signal cascade transition mechanism between the TGF-β1-mediated Smads and ERK MAP kinases signaling pathways (Huo et al, 2007); the author demonstrated that in human bronchial epithelial BEP2D cell line Smad7 is required for TGF-β1-induced activation of ERK and that this activation can be attenuated by the silencing of Smad3 and Smad4 genes, indicating that gene transcription of Smad7, driven by TGF-β1, is required for Smad3 and Smad4 expression. Imamichi and colleagues also reported that induction of focal complex formation in epithelial cells by TGF-β1 is mediated by activated ERK and
JNK MAP kinases and is independent from Smad4 (Imamichi et al, 2005); moreover Engel demonstrated that the JNK pathway is able to reinforce Smads signaling by permissive phosphorylation of Smad3 (Engel et al, 1999), while Goldberg demonstrated that overexpression of type II transforming growth factor-beta receptor is able to activate ERK and JNK MAP kinases in human fibroblasts (Goldberg et al, 2002).

1.2.2 Smads as mediators of TGF-β-induced diseases

**Fibrosis**

Destruction of the TGF-β signaling system has been implicated in embryonic anomalies (Chang et al, 1999, 2000; Galvin et al, 2000; Yang et al, 1999), cancer and tumorigenesis (Oshima et al, 1996; Dijke et al, 2002, Rahimi et al, 2007), autoimmune disease (Oshima et al 1996; Zhu et al, 1998; Yang et al, 1999; Ashcroft et al, 1999), atherosclerosis (Grainger et al, 1995a, 1995b, 1999), hypertension (Cambien et al, 1996), osteoporosis (Langdahl et al, 1997), fibrotic diseases (Blobe et al, 2000; Barànek et al, 2002) and hereditary hemorrhagic telangiectasia (Johnson et al, 1996; Gallione et al, 1998). The destruction of the TGF-β signaling system has been also shown to be involved on different pulmonary diseases such as COPD and principally pulmonary fibrosis. Injury of lung tissue leads to induction of TGF-β, which limits some inflammation reactions and is involved in mediating fibrotic tissue remodelling by increasing the production and decreasing the degradation of connective tissue and by mediating the normal tissue repair (Sporn et al, 1992). Several authors considered TGF-β as a marker of activity of tissue repairing and remodelling, actually as acute as well as chronic lung disease showed an increase of TGF-β protein and mRNA expression during the phase of tissue remodelling (Broekelmann et al, 1991; Elssner et al, 2000). The direct involvement of TGF-β in fibrosis comes from several studies of fibrotic
disease. The prominent hypothesis of pulmonary fibrosis development is that it is caused by chronic inflammation in response to an unknown etiologic agent which leads to tissue destruction, to ongoing wound healing responses and fibrosis (Bonniaud et al, 2005). TGF-β is a critical element of progression from inflammation to chronic fibrosis: the pro-fibrotic effects of TGF-β are numerous, including induction of myofibroblasts, increase of matrix synthesis and inhibition of collagen breakdown. Different studies demonstrated that TGF-β is involved in EMT by stimulating myofibroblasts transdifferentiation (Siegel et al, 2003; Zeisberg et al, 2007). The hallmarks of EMT include the destruction of cell-cell and cell-matrix interactions, degradation of the surrounding ECM and actine reorganization. However if EMT is a normal physiological process necessary for proper development, the pathological induction is associated with carcinoma and fibrotic disease (Lee et al, 2006; Radisky et al, 2007). Most of these effects are mediated through the Smad signaling pathway and even if several studies demonstrated a requirement for Smad4 in TGF-β-mediated EMT (Valcourt et al, 2005; Medici et al, 2006) Smad3 seems to be principally related to the fibrotic phenotype. Smad3 pathway is involved in pathogenic mechanisms mediating tissue destruction (lack of repair) and fibrogenesis (excessive repair); TGF-β stimulates myofibroblasts transdifferentiation through Smad3-dependent and -independent signals, contributing to the excessive matrix deposition which characterizes obliterative bronchiolitis. Recently was demonstrated that Smad3 null mice are protected from progressive fibrosis mediated by overexpression of TGF-β1 (Bonniaud et al, 2004), they do not develop lung fibrosis induced by bleomycin and are protected against radiation-induced fibrosis of the skin (Zhao et al, 2002). Experiments in vivo demonstrated that administration of active TGF-β to mice deficient in Smad3 blocks the ability of TGF-β to induce matrix and enzyme inhibitory gene expression and also matrix accumulation without any progress to scar formation or fibrosis (Gauldie et al, 2007). This indicates that TGF-β and Smad
signaling pathway, specifically Smad3, are required to initiate fibrosis and 
that the TGF-β-induced mechanism is prominent in this disease. Lost of 
Smad3 was shown to confer resistance to fibrosis and results in reduced 
inflammatory cell infiltrates, reduced autoinduction of TGF-β, important to 
sustain the process, and reduced elaboration of collagen. Recently 
Higashiyama showed that treatments with the inhibitor of activin receptor-
like kinase 5 (ALK5), a type I receptor of TGF-β, significantly attenuated 
Smad2/3 nuclear translocation and decreased myofibroblasts proliferation 
and collagen type I deposition suggesting that ALK5 inhibition is able to 
attenuate R-Smads activation and thereby pulmonary fibrosis (Higashiyama 
et al, 2007). Than a significant reduced Smad3 protein expression was 
oberved in cystic fibrotic epithelial cells of nasal epithelium and in mouse 
models, a reduction that was apparently sufficient to influence the 
transmission of TGF-β signals, including anti-inflammatory signals. 
(Kelley et al, 2001).

**COPD and asthma**

Lung tissue remodelling and airway fibrosis play an important role in the 
development of symptoms associated with lung function and loss in asthma 
and in chronic obstructive pulmonary disease (COPD). Less is know about 
the direct involvement of Smad proteins on COPD even if various studies 
demonstrated that TGF-β is involved in airways remodelling which 
characterize this disease. Increased levels of TGF-β1 protein and mRNA 
were observed in the bronchial and alveolar epithelium of COPD patients 
and correlated with the number of intraepithelial macrophages (De Boer et 
al, 1998); similarly elevated levels of TGF-β1 were found in the bronchial 
epithelium of smokers with COPD compared with healthy smokers (De 
Boer et al, 2000). Immunohistochemistry analysis demonstrated that 
cigarette smoke is able to down-regulate the transcription of inhibitory 
Smad6 and 7 in the bronchial mucosa of Severe COPD patients (Springer et
al, 2004), in the same way, a reduced Smad7 mRNA expression was observed in bronchial biopsies of Mild/Moderate COPD patients in comparison with controls; in contrast, any significative change was observed for Smad3 and Smad4. As demonstrated in a mouse model, increased presence of TGF-β1 in the parenchyma may protect against emphysema, whereas the absence of proper Smad3 signaling results in an ineffective repair response to damages in the lung, in a reduction of suppression of expression of matrix metalloproteinases (MMPs), in more susceptibility to airspace enlargement and in development of emphysema. Recently was demonstrated that Smad3-deficient animals are protected from fibrosis even if appear more susceptible to emphysema: Smad3-null mice were resistant to both bleomycin- and TGF-β-mediated fibrosis, but developed spontaneous age-related airspace enlargement, consistent with emphysema, and lacked the ability to repair tissue damage appropriately (Gaudie et al, 2006).

Studies on human asthma showed increased levels of TGF-β1 and its transductor factors in the airways (Redington et al, 1997). Airway remodelling is one of the hallmark features of asthma. TGF-β appears to be implicated in ECM proteins deposition which characterize asthma, and especially in deposition of collagen. TGF-β1 levels were significantly higher in bronchial submucosa of asthmatics if compared with subjects with COPD (Kokturk et al, 2003) and concentration of active form of TGF-β1 were higher in bronchoalveolar lavage fluid (BAL) of patients with Severe asthma compared with controls (Redington et al, 1997). Runyan and colleagues found a cross talk between Smads and Phosphoinositide 3-kinase (PI3K) pathway that enhanced TGF-β-induced collagen type I expression in human mesangial cells (Runyan et al, 2004). Recently was also observed a different Smad2 activation between asthmatic and non-asthmatic airway smooth muscle ASMC cells with levels of phosphorylated Smad2 significantly higher in the asthmatic cells in comparison with the
non-asthmatic (Runyan et al, 2004). In addition expression levels of Smad7 in bronchial epithelial cells of asthmatics were found to be inversely correlated with basement membrane thickness and airway hyperresponsiveness in asthmatic subjects, suggesting an active remodelling process resulting in a thickened of basement membrane (Johnson et al, 2006).

1.3 New perspectives for CCL5 in lung diseases

Chemokines are a family of proinflammatory cytokines that act through cell surface receptors to regulate numerous routine physiological and pathophysiological processes, such as haematopoiesis, T-cell activation, angiogenesis and inflammatory disease, as well as HIV-1 infection (Hasegawa et al, 2001; Stantchev et al, 2001). Characterized by the presence of two disulphide bonds formed between four conserved cystein residues, chemokines are classified into four subfamilies according to the pattern of conserved cysteines in their amino acid sequences: twenty-six CC chemokines, seventeen CXC chemokines, two C chemokines and one CX3C chemokine (Gale et al, 1999; Murphy et al, 2000). MIP-1α (Macrophage Inflammatory Protein-1α), RANTES also termed CCL5 (regulated upon activation, normal T-cell expressed and presumably secreted) and MCP-1 (Monocyte Chemotactic Peptide-1) are members of CC chemokines subfamily and are chemotactic and activator factors for monocytes, basophils, eosinophils and neutrophils (Alarm et al, 1993; Capelli et al, 1999; Magno et al, 2007; Yoshikawa et al, 2007). Several studies demonstrated an involvement of these chemokines pulmonary disease, such as fibrosis and COPD: MIP-1α and MCP-1 were identified as pro-fibrotic mediators, actually studies in the bleomycin model of pulmonary fibrosis showed that anti-MIP-1α antibodies, and similarly inhibition of MCP-1, could significantly reduce the development of this
disease (Smith et al, 1994; Belberio et al, 2001). Recently, we observed that in bronchial biopsies of Severe COPD there is an increase of NAP-2 and CCL5 that could explain the sustaining neutrophilia observed in airways of these patients (Magno et al, 2007); in addition, we demonstrated that in bronchial biopsies of COPD patients, levels of CCL5 immunostained cells were 2-15 times higher than those of other chemokines analyzed and that in the bronchial mucosa of Severe COPD the expression of CCL5 mRNA and the number of CCL5⁺ cells was significantly increased if compared with controls (Di Stefano et al, 2009). These results augment data in literature for which increased levels of CCL5 were observed in sputum and in BAL of patients with stable COPD (Miller et al, 2007; Costa et al, 2008). Moreover high level of this chemokine were observed in other pulmonary diseases: in asthmatics was observed an increase of CCL5 protein expression, and similarly, CCL5 protein levels appear to be higher in epithelium of patients with an exacerbation of chronic bronchitis (Chung et al, 2001) (Table 2). However the role performed by CCL5 in these diseases is not completely clear and needs more study.
<table>
<thead>
<tr>
<th></th>
<th>ASTHMA</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>+ or –</td>
<td>?</td>
</tr>
<tr>
<td>IL-13</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RANTES</td>
<td>±</td>
<td>+++*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>IL-8</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>IL-1β</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>IL-5</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>IL-3</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>EGF</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2: Expression of cytokines in asthma and in COPD (Chung et al, 2001). * during exacerbation; - reduced; + small increase; ++ large increase; ? not known or uncertain; ± no change or negligible.
OBJECTIVE

Destruction of the TGF-β signaling system has been implicated in several diseases. Recently it was considered the role of this growth factor in development of lung pathologies such as COPD and pulmonary fibrosis. The pro-fibrotic stimuli of TGF-β are numerous, including induction of myofibroblasts, EMT and regulation of extracellular matrix components, such as collagen, essential for tissue homeostasis and function. The extensive family of “COL” gene products is composed at least of 16 chain types, including fibril-forming interstitial collagens (type I, II, III and V) and basement membrane collagens (type IV). Type I collagen and ECM accumulation is one of the hallmarks of fibrosis (Verrecchia et al, 2002). It was demonstrated that Smad signaling pathway, and specifically Smad3, is required to initiate fibrosis induced by TGF-β. Indeed, loss of Smad3 was shown to confer resistance to fibrosis and results in reduced inflammatory cell infiltrates, reduced auto-induction of TGF-β and reduced elaboration of collagen; furthermore, as observed in dermal fibroblasts, Smad3 signal pathway is considered crucial for simultaneous activation of several fibrillar collagen gene promoters induced by TGF-β (Verrecchia et al, 2001). At one time it was thought that all collagens were secreted in connective tissue by fibroblasts, but now we know that numerous epithelial cells make certain types of collagens (Ward et al, 2005) playing a crucial role in the development of fibrotic disease.

On the bases of these information, the aim of this study has been: first, to set up an opportune in vitro experimental model to analyze if Smads could be abnormally expressed in the human bronchial epithelial cells after TGF-β1 stimulus, to value the involvement of epithelium in the collagen production. Second, to analyze Smads and collagen type I expression in bronchial biopsies of COPD patients, to understand their possible involvement in chronic obstructive pulmonary disease and third, according to the increased CCL5 levels recently observed in the bronchial mucosa of
Severe COPD, the aim was to check the effects of CCL5 in TGF-β/Smads-dependent collagen production to understand its possible involvement in the pathogenesis of COPD.
3.1 In vitro experiments

3.1.1 Cell culture

SV40-transformed human bronchial epithelial 16HBE cell line was used to setting an in vitro model in order to assess their behaviour after TGF-β stimulation. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B and 2mM of L-glutamine (all from Invitrogen). They were maintained at 37°C in a humidified atmosphere of 5% CO2 and media was replaced every two days until 80% confluence. For RNA and protein extraction, cells were cultured in 6-well culture plates and 25cm² tissue culture flasks (Corning), respectively, while for immunocytochemical analyses cells were plated in 8-well chamber slides (BD Biosciences). Experiments were performed using cells at passages 12-19.

3.1.2 TGF-β1 and CCL5 treatments

After reaching 80% confluence, the incubation medium was changed to serum-free DMEM, supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B and 2mM L-glutamine for 24h. Then cells were stimulated with 1ng/ml and 10ng/ml of Recombinant Human TGF-β1 (R&D System), with 10ng/ml of Recombinant Human RANTES/CCL5 (R&D System) and 10ng/ml of both TGF-β1 and CCL5, diluted opportunely in serum-free DMEM. Exposure of cells was carried out with incubation times of 0, 3 and 24 hours. Comparative dose-dependent experiments were done in triplicate, while experiments for TGF-β1 and CCL5 were repeated six times with cells at different passages.
3.1.3 MAPKs inhibitor treatments

To check MAPKs involvement in both TGF-β1 and TGF-β1/CCL5-induced effects, cells were pre-treated for 30 minutes with 3µM of SP600125, 5µM of PD059098 and 3µM of SB203580, selective inhibitors of JNK, ERK and p38, respectively (all from Calbiochem). Then a new medium with TGF-β1 and CCL5 was added to cell as previously described. Experiments were performed in triplicate with results comparison to cells not treated with inhibitors.

3.1.4 Real Time quantitative RT-PCR

Total RNA was extracted using RNeasy kit from Qiagen following the manufacturer’s instructions. Reverse transcription was carried out with superscript RT after DNase I digestion (both from Promega) according to the manufacturer’s protocols. 1µg of RNA was used for Real Time quantitative RT-PCR analysis only if the absorbance ratio ($A_{260}/A_{280}$ ratio) was $> 1.6$. Quantitative measurement of transcripts was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and Rotor-Gene6 RG-3000 detection system (Corbett) according to the manufacturer’s protocol. Amplification of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as internal standard. Specific primers pair were used for Smads (Sigma Aldrich) and for collagen type I and GAPDH (Invitrogen):

- Collagen Type I
  - Forward: 5’-CCTGGGATGCCATCAAAGTCT-3’
  - Reverse: 3’-TCTTGTCTTGGGTTCTTG-5’
- Smad3
  - Forward: 5’-CTGCGTGAATCCCTACCCT-3’
  - Reverse: 3’-GGATGGAATGGCTGTAGTG-5’;
- Smad4
  - Forward: 5’-ATCGTGACATCGAGAGACA-3’
  - Reverse: 3’-TACTGGCAGGCTGACTTGTG-5’;
- Smad7
  - Forward: 5’-GTGGGCATACTGGGAGGAGAA-3’
  - Reverse: 3’-TGTTGTCCGAATTGAGCTG-5’;
- GAPDH
  - Forward: 5’-CAATGACCCCTTCATGGACC-3’
  - Reverse: 3’-GACAAGCTTCCCGTTCTCAG-5’
The cycling conditions were: 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, Tm for each primer and 72 °C for 25 s. The results are presented as the relative expression calculated from the δδ-Ct-values.

3.1.5 Cell viability assay (MTT)

Cell viability after TGF-β1 and both TGF-β1/CCL5 exposure was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Aldrich). MTT is a yellow water-soluble tetrazolium dye which is reduced in living cells to a water-insoluble dark blue formazan precipitate by a mitochondrial dehydrogenase enzyme. After treatments for 0, 3, 24 in 24-culture well plates, culture medium was replaced by MTT previously diluted in phenol red-free DMEM. After an incubation period of 4 hours, the solubilisation of the precipitate from cells was accomplished by using acid absolute isopropanol (HCl 0.04M). The amount of formazan was therefore determined spectrophotometrically by reading $A_{570}$ with background subtraction at 650nm. DMSO 1% was used as positive control to check cytotoxicity. Triplicate assays were performed using cells at different culture passages. cells not-exposed to treatments were used as controls.

3.1.6 Total and sub-fractionated protein extraction

16HBE cells were collected after treatments. In order to obtain total proteins, cells were lysed with a lysis buffer (20mM Tris/HCl pH 7.5, 1% Nonidet P-40, 50mM NaCl, 1x Proteases Inhibitor). In parallel experiments, nuclear and cytoplasmatic proteins were sub-fractionated using Nuclear Extraction Kit (Active Motif) following the manufacturer’s protocol. Quantitative measurement of proteins was performed using the
DC Protein Assay kit (Bio-Rad), following manufactures’ instructions and determined spectrophotometrically by reading at $A_{750}$.

### 3.1.7 SDS-PAGE and Immunoblotting

30µg of total or sub-fractionated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). After blocking in 5% milk no-fat in phosphate-buffered saline (PBS) containing 0.05% Tween-20, membranes were incubated with appropriated antibodies diluted in 3% Bovine Serum Albumin (BSA) in T-PBS 0.05% Tween-20, overnight at 4°C. Specifically, to analyze nuclear fraction, membranes were incubated with anti-phospho-Smad3 at 1: 2000 dilution (rabbit polyclonal, Abcam) and anti-Lamin A/C at 1:600 dilution (mouse monoclonal, SantaCruz Biotechnology), while total proteins membranes were incubated with anti-Smad3 at 1:200 dilution (rabbit polyclonal, Abcam), anti Smad7 at 1:1000 dilution (mouse monoclonal, Abcam) and anti-β-Actin at 1:2000 dilution (mouse monoclonal, SantaCruz Biotecnology). The second day membranes were washed six times with T-PBS 0.05% Tween-20 and incubated with horseradish peroxidase-coupled secondary antibodies (anti-mouse and anti-rabbit, 1:10000 and 1:50000, respectively) for 1 hour at room temperature. After washing, the blots were visualized with ECL plus western blotting detection reagents (GE Healthcare).

#### 3.1.7 Immunocytochemical staining for assessing phospho-Smad3

In order to asses phosphor-Smad3 localization immunocytochemistry (ICC) analysis was performed on 16HBE cultured and treated in 8-well chamber slides (BD Falcon). After culturing, cells were washed with PBS and fixed in methanol for 20 minutes at -20°C. Air-dried slides were then stored at -20°C until use. For ICC procedure, the cells were permeabilized with 0.1% Triton X-100 in PBS (Sigma Aldrich) and then washed with PBS. To block
endogenous peroxidases, cells were treated with 0.3% H₂O₂ in PBS and then with 1% FBS for blocking aspecific antigens. Antibody anti-p-smad3 (rabbit monoclonal, Abcam) was used at 1:250 dilution. Antibody anti-cytokeratin 8 (mouse monoclonal, Sigma Aldrich) was used at 1:200 dilution as positive control. Negative controls were obtained substracting the primary antibody incubation step. The detection system used was the LSAB (Labelled Streptavidin-Biotin)-2 kit (Dako), an avidin-biotin complex system in which a biotinylated secondary antibody reacts with peroxidase-conjugated streptavidin molecules. The 3-amino-9-ethylcarbazole (AEC+ High Sensitivity Substrate Chromogen Ready-to-use, DakoCytomation) was used as developer. Each passage was preceded by washes with PBS. The positive reaction was observed using a light microscope (Leica DM 5000B). Percentage of positive cells was evaluated at 20x and 40x magnification.

3.1.9 Statistic analysis

Data obtained from the evaluation of the immunoreactivity, as well as from Real Time-PCR densitometry, were plotted using Microsoft Excel software (Microsoft, Redmond, WA, USA). Statistical analyses were carried out using GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA) and were performed using non-parametric tests in order to compare groups that did not fit the normal distribution. To evaluate the significance of difference between all groups the Mann-Whitney U test was used. Standard deviation of values was considered for each group of samples. Data were considered significant for p<0.05. To confirm the statistical results obtained, each experiment was repeated six times using different passages.
3.2 Experiments \textit{in vivo}

3.2.1 Subjects

All subjects were recruited from the Section of Respiratory Medicine of the Salvatore Maugeri Foundation (Veruno, Novara). Table 3 shows the clinical characteristics of the subjects recruited. The severity of the airflow obstruction was staged according to the GOLD criteria: GOLD stage I = Mild, Stage II = Moderate, Stage III = Severe and Stage IV = Very Severe COPD (GOLD 2006). Data are presented as mean (SEM).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Number</th>
<th>FEV\textsubscript{1} pre (% pred)</th>
<th>FEV\textsubscript{1} post (% pred)</th>
<th>FEV\textsubscript{1}/FVC%</th>
</tr>
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<tbody>
<tr>
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<td>115 (4)</td>
<td>ND</td>
<td>85 (3)</td>
</tr>
<tr>
<td>Healthy Smokers</td>
<td>13</td>
<td>107 (4)</td>
<td>ND</td>
<td>82 (2)</td>
</tr>
<tr>
<td>Mild/Moderate COPD</td>
<td>12</td>
<td>66 (4)</td>
<td>72 (4)</td>
<td>60 (2)</td>
</tr>
<tr>
<td>Severe/Very Severe COPD</td>
<td>13</td>
<td>30 (2)</td>
<td>33 (3)</td>
<td>42 (3)</td>
</tr>
</tbody>
</table>

Table 3: Criteria used for recruiting of COPD patients. FEV\textsubscript{1} = Forced Expiratory Volume in 1 second; FEC = Forced Vital Capacity

3.2.2 Fiberoptic bronchoscopy, collection of bronchial biopsies

A standardised procedure (Di Stefano et al, 1998) was followed for fiberoptic bronchoscopy and collection of bronchial biopsies. All subjects were premedicated intramuscularly with 0.5mg of atropine and 10mg of diazepam and orally with 10mg of dihydrocodein. Nares and oropharynx were anesthetized topically with 10% lidocaine before bronchoscopy. Bronchoscopy was performed with a flexible fiberoptic bronchoscope (Pentax FB-18P; Asahi Optical Co. LTD) in all subjects. Bronchial biopsies were taken through the bronchoscope with standard forceps from the
subcarina of a basal segment bronchus of the right lower lobe. From this area two specimens were obtained in each subject. Biopsy specimens were gently extracted from the forceps and processed for light microscopy. Samples were embedded in OCT and frozen at -80°C. The best specimen was then oriented and serial sections 4µm thick were cut. Two sections at an interval of 100µm were then appropriately stained.

3.2.3 Immunohistochemistry

For immunohistochemistry (IHC) procedure, serial sections were fixed with 4% of formaldehyde or acetone (according to the instruction relative to the primary antibody to used), and washed with a wash solution (Tris Maleato 0,05M, saponin 0,1% pH 7,6). To enhance the detection, tissue sections were firstly incubated with avidin and then incubated with biotin both for 20 minutes. To block non specific reactive antigens, sections were incubated for 20 minutes with normal serum diluted 1:20 in wash solution and stained for 2 hours with specific primary antibodies diluted in wash solution too (see Table 4); than were incubated with secondary antibodies (anti-mouse 1:200, anti-rabbit 1:200 and anti-goat 1:200, all from Vector). The detection system used was the LSAB (Labelled Streptavidin-Biotin)-2 kit (Dako), an avidin-biotin complex system in which a biotinylated secondary antibody reacts with peroxidase-conjugated streptavidin molecules. The fast-red substrate (Sigma Aldrich) was used as developer. Each passage was preceded by washes with the wash solution. Control slides were included in each staining run using human tonsil as a positive control. The positive reaction was observed using a light microscope (Leica DC 300F). Percentage of positive cells was evaluated at 400x and 600x magnification.
Table 4: Primary antibodies used for IHC analysis.

<table>
<thead>
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<th>Primary antibody</th>
<th>Code product</th>
<th>dilution</th>
<th>Fixative solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type 1</td>
<td>Sigma Aldrich mouse monoclonal</td>
<td>1:100</td>
<td>acetone</td>
</tr>
<tr>
<td>Smad3</td>
<td>Zymed rabbit polyclonal</td>
<td>1:25</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>P-Smad2/3</td>
<td>Santa Cruz Goat polyclonal</td>
<td>1:100</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>Smad2</td>
<td>Zymed rabbit polyclonal</td>
<td>1:25</td>
<td>formaldehyde</td>
</tr>
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<td>Upstate Rabbit monoclonal</td>
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<td>formaldehyde</td>
</tr>
<tr>
<td>Smad7</td>
<td>Santa Cruz rabbit polyclonal</td>
<td>1:25</td>
<td>formaldehyde</td>
</tr>
</tbody>
</table>

3.2.4 Scoring system for immunohistochemistry

Light-microscopic analysis was performed at magnification of 630x. All of the positive cells in bronchial submucosa were quantified in the area 100µm beneath the epithelial basement membrane with the use of a graded bar mounted at ocular level in several non-overlapping high power fields until all of the available area was covered. The final result, expressed as the number of positive cells per square millimetre, was calculated as the average of all of the cellular counts performed in each bronchial biopsy. The final result was expressed as the average of all scored fields performed in each biopsy. The immunostaining was also scored using a range from 0 (absence of immunostaining) to 3 (extensive intense immunostaining) in the bronchial epithelium. A mean ± standard deviation of 0.620 ± 0.220µm of epithelium was analyzed in COPD and control subjects.
3.2.5 Statistical analysis

Group data were expressed as means ± standard error for functional data or median (range) for morphologic data. Differences between groups were analyzed by using ANOVA for functional data. The ANOVA test was followed by the unpaired t test for comparison between groups. The Kruskall-Wallis test applied for morphologic data was followed, when significant, by the Mann-Whitney U test for comparison between groups. Correlation coefficients were calculated by using the Spearman rank method. Probability values of p<0.05 were considered significant. Statistical analyses were carried out using GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA).
RESULTS & DISCUSSION

TGF-β1 time/dose-induced effects on collagen type I mRNA expression

Evaluation of results obtained in three independent sets of experiments showed that cell exposure to increasing doses of TGF-β1 (1, 2.5, 5 and 10ng/ml) induced a dose-dependent enhancement of collagen type I mRNA expression after 24 hours in human bronchial epithelial cells, with significative values for 5ng/ml and 10ng/ml (p=0.0130 and p=0.0017, respectively) (Figure 5). Furthermore, treatments for different time of exposure demonstrated that TGF-β1 was also able to increase in a time-dependent manner collagen type I transcription: specifically we observed that with respect to control, 1ng/ml of TGF-β1 caused a rapid significant increase after 3 hours of exposure (p=0.0190) (Figure 6), whereas treatment with 10ng/ml caused a significant increase at 24h (p<0.0001), suggesting a time and dose-dependent TGF-β1-induced effect on collagen type I mRNA in 16HBE (Figure 7).

TGF-β1-induced effects on Smads mRNA and protein expression

In order to investigate the involvement of Smads on the collagen type I stimulation observed previously, we valued the effects of treatments on Smads mRNA and protein expression. Our results confirmed data in literature (Yanagisawa et al, 1998): actually in 16HBE treated with 1ng/ml of TGF-β1 we observed a time-dependent decrease of Smad3 mRNA expression with a significant reduction at 24h (p=0.0207) if compared with controls; furthermore, analysis of Smad4 and Smad7 transcription levels revealed that the treatment caused initially an increase of Smad7 mRNA (p=0.0043), maybe to balance the enhancing of activated p-Smad3 observed into the nucleus (see below) and a reduction of both Smad7 and Smad4 mRNA at 24h (p=0.0087and p=0.0381, respectively) (Figure 8). These results let us hypothesize that in 16HBE, treatment with 1ng/ml of
TGF-β1 do not alter the regulatory role of the inhibitor Smad7 which is able to balance the increase of p-smad3 and interfere with the induced-collagen production. In contrast, treatment with 10ng/ml of TGF-β1 caused a significant enhance of both Smad3, Smad4 and Smad7 mRNA transcription at 24h with respect to controls (p=0.0472, p=0.0433 and p=0.0422, respectively) (Figure 9) and a significant time-dependent increase of Smad3 phosphorylation (p=0.0159 at 3h and p=0.0286 at 24h) (Figure 10, A). In addition, in order to provide a quantitative confirmation to the transcriptional data observed previously we performed a semi-quantitative western blot: through the statistical analysis of both cytoplasmatic and nuclear fraction, we observed an enhancing of p-Smad3 into the nucleus after treatment with 1ng/ml of TGF-β1 (Figure 10, B-D1) while a significant increase of nuclear phospho-Smad3 protein expression was observed at both 3 hours (p=0.0079) and 24 hours (p=0.0079) (Figure 10, C-D2) after treatment with 10ng/ml of TGF-β1. These results suggested a TGF-β1-induced nuclear translocation of activated Smad3 and thereby its involvement in collagen type I transcription.
Figure 5: TGF-β1-dose-dependent collagen type I mRNA expression after 24h of exposure.
Figure 6: Time-dependent collagen type I mRNA expression after treatment with 1ng/ml of TGF-β1.

Figure 7: Time-dependent collagen type I mRNA expression after treatment with 10ng/ml of TGF-β1.
Figure 8: Smads mRNA expression after treatment with 1ng/ml of TGF-β1. A) Smad3 mRNA; B) Smad4 mRNA; C) Smad7 mRNA.
Figure 9: Smads mRNA expression after treatment with 10ng/ml of TGF-β1. A) Smad3 mRNA; B) Smad4 mRNA; C) Smad7 mRNA.
Figure 10: Effects of TGF-β1 on p-Smad3 protein expression. A) Smad3 phosphorylation level after 10ng/ml of TGF-β1; B-D1) Western blot results for nuclear p-Smad3 after 1ng/ml of TGF-β1; C-D2) Western blot results for nuclear p-Smad3 after 10ng/ml of TGF-β1.
Immunohistochemical expression of TGF-β1, collagen type I and Smads in bronchial epithelium of COPD patients

In order to assess the contribution of the bronchial epithelium in inducing collagen type I production in patients with COPD we evaluated the expression of TGF-β family members and collagen type I, in both the mature and pro-form, by immunohistochemical analysis (Figure 11, B). All of the positive cells in the epithelium were quantified using a range from 0 (absence of immunostaining) to 3 (extensive intense immunostaining) and the results were statistically analyzed to assess significance of variation of expression with respect to positive controls (Kruskall-Wallis test) and between groups (Mann-Whitney U test). No significant changes were observed for collagen and pro-collagen type I between the four groups of subjects investigated in the bronchial epithelium. In contrast, when assessing the expression of members of the transforming growth factor family and their receptors, we observed a significant increase only for TGF-β1 in Mild/Moderate COPD compared with control healthy smokers (p<0.05, Mann-Whitney U test; p=0.054, Kruskall-Wallis test). Similarly, Smads immunostaining revealed no significant variation between all the four groups of subjects analyzed (Table 5). These results confirmed the low level of collagen deposition observed in the bronchi of COPD patients, hallmark of this pulmonary disease, and open a new perspective to investigate the molecular mechanism that inhibits the pro-fibrotic stimulus induced by TGF-β1. Furthermore, the IHC results obtained for Smads demonstrated that Smad3 levels are not substantially increased in human bronchial epithelium, suggesting, as expected by our results, a modest involvement of this molecule in sustaining the pro-fibrotic process in the bronchi of these patients.
Immunohistochemical expression of TGF-β1, collagen type I and Smads in bronchial submucosa of COPD patients

TGF-β2 and TGF-β3 immunostaining levels were the two most relevant observed in this study. Specifically, counting positive cells in the area of 100 mm beneath the epithelial basement membrane, the number of TGF-β2⁺ cells in the bronchial submucosa of Mild/Moderate COPD was significantly lower than in control healthy smokers (42.5 (range 0.0-320.0), p<0.05); similarly the number of TGF-β3⁺ cells was significantly lower in both Mild/Moderate (10.0 (range 0.0-39.0), p<0.05) and Severe COPD if compared with control healthy smokers (4.5 (range 0.0-65.0), p<0.05). In contrast, even in bronchial mucosa was observed an intensive immunopositivity for collagen type I (Figure 11, A), both collagen type I and pro-collagen type I expression did not show no significant variations when control patients were compared with diseased patients, as was observed for TGF-β1 and all Smads assessed (Table 6).
| Epithelium (score 0-3) | Healthy Non smokers | | Healthy smokers | | Mild/Moderate COPD | | Severe COPD | | Kruskall Wallis p value |
|------------------------|---------------------|---|-----------------|---|------------------|---|-------------------|---|
| PP2Cα                  | 0.25 (0.25-0.5)     | 0.37 (0.0-1.0) | 0.5 (0.0-2.0) | 0.5 (0.0-2.0) | p=0.760 |
| CTGF                   | 1.5 (0.5-3)         | 2.0 (1.0-3.0) | 1.5 (0.5-2.5) | 2.0 (1.0-3.0) | p=0.431 |
| TGF-β1                 | 0.25 (0.0-1.0)     | 0.75 (0.5-3.0)* | 1.5 (0.0-2.5)* | 0.5 (0.0-1.5) | p=0.054 |
| TGF-β2                 | 0.50 (0.2-2.0)     | 0.5 (0.25-3.0) | 0.5 (0.0-1.5) | 0.62 (0.0-2.0) | p=0.850 |
| TGF-β3                 | 0.0 (0.0-0.0)      | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | p=n.v. |
| TβRI                   | 0.0 (0.0-0.0)      | 0.5 (0.0-1.0) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | p=0.334 |
| TβRII                  | 0.5 (0.2-2.0)      | 0.5 (0.0-3.0) | 0.5 (0.0-2.0) | 1.0 (0.0-2.0) | p=0.405 |
| Smad 2                 | 1.0 (0.0-2.5)      | 0.5 (0.0-3.0) | 0.0 (0.0-2.0) | 0.0 (0.0-2.5) | p=0.249 |
| Smad 3                 | 1.0 (0.0-2.0)      | 0.0 (0.0-3.0) | 0.5 (0.0-1.5) | 1.0 (0.0-2.5) | p=0.468 |
| Smad 7                 | 0.75 (0.0-2.0)     | 0.5 (0.0-2.5) | 0.35 (0.0-2.5) | 0.5 (0.0-2.0) | p=0.797 |
| p-Smad 2               | 0.5 (0.0-0.5)      | 0.25 (0.0-0.5) | 0.0 (0.0-0.5) | 0.0 (0.0-0.5) | p=0.514 |
| p-Smad 3               | 0.0 (0.0-0.25)     | 0.0 (0.0-0.5) | 0.0 (0.0-0.5) | 0.0 (0.0-0.5) | p=0.834 |
| Collagen I             | 0.25 (0.0-0.5)     | 0.5 (0.0-1.0) | 0.0 (0.0-1.0) | 0.5 (0.0-0.5) | p=0.749 |
| Pro collagen type I    | 2.75 (1.5-3.0)     | 2.5 (0.5-3.0) | 2.25 (0.0-3.0) | 3.0 (2.0-3.0) | p=0.573 |

Table 5: Statistical results for IHC in bronchial epithelium of COPD patients. * p<0.05 compared to control non smokers.

Figure 11: Photomicrographs of bronchial biopsies. (A) The bronchial submucosa of Severe COPD immunostained for collagen type I+ cells; (B) The bronchial epithelium of a Severe COPD intensely immunostained for pro-collagen type I+ cells. Both immunostainings showed no significant variations when controls subjects were compared with diseased patients. Bar = 20µm.
<table>
<thead>
<tr>
<th>Submucosa (cells/mm²)</th>
<th>Healthy Non smokers</th>
<th>Healthy smokers</th>
<th>Mild/Moderate COPD</th>
<th>Severe COPD</th>
<th>Kruskall Wallis p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (Range)</td>
<td>Median (Range)</td>
<td>Median (Range)</td>
<td>Median (Range)</td>
<td></td>
</tr>
<tr>
<td>PP2Cα</td>
<td>56.0 (0.0-137.0)</td>
<td>48.0 (24.0-250.0)</td>
<td>51.0 (11.0-247.0)</td>
<td>45.0 (0.0-303.0)</td>
<td>p=0.324</td>
</tr>
<tr>
<td>CTGF</td>
<td>219.0 (64.0-355.0)</td>
<td>222.5 (64.0-387.0)</td>
<td>178.5 (17.0-355.0)</td>
<td>224.0 (39.0-484.0)</td>
<td>p=0.693</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>53.0 (5.0-236.0)</td>
<td>33.5 (0.0-333.0)</td>
<td>48.0 (0.0-195.0)</td>
<td>30.0 (0.0-230.0)</td>
<td>p=0.705</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>40.0 (8.0-507.0)</td>
<td>103.0 (24.0-409.0)</td>
<td>42.5 (0.0-320.0)</td>
<td>57.5 (0.0-560.0)</td>
<td>p=0.074</td>
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<tr>
<td>TGF-β3</td>
<td>0.0 (0.0-13.0)</td>
<td>16.0 (6.0-58.0)*</td>
<td>10.0 (0.0-39.0)*</td>
<td>4.5 (0.0-65.0)*</td>
<td>p=0.007</td>
</tr>
<tr>
<td>TβRI</td>
<td>5.0 (0.0-52.0)</td>
<td>6.0 (0.0-75.0)</td>
<td>18.0 (0.0-97.0)</td>
<td>5.0 (0.0-73.0)</td>
<td>p=0.071</td>
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<td>TβRII</td>
<td>74.0 (0.0-225.0)</td>
<td>48.0 (0.0-216.0)</td>
<td>72.5 (8.0-505.0)</td>
<td>45.0 (0.0-376.0)</td>
<td>p=0.528</td>
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<tr>
<td>Smad 2</td>
<td>181.5 (0.0-750.0)</td>
<td>166.5 (50.0-514.0)</td>
<td>51.0 (0.0-960.0)</td>
<td>91.5 (4.0-627.0)</td>
<td>p=0.335</td>
</tr>
<tr>
<td>Smad 3</td>
<td>77.0 (0.0-690.0)</td>
<td>20.0 (0.0-754.0)</td>
<td>111.0 (0.0-909.0)</td>
<td>120.0 (0.0-353.0)</td>
<td>p=0.335</td>
</tr>
<tr>
<td>Smad 7</td>
<td>67.0 (11.0-300.0)</td>
<td>48.5 (0.0-584.0)</td>
<td>96.0 (0.0-520.0)</td>
<td>56.5 (0.0-620.0)</td>
<td>p=0.949</td>
</tr>
<tr>
<td>p-Smad 2</td>
<td>17.0 (0.0-85.0)</td>
<td>37.5 (0.0-138.0)</td>
<td>19.0 (0.0-137.0)</td>
<td>25.5 (0.0-92.0)</td>
<td>p=0.714</td>
</tr>
<tr>
<td>p-Smad 3</td>
<td>67.0 (38.0-107.0)</td>
<td>65.0 (45.0-161.0)</td>
<td>113.0 (52.0-215.0)</td>
<td>78.0 (14.0-237.0)</td>
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<tr>
<td>Collagen I Score (0-3)</td>
<td>3.0 (0.5-3.0)</td>
<td>3.0 (3.0-3.0)</td>
<td>3.0 (0.5-3.0)</td>
<td>3.0 (2.5-3.0)</td>
<td>p=0.636</td>
</tr>
<tr>
<td>Pro collagen I Score (0-3)</td>
<td>2.75 (1.5-3.0)</td>
<td>2.0 (1.0-3.0)</td>
<td>2.5 (1.0-3.0)</td>
<td>1.87 (1.0-3.0)</td>
<td>p=0.594</td>
</tr>
<tr>
<td>CTGF Score (0-3)</td>
<td>1.5 (0.5-2.0)</td>
<td>2.0 (1.0-3.0)</td>
<td>1.5 (0.5-2.0)</td>
<td>1.5 (0.75-2.5)</td>
<td>p=0.289</td>
</tr>
</tbody>
</table>

Table 6: Statistical results for IHC in bronchial submucosa of COPD patients. * p<0.05 compared to control non smokers.
**Effects of CCL5 on TGF-β1-induced collagen type I mRNA**

In order to explain the pathological differences between fibrotic-like pulmonary disease, such as asthma and fibrosis, and COPD, we analyzed the effects of CCL5 on the TGF-β1-induced collagen type I production. Recently, we demonstrated an increased expression of CCL5 mRNA and a significantly increased number of CCL5+ cells in the bronchial mucosa of severe COPD compared to controls (Di Stefano et al, 2009). Then, in order to explain the immunohistochemical results observed previously, we performed a new *in vitro* study in which cells were treated simultaneously with TGF-β1 and CCL5. Analysis of RNA revealed that collagen type I transcription decreased in a dose-dependent manner when cells were treated contemporary with TGF-β1 and with increasing doses of CCL5 and specifically, we observed a significant reduction of the TGF-β1-induced collagen type I mRNA after treatment with 10ng/ml of both TGF-β1 and RANTES if compared with control (p=0.0232) and if compared with cells treated with 10ng/ml of TGF-β1 (p=0.0015) for 24h (Figure 12). When 16HBE were treated for different time of exposure we observed that with respect to control, CCL5 was able to induce a pro-fibrotic effect on cells, with a significant stimulation of collagen type I transcription at 3h (p=0.0017) and at 24h (p=0.0433). However, treatments with both TGF-β1 and CCL5 caused a reduction of TGF-β1-induced collagen type I mRNA (p=0.0015) at 24h suggesting a down-regulatory role of CCL5 in human bronchial epithelial cells (Figure 13).

These findings indicate that TGF-β1 is able to induce the fibrotic response of 16HBE stimulating collagen type I transcription and secretion, suggesting the possible involvement of bronchial epithelial cells in maintenance of pro-fibrotic events in different lung diseases. Furthermore, these data confirm the role performed by TGF-β1 in human tissue and the importance of this cytokine in regulating ECM production and remodelling. Interestingly, when we treated cells with CCL5 there was a dose-dependent
reduction of TGF-β1-induced collagen transcription and secretion with a significant reduction, particularly at a dosage of 10ng/ml. The observed effects of CCL5 treatment in vitro could confirm data obtained in vivo in bronchial biopsies and can explain the lack of extended pro-fibrotic events and the increased levels of TGF-β-2-3+ cells observed in Severe COPD.

**Effects of CCL5 on TGF-β1-induced Smads mRNA and protein expression**

16HBE treated with 10ng/ml of CCL5 showed a reduction of TGF-β1-induced Smads transcription. Specifically we observed a significant reduction of TGF-β1-induced Smad3 and Smad4 mRNA after 24h of exposure (p=0.0293 and p=0.0052, respectively) (Figure 14, A-B); in contrast Smad7 transcription appeared to be stimulated by CCL5 even if we did not observe any significant value for treatments with simultaneous administration of TGF-β and CCL5 at 24h (Figure 14, C). Additionally, sub-fractionated western blotting analysis revealed that treatments with TGF-β1 and CCL5 caused a significant reduction of active p-smad3 into the nucleus (p=0.0381), suggesting an involvement of this chemokine in its post-translational regulation (Figure 15). These data found confirmation after immunolocalization of p-Smad3: with respect to control, we observed that TGF-β1 is able to enhance the number of p-smad3+ cells with a consistent nuclear localization (Figure 16, B-E). In contrast treatment with CCL5 is able to reduce the TGF-β1-induced number of positive cells in both cytoplasm and nucleus (Figure 16, D-F). However, precise mechanism by which CCL5 interferes on induced-Smad3 phosphorylation is not clear and lack any explanation. We need to investigate further the role of CCL5 on nuclear translocation.
Figure 12: CLL5 dose-dependent reduction of TGF-β-induced collagen type I transcription.

Figure 13: Collagen type I transcription after treatments with TGF-β1, CCL5 and both TGF-β1 and CCL5.
Figure 14: Effects of CCL5 on TGF-β1-induced Smads transcription
A) Smad3 mRNA; B) Smad4 mRNA; C) Smad7 mRNA.
Figure 15: Effects of CCL5 on p-Smad3 into nucleus. A-B) CCL5 reduced TGF-β1-induced nuclear p-Smad3 expression; C) Western blotting results for nuclear p-smad3 after 24h of treatments.
Figure 16: Immunocytochemistry demonstration of p-smad3 positive 16HBE cells after treatments. A) Untreated control; B) TGF-β1 10ng/ml; C) CCL5 10ng/ml; D) TGF-β1+CCL5 10ng/ml. Magnification 40x. E) TGF-β1 10ng/ml; F) TGF-β1+CCL5 10ng/ml. Magnification 63x.
ERK is a possible regulatory transductor of CCL5 signaling

To explain the molecular pathway by which CCL5 was able to down-regulate TGF-β1-induced effects on 16HBE we analyzed the molecular signaling pathway of this chemokine. Data in literature revealed that RANTES exhibits its effect acting through three CC chemokine receptors, CCR1, CCR2 and CCR3 (New et al, 2003). As visible in Figure 17 these are G-Protein-coupled receptors (GPCRs) which activate an intercellular signaling pathway in which are involved several MAPKs. As discussed previously the biological effects of TGF-β1 are mediated not only by Smads signaling but also by MAPKs. According to this, we evaluated the effects of JNK, p38 and ERK transduction pathway inhibition to assess a possible correlation with the observed CCL5-mediated effects. Interestingly, we observed that JNK and p-38 appeared not to be involved in collagen type I transcription (data not shown); in contrast treatments with ERK inhibitor caused a significant reduction of TGF-β1-induced collagen type I mRNA level (p=0.0020) compared with cells not treated with inhibitor, and as visible in Figure 18, a further reduction of the effects mediated by CCL5 (p=0.0020). These results suggest a possible regulatory role for ERK and could explain the molecular mechanism involved in this type of inflammation and collagen production.

Effects of TGF-β1 and CCL5 on cellular viability

In order to explain results obtained, we analyzed the effects of treatments on cellular viability and on morphological characters. Exposure of human bronchial epithelial 16HBE cells to TGF-β1, CCL5 and both together TGF-β1 and CCL5 was accomplished after a starvation period of 24h and the viability assay was performed using MTT test. As visible in Figure 19 there were no statistical changes on cellular viability after all treatments if compared with controls, and through a phenotypical analysis of cultured
cells we observed that TGF-β and CCL5 did not alter the typical features of bronchial epithelial cells. MTT test was performed also for cells treated with ERK inhibitor just to exclude any reduction of viability and have another confirmation of results obtained. Similarly the treatment with the specific ERK inhibitor had not effect on cellular viability and cells displayed no morphological changes.

Figure 17: Intracellular signaling pathway activated by CC chemokine receptors. Dashes lines indicate that the role of intermeditated proteins has not yet been elucidated (New et al, 2003).
Figure 18: Effects of ERK inhibition on collagen type I transcription.

Figure 19: MTT assay to value cellular viability of 16HBE after treatments.
CONCLUSIONS

It has been reported that destruction of TGF-β1 signaling system is involved in several pulmonary diseases, such as pulmonary fibrosis and COPD. Actually, TGF-β1 is involved in regulation of turnover of connective tissue and ECM components (Sporn et al, 1992; Blobe et al, 2000; Barànek et al, 2002; Bonniaud et al, 2005), and elevated levels of this growth factor have been observed in both bronchial epithelium (De Boer et al, 2000) and alveolar epithelium (De Boer et al, 1998) of COPD patients, leaving us hypothesize a relationship with the airway remodelling characterizing this disease. This study demonstrated that in bronchial biopsies of COPD patients there was a significant enhance of TGF-β1 immunostained cells in bronchial epithelium of Mild/Moderate COPD compared with control healthy smokers; however, we did not observe any variation for TGF-β1 in bronchial submucosa and, similarly, any change for collagen and procollagen type I immunostained cells as in epithelium as in submucosa. TGF-β1 signaling is mediated through Smads protein (Massuguè, 2005, 2006; Moustakas et al, 2009). Several investigators showed that Smad3 is principally related to the fibrotic phenotype induced by TGF-β1 and is involved in the pathogenic mechanism mediating tissue destruction and fibrogenesis (Bonnaud et al, 2004; Zeisberg et al, 2007). To value the role of Smads in mediating TGF-β1 signaling we analyzed mRNA and protein expressions in human bronchial epithelial 16HBE cells after treatments with different doses of TGF-β1 and for different time of exposure. Our study on mRNA revealed that TGF-β1 was able to stimulate significantly collagen type I transcription in a time and dose-dependent manner and confirmed that it is able to stimulate Smad3, Smad4 and Smad7 transcription causing a significative increase of mRNA level. Furthermore, we demonstrated that TGF-β1 caused a significative increase of nuclear phospho-Smad3, let us supposing a direct involvement in TGF-β1-induced collagen type I transcription. The IHC analysis on bronchial biopsies
revealed also that, in contrast with results obtained for TGF-β1 expression, there were no significant changes for all Smads analyzed in all four groups of subjects and COPD patients. These results seem to be in contrast with results obtained in vitro and suggest us a possible inhibitory mechanism of TGF-β1 stimulus in vivo. Recently, we observed that in the bronchial mucosa of Severe COPD the number of CCL5+ immunostained cells was significantly higher than controls (Di Stefano et al, 2009). These results suggested a possible mechanism in which CCL5 is involved in modulating TGF-β1-induced effects. We demonstrated that CCL5 is able to reduce in a dose-dependent manner TGF-β1-induced effects on collagen type I and Smads transcription and significantly reduce nuclear phospho-Smad3 accumulation in 16HBE cells. Our results let us hypothesized that CCL5 down-regulates TGF-β1-induced effects and that the inhibitory mechanism is, at least in part, mediated by ERK transduction pathway, as confirmed through the use of ERK inhibitor which was able to significantly reduce CCL5-mediated anti-fibrotic effects in bronchial epithelial 16HBE.

To our knowledge this is the first description of an inhibitory mechanism for TGF-β1-induced fibrotic effects. Furthermore, on the basis of our results, we hypothesised that our in vitro model could explain, a least in part, the pathological differences existing in vivo between asthma, characterized by increased bronchial fibrosis, and COPD characterized by a low level of collagen deposition in the bronchi. Further examination of this mechanism could lead to a better understanding of anti-fibrotic function of CCL5 and may represent a point of effective therapeutic intervention for fibrotic diseases. However the role performed by CCL5 and the molecular mechanism activated by this chemokine needs more study.
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ACKNOWLEDGEMENTS

Arrived at the end of these three-year research doctorate, it is quite to thank the people who stood by me. First of all, my parents and my husband, since nothing have been the same without their support and understanding of my moods. Second, those who have allowed the achievement of all the objectives which have been on the basis of this project. Moreover, I want to say ‘thank you very much’ to my tutor Prof. Giovanni Zummo, to my co-tutor Doc. Antonino Di Stefano, head of the Cytoimmunopatology laboratory of the Respiratory Section of “S. Maugeri” Foundation (Veruno), to Doc. Giampiero La Rocca and Prof. Francesco Cappello: they contributed in different way to my professional growth and development of the project. A special thanks to my colleagues Doc. Rita Anzalone, Doc. Simona Corrao, Doc. Tiziana Loria and Doc. Melania Lo Iacono for their help and friendship; Furthermore, I want to thank Prof. Ian Adcock for his support during my fellowship in the National Hearth and Lung Institute, where I started the study. Thanks also to those who have become friends even if we have not worked together (each of you knows in her/his heart whom I’m referring to).