Nanoapproaches to modifying epigenetics of epithelial mesenchymal transition for treatment of pulmonary fibrosis

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Abstract

Idiopathic Pulmonary Fibrosis (IPF) is a chronically progressive interstitial lung that affects over 3 M people worldwide and rising in incidence. With a median survival of 2-3 years, IPF is consequently associated with high morbidity, mortality, and healthcare burden. Although two antifibrotic therapies, pirfenidone and nintedanib, are approved for human use, these agents reduce the rate of decline of pulmonary function but are not curative and do not reverse established fibrosis. In this review, we discuss the prevailing epithelial injury hypothesis, wherein pathogenic airway epithelial cell-state changes known as Epithelial Mesenchymal Transition (EMT) promotes the expansion of myofibroblast populations. Myofibroblasts are principal components of extracellular matrix production that result in airspace loss and mortality. We review the epigenetic transition driving EMT, a process produced by changes in histone acetylation regulating mesenchymal gene expression programs. This mechanistic work has focused on the central role of bromodomain-containing protein 4 (BRD4) in mediating EMT and myofibroblast transition and initial preclinical work has provided evidence of efficacy. As nanomedicine presents a promising approach to enhancing the efficacy of such anti-IPF agents, we then focus on the state of nanomedicine formulations for inhalable delivery in the treatment of pulmonary diseases, including liposomes, polymeric nanoparticles, inorganic nanoparticles, and exosomes. These nanoscale agents potentially provide unique properties to existing pulmonary therapeutics, including controlled release, reduced systemic toxicity, and

combination delivery. Nanoparticle-based approaches for pulmonary delivery thus offer substantial promise to modify epigenetic regulators of EMT and advance treatments for IPF.

1 Introduction

The Interstitial Lung Diseases (ILDs) are a heterogeneous group of parenchymal diseases with various mixtures of fibrosis and inflammation. Idiopathic Pulmonary Fibrosis (IPF) is the most common and aggressive ILD subtype. IPF is a lethal diagnosis that leads to progressive dyspnea, reduced exercise capacity, and has a median life expectancy of 2-3 years after diagnosis (Raghu et al., 2011). Although there is substantial variability in case definition, comprehensive studies indicate that IPF affects over 3 M people worldwide and the incidence is increasing, particularly in ageing populations (Hutchinson et al., 2015).

Mechanistically, it is thought that environmental insults (e.g. cigarette smoking, silicates and burning biofuels) in a genetically predisposed population trigger a chronic epithelial injury-repair response, disrupting alveolar stem cell populations and leading to epithelial growth factor stimulation. In response to these factors, epithelial cells undergo cell-state changes to acquire mesenchymal characteristics and promote quiescent fibroblasts to transition into active extracellular matrix (ECM) production. Myofibroblasts perpetuate epithelial injury and further promote ECM deposition resulting in obliteration of alveolar spaces (Torr et al., 2015). Because myofibroblast numbers are inversely related to survival in human IPF (King et al., 2001), and IPF fibroblasts cause lung fibrosis in humanized mice (Geng et al., 2019), therapies reducing myofibroblast activity have emerged as a focus of recent therapeutic approaches.

Several studies have implicated the atypical histone acetyltransferase known as bromodomain containing protein 4 (BRD4) in the EMT and transforming growth factor (TGF)-induced myofibroblast transdifferentiation. In combination with preclinical studies *in vivo* that have observed small molecule BRD4 inhibitors interfere with fibrosis in mouse models of IPF, including bleomycin model (Tang et al., 2013), radiation induced fibrosis (Wang et al., 2018) and transforming growth factor (TGF)β-induced fibrosis (Tian et al., 2016a), BRD4 pathway is a mechanistically relevant and a biologically important mediator of inducible lung fibrosis. However, BRD4 is implicated in a variety of essential cellular functions, including chromatin organization, DNA-damage repair, and post-mitotic gene expression (Devaiah et al., 2016b). A major challenge with advancing epigenetic regulators for the treatment of IPF is how to provide controlled delivery to myofibroblastic foci.

In this review, we describe the mechanistic pathways how epithelial inflammation/injury promotes EMT and myofibroblast expansion and the evidence that BRD4 is an epigenetic regulator of EMT and myofibroblast transdifferentiation. Finally, we propose how nanotechnology can advance therapeutics for IPF and ILD. Encapsulation in nanoparticles enables selective drug delivery via well-accepted aerosol administration while enhancing overall pharmacokinetics. We propose that nanomedicine can advance the therapeutics of IPF by enhancing therapeutic efficacy and reducing systemic effects, thereby facilitating the development of the next generation of therapeutics for pulmonary fibrosis treatment.

2 The Role and Regulation of Fibrosis

2.1 The Injury-Inflammation-Fibrosis paradigm

Fibrosis is defined as pathological state in which tissue and organ function is hindered due to excessive scar formation during the process of wound healing, often associated with a chronic inflammatory state (Wilson and Wynn, 2009). The fibrogenic response usually consists of several phases: (1) activation

of the initial response due to organ injury, (2) stimulation of fibroblasts by inflammatory mediators, and (3) deposition of ECM components, resulting in permanent scarring(Mack, 2018).

In the second phase, after the initial injury, inflammatory and profibrogenic cytokines are released such as those from T helper type (Th)-2 cells (IL-4, IL-5, IL-10 and IL-13) or Th-1 cells (IL-2, IFN-γ and TNF) . Interleukin (IL)-4 is notable in several studies that the deletion of IL-4 reduces fibrosis in the lung (2). Encompassing a wide range of biological and immunosuppressive activities, IL-4 can activate B cells, induce differentiation of naive CD4+ T cells, contribute to the development of M2-type monocytes or macrophages, and inhibit expression of several proinflammatory mediators (e.g. TNF-α; IL-1, 6, 8,12) (Zhu, 2015). Notably, in bleomycin-induced lung fibrosis, IL-4 was found to suppress inflammation, but was profibrotic. Another important profibrotic cytokine is IL-13 (Karo-Atar et al., 2016). IL-13 has the ability to directly and indirectly activate fibroblasts along with tumor necrosis factor (TNF)-α and IL-4, to produce an upregulation of IL-13Ra2 on macrophages, allowing IL-13 to bind and induce the release of TGFβ (Mack, 2018).

Contrary to the effects of Th-2 cytokines, Th-1 cytokines can be both anti- and profibrotic. Classically, the adaptive immune response is triggered when IL-12 interacts with interferon gamma (IFN-γ); it is also important to note that macrophages can also express IFN-γ in a response to microbial stimuli (Zhu, 2015). Once, activated, IFN-γ and IL-12 combine with TGFβ to induce collagen (COL) degradation and for ECM remodeling. In contrast, Th-1 polarizing cytokines IL-6, IL-1β, and TNF-α, are released from monocytes triggering a latent transcription factors such as STAT3 and STAT1, leading to fibroblast migration and recruitment, promoting proliferation and differentiation into myofibroblasts (Mack, 2018).

2.2 Myofibroblasts

Myofibroblasts are not normally present in substantial numbers in normal adult tissues but their population rapidly expands in response to injury-repair. In normal conditions, tissue fibroblasts have little secretory activity. However, upon injury, fibroblasts actively produce ECM components [collagen (COL) and fibronectin (FN)] causing a cascade of cytokine release, resulting in chemotaxis of inflammatory cells (Hinz et al., 2007). ECM remodeling triggers local fibroblasts to produce contractile stress fibers made out of cytoplasmic actins, transforming them into "proto-myofibroblasts." In the presence of high ECM stress, TGFβ and FN splice products causing the phosphorylation of focal adhesion kinases (Phan, 2012), the proto-myofibroblast assumes a stable phenotype characterized by α-SMA replacing actin in the stress fibers (Hinz et al., 2007). Activated myofibroblasts are characterized by a spindle-like morphology with intracytoplasmic stress fibers, a contractile phenotype, expression of α-smooth muscle actin (α-SMA), transgelin (TAGLN/SM22), COL and hyaluronans. Expression of these contractile fibers by myofibroblasts enables wound contraction, and results in wound closure.

When the tissue repair process is completed, myofibroblasts disappear through apoptosis, a process driven by ECM softening (stress release) or pro-apoptotic factors such as IL-1β, fibroblast growth factor 1, and prostaglandin E2. However, under pathological conditions, such as pulmonary fibrosis, cirrhosis or hypertrophic scar, myofibroblasts persist in fibroblastic foci, producing excess scar and interfering with normal organ function. Myofibroblasts are derived from multiple mesenchymal types. In the lung, myofibroblasts are thought to derive from locally activated tissue fibroblasts, vascular pericytes or recruitment of bone-marrow derived fibrocytes (Hinz et al., 2007; Rock et al., 2011; Phan, 2012). Alternatively, it has been shown that injured epithelial cells can express mesenchymal intermediate filaments (vimentin) and α-SMA through a process of EMT, producing a

myofibroblast-like phenotype (Radisky et al., 2007). In cancer, a substantial population of myofibroblasts are thought to arise from EMT. However, because myofibroblast populations are distinct in different organs, the contributions of EMT directly to the lung myofibroblast population is controversial (Rock et al., 2011).

3 Pulmonary Fibrosis and EMT

3.1 Epithelial response to acute and chronic lung injury

Lining the airways and the respiratory compartments, epithelial cells, are exposed to a variety of environmental challenges such as microbial agents, toxic components and particulates leading to inflammation and fibrosis. The airways and distal epithelial cells comprise of a physical and chemical barrier to maintain lung function. Activated airway epithelial cells can modulate their direct barrier function through the release of a variety of defense mechanisms, such as the release of defensins and mucins, as well as by modulating fluid transport (Fehrenbach, 2001). Epithelial cells also have the ability to release multiple mediators and adhesion molecules that can mediate and activate migratory inflammatory and immunological cells. Additionally, epithelial cells interact with other resident cells, such as fibroblasts along with the ECM. The mechanism by which epithelial cells respond to different types of lung injury, acute or chronic, varies drastically from the inflammatory response to fibrosis.

Acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) can be caused by a variety of factors; pneumonia, trauma, sepsis, aspiration, post-lung transplant, or certain viral infections (Johnson and Matthay, 2010). Alveolar epithelial cells are key for alveolar homeostasis and aid in the proper alveolar fluid clearance through the direction of epithelial sodium channel (ENaC). When acute injury occurs, permeability of the alveolar-capillary membrane is caused by a disruption in the ENaC either through hypoxia such that the reoxygenation and β-agonists reverse ENaC or transcription factors and inflammation decrease the number of ENaC molecules (Lutter and Spiteri, 2005). When this occurs, fluid enters the alveoli and proinflammatory cytokines (IL-6 and IL-8) are released causing the release of proinflammatory cells such as neutrophils and macrophages. If not treated, this cascade develops into hypercytokinemia releasing IL-1 and TNF-α which will eventually lead to impaired fibrotic repair and lung failure (Johnson and Matthay, 2010).

Differing form ALI, environmental exposures of particulates and chemicals is a much more common cause of chronic epithelial damage. Particulate matter generated from cigarette smoking, diesel emissions, biofuel exposures (including burn pit exposures) contain mixtures of metal, dusta, byproducts of hydrocarbon combustion induce oxidative cellular and DNA damage. These environmental exposures induce alveolar injury and clinical exacerbations in patients with existing chronic airway disease, including IPF, asthma and COPD (Winterbottom et al., 2018). Although the initial mechanism of action is similar in that there is damage to the alveolar wall that causes recruitment of proinflammatory cells, the gradual injury of the epithelium allows time for airway remodeling (Mercer et al., 2006). In asthma and COPD, epithelial cells are exposed to a range of inflammatory mediators over a prolonged period affecting the epithelial cell integrity and metabolism (Athanazio, 2012). Changes in cellular composition of the epithelium can cause goblet cell hyperplasia and pathophysiological of the mucous glands, affecting the function of the airway mucosa (Lutter and Spiteri, 2005).

3.2 EMT and the myofibroblast expansion

EMT is a multi-step, reversible process observed in several biological contexts: embryonic development (type I EMT), malignant transformation (Type III EMT), and in normal tissues undergoing injury response (Type II EMT). For the purposes of this review, we will focus primarily on type II EMT. Type II EMT begins when oxidative or physical injury to the resting differentiated epithelial cell triggers a phenotypic change characterized by loss of apical-basal polarity and loss of intercellular tight adheren junctions (Hill, Jones, Davies, & Wang, 2019). The cells de-differentiate and become motile, enabling the cells to repopulate the injured epithelial surface. EMT can be initiated by the local production of signaling factors; most notable are TGF-β, fibroblast growth factors (FGFs), epithelial growth factors (EGFs), and the Wnt/β-catenin signaling pathways (Ijaz et al., 2014). These pleotropic signaling factors induce the expression of mesenchymal transcription factors such as Snail, Slug, and Twist whose actions repress epithelial genes (E-cadherin) and stimulate mesenchymal genes (N-cadherin, α-SMA, vimentin and fibronectin) (Hill et al., 2019). Consequently, the apical-basal polarity will switch to a front-back polarity in which the tight adherens junctions turn into N-cadherin junctions and vimentin stress fibers, promoting motility.

Systems-level studies have led to the conclusion that EMT is a multi-step, dynamic process produced by cooperative effects of master transcription factors in response to epithelial growth factor and ECM cues. Time series RNA profiles have identified cliques of master transcription factors (such as ETS2, HNF4a and JUNB) that cooperate to control partial-EMT state, as summarized in **Table 1**. These transcription factors control cell type by producing the formation of superenhancers that control mesenchymal gene-regulatory networks. Superenhancers are clusters of functional enrichers enriched in RNA Polymerase, Mediator and acetylated histones that control cell-type specification genes (Whyte et al., 2013). Of relevance here, superenhancers are stabilized by the presence of BRD4 (Shin, 2018). In studies using siRNA-mediated gene knockdowns and small-molecule BRD4 inhibitors, TGF-β-induced partial EMT was reversed. Overall, this data demonstrates that BRD4 maintains epigenetic memory of TGFβ stimulation and the EMT phenotype. While EMT is traditionally defined as the conversion of epithelial cells into mesenchymal-like cells, recent studies expanded this cellular reprogramming process to mediate changes in epithelial morphology, motility, and gene expression (Lamouille et al., 2014).

Table 1. A summary of factors and genes in myofibroblast expansion.

Factors	Representative Genes/Pathway	Action
Fibroblast and transforming growth factor signals	FGFs, EGFs, and Wnt/β-catenin pathway	Initiates EMT
Changes in ECM stiffness (COL, MMP deposition)	Integrin activation	Transition from proto- to myofibroblast
Mesenchymal transcription factors	Snail, Slug, and Twist	Reprogram epigenome: Repress epithelial genes and stimulate mesenchymal genes
Master transcription factors	ETS2, HNF4a,JUNB, NFκB	Control partial-EMT state
Superenhancers	BRD4	Control expression of gene regulatory networks specifying cell identity

3.3 EMT and myofibroblast expansion: the "epithelial injury" hypothesis

Under normal conditions, the differentiated epithelial phenotype and the mucosal barrier function is maintained through interactions with the basal lamina and supporting subepithelial fibroblasts, known as the epithelial mesenchymal trophic unit (Evans et al., 1999). Upon exposure to environmental oxidants or insults, distal epithelial injury stimulates activation of the unfolded protein response (UPR), and release of fibrogenic growth factors. The consequent EMT transition results in secretion of alternative splice variant of ECM proteins, including FN. Changes in ECM composition (stress) are an important signal for pulmonary mesenchymal cell populations to acquire myofibroblast properties (Tomasek et al., 2002).

Myofibroblasts release inflammatory/profibrotic mediators that perpetuate epithelial injury and further promote ECM deposition (Zhang et al., 1994; Torr et al., 2015). The effects of TGF-β and fibrogenic cytokines further stimulate prosurvival signaling in the myofibroblast population, leading to persistence, resistance to apoptosis and migratory invasiveness (Horowitz et al., 2006). With myofibroblast persistence, deposition of ECM stiffens the lungs, reducing normal elastic properties and pulmonary function. Not only is this cell type primarily responsible for producing ECM and interstitial fibrosis (Hinz et al., 2007; Rock et al., 2011; Hung et al., 2013), these cells form the

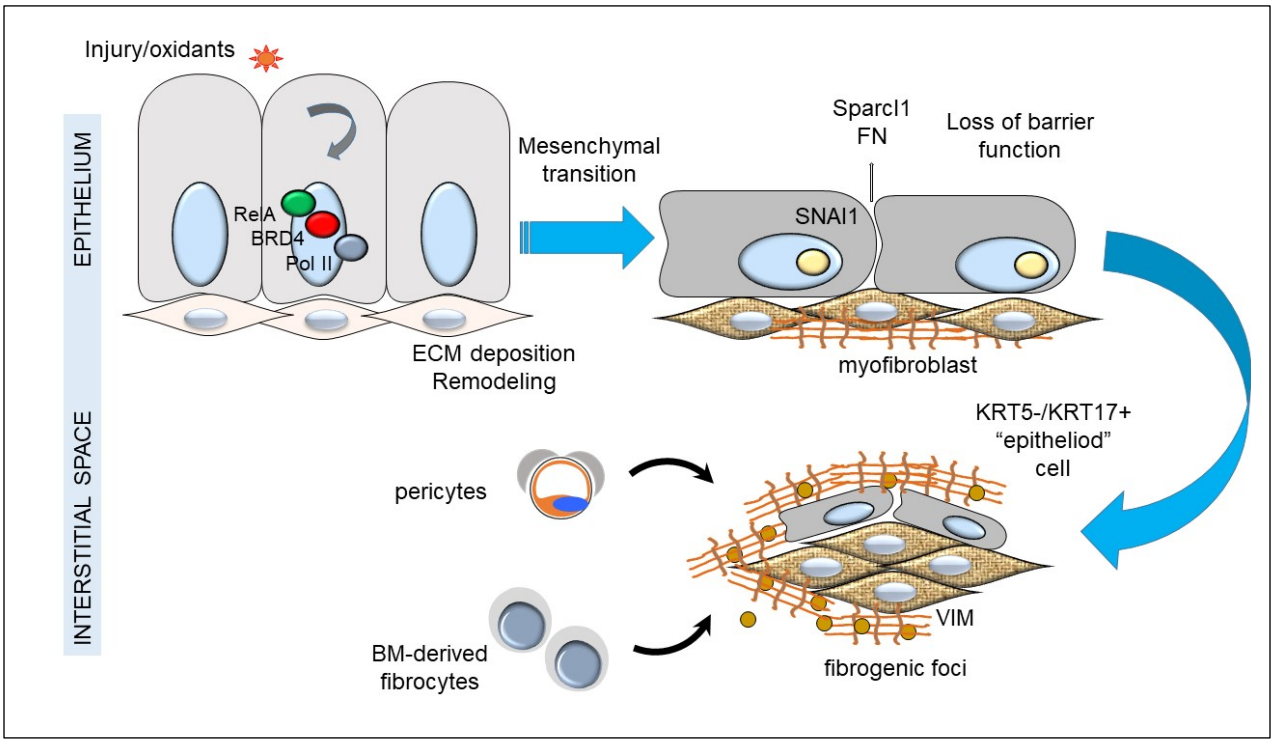

Figure 1. Epithelial injury hypothesis in IPF. Shown is a schematic view of the relationship between injury of the epithelial surface, epithelial-mesenchymal transition and formation of fibrogenic foci.

pathognomonic fibroblastic foci of human IPF (Hinz et al., 2007; Thannickal, 2012). Clinically in IPF, increased myofibroblast numbers are inversely related to survival (King et al., 2001). Therapeutics targeting the myofibroblast foci are therefore an exciting approach for treatment of ILDs.

In IPF, depletion of alveolar epithelial stem cell populations from mitochondrial dysfunction results in tonic oxidative stress, alveolar cell dysfunction, impaired renewal, senescence and production of profibrotic factors. Compensatory alveolar stress response promotes EMT and myofibroblast

transdifferentiation (Kulkarni et al., 2016). Although epithelial cells appear not to be a major source of lung myofibroblasts in IPF, this is not to say that epithelial transition is not a major driver of fibroblastic foci. Injured epithelial cells are a source of TGFβ and COL secretion that trigger myofibroblast formation (**Table 1**). Recent single cell sequencing studies have identified a keratin (KRT)5−/KRT17+ pathologic, ECM-producing epithelial cell population that was highly enriched in IPF lungs (Habermann et al., 2020). Interestingly these epithelial cells exhibited EMT signatures. This finding supports the prevailing epithelial injury hypothesis, illustrated in **Figure 1**.

3.4 New linkages of epithelial innate signaling on fibrosis programs

In addition to environmental oxidant exposure, an emerging body of work has linked respiratory virus infections with epithelial injury and remodeling. Airway remodeling is a collective term that refers to structural changes in the airways resulting in enhanced collagen deposition in the subepithelial basement membrane (lamina reticularis), disruption of the epithelial barrier, epithelial cell-state change (mucous metaplasia and/or mesenchymal transition), and smooth muscle hypertrophy (Bergeron et al., 2010). Collectively, this process narrows the small airways, producing obstruction and reduced lung compliance accounting for enhanced morbidity and mortality(Hough et al., 2020). Respiratory viruses, such as Rhinovirus (HRV) and Respiratory Syncytial Virus (RSV) are major triggers of remodeling and chronic lung disease (Jartti and Gern, 2017). These agents primarily replicate in airway epithelial cells producing innate immune response-driven remodeling (Brasier, 2019; 2020).

More recently, COVID-19 ARDS has been observed to be presaged by a hyper-inflammatory state characterized by the presence of circulating inflammatory and pro-fibrotic cytokines including IL-6, IL-7, GM-CSF, MCP and MIP1α (Ruan et al., 2020). In COVID-19 infections, circulating ECM components (hyaluronans, type III pro-collagen and laminin) are associated with increased with disease severity (Ding et al., 2020). These data suggest that COVID-19 also induces ECM remodeling, a process important in myofibroblast transdifferentiation. In survivors, radiographic evidence of pulmonary scarring (Shi et al., 2020) and functional defects in lung function (diffusion capacity) are commonly seen (Mo et al., 2020; Shi et al., 2020; Wang et al., 2020). In fatal cases, increases in the TGF-ECM networks, including upregulated TGFBR2, COL isoforms, and FN (12) are seen, and pulmonary fibrosis is found upon an autopsy (Zhang et al., 2020). These data suggest that fibrosis and impaired pulmonary function is a sequelae of severe COVID-19-ARDS infections. Although the relationship of post-COVID-19 lung scarring/fibrosis with chronic progressive, interstitial lung disease is not known due to the short-term follow-up, it is significant that post-infectious pulmonary fibrosis is also a known outcome in survivors of SARS, an infection with the closely related virus, SARS-CoV (Hui et al., 2005). We interpret these studies to indicate that viral epithelial infections may be an important modulator of airway fibrosis.

3.5 Mechanisms how innate inflammation produces EMT

Chronic oxidative stress induced by innate signaling triggers adaptive cell-state transitions of the normal epithelium to activate the EMT program. High throughput RNA-seq studies of normal human airway cells have discovered that the core regulatory network of ~3000 TGFβ-induced genes, substantially overlaps with the NFκB network (Tian et al., 2015). Using selective inducible knockouts in bronchiolar-derived basal cell progenitors, it was found that NFκB signaling drives the transition to a committed mesenchymal phenotype (Tian et al., 2018c). These findings led to the discovery that tonic (or repetitive) NFκB signaling in the airway produces EMT *in vivo* (Tian et al., 2017a). Repetitive AEs provoked by exposure to TLR3 agonists activates SNAI expression, EMT, remodeling and expansion of myofibroblast population(s).

Virus-induced NFκB complexes with the coactivator bromodomain containing (BRD4), a multifunctional protein with intrinsic RNA polymerase kinase (Devaiah et al., 2012) and atypical histone acetyltransferase (HAT) (Devaiah et al., 2016a) activity. Through its sequence specific DNA-binding activity, NFκB redistributes BRD4 to innate inflammatory genes as well as the core EMT regulators SNAI1, ZEB1 and TWIST1 (Brown et al., 2014; Tian et al., 2016b), as schematically shown in **Figure 2.** NFκB -dependent activation of the core EMT regulators results in expression of COL and FN, key ECM regulators of myofibroblast expansion, airway remodeling and expansion of the subepithelial basement membrane. Supporting this chromatin remodeling mechanism, recent analyses using transposase-accessible-next generation sequencing (ATAC-Seq) in highly differentiated lower airway epithelial cells infected with RSV demonstrate that the TGFβ-FN-JUN pathways controlling EMT are activated by chromatin decondensation at their proximal promoters (Xu et al., 2020).

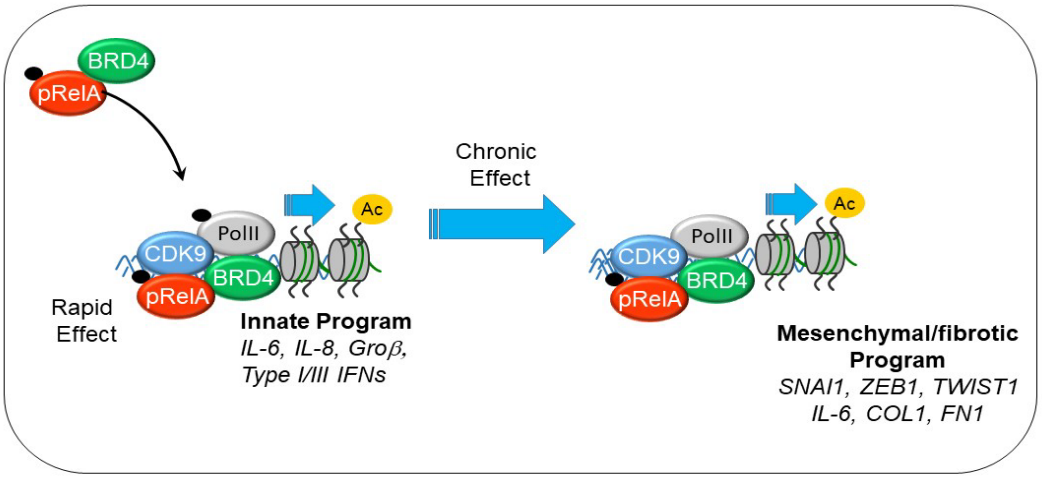

Figure 2. Mechanisms how innate signaling couples to EMT. Shown is the interaction between phosphorylated 65 kDa NFκB subunit (pRelA), BRD4. Initially this chromatin remodeling complex activates innate response genes important in viral restriction. With chronic stimulation, the complex directly activates SNAI, COL, FN and other components of EMT.

Although these fundamental studies of the NFκB -BRD4 pathway were initially developed *in vitro*, evidence that NFκB mediates mesenchymal transition, myofibroblast expansion, and pulmonary fibrosis has been developed in *in vivo* (Tian et al., 2016b). In mice, acute TLR3 activation induces CXCL8/IL-8 expression and neutrophilia, but chronic TLR3 activation produces airway epithelial mesenchymal transition, expansion of the myofibroblast population, and fibrosis (Tian et al., 2016b). Activation of the BRD4 kinase and atypical HAT activity in the airway mucosa has been demonstrated in response to TLR3 activation (Tian et al., 2017a) and viral replication (Tian et al., 2017b; Tian et al., 2018d). Here, the unique, BRD4-dependent histone acetylation on Lys 122 is induced by virus or allergen, and this induction is NFκB -dependent. Viral and allergen-induced NFκB -BRD4 complex formation was been demonstrated through proximity ligation assays, a technique to detect molecular interactions between the two proteins. Finally, using highly selective BRD4 inhibitors, it was found that inhibition of BRD4 HAT activity interferes with remodeling, airway hyperreactivity and myofibroblast transition *in vivo* (Tian et al., 2018a). These studies validate the central relevant of the NFκB -BRD4 pathway in airway remodeling.

3.6 Histone modifications underly EMT

In recent years, epigenetics has become the forefront as a major mechanism underlying fibrosis. The term epigenetics refers to altering gene expression without altering the DNA sequence. Extensive research aided by deep sequencing technology, has identified histone codes to be of interest in EMT. In eukaryotic cells, DNA is condensed in chromatin with the nucleosome, a major part of chromatin, comprised of four different histones to form an octamer (H3, H4, H2A and H2B) (O'Reilly, 2017). Although histones are globular in nature, they have a 'tails' that can possess multiple histone modifications, eight of which are known. Histone tail modifications include methylation, phosphorylation, acetylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation (O'Reilly, 2017). Additionally, histone modifications are mediated by a family of enzymes: Histone Deacetylases (HDACs), and histone acetyltransferases (HATs). HDACs remove acetyl groups on N terminal lysines, whereas HATs catalyze their addition.

First discovered in hepatic fibrosis, HDACs were shown to enhance the migratory and ECM producing properties of tissue myofibroblasts (de Ruijter et al., 2003). This idea was expanded in pulmonary fibrosis, where HDACs were extensively studied for their role in COL secretion. Inhibition of HDAC induced hyperacetylation in H3 and H4 tails. In idiopathic pulmonary fibroblasts, elevated expression of all class I (HDAC 1, 2, 3, and 8), class IIa (HDAC 4, 5, 7, 9) and class IIb (HDAC 6 and 10) HDACs were discovered to be in both tissue and isolated myofibroblasts (de Ruijter et al., 2003). During hypoxia, another regulator of EMT, cross-talk between chromatin modifiers HDAC3 and WDR5 regulates repression of epithelial genes and activation of mesenchymal genes (Lin and Wu, 2020). The histone mark histone 3 lysine 4 acetylation (H3K4Ac) was shown to bind promoters of several EMT marker genes (e.g. CDH1 and VIM) (Wang et al., 2019). Additionally, both H3K4me3 (activator) and H3K27me3 (repressor) were observed in the EMT promoters (Lin and Wu, 2020). This is of much interest as the presence of activation and repressor histone marks allows EMT markers to cycle from expressed to silenced in response to dynamic changes in the extracellular environment. Furthermore, studies using chromatin immunoprecipitation (ChIP) with anti-H3K4Ac antibodies and whole genome sequencing identified new EMT marker genes, glioma-associated oncogene homolog 1 (GLI1) and smoothened (SMO), that are regulated by HDAC3 for in migration and invasion (Wang et al., 2019). Although there has been great progress in the use of HDAC-inhibitors demonstrating anti-fibrotic activity in mechanistic studies, the advancement of HDAC inhibitors as therapeutics for IPF has been challenging, discussed below.

The ubiquitious HAT, P300, regulates hundreds of transcription factors such as TGF-β1 and can cause the hyperacetylation of H4 on the COL promoter. In response to changes in cell substrate stiffness, activated P300 accumulates in the nucleus, enhancing Smad dependent transcription (Dou et al., 2018). Smad2/3 binds directly to gene promoters to induce the transcription of profibrotic molecules, including α-SMA, COLI and tissue inhibitor of metalloprotein (TIMP), inducing myofibroblast activation and ECM deposition (O'Reilly, 2017).

A body of work has shown that transdifferentiation, anti-apoptotic activity, ECM production and migration of epithelial-like mesenchymal cells is are mediated by BRD4 (Devaiah et al., 2016a). BRD4 is a member of a family of bromodomain and extraterminal (BET) proteins, including BRD2, BRD3 and BRDt that share two tandem bromodomains (BDs) and an extra-terminal domain, from which the family was named (Taniguchi, 2016). These paralogs are thought to have arisen during a distal evolutionary duplication event. Although the functions of the BET isoforms are tissue specific and pleiotropic, BRD4 activity has been implicated in cell cycle regulation DNA repair, innate inflammation (Brasier et al., 2011; Taniguchi, 2016), and cell-state transition (Chang et al., 2016; Tian et al., 2017a); all processes that play important roles in lung diseases.

Recent work has identified a coenzyme A -dependent atypical histone acetyl transferase activity (HAT) (Devaiah et al., 2016a) and N terminal kinase activity directed for the COOH terminus of RNA Polymerase II (Devaiah et al., 2012). Through these activities, BRD4 plays a central role in the maintenance of cell-type specifying superenhancers, controlling mesenchymal gene regulatory programs by transcriptional elongation discussed above. Small molecule inhibitors and siRNA mediated knockdown experiments have shown that BRD4 plays a central role in myofibroblast transdifferentation, mediating TGF-β-induced NOX4-αSMA expression, contractility and motility as well (Ijaz et al., 2017). In this way BRD4 mediates both EMT and fibroblast-myofibroblast transdifferentiation.

3.7 DNA methylation regulates EMT

Environmental particulate matter emitted by traffic-related air pollution (TRAP); polyaromatic hydrocarbons (PAHs), black carbon, ozone (O_3), and nitrogen oxides (NOx), have been shown to be associated with changes in DNA methylation (DNAm). DNAm, one of the most well-known mechanisms of epigenetic gene regulation, is described as the attachment of methyl groups to DNA, commonly at the fifth carbon of cytosines, leading to the formation of 5-methylcytosine (5-mC)(Moen et al., 2015). Methylation on the genome predominantly occurs at C-G dinucleotides (CpGs), resulting in the initiation of transcription, elongation efficiency, and alternative splicing.

Tumor hypoxia, a known inducer of EMT through hypoxia-inducible factor-1α (HIF-1α), can directly dysregulate the activity of ten-eleven translocation (TET) DNA demethylases. A decrease in DNA demethylation triggers the accumulation of DNA methylation driven by DNA methyltransferases (DNMT) to affect genes encoding cell adhesion functions that are involved in EMT. It has been suggested that DNA methyltransferase 1 associated protein 1 (DNMT1) can directly interact with Snail and suppress E-cadherin in epithelial cells and subsequent activation of epithelial–mesenchymal transition or cell death (Espada et al., 2011). Studies have shown that TRAP and smoking can cause increased DNMT1 protein accumulation leading to misexpression of genes to cause cancer.

4 Therapeutic strategies for pulmonary fibrosis

4.1 Glucocorticoids

Glucocorticoids such as cortisone, hydrocortisone, and prednisone, have historically been used to treat lung inflammation. These drugs act by decreasing inflammation both, directly through the glucocorticoid receptor and indirect mechanisms (Barnes, 2009). However, all potent glucocorticosteroids cause side effects such as glaucoma, high blood pressure, psychological effects, osteopenia, and acne (Newman, 2018). Previously, it was thought that pulmonary fibrosis was a product of immune activity. Therefore, corticosteroids were combined with to azathioprine or cyclophosphamide and a mucolytic/antioxidant, N-acetylcysteine. These studies were stopped due to negative effects in varying patient groups and are no longer used (Newman, 2018).

Although inhaled corticosteroids have been effective for several lung diseases such as cystic fibrosis, non-specific interstitial pneumonia (NSIP) and inflammatory diseases such as asthma and COPD, however, it is not effective with IPF (Mapel et al., 1996).

In 2014, oral nintedanib, was a breakthrough therapy approved by the FDA. Nintedanib is a broad spectrum small-molecule inhibitor of receptor tyrosine kinases that reduces fibroblast proliferation and activation. Nintedanib has been subjected to a number of randomized controlled clinical trials.

These studies have shown that nintedanib slows the decrease in forced vital capacity (FVC) from IPF and other progressive fibrosing interstitial lung diseases (Richeldi et al., 2014; Mazzei et al., 2015; Flaherty et al., 2019). Pirfenidone was also licensed in the same year. Pirfenidone is a small molecule with antifibrotic, anti-inflammatory and antioxidant effects that inhibits TGF-β-stimulated collagen production. Several prospective studies have found that pirfenidone also slows the loss of FVC in IPF and improves progression-free survival (Spagnolo et al., 2010). Although these medications represent a substantial advancement for the treatment of IPF, they neither reverse established fibrosis nor are curative.

4.2 HDAC inhibitors

Trichostatin A (TSA), a non-selective HDAC inhibitor (HDACi), has been shown to attenuate pulmonary fibrosis in several models. Bleomycin-induced pulmonary fibrosis (IPF), is characterized by progressive fibrosis leading to end-stage lung disease and respiratory failure. In the bleomycin model, TSA prevented WIF-1 loss, an important Wnt signaling regulator, resulting in a reduction of collagen overexpression through b-catenin (Yoon et al., 2019). TSA has also been shown to block TGF-β mediated ECM production in corneal fibroblasts. In a separate study, epigenetic defects in COX-2 expression were restored by HDAC inhibition, protecting from pulmonary fibrogenesis (Coward et al., 2009). Although there are currently many ongoing studies with the use of TSA and the reduction of fibrosis *in vivo* with mice and rats, there has yet to be any significant human clinical trials with this drug related to pulmonary fibrosis. A major limitation of HDAC inhibitors is the lack of isoform specificity and concerns with long-term toxicity (Royce and Karagiannis, 2014), including vascular calcification (Kwon and Kim, 2017).

4.3 BRD4 Inhibitors

A number of studies have implicated BRD4 as a potential therapeutic target for modifying epigenetic responses in lung disease. It has been shown that inhibition of BRD4 blocks EMT (Chang et al., 2016) and fibroblast-myofibroblast transdifferentiation *in vitro* (Ijaz et al., 2017). Highly selective BRD4 inhibitors have the ability to block allergen, viral, and growth factor induced remodeling and fibrosis in small animal models *in vivo* (Tian et al., 2016a; Tian et al., 2018d; Tian et al., 2019; Zhao et al., 2019). Moreover, inhibition of BRD4 interferes with inflammation-induced pericyte-myofibroblast transition *in vivo* and leakage of plasma proteins into the alveolar space (Zhao et al., 2019). Finally, BRD4 activation in myofibroblasts from humans with IPF, and small molecule BRD4 inhibitors can prevent interstitial fibrosis in the bleomycin lung injury model (Tang et al., 2013). radiation induced lung fibrosis (Wang et al., 2018) and fibrosis mediated by TGFβ administration (Tian et al., 2016a).

The medicinal chemistry of BRD4 inhibitors has been rapidly advancing (Liu et al., 2018; Tian et al., 2018b; Tian et al., 2019; Brasier and Zhou, 2020; Liu et al., 2020). Recently, highly selective inhibitors of BRD4 that interact with distinct regions of the protein have been developed (Liu et al., 2020). These molecules have demonstrated efficacy and exhibit promising medicinal properties (Liu et al., 2020). However, systemic administration of BRD4 inhibitors may be problematic due to its ubiquitous expression and role in chromatin organization, cell stress and innate responses.

We contend that the advancement of epigenetic inhibitors into well-tolerated therapy for chronic lung disease will depend on advancement of strategies that can selectively deliver these agents to the diseased tissue. In the subsequent section, we will review the promise of aerosolized delivery of nanomedicines for addressing this problem.

5. Nanomedicine

Nanoparticle (NP) formulations can provide a wide variety of benefits for the delivery of existing active pharmaceutical ingredients (APIs) – that is, the efficacious molecules identified previously for their anti-fibrotic properties. This includes dramatically improved API solubility, enhanced cellular internalization, controlled or stimuli-responsive release of the API, and enhanced selectivity through targeting, ultimately increasing therapeutic efficacy while reducing toxic side effects (Alshamsan, 2010; Menon et al., 2013, 2014; Patel et al., 2013; Guo, 2014; Pradhan, 2014; Wang, 2016; Kong, 2017; Song, 2017; Yang, 2018; Peer et al., 2007). The peripheral reactive groups of many NPs also provide the opportunity for ligand conjugation and active targeting strategies (Gu et al., 2007; Bugno et al., 2015). Additionally, NPs can provide specific benefits for pulmonary delivery. For example, NPs have been found to facilitate the solubility of APIs in certain pulmonary aerosolization solvents, such as perfluorooctyl bromide and fluorocarbons (Hsu et al., 2003; Lehmler et al., 2008). NPs are also readily internalized by vascular endothelial cells, providing accumulated quantities substantially greater than their micro-sized counterparts (typically >1 μm in diameter) (Foster et al., 2001; Davda and Labhasetwar, 2002). However, despite these potential advantages, EMT-targeting NPs have so far been primarily utilized for anti-cancer strategies, and those targeting EMT in lung fibrosis currently remain limited in the literature. This is the case even as EMT becomes increasingly implicated as an important pathway for pulmonary fibrosis (Willis and Borok, 2007; Naguchi et al., 2014), with key EMT-markers, such as TGF-β (Burgess et al., 2005; Fernandez and Eickelberg, 2012) and Snail (Wettstein et al., 2013), identified as promising targets for anti-fibrotic therapies.

In the following sections, we review a selection of nanomedicines formulated for pulmonary delivery organized by NP type, with a scope limited to those specifically utilizing aerosolic delivery methods. A general overview of these strategies is presented in **Figure 3**. Nanomedicines targeting EMT-mediated pulmonary fibrosis are especially focused on, with the goal of highlighting current shortcomings in this area of research. To this end, promising directions and potential considerations for future NP formulations are also identified at the end.

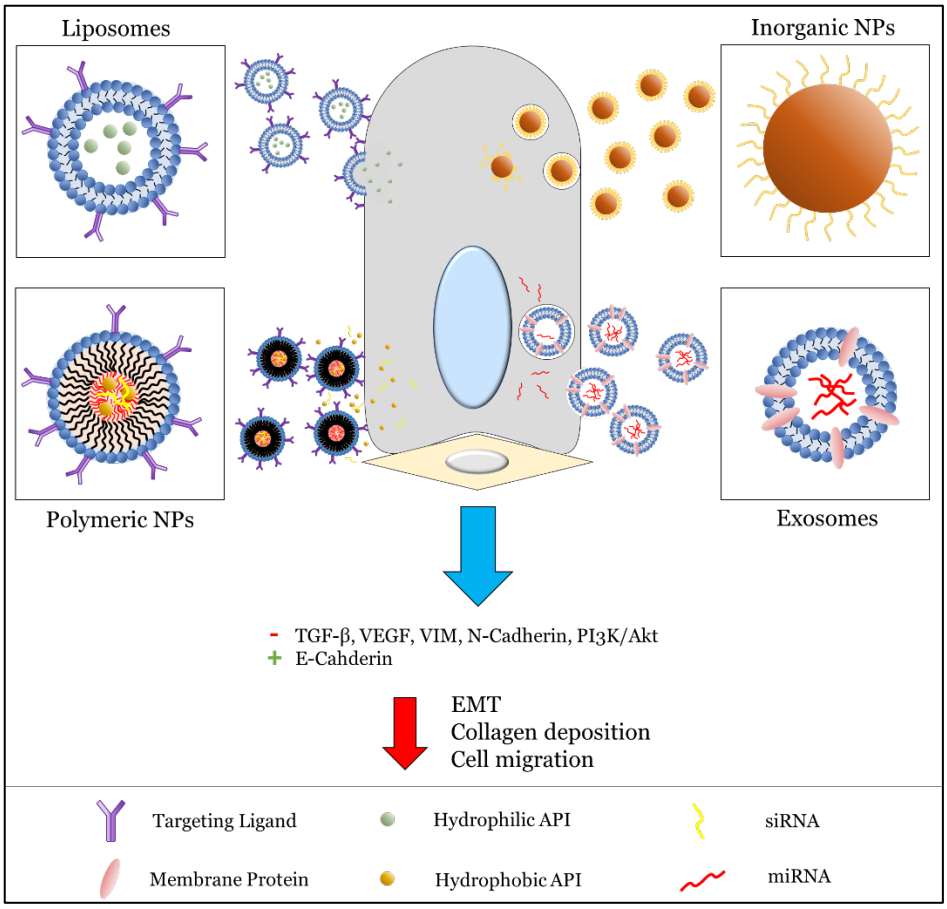

Figure 3. General strategies utilized for NP (liposomes, polymeric NPs, inorganic NPs, and exosomes) targeting the EMT pathway. For liposomes and polymeric NPs, this primarily involves active targeting via surface-bound ligands (typically proteins) followed by release of an encapsulated API. On the other hand, inorganic NPs and exosomes involve innate inhibition via their intrinsic physiochemical properties. Altogether, this leads to inhibition of key EMT biomarkers and subsequent reduction in fibrosis.

5.1 Liposomes

Liposomes are a promising nanocarrier for pulmonary drug delivery as they are primarily composed of natural or synthetic phospholipids either native to or compatible with lung tissues, such as phosphatidylcholine and egg phosphatidylethanolamine (Gao et al., 2013). These nanoformulations are also capable of including additional surfactants and cholesterol. Liposomes are comprised of spontaneously-formed lipid bilayer vesicles with diameters ranging from 30 nm to several micrometers, though those between 50-450 nm are favored for therapeutic delivery (Etheridge et al., 2013; Gao et al., 2013). Multiple forms of vesicles exist, including multilamellar vesicles (containing lamellar phase bilayers), small and large unilamellar liposomes (containing a single lipid bilayer), cochleate vesicles, and multivesicular liposomes (one vesicle containing multiple smaller vesicles). Liposomes can also be classified by preparation method, including reverse-phase evaporation and vesicle extradition (Bozzuto and Molinari, 2015). In general, liposomes consist of a hydrophobic shell encapsulating a hydrophilic solvent. This property enables loading of both lipophilic and hydrophilic APIs with relative ease. Further structural modifications provide additional benefits, such as poly(ethylene glycol) (PEG) or deoxyribonucleic acid (DNA) anchoring to embedded cholesterol for a “stealth effect” or as biosensors (Hosta-Rigau et al., 2013), or the addition of fatty acyl chains to extend transition temperature and increase stability (Bitounis et al., 2012). Circulation time in vivo

can also be extended by including hydrophilic carbohydrates, e.g., monosialoganglioside (G_{M1}), and polymers, e.g., PEG (Gabizon and Papahadjopoulos, 1988).

Aerosolized liposomes are well represented in the literature for pulmonary delivery, with promising results against multiple cancer models (including lung, renal, breast, and colon) (Knight et al., 1999; Koshkina et al., 2000, 2001; Skubitz and Anderson, 2000; Verschraegan et al., 2004), respiratory tract infections (RTIs) (Conley et al., 1997), immunodeficiency (Ten et al., 2002), pulmonary aspergillosis (Allen et al., 1994), cystic fibrosis (Davies et al., 2014), as a method of immunosuppression (Letsou et al., 1999), and as a delivery vehicle of insulin (Huang and Wang, 2006). Aerosolized liposomes have also been shown to be effectively delivered by multiple common clinical methods, including air-jet, ultrasonic, vibrating-mesh, and continuous-flow nebulizers (Waldrep et al., 1994; Elhissi and Taylor, 2005; Elhissi et al., 2007), and are clinically safe in human patients (Thomas et al., 1991; Vidgren et al., 1995; Gilbert et al., 1997; Waldrep et al., 1997). This demonstrates the high biocompatibility and adaptability of liposomes for pulmonary delivery. In fact, a liposomal formulation is the only currently approved NP formulation of aerosolized drug for pulmonary delivery. This comes in the form of Arikayce, an amikacin liposome nebulized suspension antibiotic for mycobacterium avium complex (MAC) lung disease, developed by Insmed and approved in 2018 (Cipolla et al., 2013; Insmed, 2020). Other notable clinical trials are summarized in **Table 2**.

Table 2. A summary of current clinical trials for aerosolized liposomes

NP	APIs	Target Disease	Phase	Status	Sponsor	Identifier
9-Nitro-20 (S)-Camptothecin (L9NC) Liposomes	N/A	Non-Small-Cell Lung Cancer	II	Completed in 2007	University of New Mexico	NCT00250120
9-Nitro-20 (S)-Camptothecin (L9NC) Liposomes	N/A	Metastatic Endometrial Cancer	II	Completed in 2007	University of New Mexico	NCT00249990
AmBisome	N/A	ABPA	II	Completed in 2019	Poitiers University Hospital	NCT02273661
AmBisome	Intraconazole	ABPA	III	Recruiting	Poitiers University Hospital	NCT03656081
AmBisome	N/A	Lung Transplant Infections	II	Completed in 2014	University Health Network, Toronto	NCT01254708
AmBisome	N/A	Lung Transplant Infections	III	Completed in 2007	University of Pittsburgh	NCT00177710
Arikayce	N/A	Cystic Fibrosis	I/II	Completed in 2008	Insmed Incorporated	NCT00777296

Arikayce	N/A	Cystic Fibrosis	I/II	Completed in 2009	Insmed Incorporated	NCT00558844
Arikayce	N/A	Cystic Fibrosis	II	Completed in 2010	Insmed Incorporated	NCT03905642
Arikayce	Mycobacterial Multi-Drug Regimen	Mycobacterium Abscessus Lung Disease	II	Completed in 2019	Kevin Winthrop	NCT03038178
Arikayce	Mycobacterial Multi-Drug Regimen	NTM + MAC	III	Completed in 2018	Insmed Incorporated	NCT02628600
Bupivacaine Liposomes	N/A	Coronary Heart Disease	III	Completed in 2020	Kathirvel Subramaniam	NCT03270514
Cisplatin Liposome	N/A	Osteosarcoma Metastatic	I/II	Completed in 2008	Insmed Incorporated	NCT00102531
Cyclosporin Liposome	N/A	Bronchiolitis Obliterans Syndrome	II/III	Completed in 2014	Pari Pharma GmbH	NCT01334892
Cyclosporin Liposome	Tacrolimus + Mycophenolate Mofetil Prednisone + Rampamycin	Lung Transplant Rejection	I/II	Completed in 2015	University of Maryland, College Park	NCT01650545
Pulmaquin	N/A	Non-cystic Fibrosis Bronchiectasis	III	Completed in 2016	Aradigm Corporation	NCT02104245
Pulmaquin	N/A	Non-cystic Fibrosis Bronchiectasis	III	Completed in 2016	Aradigm Corporation	NCT01515007

Abbreviations: AmBisome – amphotericin B liposome; ABPA – allergic bronchopulmonary aspergillosis; NTM – nontuberculous mycobacterial lung infection; Pulmaquin – ciprofloxacin liposome

Despite the available aerosolized liposome formulations for pulmonary delivery, comparatively few utilize an EMT-targeted approach. These are summarized in **Table 3**. The majority of these formulations utilize APIs loaded into the liposome vesicle, including PGE2 (also known as dinoprostone, an oxytocic drug used in pregnancies for smooth muscle contradictions) (Ivanova et al., 2013), paclitaxel (an anti-cancer cytoskeletal drug that targets tubulin) (Zhou et al., 2016; Jin et al., 2019), and simvastatin (a lipid-lowering drug typically used for heart defects) (Jin et al., 2019). One formulation, however, used hyaluronic acid (a ligand of CD44) embedded in the liposome bilayer as both the API and a targeting moiety for overexpressed CD44 (Pandolfi and Al., 2019). In all cases, biomarkers of the EMT pathway were subsequently inhibited by the API, including TGF-β, TNF, and VEGF. This led to significant inhibition of EMT, reducing either inflammation and fibrosis of the lungs (Ivanova et al., 2013; Zhou et al., 2016; Pandolfi and Al., 2019) or tumor metastasis (Jin

et al., 2019). Additionally, the liposomes improved the delivery efficiency through improved cellular entry, tissue retention of API, and selectivity endowed from a surface-decorated ligand.

Table 3. A summary of current aerosolized liposome formulations targeting EMT-markers in the literature

NP	API(s)	Disease	Model(s)	Effect	Ref
Liposome	PGE2	Idiopathic Pulmonary Fibrosis (IPF)	IPF Mice	Inhibition of TGFB-initiated pathways and TNF, restricted inflammation and fibrosis	(Ivanova et al., 2013)
Liposome	Paclitaxel	Pulmonary Fibrosis (PF)	PF Mice	Inhibition of TGF-β1, reduced collagen levels and alveolar thickness	(Zhou et al., 2016)
Liposome	Simvastatin + Paclitaxel	Lung Cancer	A549T Mice	Inhibition of EMT and metastasis	(Jin et al., 2019)
DPPE Liposome	Hyaluronic Acid	CTD-ILD + BOS	A549 + Calu-3 + THP-1 Cells	Inhibition of VEGF mRNA	(Pandolfi and Al., 2019)

Abbreviations: DPPE – 1,2-Bis(diphenylphosphino)ethane; CTD-ILD – connective tissue disease-associated interstitial lung disease; BOS – Bohring-Opitz Syndrome.

Even with the broad array of available aerosolized liposomes and initial promising results for EMT-targeted delivery, challenges remain if these NPs are to be further pursued. For one, liposome stability when aerosolized remains a leading concern. When passed through a nebulizer, significant loss of encapsulated API can occur from mechanical-induced fragmentation of vesicles (Taylor et al., 1990). Strategies exist to mitigate this, such as reducing vesicle size by extruding the liposomes through a membrane filter, but it remains a concern for any potential formulation. Additionally, liposomes face several biological challenges for drug delivery. This includes preferential uptake by the reticuloendothelial system (RES) and opsonization (Poste et al., 1976; Senior, 1986), high clearance of large liposomes (Oku and Namba, 1994; Ulrich, 2002), the "accelerated blood clearance" (ABC) phenomenon if PEGylated liposomes are administered repeatedly (Ishida et al.; Dams et al., 2000), and potential triggering of the innate immune response (Szebeni, 2005; Szebeni and Moghimi, 2009). While the unique anatomy and physiology of the lungs could attenuate some of these concerns, they should still be kept in mind when designing a liposome aerosol. For this reason, efforts have increasingly turned away from simpler formulations like liposomes and into more complex, and robust, nanoparticle systems.

5.2 Polymeric Nanoparticles

Polymeric NPs, which have increasingly become more explored as pulmonary drug delivery vehicles in the literature, are one such system. While liposomes are primarily composed of phospholipids with the addition of surfactants and cholesterol, polymeric NPs are instead composed of one or more synthetic polymer repeating chains linked together. The most common of these polymers utilized for pulmonary delivery include poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(lactic-*co*-

glycolic acid) (PLGA), chitosan, alginate, and gelatin bases (Menon et al., 2014). While not naturally derived, these polymers are biodegradable, which mitigates some risk of toxicity (Beck-Broichsitter et al., 2012c, 2013). Additionally, the nature of polymer chemistry (or polymerization), wherein monomer molecules are consecutively bonded together through step-growth or chain-growth synthesis techniques, affords greater control and diversity for polymeric NPs than comparable liposomal formulations (Kricheldorf, 1991; Hickey et al., 2015). This ability to finetune molecular weight (MW), chemical composition, and chain structure provides a wide diversity of potential NPs (as detailed later in this review).

The choice of polymer, polymer ratio, or polymer functional groups can also significantly change polymeric NP properties. For example, the rate of *in vivo* biodegradability can vary based on composition (pure vs hybrid NPs) (Singh et al., 2010), type of polymer (Anderson and Shive, 2012), or MW (Mohammad and Reineke, 2013), or even determine the mechanism of biodegradation itself (hydrolysis vs oxidative degradation, for instance) (Ulbricht et al., 2014). Adding another polymeric coating can also change how a polymeric NP interacts physiologically. This includes enhancing tissue penetration (for example, adding a dense PEG coating to infiltrate farther into therapeutically unsusceptible tissues like the brain or through mucus coatings) (Tang et al., 2009; Nance et al., 2012; Hong et al., 2009) and clearance (such as being susceptible to or avoiding the ABC phenomenon) (Alexis et al., 2008; Ishihara et al., 2010; Saadati et al., 2013). Other fine-tunable properties include lipophilicity and drug loading (Johnstone and Lippard, 2013), circulation time and interaction with vascular proteins (Sheng et al., 2009; Yang et al., 2014; Sunoqrot et al., 2014), NP targeting and tissue accumulation (Park et al., 2007), and NP shelf-life.

In addition to polymer choice, polymeric NPs can also be formulated with a diverse range of molecular architectures that further influence their behaviors (Menon et al., 2014), including linear (single chain) polymers, hyperbranched (multiple end group) polymers (including dendrimers), micelles, and crosslinked emulsions (**Box 1, Figure 4**). As such, polymeric NPs typically range from 1 to 1000 nm in diameter, exist in a variety of morphologies (e.g. spheres, vesicles, rods, tubules, and lamellae), and consist of homopolymers and various copolymers. Of note for drug delivery are amphiphilic copolymers. These polymer chains, consisting of a lipophilic polymer (such as PLA or PCL) conjugated to a hydrophilic polymer (e.g. PEG), spontaneously form a variety of complex morphologies in aqueous solvents due to hydrophobic interactions (Forster and Antonietti, 1999; Letchford and Burt, 2007). Altering the properties of these copolymers, such as constituent chain length, hydrophilic-lipophilic balance (HLB) , and charge, can even further alter a NP's properties, including hydrodynamic size (Wittgren et al., 1996), shell thickness (Stubenrauch et al., 2006), and exposed peripheral functional groups (Urbani et al., 2008; Pearson et al., 2016). This in turn allows further finetuning of drug delivery characteristics, such as API solubility, loading capacity, and release profiles (Yan et al., 2011; Glavas et al., 2015). Altogether, this endows polymeric NPs with advantages similar to liposomes (e.g. easy encapsulation and high loading of lipophilic APIs, API protection, enhanced half-life and lowered blood clearance, sustained and controlled release, and the availability of readily modifiable surface end groups for targeting and stealth strategies) while also providing researchers with a more adaptable NP platform for various delivery needs.

Box 1 – Various Polymeric Nanoparticle Formulations

Linear Polymer-based: A single-chain polymeric NP composed of only one polymer type, which is the simplest form of polymeric NP. Can be broadly defined as a solid colloidal particle consisting of a uniform macromolecular component, which typically forms a three-dimensional nanosphere (though can also include nanocapsules, cubes, and plates)

encapsulating therapeutic agents (or, in the case of a surface modification such as PEGylation, extend as a directly conjugated chain off the original molecule) (Chan et al., 2010; Bolhassani et al., 2014). As mentioned, these are typically synthesized using either step-growth (mostly via condensation polymerization) or chain-growth (mostly via addition polymerization such as radical, ionic, ring-opening, or living polymerizations) techniques (Kricheldorf, 1991). The polymer is then dispersed in an organic phase and cross-linked to form a spherical structure. A linear polymer can also be attached to an API through precise post-polymerization modifications of semi-telechelic chains, such as chemical ligation of specific antibodies (cysteine, tyrosine, arginine, and histidine), enzyme-mediated ligation, *in situ* polymerization initiated by an API, or through recombinant gene technology (Ekladious et al., 2019). Nanosphere diameters can range from 20-200 nm while single chain modifications can be even smaller, with some of the smallest only 1-10 nm in diameter (Bolhassani et al., 2014; Kroger and Paulussea, 2018).

Hyperbranched Polymers: Polymeric NPs composed of a hyperbranched structure, wherein exponentially increasing numbers of "branch-like" polymer chains extend from a central core to form a surrounding shell. Dendrimers are the most notable example. These complex highly branched monodisperse polymeric NPs form a spherical morphology, including a central core, a hyperbranched body, and peripheral reactive functional groups, the number of which are doubled with an increase of each dendrimer generation (for example, a generation 0 (G0) poly(amidoamine) (PAMAM) dendrimer has a theoretical four surface groups, a G1 would have 8, a G2 would have 16.) (Franiak-Pietryga et al., 2018; Hsu et al., 2016; Shi et al., 2006). Dendrimers can be synthesized through several techniques, including divergent and convergent synthesis, (Holister et al., 2003; Killops et al., 2008; Franc and Kakkar, 2009) as well as click chemistry, such as Diels-Alder (Franc and Kakkar, 2009), azide-alkyne (Franc and Kakkar, 2008), and thiol-ene reactions (Killops et al., 2008). This allows for tight control of the number of branches and results in a homologous NP with predictable properties (Landmark et al., 2008; Yang et al., 2012; Bugno et al., 2016). Unlike other polymeric NPs, the most common dendrimers for delivery are PAMAM and poly(propylene polybenzy isocyanate) (PPI) dendrimers, with other examples including poly(L-lysine), polyester, and polyether dendrimers (Kesharwani et al., 2014). Diameters can range anywhere from 1–sub-100 nm with increasing generation, but tend to favor smaller sizes of ≤20 nm for delivery (Franiak-Pietryga et al., 2018).

Micelles: NP core/shell structures, composed of amphiphilic block copolymer subunits, that self-assemble into uniform morphologies when exposed to aqueous solvents (Croy and Kwon, 2006; Zhang et al., 2014). This leads to the formation of a hydrophobic core surrounded by a hydrophilic corona, with or without peripheral reactive functional groups (that can then be further modified with polymer coatings (e.g. PEGylation) or conjugated to ligands (Choi et al., 2003)). Differences in copolymer composition, length, and solvent interaction can further alter micelle characteristics, including size and shape, leading to the formation of spheres, vesicles, rods, tubules, or lamellae (Zhang et al., 2014). The copolymer subunits themselves are synthesized similarly to linear polymers. This can involve either site selective chain-growth polymerization utilizing an existing chain as the initiator (de Espinosa and Meier, 2011), limited step-growth polymerization with two polymer species (Delaittre et al., 2009), or click chemistry between two available functional groups (such as an azide and an alkyne in the presence of cationic Cu(I)) (Li et al., 2010; Engler et al., 2013). Spherical micelles can range from 10–100 nm in diameter, while polymersomes dimensions can range from 50 nm–5 μm (Croy and Kwon, 2006; Zhang et al., 2014; Zhang and Zhang, 2017).

Emulsions: Three-dimensional networks of crosslinked hydrophilic or amphiphilic polymers, including emulsions, nanogels, and hydrogels (Kabanov and Vinogradov, 2009; Chacko et al., 2012; Kousalova and Etrych, 2018). These networks form either through physical interactions, such as hydrophobic effects, or through chemical interactions, such as hydrogen bonding, electrostatic and ionic interactions, or the formation of chemical bonds (Kabanov and Vinogradov, 2009). This generates an extensive "swollen" polymer mesh that displays the properties of both a conventional NP system and a hydrogel. This includes being highly absorbent (Hoshino et al., 2012), possessing upper diameters far larger than other polymeric NPs, an extensive capacity for API loading and release (Chen et al., 2010), viscoelasticity (Mamaghani et al., 2015), and temperature dependent phase transitions (Hoshino et al., 2012). Depending on the base network, emulsions can range widely from 10 to a few thousands of nanometers in size (Kabanov and Vinogradov, 2009).

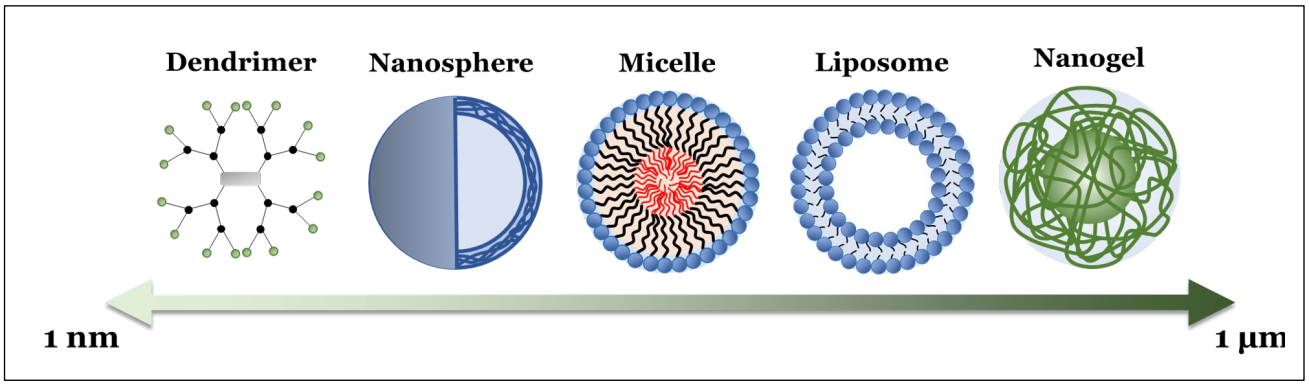

Figure 4. Schematic illustration of the different polymeric NP formulations, with their relative sizes, summarized in Box1.

The extensive variety of polymeric NPs has given rise to a broad list of aerosolized formulations in the literature. This includes NPs tested against lung tumors (orthotopic and small cell) (Bhattarai et al., 2007; Rosiere and Al., 2016; Qiao et al., 2017; Yan et al., 2017; Elbatanony et al., 2020; Vaidya et al., 2020), type 2 diabetes (Shrestha et al., 2015), biofilms and lung infections (Cheow et al., 2010; Casciaro and Al., 2019), pneumonia (Shah et al., 2013), tuberculosis (Pandey et al., 2003; Costa-Guoveia and Al., 2017), asthma (Yoo et al., 2013), and other respiratory diseases (Beck-Broichsitter et al., 2010; Kolte et al., 2017). The majority of these make use of nanospheres, including PLGA, poly(ethylenimine) (PEI), PCL, and porous silicon (PSi) nanospheres, and micelles, including PLGA-*co*-polyspermine (PSPE) and folate-PEG-dextran copolymers. Much like liposomes, polymeric NPs have also already been extensively tested with common pulmonary delivery methods, like vibrating-mesh (Beck-Broichsitter et al., 2012a, 2012b, 2014), air-jet (Beck-Broichsitter et al., 2012b), and ultrasonic nebulizers (Beck-Broichsitter et al., 2012b). Special attention has been placed on developing dry powder formulations, such as through spray freeze drying, which enhances the stability of sensitive polymer NPs and allows for finer control of delivered particles (Cheow et al., 2011; D'Addio et al., 2013; Ali and Lamprecht, 2014). However, despite these current advances, many polymeric NP aerosolization studies remain in pre-clinical *in vitro* stages. This is most visible for clinical trials, with no currently reported studies utilizing a polymeric NP aerosol. While a promising and robust alternative to liposomes, this demonstrates that polymeric NP aerosols are still in their infancy, with no sign of an approved product in the near future.

Despite this, polymer NPs are comparatively well represented when it comes to EMT-mediated pulmonary delivery, with currently studied formulations including emulsions, nanospheres, and micelles. These are summarized in **Table 4**. Like with liposomes, the majority of these utilize APIs

loaded into a lipophilic core, including pirfenidone (a drug used to treat pulmonary fibrosis) (Singh et al., 2019), MDB5 (a novel form of Hedgehog (Hh) inhibitor GDC-0449) (Kumar et al., 2019), salinomycin (an antibiotic with anti-cancer properties) (Sousa et al., 2019), and doxorubicin (an anthracycline chemotherapeutic) (Fang et al., 2014; Seifi-Najmi et al., 2016). Multiple formulations also make use of siRNA targeting genes along the EMT pathway (Fang et al., 2014; Seifi-Najmi et al., 2016; Li et al., 2019; Suresh et al., 2019), including one directly conjugated to PEI to form a polyplex (Ding et al., 2018; Wang et al., 2019), as well as an inhibitor of CXCR4 (Suresh et al., 2019), a chemokine receptor involved in the promotion of tumor migration and EMT (Lin et al., 2018). These all resulted in the inhibition of key EMT-biomarkers, including PAI-1, TGF-β, VEGF, Hh, Vimentin, and N-Cadherin. This led to decreased collagen deposition (Kumar et al., 2019; Li et al., 2019; Wang et al., 2019), cell migration and metastasis (Fang et al., 2014; Seifi-Najmi et al., 2016; Sousa et al., 2019), and tumor growth (Suresh et al., 2019). Polymeric NPs increased lung penetration and retention, provided stability by a polymer-siRNA conjugate, and facilitated codelivery further enhanced API efficacies.

Table 4. A summary of current aerosolized polymeric NP formulations targeting EMT-markers in the literature

NP	API(s)	Disease	Model(s)	Effects	Ref
Poly(ethylenimine) (PEI-C) Polyplex	siPAI-1	Pulmonary Fibrosis (PF)	PF Mice	Inhibition of PAI-1 and TGF-β, decreased collagen deposition in lungs	(Ding et al., 2018; Wang et al., 2019)
Perfluorocarbon (PFC) Nanoemulsion	CXCR4 Antagonist + anti-STAT3 siRNA	Lung Metastasis	4T1.Luc Mice	Inhibition of VEGF, decreased tumor growth and increased survival	(Li et al., 2019)
Poly(ε-caprolactone) (PCL)	Pirfenidone	Pulmonary Fibrosis (PF)	A549 Cells	Inhibition of TGF- β1 mRNA and EMT	(Singh et al., 2019)
PEG-PCC-g-DC Micelles	MDB5	Liver Fibrosis	CBDL Mice	Inhibition of Hh activity, HSC activation, and decreased collagen deposition	(Kumar et al., 2019)
Pluronic F127 (PM) Micelles	Salinomycin (SAL)	Lung Cancer	A549 Cells	Inhibition of VIM and EMT, reduced cell migration	(Sousa et al., 2019)
PEG-PLL-PLLeu Polypetide Micelles	ZEB1 siRNA + Doxorubicine	Non-Small Cell Lung Cancer	H460 Mice	Inhibition of EMT and metastasis	(Fang et al., 2014)
CMD-Chitosan Nanoparticles	siRNA + Doxorubicin	Lung Cancer	A549 Cells	Inhibition of EMT markers, reduced cell migration	(Seifi-Najmi et al., 2016)

EGFR-Conjugated Gelatin Nanoparticles	siRNA	Non-Small Cell Lung Cancer	H820 + H1975 Cells	Inhibition of Vimentin, N-Cadherin, and EMT	(Suresh et al., 2019)

While a quickly growing subfield in the aerosol delivery of NPs, concerns remain for polymeric NPs that must be addressed before they can properly compete with liposomes. Perhaps the largest of these is induced aggregation. When passed through a nebulizer, polymeric NPs, especially those with high surface hydrophobicity, can exhibit extensive aggregation within aerosol droplets (Dailey et al., 2003b). This effect can be further pronounced depending on the specific nebulizer used – for instance, NPs administered through jet nebulizers appear more susceptible to aggregation than those passed through ultrasonic nebulizers under the same conditions (Dailey et al., 2003b). Polymer NP variation can also have a profound effect. Charge, for example, influences the stability of polymeric NPs in aerosol solvents, with anionic formulations being more stable than cationic (Dailey et al., 2003a). Additionally, non-biodegradable polymer nanospheres, such as polystyrene, have been implicated in the promotion of pulmonary inflammation (Dailey et al., 2006). Though current evidence suggests that biodegradable polymeric NPs avoid this same inflammatory response, care will still need to be taken in preventing potentially irritating or toxic doses. Finally, polymeric NPs can suffer from some of the same biological limitations as liposomes, including susceptibility to opsonization and enhanced clearance (Owens and Peppas, 2006), the ABC phenomenon (Ishihara and Al., 2009; Saadati et al., 2013), and concerns over long term accumulation and toxicity in organs like the liver and brain (Raghnaill et al., 2014). Despite these potential disadvantages, the broad possibilities of polymeric NPs cause them to remain a tantalizing prospective for future aerosol delivery against EMT-mediated fibrosis. Especially as other, more conventional NPs lag even further behind in this area of study.

5.3 Inorganic Nanoparticles

Although a popular option for other routes and diseases, inorganic NPs have so far rarely been used for pulmonary fibrosis therapy. One contributing factor may be that, unlike liposomes and polymeric NPs, these NPs are composed of inorganic materials that are not readily present or biocompatible in lung tissues. For drug delivery, this often includes gold (Au), silver (Ag), iron (Fe), and zinc (Zn), and to a lesser extent titanium (Ti) and silicon (Si), either in their pure form or as a compound (e.g. oxides, hydroxides, borohydrides, chlorides, sulfides, and sulfates) (Mody et al., 2010). Inorganic NPs typically consist of a spherical, inorganic, electrostatically charged core functionalized with targeting ligands or other moieties. Other, less common shapes include diamond, octagonal, rods, and thin sheets (Xia et al., 2008; Kinnear et al., 2017). The most common formulation strategy begins with the nucleation of metal ion complexes in a colloidal solution using a reducing agent (Mody et al., 2010). The inorganic NP can then undergo different forms of surface chemistry. This can include ligand exchange reactions, wherein a inorganic NP is conjugated to any number of biomolecules (including DNA, RNA, proteins, and polymers such as PEG) utilizing weak binding between carboxyl groups and the NP surface (Kanninen et al., 2008), or ligand removal of existing surface features, such as through temperature elevation, ablation, or electrochemical etching (Niu and Li, 2014; Lu et al., 2018). This can introduce functionality to the inorganic NP, like ligand-mediated active targeting, enhance biocompatibility and stability, or facilitate the loading of APIs. Reported inorganic NPs have a wide range of sizes, though nanomedicine formulations tend to be within the range of 1-100 nm (Mody et al., 2010).

Despite the current lack of prominent aerosolized inorganic NPs being studied in the literature, they possess certain properties that makes prospective formulations appealing, particularly in targeting the EMT pathway. One intrinsic advantage of many inorganic NPs is an innate therapeutic inhibition of the EMT process. Even unmodified inorganic NPs have been found to inhibit cell proliferation, reduce expression of key EMT markers such as Snail, N-Cadherin, and Vimentin, and overall suppress oncogenic and inflammatory signaling pathways (Arvizo et al., 2013; Xiong et al., 2019). Additionally, inorganic NPs can also be used for plasmonic photothermal therapy (PPTT), wherein infiltrated cells can be locally heated by an irradiated laser (using a specific plasmon-resonant absorption wavelength) without damaging surrounding tissues (Huang et al., 2008). While primarily utilized in anti-cancer therapies, this technique has also been found to cause inhibition of the EMT pathway (Wu et al., 2018). Other advantages include excellent stability, wide availability, ease of use, and the straightforward scale-up for large scale production.

Consistent with their lack of aerosolized formulations in the literature, inorganic NPs have been used less frequently than other NPs to target EMT-mediated fibrosis. Current examples are nevertheless summarized in **Table 5**. Unlike other formulations, the majority of these to do not involve additional APIs loaded into the metallic core. Instead, the intrinsic EMT inhibition of various metals were utilized, including those of Zn (Peng et al., 2018; Wahab et al., 2016), Si (Peng et al., 2018), Au (Kaushik et al., 2016), Ti (Li et al., 2018), and Ag (Sunil Gowda et al., 2018). Two APIs that were used, however, include cold plasma (a partially ionized gas (that is externally discharged using an air plasma device with antibacterial and wound healing properties) (Kaushik et al., 2016) and gallic acid (an antioxidant capable of inhibiting glioma cell metastasis and radiation-induced reactive oxygen species (ROS)) (Sunil Gowda et al., 2018). Both were added as promising novel inhibitors of EMT markers (specifically PI3K and vimentin, N-cadherin, and Snail-1, respectively) to enhance the existing anti-fibrotic efficacy of their inorganic NPs. In general, common EMT markers targeted by inorganic NPs included N-cadherin, E-cadherin, and TGF-β (Peng et al., 2018; Li et al., 2018; Wahab et al., 2016). This in turn led to significant inhibition of EMT, reducing cell migration, tumor growth, and treatment-induced radiation toxicity and resistance. Thus, unlike other NP formulations which only promoted the delivery of APIs, the inorganic NPs here provided key inhibition themselves, in addition to other advantages like enhanced stability and cell penetration and accumulation.

Table 5. A summary of current aerosolized inorganic NP formulations targeting EMT-markers in the literature

NP	API(s)	Disease	Model(s)	Effect	Ref
ZnO_2 and SiO_2 NPs	N/A	Fibrosis	LX-2 Cells	Inhibition of N-Cadherin and EMT, elevation of E-Cadherin	(Peng et al., 2018)
PEG-coated Au NPs	Cold Plasma	Lung Cancer	T98G + A549 Cells	Inhibition of PI3K/AKT axis and EMT	(Kaushik et al., 2016)
TiO_2 NPs	N/A	Lung Cancer	A549 Cells	Inhibition of TGF-β and EMT, reduced cell migration	(Li et al., 2018)
ZnO Nanostructures	N/A	Lung Cancer	H460 + MRC5 Cells	Inhibition of N-Cadherin and EMT	(Wahab et al., 2016)

Ag NPs	Gallic Acid	Lung Cancer	A549 Cells	Inhibition of Vimentin, N-Cadherin, and Snail-1	(Sunil Gowda et al., 2018)

Despite the unique and promising advantages provided by inorganic NPs, however, significant disadvantages remain which challenge their application for pulmonary fibrosis treatment. Perhaps the most significant is localized toxicity. As previously mentioned, inorganic NPs are composed of metals which are not naturally found in significant quantities in the lungs. Exposure to these NPs (at industrial and biomedical levels) has been found to cause acute pulmonary inflammation (Cho et al., 2010; Cho et al., 2012a; Cho et al., 2012b) and oxidative stress (Limbach et al., 2007). Specifically, exposure to common inorganic NPs led to elevated neutrophil and eosinophil recruitment and infiltration into lung tissues (Cho et al., 2010; Cho et al., 2012a), the extent of which appears dependent on the relationship between zeta potential and solubility (Cho et al., 2012b). This includes for carbon (C), cerium (Ce), Ti, Si, nickel (Ni), Zn, and Cu NPs, especially in their pure or oxide forms. However, other studies have demonstrated that biomedical quantities of inorganic NPs are no more toxic than naturally occuring dusts and other aerosols (Veranth et al., 2007). In either case, pulmonary toxicity will remain a concern for any potential aerosol formulation. Additionally, inorganic NPs in general can exihibit elevated systemic clearance by the kidneys and the reticuloendothelial system (RES) in the liver, spleen, and lungs, penetration through vascular endothelium and the blood brain barrier (BBB), and potentially toxic tissue accumulation (Li and Chen, 2011). Finally, due to the current lack of relevant examples in the literature, the potential for aerosolized delivery of inorganic NPs is largely unknown currently. However, the existing use of aerosol vapor deposition for inorganic NP films (Palgrave and Parkin, 2006) and other naturally occurring aerosols (Quadros and Marr, 2012) both suggest its feasibility. For these reasons, future treatments for pulmonary fibrosis may lie in formulations that are closer in design, not farther, to naturally occurring lung vesicles.

5.4 Exosomes

Exosomes represent perhaps the most novel formulations currently being explored for pulmonary delivery. A type of membrane-bound extracellular vesicle (EV), small vesicles naturally derived from pro- and eukaryotic cells, exosomes are a more homogenous population of EVs – sharing a similar spherical shape and sizes that range from ~50-150 nm – that are uniquely rich in cholesterol and diacylglycerol (Abels and Breakefield, 2016; Silverman and Reiner, 2011; Ellis and Kuehn, 2010). They are generated when the plasma membrane of a cell "buds" inwardly to form an intermediate endosome-vesicle known as the multivesicular bodies (MVBs). These MVBs are then either degraded by lysosomes or fuse with the plasma membrane to be released as exosomes, which in turn intersperses endosomal surface proteins into the exosome bilayer (Fevrier and Raposo, 2004; Colombo et al., 2014). This helps differentiate exosomes as well as provides unique characteristics depending on the origin cell (Thery et al., 2002). However, there is still some debate on the exact definition, and there is considerable overlap in size and morphology between exosomes and the two other categories of EVs: apoptotic bodies and micro vesicles. Additionally, the exact biological functions of exosomes remain somewhat unclear, though they are known to act as miRNA transporters between cells with important regulatory effects for intracellular mRNA activity (Valadi et al., 2007). Other suggested functions include elimination of undegraded endosomal and lysosomal proteins and membranes, regulation of T cell activity, and antigen transfer to dendritic cells (Thery et al., 2002)**.**

As delivery vehicles, exosomes share many similarities with liposomes, including similar size and morphology (as lipid bilayers) and the ability to encapsulate both hydrophilic and lipophilic APIs

(Johnsen et al., 2014; van der Meel et al., 2014). However, the complex composition of surface proteins on exosomes provides additional benefits, which most notably includes enhanced organotropism – that is, exosomes are capable of efficient targeting and uptake by specific recipient cells related to the surface proteins on the original plasma membrane (Antimisiaris et al., 2018). Additionally, the unique natural lipid composition of exosomes makes them highly biocompatible, while selection of autologous exosomes can further prevent any adverse patient immune responses (Antimisiaris et al., 2018).

Examples of aerosolized or inhaled exosomes, though limited, do exist in the literature, including as a potential therapy for tuberculosis (Cheng and Schorey, 2013), lung inflammation (Zhang et al., 2018), pulmonary fibrosis (Dinh and Al., 2020), and the general delivery of therapeutic miRNA (Zhang et al., 2017). Overwhelmingly, the exosomes used were derived from alveolar macrophages (Cheng and Schorey, 2013; Zhang et al., 2018; Zhang et al., 2017), though one study did utilize exosomes derived from mesenchymal stem cells instead (Dinh and Al., 2020)**.** This same study also utilized a compressed air nebulizer for successful aerosol delivery (Dinh and Al., 2020), suggesting that clinically relevant nebulizer systems could be viable for a future exosome formulation. However, comprehensive studies characterizing the physical properties of aerosolized exosomes still appear to be missing. Interestingly, despite the overall current lack of studies on the efficacy and patient safety of aerosolized exosome formulations, several clinical studies have recently surfaced due to the coronavirus disease 2019 (COVID-19) pandemic. These are summarized in **Table 6**.

Table 6. A summary of current clinical trials for aerosolized exosomes

NP	APIs	Target Disease	Phase	Status	Sponsor	Identifier
CSTC-derived Exosomes	N/A	Coronavirus + Pneumonia	I	Active	TC Ericyes University	NCT04389385
MPC-derived Exosomes	N/A	Drug Resistance	I/II	Recruiting	Ruijin Hospital	NCT04544215
MSC-derived Exosomes	N/A	Coronavirus	I	Completed in 2020	Ruijin Hospital	NCT04276987
MSC-derived Exosomes	N/A	Healthy Tolerance	I	Recruiting	Ruijin Hospital	NCT04313647
MSC-derived Exosomes	N/A	ARDS	I/II	Not Yet Recruiting	Ruijin Hospital	NCT04602104

CSTC – COVID-19 specific T cell; MPC – mesenchymal stem/progenitor cell; MSC – mesenchymal stem cell; ARDS – acute respiratory distress syndrome

Similarly, the use of exosomes for EMT-targeting appears to still be in its infancy, with no current examples in the literature of aerosolized exosomes specific for the pathway. However, examples of non-aerosolized formulations do exist. This includes for intrauterine adhesion (IUA) (Yao et al., 2019), liver fibrosis (Li et al., 2012), acute lung injury (ALI) (Xiao et al., 2020), and cancer (including colorectal (Zhang et al., 2020), liver (Chen et al., 2018), thyroid (Hardin et al., 2018), and lung (Zhao et al., 2018)). In the majority of these studies, MSC-derived exosomes were used

(including from bone marrow (Yao et al., 2019) and umbilical cord (Li et al., 2012; Zhao et al., 2018)), though others involved exosomes derived directly from carcinomas (Zhang et al., 2020; Chen et al., 2018) or stem-like cells (Hardin et al., 2018). Like inorganic NPs, most of the exosomes involved no additional API and instead relied on the intrinsic EMT-inhibition of the derived surface proteins. However, one exception did utilize the additional delivery of an exosomal miRNA (Zhang et al., 2020). In all instances, EMT inhibition was achieved through the regulation of key biomarkers, including VIM, TGF-β, Smad2/3, FSP-1, E-Cadherin, CK19, NF-κB, ERK, JNK, and the Akt and hedgehog pathways. This highlights the inherent advantages to using an exosome formulation directly cultured from EMT-associated cells, with the potential for similar results for exosomes derived from fibrotic lung epithelial cells.

Even with the unique advantages provided by exosomes, concerns remain for any future aerosol formulations that must be addressed. For one, exosomes have also been linked to the promotion of EMT for a variety of diseases, notably for cancers (including renal (Wang and Al., 2019), esophageal (Min et al., 2018), pancreatic (Li et al., 2018), breast (Wang et al., 2019), and lung (He et al., 2019)). Care will need to be taken in selecting a model exosome for fibrosis therapy which does not induce further long-term tissue injury, especially given the unknown physiological functions of many EVs. Additionally, the exosomal surface can quickly become a disadvantage for the design of more robust formulations or combination therapies – due to the complex nature of surface proteins and the current lack of synthetic alternatives to natural biogenesis, the controlled modification of exosomes with other NP strategies, such as PEGylation, is difficult if not impossible (Antimisiaris et al., 2018). This has led to increased interest in engineered exosomes, so called “EV-mimetics”, in recent years as one potential solution to limited natural yields and to facilitate more varied chemistry. Finally, exosomes in general suffer several therapeutic limitations, including rapid *in vivo* blood clearance, methods which result in low drug loading and retention, and difficult and costly synthesis strategies with limited yields (Antimisiaris et al., 2018).

5.5 Other Directions and Considerations

Although promising NP formulations for targeting EMT-mediated pulmonary fibrosis already exist, there are additional directions which could be pursued for further benefits. For example, current therapies largely rely on the passive delivery of APIs (whether drugs or genetic material) or the active targeting of the encapsulated moiety itself. Instead, ligands, especially antibodies and synthetic peptides, could be conjugated to the surface of NP formulations to facilitate active targeting. These sequences could be derived from a multitude of EMT-related axis and phenotypes. For example, a ligand utilizing a passive targeting approach could target an overexpressed protein characteristic of fibrosis, such as fibronectin (Torikata et al., 1985), while a ligand inhibitor utilizing a more active targeting approach could instead target a receptor along the EMT pathway itself, such as TGF-β family receptors, tyrosine kinase receptors, and numerous integrins (Stone et al., 2016). Similar targeting strategies have already demonstrated success in anti-cancer NP formulations, many of which could easily be translated to target the EMT pathway (Shargh et al., 2015; Jeong et al., 2018).

Additionally, more focus could be placed on developing combination therapies. Drug molecules (anti-inflammatory, fibrotic, and oxidation), genetic material (siRNA), and artificial proteins (antibodies and peptides) could all be combined utilizing a robust NP platform. For example, protein-NP conjugates have demonstrated the ability to significantly increase the potential efficacy of existing therapeutic formulations (Jeong et al., 2020). This could lead to the development of more adaptable therapies capable of overcoming the efficacy and toxicity limitations of other, more conventional therapies. This has become even more important in recent years as increased awareness

of genetic variation in patients, including for pulmonary fibrosis, has brought into question the past rationale of a "one-size-fits-all" therapeutic (Kaur et al., 2017). Again, this approach has already found promising success in the development of anti-cancer formulations (Nam et al., 2019).

Despite the many promising advantages of nanomedicines, the treatment of EMT-mediated pulmonary fibrosis provides unique challenges for NP therapeutics. For one, NPs have been linked to the facilitation of EMT and lung fibrosis themselves. Though most frequently caused by industrial pollutants such as cerium oxide, silver, and silica NPs (Ma et al., 2017; Wang et al., 2017; Gliga et al., 2018), the elevated infiltration and retention of other NP formulations can still toe the line between beneficial and toxic and must be carefully analyzed (Fattal et al., 2014). Inorganic NPs in particular run the risk of becoming acutely toxic to local tissues, but polymeric NPs and even liposomes and SLNPs can still lead to lung damage if not safely absorbed and eventually cleared.

The ability of NP formulations to be properly administered using common pulmonary delivery methods, such as through nebulizers, pressurized metered-dose inhalers, and dry powder inhales (DPIs), can also be a limiting factor (Dailey et al., 2003b). Aggregation, especially, becomes a frequent concern when NPs are aerosolized through fine pores. Liposomes, micelles, and other spontaneously assembled NPs, which rely on maintaining specific concentrations or solvent conditions, can also become unstable when subjected to the aerosolization process. Despite this, however, numerous NP formulations have already demonstrated successful aerosolization, including liposomes (Zaru et al., 2009; Arora et al., 2010), polymeric NPs (Doan and Oliver, 2009; Ungaro et al., 2009; Beck-Broichsitter et al., 2010), and even Cu/Zn NPs (Langenback et al., 1999). This indicates that the aerosolization of these novel formulations is certainly a surmountable obstacle worth investigating.

Conclusion

In conclusion, the IPF has substantial morbidity and mortality for which no curative treatments are available. In this chapter, we have reviewed the prevailing epithelial injury hypothesis. In this model, epithelial injury-induced EMT drives the expansion of pathogenic myofibroblast populations. Myofibroblasts are principal components of extracellular matrix production that result in airspace loss and mortality. We review the substantial mechanistic understanding of EMT, and the recent work demonstrating that reversible histone modifications are an important process regulating gene expression programs controlling mesenchymal transition. This mechanistic work has focused on the central role of BRD4 in mediating EMT and myofibroblast transition and initial preclinical work has provided evidence of efficacy in multiple models of fibrosis, including bleomycin, radiation, TGF-β induced and viral induced remodeling. Advancing epigenetic regulators as therapeutics will be challenging. Additionally, we review the current state of nanomedicine with a focus on formulations that hold promise for aerosol delivery and EMT-targeting, including liposomes, polymeric NPs, inorganic NPs, and exosomes. These agents can provide a host of benefits, including controlled release for enhanced therapeutic efficacy, reduced systemic toxicity, and intrinsic inhibitory effects against common EMT biomarkers. Many of these NP formulations have already demonstrated success as aerosolized formulations in both the lab and clinic. With further development, NP-based approaches for pulmonary delivery offer the substantial promise to modify epigenetic regulators of EMT and advance treatments for IPF further than current strategies allow. This could open up a new field of study centered on the EMT pathway which combines new physiological understandings and state-of-the-art advances in nanoparticle delivery.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

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