Animal Models of COPD: What Do They Tell Us?

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a major cause of global mortality and morbidity but current treatments are poorly effective. This is because the underlying mechanisms that drive the development and progression of chronic obstructive pulmonary disease (COPD) are incompletely understood. They differ depending on exposure to various causative agents like cigarette smoke or air pollution. Animal models of disease provide a valuable, ethically viable and economic platform with which to examine these mechanisms and identify biomarkers that may be therapeutic targets that would facilitate the development of improved treatments. Here we review the different established animal models of COPD and the various aspects of disease pathophysiology that have been successfully recapitulated in these models including; chronic lung inflammation, airway remodeling, emphysema and impaired lung function. Furthermore, some of the mechanistic features, and thus biomarkers and therapeutic targets of COPD identified in animal models have been outlined. These include recent studies of oxidative stress, mast cell proteases, circadian rhythm, epigenetic changes and microRNAs. Most therapeutics currently in clinical trials originated from studies on animal models, yet there is still a lack of therapies that halt the progression of COPD once it is established, and none that reverse its disease features. Some of the existing therapies that suppress some disease symptoms that were identified in animal models and successfully applied to the clinical setting have been outlined. Further studies of representative animal models of human COPD have the strong potential to identify new and effective therapeutic approaches for COPD.

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SUMMARY AT A GLANCE

Here we review the current animal models that are widely used to investigate the pathogenesis of COPD. Recent studies that have revealed new mechanisms and potential treatments using these models are highlighted.

Keywords: (5 keywords in alphabetical order) animal models, COPD, disease mechanisms, therapeutic targets, therapies.

Abbreviations:

-/-	homozygous knockout
AHR	airway hyperresponsiveness
BALF	bronchoalveolar lavage fluid
CLOCK	Circadian locomotor output cycles kaput
COHb	Carboxyhemoglobin
COPD	Chronic obstructive pulmonary disease
CORT	Corticosterone
CRY	Cryptochrome
CS	Cigarette smoke
DNMT	DNA methyltransferase
EGCG	epigallocatechin 3-gallate
FEV_1	Forced expiratory volume in one second
FOXO3	Forkhead box O3
HDAC	Histone deacetylase
IFN	Interferon
IL	Interleukin
LT	Leukotriene

LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCs	Mast cells
miRNA	MicroRNA
mMCP	Mouse mast cell protease
MMP	Matrix metalloproteinases
MMP-1	Collagenase-1
NAC	N-acetylcysteine
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NF-kB	Nuclear Factor-kappaB
NO	Nitric oxide
PDE	Phosphodiesterases
PER	Period
PI3K	Phosphoinositide 3-kinase
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive Oxygen Species
SIRT1	Sirtuin1
TNFα	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third leading cause of chronic morbidity and death worldwide and its prevalence is continuing to rise.[1] Recent estimates suggest that its prevalence may reach 9–10% in adults over the age 40 and has a global cost

of >$2 trillion.[2] Cigarette smoke (CS) is the leading cause of COPD in Western societies although exposure to air pollution and occupational exposures to dusts and fumes are also risk factors. In developing countries, exposure to biomass fuels used for cooking is a major precipitant.[3] Only 25% of smokers develop COPD and genetic predisposition likely plays a role.

COPD is an inflammatory lung syndrome that is characterised by the limitation of expiratory airflow that deteriorates over time. Although heterogeneous, it is characterised by the common pathologies of chronic bronchitis and/or emphysema that lead to reduced lung function. These pathological features are associated with frequent infection-induced exacerbations of chronic airflow limitation and breathlessness.[4] Chronic inflammation is characterised by increased levels of neutrophils, macrophages and $CD8^+$ T cells throughout the airways that together with the injured airway epithelium release a variety of inflammatory mediators including leukotrienes, interleukin (IL)-8 (CXCL8), Tumour necrosis factor alpha (TNFα) and reactive oxygen species (ROS).[5-7] These events promote further inflammation forming a feedback loop that promotes chronic inflammation. Once induced the patients' condition progressively deteriorates with worsening inflammation, emphysema, declining lung function and increased breathlessness. Importantly, the mechanisms that drive the induction and progression of chronic inflammation, emphysema and altered lung function are not well understood and this has hampered the development of effective treatments for COPD. There is a strong systemic component to the disease with cachexia and cardiovascular involvement and it is emerging that there is lung-gut crosstalk that is a contributing factor.[8] These factors need to be taken into account when developing new therapies.

Current treatments for COPD use glucocorticoids and bronchodilators to suppress the symptoms of disease but have limited clinical efficacy. There are no treatments that effectively halt the induction or progression of COPD. Increasing our understanding of the

molecular pathways and responses that contribute to the initiation and progression of disease features will facilitate the development of novel therapies. Human studies are complicated by individual genetic background, environment, smoking habits, the gradual long-term progression of disease and limitations in the samples that can be collected. The development of animal models of COPD that accurately recapitulate the critical features of the human disease in a short time-frame will be useful in efforts to develop effective treatments. Here we summarise the available animal models that recapitulate airway disease obtained from exposure to CS, air pollution and ozone. We then review what we have learnt so far from these models in regard to underlying disease mechanisms, biomarker discovery and therapeutic development.

ANIMAL MODELS

The interrogation of animal models of COPD plays an important role in determining the mechanisms leading to the development and progression of COPD as they enable the analysis of pathways involving integrated whole body responses in a reasonable timeframe, and the use of in-bred strains removes issues of genetic variability. Animals that accurately display the hallmark features of the disease are key in the drug discovery process as they facilitate the testing of novel therapeutics. There are some issues with differences in respiratory physiology between humans and animals that need to be taken into account, such as the reduced numbers of bronchial branches in mice.

The ideal model would possess the hallmark features of the human disease, be induced by the same aetiological agent and be reasonably short-term to allow rapid progress.

CS-INDUCED ANIMAL MODELS

105 The use of tobacco, primarily CS, causes >5 million deaths/year, and CS is the main risk
106 factor for the development of COPD. CS contains >7,000 chemicals, of which >250 are
107 hazardous and >60 are carcinogenic, 20 carcinogens cause lung tumours in laboratory
108 animals or humans and are, therefore, likely to be involved in the induction of lung cancer in
109 humans.[9] Collectively these factors induce inflammation (inflammatory cell influx and
110 increases in cytokines and chemokines in the airway and parenchyma), mucus hypersecretion
111 (goblet cell metaplasia), airway remodelling (smooth muscle deposition, matrix deposition,
112 and fibrosis), emphysema and impair lung function. These are the major features of COPD
113 that restrict the life quality of the patients. Nevertheless animal models of CS-induced disease
114 have only recently been developed and have used Guinea pigs, rats and mice (Table 1). Mice
115 have become the most popular because of cost, ease of housing, and the availability of a
116 plethora of molecular and immunological reagents and genetically modified strains.

117

118 **Guinea pigs**

119 Guinea pig models of CS-induced COPD develop disease features such mucus-secreting
120 goblet cell metaplasia in the airways, small airway remodelling, inflammation, altered lung
121 function and emphysema.[10-12] The development of mucus hyper-secretion and emphysema is
122 more prominent than in other models. Serum markers such as cotinine or blood
123 carboxyhemoglobin (COHb) are useful for confirming the relative amount of smoke
124 exposure. Heck *et al*., showed levels of COHb in the blood of ~15–20% for an acute model
125 and ~5% for a chronic model,[13] which is similar to that detected in humans. Their main
126 disadvantages are high cost and the lack of molecular and immunological tools such as
127 antibodies and factor deficient and transgenic strains for performing molecular studies, and
128 lung function is not generally assessed.

129

Rats

Rats are becoming more prominent in studies of CS exposure and COPD. A wealth of information including genetic mapping has been gathered that allows the development of genetically modified strains of rats, although this is not routine as it is for mice. Rats and mice share ~90% of their genes with humans, and many of the physiological pathways and processes can be related clinically. Several rat models recapitulate some features of human COPD. Side-stream whole body CS exposure is the method of choice as the relatively large size of rats reduces the viability of large-scale mainstream nose-only smoke exposure methods. A 30-week protocol of side-stream CS exposure induced parenchymal destruction and altered lung function with increased tissue dampening and respiratory system resistance and compliance.[14] The extensive time frames involved in these models reduces viability and progress. To address this a 12-week side-stream CS exposure protocol coupled with repetitive bacterial infections to the airways was developed to induce COPD.[15] Several features of COPD were observed including pulmonary hypertension, and airway remodelling, and reduced alveolar number and pulmonary function. The similarities in the COPD features observed and their relevance to the clinical setting may allow for more comprehensive studies of the mechanisms underpinning the initiation and progression of COPD in rats, and facilitate the development of effective therapies.

Rat specific nose-only exposure systems have been developed, however, most studies have not been aimed at elucidating CS-induced COPD and its mechanisms, but rather the short-term effects of exposure. Stinn, *et al*., used a two year nose-only smoke exposure regime to show that exposure to diesel exhaust but not sidestream CS resulted in lung pathophysiology in terms of lung weight, cell proliferation, inflammation and tumorigenesis.[16] van Miert, *et al*., used an acute model with 2x1hr exposures of diluted mainstream CS to deliver varying concentrations of particulate matter to show dose

dependent increases in lung epithelial hyperpermeability.[17] A similar study over 13 weeks showed that mainstream CS exposure upregulated nicotinic acetylcholine receptors in the brain.[18]

Mice

The majority of recent models of CS-induced models use mice. They offer the advantages of low cost and ease of housing, the availability of extensive genomic data, a wide array of molecular and immunological tools and the potential for nose-only exposures. Importantly, a plethora of factor-deficient or over-expressing mouse strains are available or can be easily and rapidly produced with new CRISPR technology. They are valuable in assessing the pathogenesis of COPD. These models and strains can be used to assess the impact of short-term CS or other exposures (4 days to 4 weeks) or the processes involved in the generation of COPD features (8 weeks to 6 months). Many of the characteristic features of human COPD, such as chronic lung inflammation, pulmonary hypertension, airway remodelling, emphysema, and impaired lung function, can be generated in CS exposed mice.[19-21] CS exposure can be combined with mouse models of respiratory infections to study the impact of infections on pathogenesis and their roles in exacerbations.[19-25] In one model, mice were exposed to side-stream CS for 36 weeks that induced various hallmarks of human COPD, including increased airway resistance and respiratory system elastance.[26] However, this is a long model and shorter models that have the hallmark features of disease would enable rapid progression of our understanding of pathogenesis and development of new treatments. We have recently developed a novel short-term mouse model of CS-induced experimental COPD, using nose-only exposure that generates the major features of the human disease in 8 weeks.[22,23,27,28] Mice are exposed to the CS of 12 cigarettes for 75 minutes per day, twice per day for 5 days per week. Exposure consists of normal air interspersed with puffs of CS and is

representative of a pack-a-day smoker.[29] Cotinine levels found in these models are around 100ng/ml immediately after exposure, which is similar to that found in patients saliva (smokers approx. 113ng/ml).[30] This regime results in acute and chronic airway and parenchymal inflammation, goblet cell metaplasia, airway remodelling, emphysema and impaired lung function,[22,23,27,28,31] i.e. the major hallmarks of human COPD. Disease features progress over 8-12 weeks of CS exposure.[22] Like in humans; features are not suppressed by corticosteroid treatment and do not resolve over time, mice with experimental COPD are more susceptible to viral (influenza) and bacterial (*Streptococcus pneumoniae*) infections, and have systemic involvement with skeletal muscle loss, and effects on the reproductive tract.[22,32,33]

AIR POLLUTION MODELS

Air pollution-induced models exist for Guinea pig, rat, and mouse,[34] and typically use particulate matter (e.g. urban particulate matter),[35] gases (e.g. ozone),[36-38] or a combination of the two (e.g. freshly generated diesel exhaust).[39] They are employed to understand toxicological effects of pollution on the lung and the impacts on the development of allergic airways disease.[39-41] Innate immune activation[42] and induction of oxidative stress[43] are frequently observed, which are directly relevant to the development and exacerbations of COPD. Typical outcome measures include lung inflammation, goblet cell metaplasia, and lung function alterations (including responsiveness to methacholine). These methods can be applied to investigate how air pollution contributes to a COPD-like pathology in animals. Nevertheless few studies have been performed. Those that have been undertaken along with the clinical epidemiology data that suggests air pollution is a contributing factor to the development[44] and exacerbation of COPD.[45] Acute (24 hours) and chronic (6 weeks) ozone exposure models are used to investigate lung inflammation and remodelling processes in

mice. Ozone initiates intracellular oxidative stress through the formation of ozonide and hydrogen peroxide,[46] which induces a COPD-like phenotype in 6 weeks.[38] Ozone exposure in mice induces airway inflammation, airway hyperresponsiveness (AHR)[47] and lung destruction similar to that observed in patients with COPD.[48] These effects are in part reversible by treatment with the antioxidant N-acetylcysteine (NAC)[49,50] and the MIF inhibitor (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1).[51] The effects of ozone exposure are associated with mitochondrial dysfunction and reflected by decreased mitochondrial membrane potential ($\Delta\Psi m$), increased mitochondrial oxidative stress, and reduced mitochondrial complex I, III, and V expression in the lung. Reversal of mitochondrial dysfunction by the mitochondria-targeted antioxidant MitoQ reduced inflammation and AHR.[38] Furthermore, chronic ozone exposure induces a steroid insensitive phenotype, where inflammation and remodelling are not prevented by dexamethasone pre-treatment in chronically exposed mice.[51] Animal models of exposure to air pollution exposure alone or in combination with CS exposure will be valuable in exploring how this environmental risk factor impacts the development and exacerbations of COPD.

OTHER MODELS

A variety of other models exist that can be used for specific purposes. The use of factors that are known to play specific pathogenic roles in COPD, such as elastase and lipopolysaccharide/endotoxin can be used to induce specific features.[52]

Transgenic and gene deficient mice have been used to investigate the roles of specific factors in COPD pathogenesis. Transgenic mice that overexpress a particular gene product have been used to demonstrate that some factors are involved in promoting COPD features,

usually alveolar enlargement/emphysema (Table 2). For example, the constitutive overexpression of collagenase-1 (MMP-1) resulted in alveolar enlargement[53]. A limitation of the study was that the expression of collagenase-1 was not inducible, although it was lung specific in some lines; furthermore there was no detection of expression during early development. The use of inducible transgenic factors enables the elucidation of their function in adulthood, which excludes any effects on development. Overexpression of the Th2 and Th1 cytokines IL-13[26] and interferon (IFN)-γ[54] are two important examples of the use of inducible transgenes. Their temporal overexpression leads to emphysema. Overexpression of IL-13 resulted in inflammation and lung destruction in a MMP-9, MMP-12 dependent manner[55]. Overexpression of IFN-γ resulted in inflammation and proteinase-dependent emphysema.

Gene deficient mice have been used to demonstrate complex roles for TGF-β in COPD. TGF-β deficient ($^{-/-}$) mice have high mortality within 1 month of birth due to the chronic inflammation, hence limiting their utility in COPD studies. However, Avb$^{-/-}$ mice are deficient in β_6-integrin, and fail to activate TGF-β within the lung. These mice develop emphysema over time with excess MMP-12 production and macrophage rich inflammation.[56] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine that induces both inflammation and apoptosis.[57] We recently showed that total TRAIL deficient mice (*Tnfsf10*$^{-/-}$) chronically exposed to CS had reduced inflammation in both the bronchoalveolar lavage fluid (BALF) and parenchymal tissue, and suppressed expression of pro-inflammatory cytokines (TNFα, IL-33), chemokines (CXCL1, -3, CCL4, -22) and other COPD-related factors (MMP-12, SAA3, active NF-kB p65)[31]. These reductions in inflammation were accompanied by decreased emphysema-like alveolar enlargement which all combined to improve lung function outcomes such as lung compliance. Most importantly therapeutic neutralisation of TRAIL induced a reduction in pulmonary inflammation, emphysema-like

alveolar enlargement, and small airway changes[31]. This prompted us to examine human tissues, in which we observed TRAIL and its receptors were also elevated in bronchial brushings and parenchyma of COPD patients also. Thus targeting TRAIL may be a potential new therapeutic approach in humans.

PATHOGENIC MECHANISMS IDENTIFIED IN ANIMAL MODELS

Oxidative Stress

The causative risk factors CS and environmental pollutants induce the generation of excessive oxidative stress from inflammatory cells, which plays an important pathogenic role in COPD.[58-60] Increases in ROS, have been identified in mice in endothelial cells in response to CS that is mediated by the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[61] Increases in mitochondria-specific ROS has been shown to accompany lung inflammation and AHR with ozone exposure of mice.[38] This was associated with mitochondrial dysfunction. The mitochondria targeted anti-oxidant reversed these features.

Short-term CS exposure (4 days) of mice induces systemic oxidative stress, indicated by elevated levels of ROS, lipid peroxidation and superoxide dismutase, in the heart, liver and kidney.[62] These data are supported by another short-term study, where they found that both short-term (6 weeks) and long-term (16 weeks) CS exposure cause increases in arterial pressure and a marked decreases in nitric oxide (NO). They also reported a correlation between NO and changes in the structural and mechanical status of arterial walls in response to CS.[63]

FOXO3 is a transcription factor that protects against oxidative stress by promoting the transcription of antioxidants such as catalase.[64,65] Activation of the phosphatidyl-inositol 3-

kinase (PI3K) signaling pathway leads to phosphorylation of FOXO proteins by the kinase AKT.[66] Phosphorylated FOXO3 then translocates from the nucleus to the cytosol, where it becomes ubiquitinated, leading to its degradation by the proteasome.[67] In the absence of external growth signals, the PI3K–AKT axis is inactive, and unphosphorylated FOXO3 binds to its DNA consensus sequence to promote target gene transcription. A novel role in regulating lung inflammation and COPD pathogenesis was identified in CS-exposed FOXO3$^{-/-}$ mice. These mice had reduced antioxidant gene expression in the lungs that was associated with exaggerated inflammatory responses and increased alveolar enlargement compared to CS-exposed wild-type mice.[68] Furthermore, FOXO3 has been shown to act as a fine-tuner of NF-kB activity, and also modulates CS-induced lung inflammatory responses and COPD in this way.[68]

Sirtuin1 (SIRT1) is a NAD^+-dependent deacetylase and has been shown to be decreased in the lungs of rodents exposed to CS.[69,70] SIRT1 deacetylates FOXO3 through direct protein-protein interaction. This increases the activity of FOXO3 thereby tipping the balance to cellular survival in response to oxidative and carbonyl stress. A study of lung senescence using CS- and elastase-induced alveolar enlargement in mice, demonstrated that SIRT1 protected against emphysema and a decline in lung function through a FOXO3-dependent anti-senescent mechanism.[71] A potential therapy is resveratrol, which has been demonstrated to activate SIRT1.[72] Recent studies have suggested that resveratrol attenuates oxidative stress-induced damage to the lung, as well as decreasing the levels of NF-kB activity and increasing HO-1 expression.[73]

Circadian rhythm

An internal molecular clock exists that drives intrinsic circadian rhythms of physiology and behaviour. It is defined as a transcriptional and translational feedback loop oscillator.

Emerging evidence suggests that the molecular clock is intimately associated with responses to environmental stimuli. The positive inductive elements include the transcription factors CLOCK and BMAL1, which form a heterodimer and initiate gene transcription including of Period (PER) and Cryptochrome (CRY).[74,75] Conversely, negative feedback is promoted by PER:CRY heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK:BMAL1 complex.[76,77] BMAL1 may also have a role in oxidative stress-induced inflammation.[78-80] Patients with COPD display abnormal circadian rhythms in their lung function including variations in inspiratory capacity (IC), forced expiratory volume in 1 second (FEV_1) forced vital capacity and peak inspiratory flow.[81-83] Hence, CS exposure may affect circadian clock function in the lung leading to inflammatory and injurious responses. SIRT1 affects clock function by binding to CLOCK:BMAL1 complexes and deacetylating BMAL1 and PER2 proteins.[84,85] CS exposure of mice alters the expression of the clock gene and reduces locomotor activity by disrupting the central and peripheral clocks, and increasing lung inflammation.[84] Furthermore, BMAL1 has been shown to be acetylated and degraded in mouse lungs in a CS exposed model, mechanistically linking this factor to the CS-induced reduction of SIRT1.[84]

Further studies in this area have revealed that two stress hormones, corticosterone (CORT), an adrenal steroid that plays a substantial role in stress and anti-inflammatory responses, and serotonin (5-hydroxytryptamine; 5HT), a neurohormone that contributes to sleep/wake regulation, are altered in the plasma of CS-exposed mice. This suggests that CS exposure affects the rhythms of stress hormone secretion, which may have subsequent detrimental effects on cognitive function, depression-like behaviour, mood/anxiety and sleep quality in smokers and COPD patients.[86]

Understanding the contributions of the molecular clock function to the physiology and function of the lung, particularly in response to tobacco, may inform the schedule of treatment in the management of COPD.

Epigenetics

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that does not involve changes in DNA sequence. These changes influence gene expression and can result from three mechanisms; DNA methylation, histone modification and non-coding RNA interference. Epigenetic modifications have been linked to a number of diseases such as asthma and COPD (Table 3).

In one study mice exposed to CS had global DNA methylation patterns that were altered prior to changes in histopathology,[87] suggesting that these changes may precede disease development and could therefore be a potential biomarker. In addition DNA methylation may prime for a second insult, such as infection, that may increase susceptibility to COPD. DNA methyltransferases (DNMTs) are regulatory enzymes that are responsible for DNA methylation that silences gene transcription. The use of the DNMT inhibitor; epigallocatechin 3-gallate (EGCG), found in green tea, has been demonstrated to abrogate the alveolar enlargement and goblet cell hyperplasia in rats exposed to CS.[88] In a mouse model, EGCG, has been shown to decrease inflammatory cell number in the lavage fluid but had no effects in halting the development of alveolar enlargement.[89]

In regard to histone modifications, a mass spectrometry analysis approach in CS-exposed mouse lungs identified potential novel histone marks including acetylation, as well as mono- and di-methylation of specific lysine and arginine residues of histones H3 and H4. Furthermore, histones H3K27me1 and H3K27me2 were only detected in the CS-exposed

group suggesting that gene transcriptional regulation was affected.[90] A rat model of CS exposure was interrogated to show increased acetyl-H4 and phosphorylation of a specific histone 3 serine residue, H3S10p, compared to non-exposed groups both of which were thought to trigger inflammatory gene transcription.[91] Histone deacetylase (HDAC) activity and in particular HDAC-2, is reduced in CS-exposed mice. This was associated with a reduction in glucocorticoid function, which was restored when mice were treated with the PI3K inhibitor theophylline.[92,93] Decreased HDAC2 activity and expression was also detected in the lung tissue of CS-exposed rats.[91] From a therapeutic perspective it has been shown that low levels of theophylline can restore HDAC2 activity and therefore GR function in macrophages.[94]

MicroRNAs (miRNAs) are noncoding sequences that post-transcriptionally regulate messenger RNAs (mRNAs).[24,95] In this way miRNAs contribute to the basic regulatory mechanisms of gene translation in cells including those that control inflammation. Thus, dysregulation of miRNAs, resulting in aberrant gene expression may play important roles in COPD pathogenesis. Analysis of miRNA in the lungs of rats exposed to CS extract (CSE) showed that most were down-regulated. Out of 484 miRNA analysed, 126 were downregulated including Let-7c, miR-34c and miR-222.[96] In contrast, in the lungs of mice exposed to CSE only 15 were downregulated including, Let-7a, -7b and -7f, miR-124a and -122a.[97] Several of these miRNAs, such as miR-30, -146, -132 and -155, have roles in the activation of the NF-κB pathway, and their downregulation would increase inflammatory responses in the lungs and may contribute to COPD pathogenesis. In our CS-induced model that was followed by infection with *Haeomphilus influenzae*, the inhibition of miR-328 with an antagomir reduced infection without increasing inflammation, inhibited excessive mucus production and improved lung function.[24] This was likely the result of augmented macrophage phagocytosis.

With evidence of aberrant epigenetic alterations occurring in response to CS and in the pathogenesis of COPD, targeted inhibitors and/or activators may restore the balance of regulatory enzymes and miRNAs. This would reduce pro-inflammatory gene transcription, and disease pathogenesis.

Mast cell proteases

Mast cells (MCs) have potent pro-inflammatory properties. Upon activation, they release newly formed and preformed mediators from their granules. Around 50% of human MCs consist of 16 neutral proteases that have various overlapping and unique roles in acute inflammation, blood coagulation and in protecting against infection.[98] MC factors have highly potent effects and the influx and activation of small numbers of these cells can have a massive impact inducing life-threatening anaphylaxis. Genomewide association studies have not found a link between mast cell proteases and COPD, however this is likely because of their overlapping activities. Animal models can be used to delineate their roles that cannot be studied in humans. We have used our mouse model of CS-induced experimental COPD and factor deficient mouse strains to show that MC proteases play important roles in pathogenesis. The murine orthologs of human mast cell tryptase-β and tryptase-γ are mouse mast cell protease (mMCP)-6 and Prss31, respectively. When exposed to CS mMCP-6$^{-/-}$ mice had an equivalent elevated influx of mast cells into the airways as wild-type mice, but had reduced macrophage and neutrophil influx and parenchymal inflammation, and were protected against airway remodeling, emphysema and impaired lung function.[22,28] Similarly Prss31$^{-/-}$ mice were also protected against airway and lung inflammation, airway remodeling and a measure of impaired lung function. These studies identify mast cell proteases as pathogenic factors and potential therapeutic targets in COPD. The development of inhibitors could suppress their activity and may have therapeutic benefit for patients.

Other mechanisms

We describe a selection of mechanisms of interest identified using animal models that are likely involved in COPD pathogenesis. Many other studies have been performed, mechanisms and therapeutic targets identified and drugs trialed. These models can also be used to study other features of COPD including systemic effects, pulmonary and gut cross talk and the roles of microbiomes.[8,99] Genomic and epigenetic profiling and next generation sequencing would provide valuable libraries of data that could be interrogated to find broader disease pathways that could also be targeted.[100]

BIOMARKER DISCOVERY

In COPD the mechanisms that drive and mark the development and progression of disease remain poorly understood. As a result there are currently no reliable biomarkers of disease that can be used for non-invasive screening. Long-term monitoring of declines in FEV_1 has been used to identify risk factors and gauge the efficacy of potential therapies, however this approach is slow and expensive. The identification of defined biomarkers would be valuable in the investigation of the natural history of COPD, the development of rapid and accurate diagnostic techniques, as well as provide a means for identifying those most at risk of disease development or progression. They could also serve as markers for the evaluation of efficacy and appropriate dosage of treatment in relatively short-term studies. The use of whole genome arrays or proteomics to identify biomarkers of disease has increased recently. A proteomic analysis of lung tissue from CS-exposed rats found two antioxidants, thioredoxin and peroxiredoxin-6 were increased whereas enolase, a multifunctional protein with roles in glycolysis, tolerance of hypoxia and allergic responses was decreased.[101] Moreover, another

similar model showed that in lung tissue the receptor for advanced glycation endproducts (RAGE), calcyclin and thioredoxin were all increased.[102] A benefit of discovering biomarkers in animal models is that the nature of their involvement can be assessed using interventions or genetic modifications (deletion or over-expression) and potential for therapeutic intervention can be studied. Nevertheless these findings are limited until they have been validated in clinical samples.

THERAPEUTIC DEVELOPMENT AND TESTING

Current treatments for COPD are poorly effective at inhibiting chronic inflammation, and do not reverse pathology or modify the factors that initiate and lead to disease progression in the long term. Therefore, it is clear that there is a need to develop new therapies to prevent the initiation and the progression of COPD, and an effective option is through the use of animal models that accurately reflect the physiopathology of the disease. Indeed, many COPD drugs that are currently in clinical development, such as inhibitors of inflammatory mediators, oxidative stress, kinases, phosphodiesterases (PDE) and proteinases, were originally identified in studies using animal models.

Various inhibitors of inflammatory mediators are being developed and tested for the treatment of COPD. Inhibitors of TRAIL, leukotriene B4 (LTB4), TNF-α, IL-1, IL-8, and epidermal growth factor have shown strong indications when used in animal models, however the translation into the clinic has been disappointing with little to no sign of improved disease outcome in patients.[103] For example, studies exposing TNF-α receptor knockout mice to CS resulted in reduced inflammatory cells in lavage fluid and attenuated alveolar enlargement compared to wild-type mice.[104] These findings were supported by another knockout mouse study where both TNF-α receptors were shown to contribute to the

447 pathogenesis of murine COPD, with TNF-α receptor-2 being the most active receptor in the
448 development of inflammation, emphysema and systemic weight loss.[105] However, as
449 occurred with asthma, where mouse studies were not interpreted properly or transferred
450 effectively into clinical studies, it is likely that selected groups or phenotypes of patients may
451 respond better to specific treatments.

452 Anti-oxidants, particularly those that target specific processes in COPD have shown
453 some promise. For example, in addition to reserveratrol, the antioxidant enzyme Gpx-1 has
454 been shown to protect against lung inflammation and CS-induced emphysema in mice, and a
455 Gpx mimetic also reduced lung inflammation when administered both prophylactically and
456 therapeutically.[106]

457 Studies of animal models of CS-induced airway inflammation support the potential
458 therapeutic use of kinase inhibitors, such as those that inhibit p38 mitogen-activated protein
459 kinase (MAPK) and PI3K, in COPD.[107] MAPKs plays key roles in chronic inflammation,[108]
460 and the p38 MAPK pathway is activated by cellular stress and regulates the expression of a
461 wide variety of inflammatory cytokines and remodeling factors including IL-8, TNF-α and
462 MMPs.[109] Small molecule inhibitors of p38 MAPK have been developed, such as SB239063
463 and have been shown to have anti-inflammatory and -remodelling effects.[110] SB239063
464 reduces neutrophil infiltration and the concentrations of IL-6 and MMP-9 in the BALF of rats
465 after endotoxin inhalation, suggesting its potential as an anti-inflammatory agent in COPD.[111]
466 PI3Ks play a role in controlling a wide variety of intracellular signaling pathways. Recent
467 studies suggested that numerous components of the PI3K pathway play a crucial role in the
468 expression and activation of inflammatory mediators, inflammatory cell recruitment, immune
469 cell function and airway remodeling as well as corticosteroid insensitivity in chronic
470 inflammatory respiratory disease such as asthma.[112] It is emerging that PI3K also plays a
471 pivotal role in the pathogenesis of COPD. It is important in the activation of macrophage and

neutrophils, which are key players in COPD inflammation.[113] We have shown that influenza infection is more severe in CS-induced experimental COPD that is associated with increased PI3K activity.[23] Treatment with the PI3K inhibitor LY294002 suppresses this activity, and enhances anti-viral responses that attenuate the infection leading to improved lung function.

The PDE4 inhibitor Roflumilast, a licensed treatment for severe COPD, was originally identified as a potential therapeutic in acute and chronic murine models of CS-exposure.[114] PDE4 degrades the anti-inflammatory cyclic adenosine monophosphate and its inhibition in mice has been shown to have numerous protective effects including reversing the loss of lung desmosine, a breakdown product of elastin, reducing neutrophil and macrophage influx, increasing the anti-inflammatory cytokine IL-10, and improving emphysema.[114]

Serine-, metallo- and cysteine proteinases are the primary proteinases implicated in the development of COPD.[115] In studies aimed at preventing the destruction of alveolar walls by proteolysis, and ultimately the development of emphysema, inhibitors of various proteinases have been trialed in animal models with varying levels of success. Guinea pigs were subjected to acute CS-exposure to induce increases in lavage neutrophils, desmosine, and hydroxyproline, and elastine and collagen breakdown. Subsequent treatments with the neutrophil elastase inhibitor ZD0892, reduced all of these factors, highlighting proteinase inhibitors as promising therapeutics for further studies.[116]

Collectively studies show that animal models of COPD are valuable tools that further our understanding of the pathogenic aspects of the disease and can be used to identify novel therapeutic targets and develop and test new therapies. The inherent heterogeneity of the disease can also be reproduced and studied in animal models that are induced using different combinations or doses of induction agents. In such studies it is important to choose the model according to whether the research is focused on pathogenesis, diagnosis or treatment.

CONCLUSIONS

The current therapies for COPD are poorly effective because we do not understand how the disease develops and progresses. Animal models have been established that develop the hallmark features of human COPD. The use of mice and CS exposure are the most common and representative of the causal factors, respectively. They develop pulmonary and systemic inflammation, small airway remodeling, emphysema and impaired lung function, some within the relatively short time frames of 8 weeks. These models are used to find factors that may be important in the pathogenesis and progression of COPD that identifies potential new therapeutic targets that are common between animal models and human disease. They can be also be used to discover biomarkers and test new treatments. Whole genome studies are now easily and economically achievable opening up this avenue for analysis of new representative animal models. Advancements in protein analysis have also allowed us to assess protein changes and post-translational modifications that may be important drivers of COPD. The interrogation of animal models has identified specific roles for inflammatory factors and immune cells. Numerous mechanisms associated with COPD have been identified such as, oxidative stress, circadian rhythms and epigenetic changes. These studies have opened up avenues for therapeutic development that target these mechanisms. Studies have aimed to develop more effective therapies, which can be tailored to the disease profile of patients leading to the future of "personalised medicine". Since there are currently no effective therapies that halt the progression of disease, development of biomarkers for early detection of disease would dramatically improve the therapeutic outcome of these novel therapies. The use of a short-term animal model of COPD disease features, allows the identification of biomarkers, the possible targets of novel therapies and to test and assess the effects of novel therapeutic agents.

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Species	Inflammation	Mucus hyper-secretion	Small airway remodelling	Emphysema	Impaired lung function	Smoking cessation	Steroid resistance	Ref
Guinea Pig	+			+				117
	+	+	+	+	+	+		11,116,118-122
	+		+					123

	+	+	+	+				123-125
Rat	+			+				126,127
	+			+				128,129
	+		+	+				130
	+	+		+	+		+	131
		+						132
Mouse	+			+	+			21,133
	+		+	+				104,134
	+			+	+			135
	+		+	+				136-138
	+			+		+		89
	+			+				139
	+			+		+		140
	+			+	+			141
	+	+	+	+	+	+	+	22,23,27,28,31

914

915 **Table 1.** CS-induced animal models of COPD**.** Animal models that display features of
916 chronic obstructive pulmonary disease (indicated by +). Only the last model by Beckett *et al*.,
917 used in other studies, displays the important aspects of disease phenotype, including steroid
918 insensitivity.

919

	Mutation	Phenotype	Ref
Transgenic mice			
Collagenase-1	MMP-1	Alveolar enlargement	53
Interleukin-13	IL-13	MMP-9, MMP-12 dependent lung destruction, airway inflammation, and airway remodelling.	26,55
Interferon (IFN)-γ	IFN-γ	Inflammation and proteinase-dependent emphysema	54
ApolipoproteinA-1 (ApoA1)	Doxycycline Induced ApoA1	Protection against lung inflammation, oxidative stress, apoptosis and metalloprotease after exposure to CS	142
"Knock-out" mice			
Transforming growth factor beta (TGF-β)	Avb-/-	Display a development in emphysema and macrophage rich inflammation	56
Nrf2	Nrf2-/-	Susceptible to CS-induced emphysema	143,144
Macrophage elastase (MMP12)	MMP12-/-	Protection against emphysema after exposure to CS	21
Neutrophil Elastase (NE)	NE-/-	Protection against emphysema after exposure to CS	145
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Tnfsf10 -/-	Protection against inflammation, emphysema and lung function after CS exposure	31

Table 2 Genetically modified mice that develop or are protected against COPD-like features.

	Animal model	Epigenetic modification	Relevance/Findings	Ref
DNA methylation	Mouse (CS)	Altered Global DNA methylation	Potential biomarker	[87]
Histone modification	Mouse (CS)	**Increased:** *Lysine methylation sites:* H3K27me2; H3K36me; HK56me2; H4K20me2; H4K31me2; *Arginine methylation sites:* H4R35me2; H4R35me2; H4R36me1 *Lysine acetylation sites:* H3K79ac; H4K12ac; **Decreased:** H3K23me2; H3R72me2; H4K16ac	Smoke affects gene transcriptional regulation	[90]
	Rat (CS)	Increased acetylated histone H4	Inflammatory gene transcription	[91]
	Mouse (CS)	Decreased HDAC (total and -2) activity	Correlated with reduction in Glucocorticoid function	[92,93]
	Mouse (CS & elastase)	Decreased SIRT1	Potential therapeutic avenue by activating SIRT1	[71]
	Rat (CS)	Decreased HDAC2	Increased inflammatory gene transcription	[91]
miRNAs	Rat (CS)	Decreased let7c, miR-34c and miR222		[96]
	Rat (CS)	Increased miR146a, miR-92a-2, miR-147, miR-21 and miR-20		[146]
	Mouse (CS)	Increased miR-135b		[147]
	Mouse (CS)	Decreased miR-34b, miR-345, miR-421, miR-450b, miR-466 and miR-469	Not reversed after 1 week CS cessation	[148]

Table 3 Altered epigenetic mechanisms discovered in animal models of COPD.