Chapter 8

Summary and General Discussion

8.1 Overview

COPD is a multifaceted disease, with underlying pathophysiology not clearly understood. It is pathologically and physiologically complex with a number of overlapping and interacting components. However, physiologically it has been most defined by irreversible airway obstruction and pathologically by chronic airway inflammation leading to airway wall remodelling and thickening. Symptoms may include impaired exercise capacity and dyspnoea and if associated with chronic bronchitis, patient will also have chronic productive cough. Tobacco smoking is considered as the main aetiological factor associated with the disease at least in western countries, but why only a minority of smokers develop COPD is not clearly understood (details of this discussion are given in Chapter 2).

My thesis reports very novel findings of Rbm fragmentation which I propose as the “hallmark” of EMT in COPD, not described in the literature before. This will add significantly to the body of literature on airway remodelling. My finding will also add to the corpus of knowledge on the status of anti-inflammatory HDAC2 in COPD.

The thesis is divided into five main chapters of results. The first chapter involved a preliminary study into EMT in COPD current smokers followed by a more definitive cross-sectional study into EMT markers in Chapter 4. In Chapter 5, I further confirmed EMT by taking into account potential confounding by inflammatory and immune cells. Chapter 6 was a longitudinal analysis into the effects for potentially normalising Rbm fragmentation. Chapter 7 involved a cross-sectional and longitudinal analysis looking at the status of HDAC2 expression in COPD and its potential modulation with ICS and/or smoking cessation.
8.1.1 Preliminary observations

In the preliminary observations described in Chapter 1, I found that the Rbm in COPD current smokers has highly fragmented with elongated spaces and cracks (termed “clefts”) with cells within them. The literature strongly suggested that this could be a hallmark of EMT (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). Following this initial exciting finding I undertook a preliminary study using the classic EMT markers most favored in literature (S100A4, MMP-9 and EGFR) applied to sections from bronchial biopsies obtained from COPD current smokers, as I thought it likely that EMT would be most active in these individuals.

This preliminary study demonstrated that the Rbm in subjects with COPD who are current smokers is indeed consistently highly fragmented and that this appeared to be associated with cells expressing the proteolytic enzyme MMP-9. Epithelial expression of EGFR suggested that epithelial cells may be primed for migration, and increased expression of the fibroblast marker S100A4 suggested phenotypic transition to a mesenchymal cell type. These preliminary data suggested that EMT could be active in COPD pathology. Thus, I designed a comprehensive cross-sectional study into the potential for active EMT in COPD, asking whether it is specific to COPD and/or smoking or both, and used material from a longitudinal therapeutic study to see if ICS has any potential for reducing Rbm fragmentation (which is a central feature of EMT).

As a part of my thesis, I also decided to study the status of HDAC2 in COPD. It is suggested that expression and activity of HDAC2 is decreased in COPD due to increased oxidative stress, secondary to airway inflammation, it has also been suggested this may induce (relative) resistance to inhaled corticosteroid therapy in COPD/smokers but there is little confirmatory data on this, especially outside of the Peter Barnes group in London, which is highly committed to this theory. In fact, COPD is responsive to ICS though relatively less clinically, compared to asthma, but this is largely ignored in this conceptual model. Moreover, the methodology used by the Barnes group is questionable and largely dependent on molecular RNA quantitation and protein analysis which could not take into account relative
differences in cellular profiles in airway tissues in different disease and control groups, where biases could arise due to differences in total and differential cellularity. For the protein assays Barnes team attempted to normalise biopsy-extracted proteins against bovine serum albumin (BSA) and mRNA RT-PCR assays with a so called house-keeping gene GAPDH (glyceraldehyde-3-phophate dehydrogenase) and beta-actin, but it is not clear to me, if this is an adequate way to allow for a change in the cell sub population of interest, for example inflammatory cells in the lamina propria. These house-keeping genes can cause confounding (Glare, Divjak, Bailey, & Walters, 2002), but they would also reflect the more global environment of the airway with all its complexity. Therefore, the second aspect of my thesis involved a comprehensive immunohistochemical cross-sectional and longitudinal analysis investigating the status of HDAC2 expression in COPD and asking whether smoking cessation and/or ICS therapy have any potential to raise HDAC2 airway levels in COPD.

8.1.2 Rbm fragmentation and potential EMT in smoking and COPD

This study as introduced above and described in detail in Chapter 4 and Chapter 5, was a comprehensive cross-sectional analysis into the potential for active EMT in COPD, whether it is specific to COPD and/or smoking or both. In this study I found that Rbm is highly fragmented in both smokers and COPD patients, but especially in current smokers with COPD Rbm fragmentation positively correlated with smoking history in the smoking COPD subjects.

Markers of EMT were evident in bronchial biopsies from smokers and COPD patients. Dual immunostaining for S100A4 and vimentin in the basal epithelium and Rbm further strengthened the likelihood that these cells are undergoing transition to a mesenchymal phenotype. COPD ex-smokers were more variable but mostly quite similar to COPD. For S100A4 cells in the Rbm, there were significant more positive cells in current smoking COPD than both other comparator groups (COPD-ES and NS).

The only significant relationship with degree of physiological fixed airflow obstruction was with S100A4 cells in the basal layer of the epithelium in the current
smoking COPD group. These data taken together suggested that the remodelling changes are more exaggerated in established COPD as might be expected, especially in COPD with active current smoking, and that the changes we describe and attribute to likely EMT, may well be of pathophysiological relevance.

The positive findings for EMT markers in smokers with normal lung function, compared to normal non-smokers, suggest that smoking itself has the potential to initiate EMT. Furthermore, our data in the ex-smoker COPD group suggest that once these changes are initiated they may be irreversible. However, the observations that there fewer cells expressing S100A4 in ex-smokers in COPD ex-smokers in the Rbm but no difference in basal epithelial cell S100A4 staining, may suggest that migration from the epithelium across the Rbm decreases following smoking cessation, to confirm this I need long term smoking cessation.

In summary, the main message of the study was that smoking has potential to initiate EMT, including Rbm fragmentation, but that this is most marked in currently smoking COPD. It also suggested that smoking cessation has the potential to reverse the process to some extent.

The potential criticism of the study is that the cells in the Rbm positive for S100A4 could be infiltrating inflammatory or immune cells rather than epithelial cells undergoing transition. So, to confirm the epithelial origin of these cells in the Rbm and to address the potential for our data to be confounded by infiltrating inflammatory or immune cells, I undertook double immunostaining with epithelial and mesenchymal markers and also specific comparative staining for infiltrating inflammatory or immune cells in matched slides.

8.1.3 Confirming EMT
In Chapter 5, I followed on from Chapter 4 and undertook double immunostaining with epithelial and mesenchymal markers and also specific comparative staining for infiltrating inflammatory or immune cells in matched slides. The comparative analysis demonstrated that these cells in the Rbm are of epithelial origin (by double staining for cytokeratin) and they stained with a mesenchymal marker (vimentin)
suggesting phenotype change; and they are not infiltrating macrophages or fibroblasts, nor CD4+/CD8+ T lymphocytes or B-cells or dendritic cells. This further confirmed EMT and also demonstrated that my data is not confounded by infiltrating inflammatory or immune cells.

8.1.4 Effect of ICS on Rbm fragmentation: potential for the amelioration of EMT

In the previous chapters (as shown above), I concluded that EMT is likely to be an active process in COPD. A key question is whether ICS have any potential for amelioration or even reversal of EMT in COPD. To answer this question I performed a longitudinal analysis, described in Chapter 6. This analysis revealed that fluticasone propionate has potential for normalising EMT in COPD using quantitation of Rbm fragmentation as the illustrative and “classic” manifestation of the process. The study was limited by the fact that I haven’t yet undertaken comprehensive longitudinal analysis with direct cellular markers of EMT as described in Chapter 4, but this was due to the time limits I had with my PhD submission. However, I need now complement this work with a wider range of markers of EMT to confirm and extend these preliminary findings.

8.1.5 HDAC2 in COPD

As part of my thesis I also looked at the status of HDAC2 expression in COPD and smoking using immunohistochemistry and its potential modulation with ICS and/or smoking cessation, described in detail in Chapter 7. This was the first comprehensive airway biopsy study looking at the status of HDAC2 in COPD and smoking.

The data regarding HDAC2 was very striking and quite different to what others have reported using molecular techniques. Interestingly, HDAC2 expression was not different in the epithelium between the groups. The fact that HDAC2 expression in the epithelium is well preserved in smokers (even if little less in current smokers COPD subjects) suggest that ICS could still be effective in this tissue compartment, as the CS-GCR complex should be able to access its transcription sites on CS-sensitive genes.
I found that smoking has the potential to stimulate total HDAC2 expression, potentially increasing the anti-inflammatory environment in the airway wall, as shown in normal lung function smokers. This does seem to be through a direct action on HDAC2 status itself, as the total numbers of cells in lamina propria were lower than normal in number while total HDAC2 positive cells were higher in number (significantly) and percentage HDAC2 positive cells therefore were also increased (significantly). In contrast, a decrease in HDAC2 was observed in COPD current smokers (described in terms of reduction in HDAC2 positive cells in the lamina propria), but this was largely due to confounding by a decrease in total lamina propria cellularity compared to normal, this lowering in cell number was strongly negatively related to smoking history. The percentage of HDAC2 positive cells in COPD current smokers was down slightly compared to normal but not significantly so. There was a significant difference in HDAC2 percentage cell staining between smokers with COPD (lower than normal) and physiologically normal smokers (higher than normal, as above).

Quitting smoking did seem to have a potential for up-regulating HDAC2 at a cell level as shown by an increase in number of cells staining positive for HDAC2 in the lamina propria of COPD ex-smokers and also a slight increase in the percentage cells staining. However, we need long term smoking cessation studies with a larger cohort/size to confirm these findings.

A key finding, I believe, is the difference in HDAC2 expression between normal lung function smokers and COPD current smokers. This may reflect a fundamental difference between individuals in response to cigarette smoke and through this vulnerability to developing COPD ie if in an individual smoker decreases cells more than stimulating anti-inflammatory HDAC2, then COPD might ensue. Moreover the status of HDAC2 is not different from normal in the epithelium in either smokers or in COPD. ICS made no difference to HDAC2 status in COPD, with little likelihood of a type-2 error in this finding.

We now need comprehensive immunohistochemical studies to fully understand smoking-related cellular changes in the lamina propria especially in COPD, and prospective long term smoking cessation studies with large cohorts to confirm these
findings. Molecular methods must take such changes in the cellular environment into account and cannot be taken on simple face value; indeed the current published data is likely to be misleading because this may not have been taken adequately into account.

8.2 Final Conclusions

My findings reported in this thesis have confirmed that EMT is an active process in COPD and is most likely initiated by smoking, but most exaggerated in active smoking COPD. Smoking cessation and ICS both have the potential to reverse the process of EMT. This finding of EMT in COPD adds to understand about the remodelling process in COPD and to knowledge on pathophysiology of COPD. It may also help to understand why lung cancer is so common in smokers with COPD, and indeed why it is so aggressive, invasive and fatal in over 80% of cases. Observations regarding the status of HDAC2 in COPD reported in this thesis are novel and will rebalance the bias currently in the literature. The data confirm that HDAC2 expression is stimulated in physiologically normal smokers, but down-regulated in smoking COPD individuals but the latter effect is due to smoking-related decrease in lamina propria cellularity.