Lung Epithelial Wound Healing in Health and Disease

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Abstract:

The process of physiological lung epithelial wound repair is a complex highly orchestrated process presenting numerous points where dysregulation may occur, leading to the development of several pulmonary disorders. Current studies are limited by a lack of lung relevant injury models, with much work relying on other organ models such as the skin or in vitro cultures. However, much promising investigative work is being undertaken, of which some is described in this review. This review attempts to describe the processes required to heal a severe wound to the airway epithelium, characteristic of several chronic pulmonary disorders; highlighting areas where dysregulation may occur which in turn leads to the development/continuation of a disease state.

Key Issues:

• The healthy lung epithelial wound repair process is incredibly complex.
• This complexity presents many opportunities for dysregulation which leads to the development of numerous pulmonary disorders.
• Current studies of lung epithelial wounding are limited by a lack of suitable models.
• Correspondingly our understanding of healthy lung epithelial wound resolution is poor, as are the areas in which dysregulation can occur, leading to a diseased state.
• Whilst the most common wound event to the lung is likely to be minor, several pulmonary disorders feature a chronic micro-wounding milieu which can lead to very severe damage.
• Recovery from this state will be key in correcting these disorders, and a description of the processes involved in this recovery are described in this review.
The Physiological Importance of the Lung Epithelium

At its most basic we can think of the lung as comprising of a continuous epithelial barrier of varying function; the lower divisions of which are closely associated with the circulatory system to facilitate gas exchange, with this network existing in a supportive, metabolically and immunologically active matrix. In the development of the lung these tissues are formed by cell migration and trans-differentiation in a heavily orchestrated manner producing branching morphogenesis to produce the final complex structure.

As a result of its intimate contact with the external environment, the lung is protected by a number of innate defence mechanisms. Desmosome mediated epithelial cell adhesion prevents the mechanical invasion of pathogens into the parenchyma, whilst gap junctions facilitate co-ordinated immune cell responses against detected pathogens. This tight adhesion also preserves the ionic gradient which is key to the maintenance of the airway mucus layer and the mechanistic ciliary clearance that it facilitates. As well as acting as a barrier and means of mechanistic clearance the mucus also acts as a medium for immune cells such as macrophages and granulocytes within the airway. In addition the airway epithelium actively secretes innate defence molecules to help defend, and if required, restore epithelial integrity. These elements of innate immunity include anti-microbial peptides such as Beta-defensins \(^1,2\), anti-proteases such as Secretory Leucoprotease Inhibitor (SLPI) \(^3\), inhibitors of microbe colonisation and development such as lactoferrin \(^4\) and components of the complement system \(^5\).

Lung Epithelial Injury

Passing down the bronchial tree from the stratified airways to the terminating alveolar regions there is a great variability in constituent cells, thickness and function; this variation impacts on the type of injuries sustained and their subsequent resolution. Due to issues of scale it becomes progressively more difficult to investigate wound resolution in the lower regions of the bronchial tree, therefore throughout this review the mechanisms described should be thought of as airway descriptive unless otherwise specified. The mechanism described may be similar in the small airways and alveolar regions
of the lung and *in vitro* studies of cell responses would seem to confirm this, however it is impossible to confirm with our current techniques.

However this issue with a lack of suitable models is not small airway and alveolar specific, the entire field of pulmonary epithelial wound repair research is limited by a lack of suitable models. The majority of wounding work has been carried out in animal skin and corneal models or in human cells *in vitro* due in part to the ease of access and visualization. However, to translate these studies into an *in vivo* pulmonary model is much more difficult; despite this there are a still a significant number of studies performed in the lung, although as described above few of these focus on the lower regions of the bronchial tree. This has resulted in a reliance on the use of alternative *in vitro* models or new *in silico* models of wound repair. Where possible in this review pulmonary *in vivo* studies have been referenced ahead of *in vitro* and other organ system models.

**Figure 1** describes the currently available model systems for wound research along with some of the methodologies used to re-create wounding events. There are strengths and weaknesses to all of the current model systems and so a combinatorial approach is required to produce the most robust data. It is the author’s opinion that the developing areas of 3D multi-cellular cultures, explants of tissue and whole organs along computer based organ modelling are the areas which are likely to deliver the greatest insights into wound healing. However as mentioned these are developing areas and there still are, and likely always will be, a requirement for traditional 2D culture and *in vivo* animal modelling. Sources of epithelial injury include environmental particulate matter, viral and bacterial infection, oxidative stress and damaging protease enzymes. To maintain effective lung function and to prevent progressive infection and further damage any epithelial injury must be repaired and epithelial integrity restored, as quickly as possible. In health, this process is likely to happen continuously at a background level in order to maintain homeostasis. However in chronic lung disease the repair processes are not able to adequately offset the injurious process and aberrant repair causes a failure to restore normal epithelial integrity leading to loss of lung function.
This review attempts to describe the repair process starting from an initial deep wound which has destroyed the basal lamina and exposed the underlying extra cellular matrix (ECM). As individual wound events these acute severe occurrences are unlikely in the lung due to the protection afforded to the lung epithelium, and instead the most common wounding event in the lung is likely to be a shallow injury to the epithelium resulting in a relatively small area of denuded epithelium. However in numerous disease states a chronic micro-wounding process can occur resulting in a more severe wound, exposing the basal lamina or in the most severe cases the underlying ECM itself Figure 2. In order to rescue the lung from this often disease driven state we will need to understand and be able to manipulate the linked events occurring in the mesenchyme, epithelium and airway space. The hypothesis of this combined activity was first put forward in the asthma field and was titled the epithelial mesenchymal trophic unit (EMTU), in this case describing the dysregulated actions of the epithelium in disease progression. However even in mild wound events the interaction between the various airway constituents is still key to successful resolution, and a lack of this interaction is implicated in the development of several disorders including chronic obstructive pulmonary disease.

**The Normal Epithelial Wound Repair Process**

In normal tissue in a healthy subject, epithelial injury should repair effectively in a physiological manner returning it to functional homeostasis. The normal wound response requires a spatially and temporally orchestrated response from key cells within the airway or alveolus but also from immune and progenitor cells drawn from the circulatory system. The physiological response to wound repair can be characterised by three overlapping phases, an initial response, a recovery of integrity followed finally by resolution of the wound back to a functional epithelium, for a generic diagrammatic representation of this process see **Figure 3**.

**Factor Release**
Damaged epithelial cells, surrounding structural cells and immune cells, including macrophages and dendritic cells, respond to injury by the release of a cocktail of factors and cytokines including Epidermal Growth Factor (EGF) \(^{11}\), Fibroblast Growth Factors (FGF) such as Keratinocyte Growth Factor (KGF) \(^{6}\), Transforming Growth Factor-Beta (TGF-\(\beta\)) \(^{12}\), Tumour Necrosis Factor-alpha (TNF-\(\alpha\)) \(^{13}\), Interleukins (IL) \(^{14}\) and proteases such as Matrix metalloproteinase’s (MMPs). A more in-depth review of the diversity of factors involved in wound healing and the roles that they play is given by Crosby et al \(^{15}\). The production, release and response to these factors by the cells involved in the healing response varies greatly, accounting for the often conflicting actions described in the literature.

Two key factors that appear frequently in the existing literature are TGF-\(\beta\) and the proteolytic subgroup of MMPs. The TGF-\(\beta1\) isoform plays a key physiological role throughout the wound healing process whilst also being implicated in the pathogenesis of dysregulated wound healing in numerous pulmonary diseases \(^{16}\) such as Idiopathic Pulmonary Fibrosis (IPF) \(^{12}\), Asthma \(^{17}\), Obliterative Bronchiolitis (OB) \(^{18}\) and Chronic Obstructive Pulmonary Disorder (COPD).

In homeostasis, TGF-\(\beta\) is usually held inactive by the latency-associated protein (LAP), with disassociation allowing TGF-\(\beta\) to bind predominantly to its receptors TGF-\(\beta\) Receptor 1 and 2. This binding can induce signalling along the canonical Mothers against decapentaplegic homologs (SMAD) pathway, the Mitogen-activated protein kinase (MAPK) cascade along with potential roles in several other pathways. The exact response depends on receptor binding, cell type, co-signalling and co-factor binding and it is this diversity that allows TGF-\(\beta\) to induce its pleiotropic effects \(^{19}\).

In the wound response, MMPs were for a long time thought to be responsible solely for the degradation of old ECM, which although an important part of their function, is not their only role. MMPs are responsible for maintenance and modelling of the ECM during its formation and maturation via control of cytoskeletal constituents involved in epithelial motility, mediating wound contraction and maturation of the new epithelium in addition to a critical role in the regulation of chemokine function.
Produced by stromal cells, immune cells and the epithelium itself, MMPs have the ability to increase chemokine activity (MMP-3 for neutrophil-activating protein-2), decrease activity (MMP-13 for Stromal cell-derived factor-1), degrade the chemokine completely (MMP-9 for PF4) or create receptor antagonists (MMP-3 for MCP4) via cleavage of the mature or pro-chemokine. This interaction is key for the development of chemokine gradients required throughout the wound healing process, for example MMP-7 has been shown to be required for the chemotactic attraction of neutrophils into the airway space after injury by cleaving syndecan-1 which is strongly associated with the mouse chemokine KC. In addition the actions of MMPs are modulated by their inhibitors Tissue Inhibitor of Metalloproteinase’s (TIMP), which competitively bind with the active site of the MMP inhibiting its function. Review articles by Brew et al and Murphy et al give in-depth review of the current understanding of MMP/TIMP interactions.

**Extra Cellular Matrix Response**

If the vasculature is breached during lung epithelial injury, the coagulation system is activated with factors such as Thrombin driving the formation of a platelet rich plasma derived fibrin-fibronectin plug. Resident fibroblasts use this plug to begin quickly laying down a provisional ECM rich in collagen III, which is able to cross link and form a disorganized matrix that has greater contractile and motile ability than a mature type I collagen rich ECM.

An excess of ECM deposition is a hallmark of chronic progressive scarring conditions that fall under the fibrotic interstitial lung disease umbrella including Idiopathic Pulmonary Fibrosis (IPF). As the name suggests the induction and progression of IPF remain unknown, two hypotheses to explain disease progression have been described. Firstly persistent chronic inflammation from a recurrent external injury may act as a driver of fibrosis, or alternatively a recurrent, possibly autologous, injury to the alveolar or terminal bronchiolar epithelium which induces dysregulated wound responses with either a normal, reduced or absent inflammatory response. Regardless of the driving mechanism, the response is the same with the wound repair response becoming dysregulated. At this point hyper-
fibro-proliferation and greater occurrence of myofibroblasts, potentially formed via Epithelial to Mesenchymal Transition (EMT), in the wound will result in the formation of the characteristic fibroblastic foci and accumulation of corresponding ECM 29.

A major driver of this dysregulated repair in IPF is TGF-β1, demonstrated via transient expression of TGF-β1 in rat 30 and mice 31 lungs to produce a severely fibrotic lung, with inhibition of TGF-β1 via SMAD-3 knockout blocking this development 31. Studies of human bronchoalveolar lavage (BAL) have detected elevated levels of TGF-β1 in the IPF lung 32, and work performed in our lab has shown a pro-fibrotic role for TGF-β1 in primary human pulmonary epithelial cells. In a chronic wound environment it is believed that constant TGF-β1 expression, combined with the high dissociation constant of TGF-β and its receptors, induces fibro-proliferation in the wound area, an increased recruitment of fibrocytes and also drives EMT 33.

Early Immune Cell Responses

The aforementioned released factors, along with the chemo-attractive properties of fibronectin itself, facilitate the entrance of non-resident neutrophils, monocytes and fibroblasts into the wound. The first non-resident responders are the neutrophils, eosinophils and basophils that are attracted to the wound area via chemotaxis; which are then responsible for degrading any cellular debris by phagocytosis, and/or neutralizing invading pathogens via an oxidative burst response. The granulocyte count in the wound area peaks rapidly, but is followed by an equally fast decline in number due to apoptosis and non-inflammatory phagocytosis by resident macrophages and responding monocytes 34,35.

Prolonged survival of granulocytes in the wound area, especially eosinophils and neutrophils, is seen in chronic asthma and has been implicated in the development of sub-epithelial fibrosis and increased smooth muscle and mucus production, resulting in obstruction and hyper-responsiveness of the airways 36. Factors such as granulocyte/macrophage colony stimulating factor can be used to generate animal models of asthma and have been shown to aid in the maintenance of granulocytes in the airway
Tying in the role of MMPs and cytokines in the early response is the murine cytokine KC. KC needs to bind with an MMP-7 cleaved form of the epithelial bound protein syndecan-1 in order to attract neutrophils via chemotaxis. In lung injured MMP-7 knockout mice the neutrophils remained in the interstitium and did not migrate into the alveolar space. This resulted in a minor reduction in collagen deposition and increased survival in response to bleomycin injury. Increased levels of MMP-7 in lung tissue samples have also been shown to correlate with diagnosis of IPF, although the sample sizes used in the study were small.

This granulocyte response is especially important at this early stage as the provisional ECM which provides an ideal motile platform for resident cells, and is susceptible to opportunistic bacterial colonization by organisms such as *Pseudomonas aeruginosa*. In physiological repair this process is either blocked or inhibited to such an extent that the immune response can eradicate the infection. In contrast it is thought that a less effective immune response, leading to chronic infection may be a cause of dysregulated wound response and the development of related diseases.

**Macrophage Response**

Maturation of recruited monocytes in response to the wound area milieu increases the total macrophage count in the wound. These cells will perform a similar function to that previously described for infiltrating neutrophils with the additional action of promoting the formation of new ECM via the release of factors such as TGF-β1 which in this instance stimulates resident fibroblasts to up-regulate production of provisional ECM constituents specifically collagen III.

This apparent duality of constructive and destructive function of macrophages may be explained via the hypothesis of a plastic spectrum of differentially functioning macrophages. Two phenotypes, termed classically activated macrophages (CMΦ) and alternatively activated macrophages (AMΦ) are used to define the differing responses. The CMΦ, often defined by CD40 expression are typically induced via interferon-γ in the wound area and take on a pro-inflammatory role, releasing cytokines such as TNFα and IL-8 along with Reactive Oxygen Species (ROS) and demonstrate a high phagocytic
capability. The AMΦ are thought to be more heterogeneous in function with an anti-inflammatory pro-repair capacity mediated via the release of factors including the fibroblast stimulating TGF-β, the loss of inflammatory cytokine and ROS release and a much reduced phagocytic capacity 43. AMΦ are often defined by expression of CD163, CD206 or CCL18 45 and are induced via IL-4, IL-13 and IL-10 46. IL-10 induced macrophages display a more profound shift towards the anti-inflammatory end of the spectrum compared to IL-4 and IL-13 which maintain some inflammatory capacity 43. It is thought that a shift from CMΦ to AMΦ is one of the main drivers in the resolution of the immune response allowing the physiological healing response to proceed 47. It is currently unknown whether IL-4/IL-13 induced macrophages are precursors to the strongly anti-inflammatory IL-10 induced macrophages, also the triggers for, and sources of IL-4, IL-13 and non-AMΦ derived IL-10 responsible for driving the in vivo shift from CMΦ to AMΦ have yet to be determined.

Along with this shift in function, studies have also suggested that macrophages present in the wound area may have a deleterious effect on the process of re-epithelialization 48. Correspondingly macrophage number is significantly reduced via egress into the lymphatic system 49, the timing of which is important due to the heightened risk from pathogen colonization of the exposed ECM and also epithelium (see below). Apoptosis is not thought to occur in situ partly to avoid triggering of an immune response, and also because these macrophages in the wound area are primed to resist apoptosis, although several types of bacterium have shown the ability to subvert this resistance 50.

The importance of the macrophage’s role in the epithelial wound repair in chronic lung disease is demonstrated by the inflammatory hypothesis of IPF. The basis for this hypothesis is the increased number of macrophages found both in the alveolar spaces and interstitium of the lung in these patients. It is thought that key factors released from activated macrophages including TNFα, ROS and TGF-β1 along with their phagocytic action degrades the injured epithelium whilst concurrently inducing the hyper activation of fibroblasts present in the wound area. However this hypothesis is challenged due to the poor efficacy of anti-inflammatory treatments in trials, including corticosteroids, cyclophosphamide, cyclosporine, or pirfenidone, none of which produced reproducible benefits
although some positive trends were observed. Conversely the TNFA-308G>A polymorphism which has previously been reported to produce elevated free TNF-α levels in sarcoidosis patients was also found to be significant in IPF, suggesting that our understanding of the role of inflammation in IPF is still significantly lacking. An interesting development in this field is the apparent synergy between the pro-fibrotic actions of TGF-β1, and the pro-inflammatory actions of TNFα in the development of fibrosis. Originally described by our group in an EMT model for the development of OB it would be plausible to expect a similar response in IPF, although no data has yet been reported on this. For a discussion of the role of inflammation in IPF see the following review by Behr et al.

Re-constitution

After the initial inflammatory response has attracted and activated immune cells, which clear the wound site and prevent colonisation of the wound area, the formation of a provisional ECM is the next critical stage in re-epithelisation of the wound.

A Provisional ECM

Resident fibroblasts respond to factors including IGF, TGF-β and FGF in the wound area by proliferating and beginning to lay down a provisional ECM of disordered collagen III fibres and α-smooth muscle actin (α-SMA). This matrix strengthens the wound and also acts as a scaffold facilitating easier cell motility as well as future wound contraction. This provisional ECM becomes populated with an increasing numbers of fibroblasts due in part to fibroplasia but also potentially via recruitment of circulating fibrocytes from the vascular system, along with macrophages and platelets already present in the wound. The cells embedded in this provisional matrix along with the matrix itself are key players in mediating the milieu of the wound and therefore the success of wound resolution.

The theory of circulating fibrocytes contributing to repair of lung epithelial wounds is a relatively new concept, suggesting that bone marrow derived fibrocytes can be recruited from the vasculature in
response to a chemotactic gradient emanating from the wound. These cells differ from resident fibroblasts in that they express haematopoietic markers such as CD34, CD45 and CD54. CD34 was the first marker described for fibrocytes and has therefore received the most attention. The signal has been shown to decrease \textit{in vitro} however it is thought that those in the wound environment maintain expression.

The factors responsible for the chemotactic recruitment of these fibrocytes is not completely understood, however expression of the CCR3, CCR5, CCR7, and CXCR4 receptors has been demonstrated along with a response to the CCR7 ligand SLC. Once resident in the wound site the fibrocytes are capable of producing collagen and $\alpha$-SMA, a process which is increased in the presence of TGF-$\beta$1. Whilst these fibrocytes may play a role in physiological wound repair, an over-recruitment is associated with numerous pulmonary disorders. An increased number of fibrocytes is associated with IPF with an even greater abundance in acute exacerbations, although the mediator of their recruitment is unknown. In asthma an increased number of fibrocytes are present in the hyperplastic airway smooth muscle, although it has not been determined if they contribute significantly to disease progression or severity, or are attracted by the already hyperplastic tissue.

Resident and recruited fibroblasts in the matrix continue to proliferate and lay down new ECM, using the initially deposited fibronectin as a scaffold on which to lay progressively more collagen III in a disorganised fashion. This acts to strengthen the wound but is also important in the final stages of wound resolution. The high proportion of fibronectin initially present is important as it creates a more extensively hydrated matrix allowing for easier cellular motility, due to a swelling of the ECM, thus creating large spaces for cells to pass through. This hydration is thought to be due to the increased presence of hyaluronic acid within the fibronectin and elastin rich matrix of the wound, which maintains a more open matrix allowing for greater water content. Interestingly hyaluronic acid also interacts with CD44 and RHAMM, cell surface markers implicated in cell motility. Whilst initially important in the normal wound healing process fibronectin is gradually replaced, producing a more...
stable, tighter ECM reducing cellular motility. However, continued production of fibronectin has been demonstrated in histological sections from IPF, potentially allowing for motility of myofibroblasts and the spreading of fibrosis throughout the lung.

Contraction of the Wound

Concurrently with the strengthening of the provisional ECM, contraction occurs mediated by myofibroblasts present in the wound. These are either already resident in the lung or, as in the majority of cases, have been induced via factors such as Thrombin into forming myofibroblasts from the resident fibroblast population. These myofibroblasts traverse the initially fibronectin rich provisional ECM until they reach the edge of the wound where they tether to the stronger collagen and α-SMA rich maturing ECM at locations known as fibronexi and then to each other via adherens junctions forming foci. The myofibroblasts’ MMP-3 re-ordered actin cytoskeleton then contracts closing the wound. The exact initiation and termination of this process is poorly understood, although once again a major role for TGF-β1 has been described.

With contraction complete the majority of these myofibroblasts undergo apoptosis, a process which is thought to be triggered by the formation of a provisional ECM great enough to maintain integrity in its own right. Although this process is accepted the mechanism for action is unknown; although both the loss of contact mediated survival signalling and de-sensitization to the previously proliferative TGF-β1 signal have been postulated.

Whether they are derived from either resident fibroblasts, circulating fibrocytes or via the process of EMT, an excess of myofibroblast foci are considered one of the hallmarks of IPF pathology. Previously it was thought that these foci were independent, but recent studies have shown that these foci form part of an interlinked fibrotic network across the affected area of the lung. The excess of myofibroblasts are then responsible for the dysregulated excessive deposition of ECM proteins into the alveolar septal walls seen in IPF.
Re-epithelization

The loss of contact inhibition and apical-basal polarity in healthy cells at the edge of the wound site, along with a chemotactic gradient of factors including KGF and EGF from the wound site, introduces polarity to the wound healing process demonstrated by a leading edge of CDC42 expression, a key regulator of cellular polarity. This polarity induces healthy epithelial cells, several ranks back from the edge of the wound to loosen but not completely release adherens junctions with their neighbours, along with a loosening of desmosome attachment to the basal membrane. The desmosome loosening allows a redistribution of the actin cytoskeleton and related integrins such as α5β3 which now form lamellipodia and filopodia mediated by Rho-GTPases including the aforementioned CDC42. The actin cytoskeleton of these protrusions interacts with the provisional matrix via a polarised integrin bind and release cycle, thus dragging the epithelial sheet behind them towards the centre of the wound as a continuous sheet. This motility can be further enhanced by feedback cycles involving TGF-β and integrins such as the aforementioned α5β3 and α5β5. There are numerous ways this process can be achieved including activation of latent TGF-β via conformational changes through direct integrin binding, a release of soluble TGF-β via integrin associated cleavage or modulation of the SMAD pathway itself. Conversely TGF-β signalling can both repress and induce production of further integrin molecules, the exact control mechanisms that determine this are not completely understood but this interplay has received much attention as it is thought to be a key area in the dysregulation of wound healing.

The epithelial membrane bound integrin α3β1, has been implicated in playing a key role in the development of IPF. α3β1 knockout mice displayed a normal response to bleomycin lung injury, however they did not develop the characteristic excess of myofibroblasts and ECM deposition. It has also been demonstrated that α3β1 can act via phosphorylation of β-catenin, which then induces SMAD signalling with a potential induction of EMT both in vivo and in vitro. Another example of the important role of integrins in pulmonary disease is seen in asthma. Several genotyping experiments have associated Single Nucleotide Polymorphisms in the integrin β3 gene (ITGB3) with the
development of asthma, particularly at an early age and in severe cases. A mouse knockout for ITGB3 has demonstrated increased levels of TGF-β1 which would seem to fit in with a pro-fibrotic outcome, however these knockout mice actually demonstrated accelerated re-epithelization \(^{101}\), further emphasizing both the pleiotropic nature of TGF-β1 and our poor understanding of the wound healing process.

Traditionally asthma was thought of as a purely inflammatory disease, yet longitudinal studies have shown that whilst anti-inflammatory treatments are able to suppress the disease and moderate exacerbations they require continuous usage, and appear to not influence the factors required for disease initiation and progression \(^{102}\). This lack of response to anti-inflammatories led to the formation of the dysregulated EMTU hypothesis; whereby a dysregulation in the actions of the epithelium and mesenchyme and/or the interactions between the two leads to the development of asthma \(^9\).

This paradigm shift away from a purely inflammatory disease model is strongly supported by the discovery of several epithelial specific markers of susceptibility, such as the previously mentioned ITGB3 along with IL-10, IL-4 receptor and Interleukin-1 receptor-associated kinase 3 \(^{100}\) among others. Numerous factors are known to be released from the epithelium in physiological repair, and there are several examples of a dysregulated release corresponding with the development of asthma including the protease MMP-9 and its inhibitor TIMP-1. MMP-9 is detected at elevated levels in the BAL \(^{103}\), with increased serum levels correlating with disease severity \(^{104}\). This increase is primarily though to be driven by granulocytes \(^{105}\), although the bronchial epithelium is also thought to play a role \(^{106}\). A key role for MMP-9 was confirmed in a mouse model of asthma, where MMP-9 knockout mice did not develop the hyper-responsive airways characteristic of asthma after challenge \(^{107}\).

The role of TIMP-1 in this process remains poorly understood due to the conflicting reports of several studies leading to opposing hypotheses. Increased TIMP-1 expression relative to MMP-9 \(^{108}\) led to the hypothesis that even though MMP-9 was present in greater amounts it’s ECM degrading activity was being inhibited allowing an excess of ECM deposition. Conversely data showing a relative increase of
MMP-9 levels relative to TIMP-1 \textsuperscript{109} led to the hypothesis that increased MMP-9 activity inhibits the resolution of the airway leading to a chronic wound environment.

The interactions of MMP-7 and TIMP-1 further display the importance of this MMP/TIMP balance in the wound resolution process. MMP-7 has been shown to be important in the process of re-epithelization. MMP-7 is secreted from the epithelium itself and is responsible for the cleavage of cell to cell adherens proteins such as E-cadherin allowing the required mobility needed for wound closure \textsuperscript{110}, with over expression leading to overly motile epithelial cells and poor wound resolution \textsuperscript{111}. TIMP-1 has been implicated as playing a key role in the regulation of MMP-7 activity due to its ability to suppress the migration of epithelial cells in culture \textsuperscript{112}. An increase of TIMP-1 expression is elevated in sections taken from post-transplant lungs with a co-localization of MMP-7. Wounding experiments in air liquid interface cultures also displayed this co-localization at the wound edge. A TIMP-1 deficient mouse model of OB displayed a reduced fibro-proliferative response, and correspondingly reduced airway obliteration \textsuperscript{113}. These findings were reinforced by two skin model studies describing the ability of a synthetic MMP inhibitor GM 6001 \textsuperscript{114}, or transgenic TIMP-1 over-expression \textsuperscript{115} to significantly reduce the motility of keratinocytes in the epithelial sheet and thus retard the normal wound healing process.

The discovery of this epithelial sheet movement was important as it demonstrated that the first action to cover the wound is one of hypertrophy \textsuperscript{85} (at least over the wound area) as the motile epithelia extends and flattens, and not hyperplasia as was expected (although this does occur away from the wound edge) see \textbf{Figure 4}. These motile epithelial cells have the ability to degrade any remaining debris lying on top of the provisional matrix in order to create a smooth and continuous epithelium, this process is mediated by the release of MMPs from both cells of the epithelial sheet and non-epithelial cells in the wound micro-environment. It is important to note that whilst hypertrophy is the major driver over the wound site, hyperplasia is initiated in progenitor cells near the edge of the wound soon after mobilisation occurs. This hyperplasia near the wound edge is most likely induced via previously discussed growth factors, their receptors and the stretch response \textsuperscript{116}. The role of EGF
as a stimulant for wound healing has been previously mentioned. The expression of the EGF Receptor is increased over a very tight temporal period, with a subsequent de-crease in expression, and this is a putative explanation for the shift from hypertrophy to hyperplasia ¹¹.

As the wound closes, epithelial migration is stopped via Pak1 mediated contact inhibition between the leading edges of the epithelial sheet. At this point the pseudopodia regress and the cells in conjunction with the provisional matrix form a new basal membrane to which they re-attach whilst also reforming the adherence junctions that previously characterized them ⁸⁰. At this point a continuous epithelium has been restored however the epithelium present over the wound area is not functionally active and requires further maturation during resolution stage. At this stage and throughout maturation these cells are more susceptible to infection from pathogens, especially P. aeruginosa which is thought to bind to the surface marker Asialo Ganglioside M1 which is expressed on the apical surface of hypertrophic epithelial cells during the repair process ¹¹⁷, and may help explain why these cells express elevated levels of anti-microbial agents ².

**Progenitors and Differentiation**

The actual epithelial progenitor cell type involved in the hyperplasic action depends on the location of the wound. Transgenic mouse lineage tracing experiments have identified basal cells as playing a key role in epithelial migration over the wound area as described above in stratified and pseudostratified regions of the lung. These experiments also identified a basal cell specific receptor combination in Integrin alpha-6 and Nerve Growth Factor Receptor ¹¹⁸. In the smaller airways the uteroglobin expressing Clara cell has shown progenitor capacity ¹¹⁹,¹²⁰, with a detectable loss of uteroglobin occurring during the repair process, suggesting some form of de-differentiation has occurred. Uteroglobin acts as a potent anti-inflammatory in the lung and uteroglobin knockout mice can sporadically develop localized pulmonary fibrosis ¹²¹ suggesting a possible mechanism for the spread of fibrosis throughout the lung.
In the alveolus the mechanism is less well understood due to the difficulties in observing responses in vivo. However there is strong in vivo and in vitro evidence that the surfactant protein expressing type II (TII) pneumocyte can hyper-proliferate to repair damaged or destroyed epithelium with KGF strongly implicated in this process. These cells make desirable therapeutic targets and with knowledge of these specific markers it is now becoming possible to directly target these cells for treatment.

At this point it is worth discussing the topic of plasticity and in particular the phenomenon of EMT which as a process has received much attention in cancer development and numerous fibrotic disorders including those of the lung. If we think of a mature static pulmonary epithelial cell and its motile mesenchymal counterpart as endpoints on the spectrum of EMT then the description of the motile loosely bound epithelial sheet would seem to fit well in the EMT hypothesis. Cells lose tight cellular adherence, gaining motility and all via a transition event as part of the normal wound repair process. Perhaps EMT represents a spectrum of responses which at one end is the physiological process involved in wound repair, whilst at the other end a dysregulated form giving rise to a more severe and or permanent shift towards the mesenchymal endpoint.

TGF-β1 has been implicated as a key driver of EMT in alveolus associated IPF both in vivo and in vitro with the TII pneumocyte identified as the trans-differentiating cell. Whilst the in vivo relevance of EMT has been questioned in the literature recently due to a lack of response in in vivo lineage tracing models, this is an area in which the lung is ahead of the field. A triple transgenic mouse model was developed that demonstrated the ability of mouse TII pneumocytes to trans-differentiate and begin expressing the myofibroblast marker α-SMA, whilst maintaining TII specific markers such as SPC in response to TGF-β1; thus providing a robust base for EMT work, especially in the alveolus.

Resolution

During the re-establishment of the epithelial sheet the basal lamina is also reformed underneath the migrating epithelial and basal cells and is derived predominantly from the epithelium. Whereas the provisional ECM is composed mainly of collagen III, the basement membrane has a higher proportion...
of type IV collagen which due to different post translational processing forms a single thin sheet as opposed to the thicker matrix below. The function of this membrane is two-fold, to anchor the cells in place and also act as a physical barrier to invading pathogens.

Concurrent with this basal lamina formation is the maturation of the ECM. Evidence from non-pulmonary models suggest that the more flexible type III collagen, quickly deposited by myofibroblasts in the previously described disorganized matrix is gradually replaced by the stronger collagen I over a period of several weeks. This maturation also signals the point where total collagen production and degradation equalize as existed before wounding, due to a normalising of the MMP to TIMP ratio. This shift results in the return to a more normal ECM composed predominantly of collagen I which is aligned via the action of myofibroblasts along stress lines and cross linked in such a way so as to return the tensile strength of the wound back to near normal levels. A repeated but non-chronic wounding of the same area results in a progressively weaker epithelium and underlying ECM, and often the formation of non-functional scar tissue in the place of the epithelium and in previously discussed chronic wound environments this can lead to widespread fibrosis and loss of function.

The majority of cells left embedded in the ECM such as fibroblasts and macrophages either apoptose in situ or egress into the airway lumen before apoptosing, thus returning the ECM back to a less active state. It is not known if a similar mechanism to the first reduction of macrophages in the wound via the lymphatic system occurs as it this point.

The naïve hypertrophic epithelial cells now covering the wound area respond to the maturation of the ECM, reformed basal lamina and contact inhibition signalling by beginning the process of maturation with cells returning to their normal un-stretched phenotype. However, this process alone would leave the epithelium susceptible to tearing so hyperplasia of progenitor cells at the wound edge ensures that sufficient cells are produced to maintain integrity. The mechanism for this maturation is poorly understood, but it has long been thought that EGF signalling through its receptor plays a key role in stimulating this transition. Evidence for the importance of EGF comes from two sources; firstly that
over expression of EFG-R is one of the hallmarks of over-proliferative non small cell lung cancer 132. Secondly, and in contrast to the previous, IPF patients exhibit significantly decreased levels of EGF mRNA expression compared to controls 133. A mediator for this process has been described in Mitogen-inducible gene 6 which plays a key role in the correct early development of the lung in mice 134. EGF has also been shown to be important for the maintenance of lung homeostasis in adult mouse models, with EGF knockout mice developing a COPD analogue, a result was also demonstrated in human bronchial cell lines 134. A potential marker for the maturation of epithelial cells has been described in vitro in the epithelial cell specific Zona Occludens-1. In non-mature epithelial cells it is predominantly located in the nucleus, but during the process of maturation it is trafficked to the cell-membrane aiding in the formation of the close cell to cell binding typical of the mature epithelium 135.

Epithelial cell proliferation at this point can be inhibited by MMPs in the wound space, such as MMP-2. Often this occurs when the wound area is not completely closed, or a chronic wounding process is occurring leaving the ECM exposed resulting in the maintenance of MMPs in the airway. Epithelial differentiation is also influenced by the action of MMPs, as an example MMP-7 is present at elevated levels during the differentiation stage of wound healing whereas the TIMP-1 inhibitor remained stable 20,129 facilitating a greater action from MMP-7. Although it is unknown precisely what the role of this imbalance is, it is likely that MMP-7 facilitates some form of cytoskeletal re-ordering resulting in a reformation of cell to cell binding and desmosome attachment to the basal lamina.

**Expert Commentary and Five Year View**

As mentioned in the introduction a limiting factor of epithelial wound repair research in the lung epithelium (and other internalized epithelia) is the lack of suitable models. A combination of animal model in vivo work, potential human ex vivo lung studies along with in vitro cultures of primary tissue and cells aligned with ever more complex in silico models should prove key in improving our understanding of the mechanisms involved.
The wound repair process is a complex highly orchestrated process with numerous levels of control that operate in synch to facilitate physiological wound repair. The examples provided show that dysregulation at any stage of this process could cause pathological wound repair which may be critical in the pathophysiology of a number of chronic lung diseases. Due to the complex and often pleiotropic nature of factors in the wound process it is likely that any therapeutics developed will have to be highly targeted towards specific cell populations as well as temporally and spatially accurate. To this end development of specific markers that identify specific cell types in the lung is a key area requiring more research, concurrently with the development of new methodologies for delivering therapeutics specifically to these populations.


