

**THE EFFECT OF BRUSH-INDUCED INJURY ON MOLECULAR
EXPRESSION PROFILE OF RABBIT AIRWAY EPITHELIUM DURING
REGENERATION AND REPAIR**

by

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LIST OF ABBREVIATIONS

AB-PAS	Alcian blue-periodic acid Schiff
ADAMTS5	ADAM metalloproteinase with thrombospondin type 1 motif, 5
ALI	Acute lung injury
AQP1	Aquaporin 1
AQP5	Aquaporin 5
ASL	Airway surface liquid
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
CCL21	Chemokine (C-C motif) ligand 21
CCNA	Cyclin A
CCNE	Cyclin E
CD38	CD38 molecule
CD59	CD59 molecule
CDK2	Cyclin-dependent kinase 2
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CF	Cystic fibrosis
COPD	chronic obstructive pulmonary disease
C_t	Number of cycle at the threshold level
CXCL13	Chemokine (C-X-C motif) ligand 13
DA	Damaged area

ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EMT	Epithelial mesenchyma transition
ET	Endotracheal tube
Etv5	E26 transformation specific variant 5
FOXJ1	Forkhead box J1
FGF	Fibroblast growth factor
GSK3B	Glycogen synthase kinase 3 beta
H&E	Haematoxylin and Eosin
HBEC	Human bronchial epithelial cell
HGF	Hepatocyte growth factor
Id2	Inhibitor of DNA binding 2
hrs	Hour/hours
IFNG	Interferon gamma
IHC	Immunohistochemistry
IL4	Interleukin 4
IL8	Interleukin 8
ITGB1	Integrin- β 1
KRT10	Keratin 10
LEF	Lymphoid enhancing factor
LPS	Lipopolysaccharide
min	minute
MMP	Matrix metalloproteinase

MMP9	Matrix metalloproteinase 9
MMP12	Matrix metalloproteinase 12
OCT	Optical coherence tomography
PCL	Periciliary liquid layer
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
POU5F1	POU class 5 homeobox 1
Rho-GTPases	Ras homolog family member GTPases
RIN	RNA integrity number
RNA	Ribonucleic acid
RT	Reverse transcriptase
SCGB1A1	Secretoglobin, family 1A, member 1
SFTPA1	Surfactant protein A1
TCF	T-cell factor
TGF- β_2	Transforming growth factor, beta 2
TIMP1	TIMP metalloproteinase inhibitor 1
TNF	Tumour necrosis factor
Trp63	Tumour protein p63
VEGFA	Vascular Endothelial Factor A
WHO	World Health Organisation
Wnt	Wingless-type MMTV integration site family

LIST OF SYMBOL

%	percent
μl	microlitre
μm	micrometre
$^{\circ}\text{C}$	degree of celcius
cm	centimetre
kg	kilogram
\log_{10}	logarithm 10
m/s	milimetre per second
mg/kg	milligram per kilogram
mm	millimetre
ng	nanogram
RPM	revolution per minute
sec	second

**KESAN KECEDERAAN DISEBABKAN BERUS KE ATAS PROFIL
EKSPRESI MOLEKULAR SEMASA PROSES PERTUMBUHAN DAN
PEMBAIKPULIHAN EPITELIA TRAKEA ARNAB**

ABSTRAK

Epitelium trakea mempunyai ciri-ciri pertahanan yang mampu untuk melindungi dari partikel-partikel berbahaya. Semasa berlakunya kecederaan, proses pembaikpulihan sel-sel yang bertanggungjawab yang mana ianya melibatkan proses-proses seperti sel migrasi dan penyebaran, proliferasi, dan pembezaan semula sel-sel. Selain daripada itu, aspek molekular juga tidak boleh diketepikan. Ianya berfungsi penting dalam proses pembaikpulihan ini. Gen-gen yang terlibat dalam proses molekular boleh dibahagikan kepada beberapa bahagian mengikut fungsi masing-masing. Fungsi-fungsi tersebut termasuk proses migrasi, proliferasi, inflamasi, penanda sel-sel tunjang, dan penanda sel-sel gen khusus. Interaksi di antara komponen selular dan molekular semasa proses pembaikpulihan memberikan kita satu pemahaman bagaimana sel-sel itu berfungsi. Oleh itu, objektif penyelidikan ini adalah untuk mengkaji bagaimana sel-sel bertindak balas terhadap kecederaan yang disebabkan oleh berus pada trakea arnab. Tiga puluh enam ekor arnab berspesies 'New Zealand white' dibahagikan kepada dua kumpulan iaitu kumpulan tidak tercedera dan tercedera (pada selamasa 0.5, 1, 6, 12 jam, 1, 2, 3, 4, 7, 14, dan 21 hari). Selepas melakukan teknik pemberusan, arnab dipelihara seketika sebelum dikorbankan mengikut masa yang telah ditetapkan. Seterusnya trakea diambil dan disimpan sebelum dilakukan teknik pewarnaan 'Haematoxylin and Eosin' dan 'Alcian-bue Periodic Acid Schiff'. Selain itu trakea ini juga dianalisa menggunakan teknik

molekular 'real-time PCR'. Teknik pemberusan yang digunakan dalam penyelidikan ini berjaya merosakkan lapisan epitelia trakea. Jarak kawasan kecederaan yang disebabkan oleh berus berkurangan apabila titik masa meningkat. Kawasan kecederaan ini tertutup pada hari ke-21. Jumlah sel-sel goblet meningkat dan mencecah aras tertinggi pada titik masa hari ke-3 sebelum berkurangan pada titik masa berikutnya. Analisa molekular menunjukkan pelbagai ekspresi gen-gen yang terhasil pada titik masa yang berlainan. Manakala ekspresi gen-gen yang berkaitan dengan sel-sel proliferatif dan migrasi pula mempunyai ekspresi yang tinggi pada awal titik masa sebelum berkurangan pada waktu berikutnya. Penyelidikan ini berjaya menghasilkan teknik pemberusan yang menyebabkan kecederaan pada epitelia trakea. Teknik ini dilihat lebih praktikal, murah dan dapat mengurangkan risiko infeksi. Jarak kawasan kecederaan berkurangan apabila titik masa meningkat menunjukkan proses pembedahan juga turut berlaku. Pada aspek selular pula, sel-sel goblet meningkat pada awal titik masa bagi memberikan perlindungan lapisan tercedera oleh mukus. Penemuan ini disokong oleh ekspresi gen yang terlibat dalam proliferasi dan migrasi. Aktiviti sel ini sangat penting pada awal titik masa dalam menggalakkan pertumbuhan sel-sel yang tertinggal pada kawasan kecederaan. Penemuan kami juga menunjukkan populasi sel-sel basal mendominasi pada awal titik masa. Manakala, sel-sel cilia pula terhasil pada lewat titik masa. Menariknya, sel-sel Klara tidak terlibat secara langsung dalam proses pembedahan selepas kecederaan berlaku. Kelebihan teknik ini yang sesuai digunakan dalam makmal berskala kecil menjadikan ianya sebagai satu alat yang mampu mencapai kelestarian. Komponen selular dan molekular berkerjasama dalam pelbagai fungsi di mana

sebahagian komponen tersebut penting dalam mekanisma pertahanan manakala sebahagian yang lain menyumbang ke arah pertumbuhan semula epitelia.

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ABSTRACT

Tracheal epithelium is equipped with well-balanced defensive features to keep the body away from harmful particles. Upon an injury, the cellular repairing process undergone with the involvement of cell migration and spreading, proliferation, and redifferentiation. Molecular aspect also play important role in the repairing process. The genes involved in the process can be grouped based on their roles, which include migration, proliferation, inflammation, stem cell marker and cell-specific gene marker. Understanding the interaction between cellular and molecular components during repair will give a better view on how cells behave and function. Thus, in this study, cellular and molecular responses following tracheal brushing-induced injury using rabbit model were investigated. Thirty six New Zealand white rabbits were divided into uninjured and injured (0.5, 1, 6, 12 hours and 1, 2, 3, 4, 7, 14, and 21 days) groups. After brushing, the animals were maintained before being sacrificed according to their respective time point. The trachea was retrieved and preserved before subjected to haematoxylin and eosin (H&E) staining, Alcian blue-periodic acid Schiff (AB-PAS) staining, and real time PCR. The technique used in this study was proved to cause removal of the epithelium. The extent of damage reduced as the time point increased and completely closed on day 21. The number of goblet cells was observed increased and reached the peak level after 3 days and subsided on later time point. Molecular analysis revealed that the genes were expressed differentially

in various time points. Genes related to cellular proliferative and migration activity expressed in the early time and reduced on the other end. We have successfully developed a blinded brushing technique to cause injury on the tracheal airway epithelium. This technique is practical, inexpensive, and can reduce the risk of infection. Reduction of the extent of damage over the time point indicated that regeneration process was undertaken. By cellular response, the number of goblet cells increased on the early time point, giving an instant response by providing a mucus protection barrier. This finding was in accordance to the proliferative and migration activity related gene expression. The activity was crucial in the early time point to encourage the remaining cells to repopulate the damaged area. We also found that the basal cells were predominantly involved during the early stage of repair, while ciliated cells rose on the later stage. Interestingly, the Clara cells did not participate in repairing the injured tracheal epithelium. Our technique provides approach in achieving sustainability goal with its suitability use in simple laboratory setting. The number of goblet cells increase on the early time point provides importance defensive mechanism against harm particles. Molecular together with various functions in regulating the protective mechanism and while on the later stage contribute to formation of regenerated epithelium.

CHAPTER 1

INTRODUCTION

1.1 Respiratory diseases

Globally, it is estimated that over 1 billion people suffer from chronic respiratory diseases (Suzanne and Elisabetta, 2008). Among the chronic respiratory diseases, asthma has the highest incidence with about 300 million patients, whilst 210 million patients suffer from chronic obstructive pulmonary disease (COPD) (WHO, 2007). It is predicted that the disease will become the third leading cause of death in the world by 2030 (WHO, 2008). This imminent fact may lead to increased burden on healthcare system, economy, and capital human resources, thus urging personnel in medical field to take endeavour in finding a better treatment for these diseases.

1.2 Structure and physiology of tracheal airway

1.2.1 Structure of tracheal airway

In normal physiology, the airway epithelium structure consists of several types of cells, which include ciliated, goblet, basal, and Clara cells. Goblet cells populate in the tracheal airways. The population decreases as the structure reaches bronchus and is replaced gradually by Clara cells. These Clara cells are mainly present from distal

position of the tracheal airway until bronchial region (Crystal *et al.*, 2008). On the other hand, ciliated and basal cell population can be found throughout the airways (Crystal *et al.*, 2008). Underneath the airway epithelial layer is a submucosa layer. This layer contains submucosa gland, blood vessel, and smooth muscle. The submucosa gland consists of two types of cells, mucus cells or serous cells (Puchelle *et al.*, 2006). Goblet cells, Clara cells, and submucosa gland are known as mucus-producing structures due to their physiological role in producing mucus, which is essential for defensive barrier (Puchelle *et al.*, 2006).

1.2.2 Normal physiology of tracheal airway

Tracheal airway is often exposed to foreign particles. These particles are harmful and irritant to the tracheal wall. Tracheal airway is equipped with airway surface liquid (ASL) as a primary defense mechanism against inhaled particles (Matsui *et al.*, 1998). ASL consists of upper and lower layers (Matsui *et al.*, 1998; Fahy and Dickey, 2010). The upper layer, which is also known as mucus layer, contains enzyme to digest the foreign particle and has antimicrobial properties. On the other hand, the lower layer, which is also known as pericilliary liquid layer (PCL), is a water-like layer that surrounds the apical surface of ciliated cell. The liquid property of this layer facilitates the mobility for the cilia movement. Collectively, mucus and PCL layers help the movement of digested harmful substance towards the proximal area out from the respiratory system. Eventually, as this substance moves out from the respiratory system, the cough mechanism would be triggered.

1.2.3 Cellular composition of normal tracheal airway

The cellular composition in airway varies between animal models. The distribution of the cells in the tracheal epithelium follows the common pattern among animal models. Ciliated cells populate most areas of the epithelium, followed by basal and goblet cells that are present less in number (Plopper *et al.*, 1983). The following are some examples of mammalian models with their respective population of ciliated, non-ciliated, basal, and goblet cells: hamster (47.5%, 46.7%, 5.6%, 0%), rat (40.6%, 45.9%, 13.4%, 0.5%), rabbit (43%, 28.3%, 28.2%, 1.3%), and sheep (30.6%, 41%, 28.5%, 5.1%) (Plopper *et al.*, 1983).

The density of submucosal gland varies between species. This gland, which is situated in the submucosal layer, produces more mucus compared to goblet cells. The submucosal gland is frequently found in human and large animals while barely seen in small animals. Nonetheless, this gland still can be found in most part of the proximal airway of small animals (Choi *et al.*, 2000). For example, submucosal gland in rats is only found restricted to the proximal area of their tracheal airway (Choi *et al.*, 2000). To a greater extent, this gland is absent or relatively small in number at the larynx tracheal junction area of mouse and rabbit (Choi *et al.*, 2000). However, the situation differs in larger animals such as sheep, ox, and pig. Their submucosal gland resides throughout the airways (Choi *et al.*, 2000). It is suggested that bigger animals relatively need more volume of mucus due to higher particle deposition from higher maximal air velocity (Choi *et al.*, 2000).

1.2.4 Pathophysiology

1.2.4.1 Structure and characteristics of pathological tracheal airway

Harmful particles, viruses, bacteria, and other foreign materials cause disturbances on the respiratory airway. To prevent such disturbances, respiratory airway is equipped with dynamic and distinctive defence mechanisms that line the wall of respiratory airway. Mucus production, cilia movement, and a tight junction between epithelial cells are among these defence mechanisms at the respiratory airway wall. However, constant exposure and overexposure to foreign substance and failure of defensive mechanisms in combating the imminent threat lead to pathological development. A common defensive failure that leads to pathological disease is the loss of epithelial integrity. A study involving human chronic obstructive pulmonary disease (COPD) subject found that the airway epithelial wall of the patient was shedding and sloughing (Naylor, 1962). In asthmatic patient, the epithelial integrity of the airway epithelium, in which the inflammatory factors such as TNF- α , IL13, and IFN- γ are responsible for the phenomenon, is found to be defective (Xiao *et al.*, 2011). This pathological phenomenon is due to the cleavage of junction between basal cell layers that loosens the interaction between these cells thus resulting in the shedding of epithelial cells (Montefort *et al.*, 1992). In addition, impaired epithelial tight junction also facilitates the invasion of viruses, bacteria, and inflammatory cells infiltration (Heijink *et al.*, 2012). The penetration subsequently exacerbates the inflammatory response (Heijink *et al.*, 2012). The epithelium integrity depends on various numbers of adhesion molecules(Roche *et al.*, 1993).For example, Integrin64

is predominant on basal cell layer while desmosomal protein 1 and 2 are prevalent between columnar and basal cell layers (Bhattacharya et al., 2009).

In a pathological condition, the layer of epithelial cells that lies above the basement membrane shows thickening. This apparent thickening of the basement membrane is a result of subepithelial fibrosis at the level of lamina reticularis, and this fibrosis may extend into the submucosal layer. The fibrosis consists of increased deposition of collagens I, III and V, fibronectin, tenascin, lumican, and biglycan (Huang *et al.*, 1999).

Goblet cells and submucosal gland hyperplasia are observed together with mucus overproduction in airway injury. Mucus overproduction is an important pathological remark of respiratory diseases. Persistent mucus overproduction contributes to reduced airway calibre, which further leads to the occlusion of small airways (Jackson, 2001). Individuals with chronic mucus overproduction also suffer from recurrent respiratory infection for persistent duration, causing further exacerbation of their original respiratory pathology (Jackson, 2001).

Two major sources of mucus production in the respiratory tract are the surface epithelial goblet cells and the submucosal glands. In the respiratory tract, goblet cells populate in the trachea and become increasingly sparse towards the bronchioles. The submucosal glands are restricted mostly to the large airways and are absent in the bronchioles. Under the conditions of mucus overproduction, the density of goblet cell increases thus causing further impairment of mucus clearance by mucociliary mechanism.

An increase in smooth muscle mass resulting from hypertrophy and hyperplasia shows that the smooth muscle may play a role in pathological condition of the airway. Hypertrophy is the phenomenon where the cell increases in size as a result to compensate the needs of enlargement of the cell component while hyperplasia is increase in cell number. This pathologic condition, which may cause a respiratory constriction, is commonly observed in asthmatic airways. Furthermore, the airway constriction is also influenced by the changes of cartilage volume. Cartilage is an important structure that determines the airway wall's stiffness and integrity. Decreased cartilage volume and increased cartilage proteoglycan degradation are seen in asthmatic airways. Reduced cartilage integrity may result in a more powerful bronchoconstriction.

1.2.4.2 Cellular response during pathological development

In airway pathological development, the airway tract is constantly exposed to foreign irritant. The persistent exposure forces the airway epithelial cells to respond to protect the epithelial integrity. The airway epithelial cells vigorously participate in repairing mechanism. However, the repairing mechanism is limited. In the case of chronic respiratory disease, the repairing process is undermining. Repeated exposure to irritant develops severe injury, which forces remodelling mechanism instead of normal repairing process. Remodelling impairs the normal airway function by narrowing the lumen in consequence from formation of fibrous tissue. Thus, enhancing, maintaining, and restoring the normal repairing process are indeed

practical approach in preventing the development of the disease (Puchelle *et al.*, 2006).

Indeed, the cell proliferative activity of the airway is very low with less than 1% turnover (Boers *et al.*, 1996). To investigate the potential of the cell in the airway, induced-injury is a prerequisite to provoke an injury to this area. This injury will cause the cells to actively regenerate to form normal airway. Thus, this process allows assessing the cells that have stem cell-like features. For example, goblet cells that line up on the pseudostratified layer of airway epithelium have the potential to give rise to other cell types in response to airway injury and repair (Crystal *et al.*, 2008). This process in turn makes the goblet cells a potential target for cellular therapy. Since abundance of the goblet-mucus cells can be found in the upper region of airway and they are also capable of self-renewable regeneration, it is interesting to study the ability of these cells in regeneration and in repair process of the airway.

Although it has a great defence mechanism against irritant particles, epithelial airway is still prone to injury due to continuous exposure to irritant, which can come in various forms such as chemical poisoning, infectious agent, and immunological response. These disturbances produce similar response towards the cellular changes by increasing inflammatory reaction, cytokine production, and thickening of the epithelial layer itself (Erjefält *et al.*, 1995). For instance, in asthma disease caused by allergens, the immunological system is triggered to respond and thus some pathological features will be shown such as increased airway resistance, chronic inflammation, cytokine production, increased mucus secretion, acute bronchoconstriction, and some other pathological features (Keir and Page, 2008).

Asthma is a complex disease that involves multiple pathological features. This disturbances includes removal of an epithelial layer by physical means, leaving the underlying layer exposed and leading to pathological manifestation of neutrophil infiltration and submucosal oedema (Yahaya *et al.*, 2011). Significant increase in the thickness of the airway epithelium is shown during the administration of smoke in the respiratory system (Brenner *et al.*, 2007). Acute lung injury (ALI) induces in the animal model by instillation of the bacterial endotoxin known as lipopolysaccharide (LPS), which causes oedema, inflammatory reaction, and interalveolar septal thickening (Mei *et al.*, 2007).

1.3 Expression of gene specific to airway respiratory cell types

1.3.1 Lung development

The respiratory system arises from ventral foregut endoderm. The process initiates with the establishment of primary lung bud in primitive foregut separate from oesophagus on the ventral. The bud elongates downward and arises as an early trachea (Cardoso and Lü, 2006; Morrisey and Hogan, 2010). Then, bud develops a bilateral expansion of two bronchi and later forms five major lobes, three on the right bronchus and two on the left bronchus (Cardoso and Lü, 2006). The cellular fate on this early stage development is under the responsibility of surrounding mesenchyme. The mesenchyme, which is originated from foregut mesoderm, differentiates into various cellular fates through epithelial-mesenchymal-transition (EMT) (Beers and Morrisey, 2011). Right interaction and balance of EMT are crucial in determining the

cellular fate. Regulation of EMT is played by several signalling pathways including Wnt, BMP, TGF- β , and FGF (Beers and Morrisey, 2011).

Wnt acts through β -catenin-mediated pathway. Once Wnt binds to Frizzled/co-receptor domain, it leads to inhibition of GSK3 β protein. Absence of Wnt permits GSK3 β to allow β -catenin degradation. Thus, the activation of Wnt causes β -catenin accumulation, stabilisation, and translocation to the nucleus, which in turn binds into TCF/LEF transcription factors to activate different target gene expression (Barker, 2008). Loss of Wnt leads to a complete loss of lung endoderm progenitor specification and retards lung and trachea formation (Goss *et al.*, 2009). Uniformly, the same result occurs with intentional impairment of β -catenin expression (Harris-Johnson *et al.*, 2009). Wnt/ β -catenin signalling activity is critical in this early stage of lung development which on the later stage the activity is decreases due to lack of involvement in those development process (Goss *et al.*, 2009).

The development proceeds in pseudoglandular phase with the bronchiole formation through four repetitive processes namely bud elongation, cessation of outgrowth, expansion of the tip, and bifurcation (Metzger *et al.*, 2008; Morrisey and Hogan, 2010). These progressive processes end up with the formation terminal bronchioles. Limited numbers of studies have been conducted in finding the explanation of molecular mechanism during bud morphogenesis. Tips of the bud constitute multipotent progenitor cells and are maintained by a coordination of Wnt and Fgf significant pathway (Morrisey and Hogan, 2010). Involvement of Wnt signalling is still very much affected with the canonical and noncanonical pathway. Canonical pathway is a pathway normally the cell will choose to undergone certain biological

process. In opposite, noncanonical is phenomenon where the cell choose to deviate from common pathway. Noncanonical pathway like Rho-GTPase and Jun-Kinases is important in expansion of the tip and in modulating the length and diameter of epithelial tubes at different stages (Yu *et al.*, 2009). On the other hand, canonical pathway or β -catenin drives bifurcation of the tips bud (Li *et al.*, 2002). Attenuation on both pathways leads to abnormal morphogenesis of branching airways (Li *et al.*, 2002; Yu *et al.*, 2009).

Meanwhile, as the tips bud outgrows and elongates, the stalk bud, which consists of left progeny, gives rise to all different cell types of the future trachea, bronchi, and bronchioles. These progeny cells are derived from multipotent progenitor cells located in the tips bud. Tracheal surface epithelium mainly consists of basal, goblet, and ciliated cells. It is known that basal cell is a progenitor cell, characterised by Trp63 and Keratin 5/14, which give rise to ciliated and Clara cells (Rock *et al.*, 2009). Clara cells then differentiate into a mucus-producing cell. NOTCH signalling pathway is involved in orchestrated differentiation of basal cells (Rock *et al.*, 2009). Increasing expression of NOTCH leads to the raise of the mucus-producing cells while attenuation results in more number of ciliated cells (Guseh *et al.*, 2009).

After pseudoglandular phase, canalicular phase continues with the initiation of morphogenesis on respiratory part. The development starts on terminal bronchiole until formation of alveolar duct until surfactant protein begins to be secreted in saccular phase. Finally, alveolarisation occurs after birth where respiratory exchange unit is developed and this development leads to the formation of secondary septae (Morrisey and Hogan, 2010).

The developmental pathways namely NOTCH, BMP, and SOX2 are not only confined to this group of genes. Molecular mechanism involved is complex with many intertwined, overlapped genes, which regulate each other. A few reviews have documented list of genes ranging from signalling molecules, transcription factors, and other groups (Costa *et al.*, 2001; Cardoso and Lü, 2006). Nonetheless, few of them have been well studied and interestingly, evidence has shown that the developmental pathway may recapitulate in adult injury repair induced during diseases state (Beers and Morrisey, 2011; Masterson *et al.*, 2011; Rock *et al.*, 2011). However, catalogued genes involved in the lung developmental process especially on the early stage of expression proceed along the process and are utterly terminated in adult cells injury (Cardoso and Lü, 2006). Among the genes are Id2, SOX9, and Etv5(Morrisey and Hogan, 2010). This suggests that some of the genes are restricted to participate during developmental process. How these genes behave differently from others remains elusive.

Several genes involved in lung developmental process have been studied in repair and regeneration process of airway epithelial injury (Rosendahl *et al.*, 2002; Tompkins *et al.*, 2009; Song *et al.*, 2010; Masterson *et al.*, 2011; Whitsett and Kalinichenko, 2011; Xing *et al.*, 2012). The findings show that these genes recapitulate and play important roles in the repair process after adult tissue injury. The study on repair and regeneration process also finds the specific regulation and involvement of these genes. Expression of SOX2 is required in maintaining the pluripotent and undifferentiated state of embryonic stem cell during lung developmental process (Tompkins *et al.*, 2009; Song *et al.*, 2010). During repair and

regeneration of airway epithelial injury, SOX2 has been found to be involved in the specific regulation of Clara cell differentiation in the bronchioalveolar region (Tompkins *et al.*, 2009; Song *et al.*, 2010). Other studies state that proliferation and differentiation of Clara cell are regulated by the NOTCH signalling pathway through NOTCH1 receptor, which is abundant in the airway epithelial cell (Whitsett and Kalinichenko, 2011; Xing *et al.*, 2012). These evidences demonstrate that the role of genes during embryonic period of lung development process is conserved in the repair and regeneration of airway epithelial injury.

1.3.2 Regeneration and repair

Regeneration and repair is a complex process that involves various components and regulations governing the process, including molecular process with huge involvement of thousands of genes (Heguy *et al.*, 2007). The genes participate in cell cycle, signal transduction, metabolism, and transportation (Heguy *et al.*, 2007). During the repair process, change in the expression level occurs simultaneously too with many of the genes up regulated and down regulated. As the healing is almost completed, the expression patterns are similar with the resting epithelium.

Cell migration is an essential activity in a dynamic repair process. Following removal of epithelium, remaining cells have to move into denuded area in order to replace the lost cells and start to regenerate the normal epithelium (Keenan *et al.*, 1982). Locomotion of the cells involves attraction and breakage of the adhesive contacts between cells and extracellular matrix (ECM), which acts as a floor for the cells to

adhere and subsequently exert the attraction force that enables cell to migrate (Greenlee *et al.*, 2007).

Proteolytic enzyme, MMP group serves a very important role in facilitating the movement of the cells. Upregulation of MMP during the time of disease is extensively important for the healing process in the later stage (Greenlee *et al.*, 2007). Nevertheless, increased regulation beyond the normal state can cause tissue to deteriorate instead of getting cured (Greenlee *et al.*, 2007). In particular, MMP9 has been regarded as a crucial component in epithelium repair in many studies been conducted (Coraux *et al.*, 2005b). MMP9 specifically degrades gelatinase b, which is also known as type IV collagen of ECM. MMP9 is strongly expressed by human bronchial epithelial cells (HBEC) during repair process (Legrand *et al.*, 1999).

Knockout MMP12 mice exposed to the prolonged cigarette smoke fails to recruit inflammation to irritated area compared to wild type. Thus, no development of the disease could be seen (Greenlee *et al.*, 2007). Similar study states that the lack of MMP9 production in mice reduces the number of eosinophils and neutrophils (Corry *et al.*, 2004). These evidences show that MMP does regulate traffic movement of inflammatory cells from adjacent bloodstream to mucosa and submucosa of epithelial airway.

1.4 Animal model system

1.4.1 Rationale of using animal as a model

In the field of respiratory disease research, subjects range from fundamental, developmental, and application are required in creating a better approach to combat the disease. Human is an ideal subject to fulfil the research need. However, human subject faces some limitations. Ethical and health concerns are the main issues that render the human subject from being utilised in research application. Thus, in many studies, animal model is used to replace human as a subject. An animal model recapitulates the mechanics of human with research finding being reflected on the human perspective. Hence, several factors must be considered for a research to choose the best animal model in the experiment. In that sense, the finding demonstrated by the animal study should reflect the nature of human body.

1.4.2 Cellular mechanisms towards airway regeneration and repair using animal model system

An animal model is used to recreate the event of human lung mechanics in order to have a reliable result that reflects the human. Several factors must be considered for a research to choose the best animal model in the experiment. There are many animal models used in the airway respiratory disease research, ranging from sheep, guinea pig, hamster, mice, mouse, and rabbit (Keenan *et al.*, 1982; Barrow *et al.*, 1992; Kim *et al.*, 1997; Brenner *et al.*, 2007; Yahaya *et al.*, 2011). Anatomically, large animals are more similar to human. Another advantage of using large animal model is that the

researcher is able to practically measure the lung function. However, large animal is expensive and difficult to handle. On the other hand, small animal models such as rat and mouse are available for genetic manipulation, which enables tracking a specific cell during regeneration process using a specific marker.

The aim in studying regeneration and repair process of airway epithelium using animal model system is to acquire the fundamental aspect in understanding the process involved in the event. Regardless of the type of animal model used in the study, this process follows some common stages. There are five stages of cellular event during the repair and regeneration of airway epithelium (Coraux *et al.*, 2005a): 1) Spreading and migration of the cell to the damaged area; 2) Cell proliferation and single squamous epithelial formation; 3) Cell proliferation and formation of multilayered epithelial cell; 4) Redifferentiation of terminally differentiated cell; and 5) Ciliogenesis. Eventhough this process has these common stages, the time frame needed for each of the stages differs depending on the animal model chosen. For hamster, the regeneration begins with the spreading and migration of the basal cells neighbouring the wounded area beginning 6 to 12 hours. On day 1, proliferation with active formation of new cells forms multilayered squamous metaplasia. Then, redifferentiation takes place with the emergence of precipitated cells on day 2 time point. Finally, after 3 days, formation of cilia through ciliogenesis rises to complete the regeneration (McDowell *et al.*, 1979). Similarly, a study using dog model showed the repair activities at 6 hours, in which the flattened cells were found migrating and residing on the denuded area after tracheal injury from SO₂ exposure (Hulbert *et al.*, 1989). In another experiment that used hamster model, the border of

the damaged area was populated by the flattened cells observed at 6 hours after the physical injury on the trachea. By 12 hours, the damaged area was fully covered with single layer of flattened cell. On day 1, 2, and 3 multilayered cells formed. In contrast with previous finding, the normal regeneration of hamster tracheal epithelium was completed on day 5 after injury (Keenan *et al.*, 1982). Meanwhile, in unrelated study using guinea pig as an animal model, the physical damage was applied on the tracheal airway and the damaged epithelium recovered on day 7 (Kim *et al.*, 1997). After prolonged exposure to chlorine on the mice airway, it was found that the remaining cells after injury required 7 days to achieve the complete regeneration (Mo *et al.*, 2013). Thus, in small animal model of epithelial airway injury, the cellular response needs between 5 to 7 days to achieve well-balanced normal restitution of epithelium.

In contrast, human airway regeneration takes long time to complete. Human airway xenograft model needs 35 days to complete the regeneration of the epithelial tracheal tissue (Dupuit *et al.*, 2000). Pseudostratified epithelium can be observed after 25 days and complete epithelium redifferentiation can be observed on day 35. This process is similar in human airway xenograft model where the pseudostratified epithelium are seen as early as day 25 and the regeneration continues until day 35 (Coraux *et al.*, 2005a; Hajj *et al.*, 2007). However, the time frame needed for the regeneration of primary epithelial airway culture harvest from cystic fibrosis (CF) patient is slightly delayed (Hajj *et al.*, 2007). The pseudostratified layer is only observed after 35 days. The regeneration process in human xenograft model follows some common steps. Three days after injury, the trachea is populated with flattened,

nonciliated, and poorly differentiated cells. The cells are derived from airway epithelial and seen to have expanded cytoplasm. In the meantime, migration happens on these cells towards denuded area. After 2 weeks, the whole injured area is covered by abundant proliferative squamous epithelial cells in the stratified layered pattern (Dupuit *et al.*, 2000; Coraux *et al.*, 2005a; Hajj *et al.*, 2007). This finding of using human xenograft model is supported by the study on the sheep bronchi after being injured using brushing technique (Yahaya *et al.*, 2011). It was observed that the regeneration was still in progress on day 7 after injury where the substantial evidence of redifferentiation took place (Yahaya *et al.*, 2011).

From the various studies on the different experimental system, it can be concluded that the regeneration period is different from one system to another especially in human, which needs at least 35 days to complete the stages. In spite of these differences, they still have a common repairing pathway. The pathway starts with flattening-dedifferentiated cells bordering wounded area, followed by migration to the denuded area and massive proliferation, finally redifferentiation that takes place until formation of pseudostratified epithelial cells and other specialised type of cells. This final formation remarks the completion of the regeneration (Coraux *et al.*, 2005a).

1.4.3 Methods to induce injury

Physically induced injury on the airway epithelium is defined as an injury manifested by the removal of epithelium and epithelial shedding (Raub *et al.*, 2010). Epithelial layers consist of different types of cell. Basal cells situated on the basement

membrane are one of the marks used to differentiate degrees of injury. Barrow et al. classify the epithelial airway injury into four levels (Barrow *et al.*, 1992): 1) The reversible injury; 2) The irritant causes the exfoliation of individual cells; 3) As the injury becomes more extensive, there will be desquamation of cells with the intact basal cell and basement membrane; and 4) Far more severe injury will cause a complete removal of epithelium and basement membrane. On the other hand, Coraux et al. propose three levels of injury (Coraux *et al.*, 2005a): 1) The injury causes a mild disturbance and thus causes epithelial surface integrity loss; 2) The injury demonstrates partial shedding of epithelial cells; and 3) A more severe injury causes a completely denuded basement membrane.

There are many techniques to inflict injury on the epithelial airway by physical means. The suitable technique must be chosen according to criteria such as accessibility of the technique to the target site, availability and cost of the equipment, and ability of the technique to reduce or eliminate chances of introduction of any other possible causes of injury. Nylon brush has been established in the removal of epithelial layer by scraping the rabbit trachea that has previously been incised using surgical technique (Nakagishi *et al.*, 2005). The airway shows inflammation, proliferation of fibroblasts, and thickening of the collagen after nine days of post-scraping.

On the other hand, sophisticated technique that combines between bronchoscope and bronchial brush successfully produces an injury in bronchi stem of sheep (Yahaya *et al.*, 2011). By utilising the bronchoscope, the predetermined area for brushing can be selected and the injury can be verified by the mucosal bleeding immediately. After 6

hours following injury, complete disturbances of epithelial layer and neutrophil infiltration can be seen on the affected area. In addition, inflicted injury using steel probe can be executed in either pre-exposed airway structure with tracheotomy or combined with fibre optic that passes through the mouth; both methods can be carried out in hamster and guinea pig (Keenan *et al.*, 1982; Erjefält *et al.*, 1995; Kajstura *et al.*, 2011). This technique causes de-epithelisation without disrupting the submucosal layer. Researchers use curettage and cotton swab in combination with surgical technique is the early physical technique used to inflict injury in the airway (Wilhelm, 1953; Hilding, 1965).

However, most of these techniques employ surgical approach to pre-expose the airway system before inflicting injury. Such an approach may expose the injured area to infection that can interfere with the repair mechanism and can lead to the death of the animal. Although the techniques can be assisted by an optical approach to avoid surgical technique especially in sheep and guinea pig, due to limited financial resources, the optical equipment cannot be afforded (Erjefält *et al.*, 1995; Yahaya *et al.*, 2011).

Despite its wide utilisation in research, surgery-related techniques pose some disadvantages. Dedicated time and personnel with surgery skills are required since correct incision is mandatory. In addition, the surgical wound would potentially expose the animals with high risks of infection. Therefore, additional treatments are required to ensure that animals stay healthy and alive. Even though bronchoscope utilises more direct approach to the injury site, the equipment itself is expensive and researchers find financial difficulties to buy it especially if there are limited financial

resources. Due to these main factors, our study has developed a brushing technique, which does not require surgical opening of the trachea and is simpler than bronchoscope. The technique is expected to overcome disadvantages incurred by the surgery-related techniques. Personnel with specific surgical skills are no longer required, making the procedure much more user-friendly. Moreover, the absence of surgical wound would be expected to significantly reduce the risk of infection to the animals.

1.4.4 Rabbit as a model in tracheal airway induced injury

In order to select the suitable animal model system, some of the features that need to be considered include anatomical similarity to the human, cost and setup of the animal, handling technique, and suitability of the technique (Keir and Page, 2008). Several studies on the effect of physically induced injury towards repair and regeneration of tracheal epithelium use rabbit as a model. Due to the smaller diameter of rabbit bronchial lumen as compared to large animal such as sheep, the physical tools applied to produce injury could only reach as far as the tracheal region. Nevertheless, it makes the rabbit relevant animal to study the tracheal region. In addition, the absence of submucosal gland in the rabbit tracheal airway makes the model suitable to the study of the response of mucus cell production, i.e., goblet cell population without interference with submucosal gland towards tracheal airway injury (Choi *et al.*, 2000). This limitation is however absent in large animal. Unlike rabbit, studies that use large animal model have superior advantage in terms of the size of respiratory system of the large animal, which is similar to the human lung

mechanics. Eventhough rabbit has a smaller size than sheep; previous study has successfully proven the feasibility of using rabbit model in measuring the lung function in the aspect of airway pressure and gas exchange efficiency (Hon *et al.*, 2000).

To date, sheep is the only large animal model system used in the study of bronchial airway epithelium regeneration and repair after physical injury. Sheep as a large animal model system allows visualisation by insertion of the optical equipment through respiratory lumen (Yahaya *et al.*, 2011). The similar approach has been applied by using Optical Coherence Tomography (OCT) to provide the real-time *in vivo* monitoring of tracheal airway mucosa (Brenner *et al.*, 2007). Hence, using rabbit as an alternative model to replace large animal model is relevant and practical in the sense of lung mechanics and application of physical induce injury technique.

In terms of animal handling, lack of the required laboratory setup to restrain sheep while performing anaesthesia and induced-injury technique may be the shortcomings of using large animal model. In addition, this approach may need extra personnel to handle the animal during performing the experiment. In small animal study, rat, hamster, and guinea pig have been widely utilised in the study of pathological changes of the airway injury (Keenan *et al.*, 1982; Erjefält *et al.*, 1995; Kajstura *et al.*, 2011). The laboratory equipment used to handle the animal is simple and cost-effective, making small animal model a better choice than large animal model.

Physical technique to induce injury on tracheal epithelium using smaller animal has been implemented in many studies (McDowell *et al.*, 1979; Keenan *et al.*, 1982; Kim *et al.*, 1997; Nakagishi *et al.*, 2005; Raub *et al.*, 2010; Kajstura *et al.*, 2011). Surgical technique is used to expose the trachea before the technique is applied on the epithelium. Cotton swab and brushing are among the tools used. The surgical technique has some disadvantages. Injury produced from surgical intervention increases the risk of infection. Thus, the chance for the animal to die during the experiment is high. Because the size of rabbit is bigger than any other small animal model, recent study has utilised a direct approach to induce injury on tracheal region. The technique uses cytology brush by inserting the brush to the trachea via mouth opening with the guidance of an endotracheal tube (Raub *et al.*, 2010). This technique is an improvisation from the previous technique to avoid using surgical technique (Nakagishi *et al.*, 2005).

1.5 Rationale of the study

Techniques used to induce airway injury face few challenges which include exposed to infection due to its requiring surgical intervention. Therefore, the development of non-surgical based method with simple laboratory setting are required as an alternative strategy that is capable to produce injury to the target area (the airway) whilst minimising the risk of infection. Understanding the biological responses of airway epithelium following physical-induced injury is the fundamental aspect of this study where the molecular and cellular responses towards the airway epithelium regeneration and repair will be studied. As the rabbit is well known model that

mimics the human condition of lungs and represents well as a model for asthma and other chronic lung diseases, thus this study could provide strong foundations on how the rabbit airway responses towards physical-induced injury. The involvement of goblet-producing cells as a hallmark for asthma-related symptoms (Aikawa *et al.*, 1992; Ordoñez *et al.*, 2001). will be investigated, to be the future target for cell-based therapy in treating asthma.

1.6 Objectives of the study

1.6.1 General objective

To investigate cellular response and molecular expression during repair process of rabbit's tracheal airway epithelium following brushing-induced injury.

1.6.2 Specific objective

- To develop a blinded brushing technique that could produce a physical injury on the tracheal epithelium using rabbit as a model.
- To study the pathophysiological and goblet cells changes of the airway trachea in response to the repair of tracheal airway epithelium following brushing-induced injury using a haematoxylin and eosin (H&E) and Alcian blue-periodic acid Schiff (AB-PAS) staining technique.
- To assess the molecular gene expression profiles of the genes involved in inflammation response, proliferation activity, extracellular matrix (ECM), stem cells recruitment, and cell specific gene in response to brushing-induced injury of airway epithelium using real time PCR.

1.7 Flow chart of the study

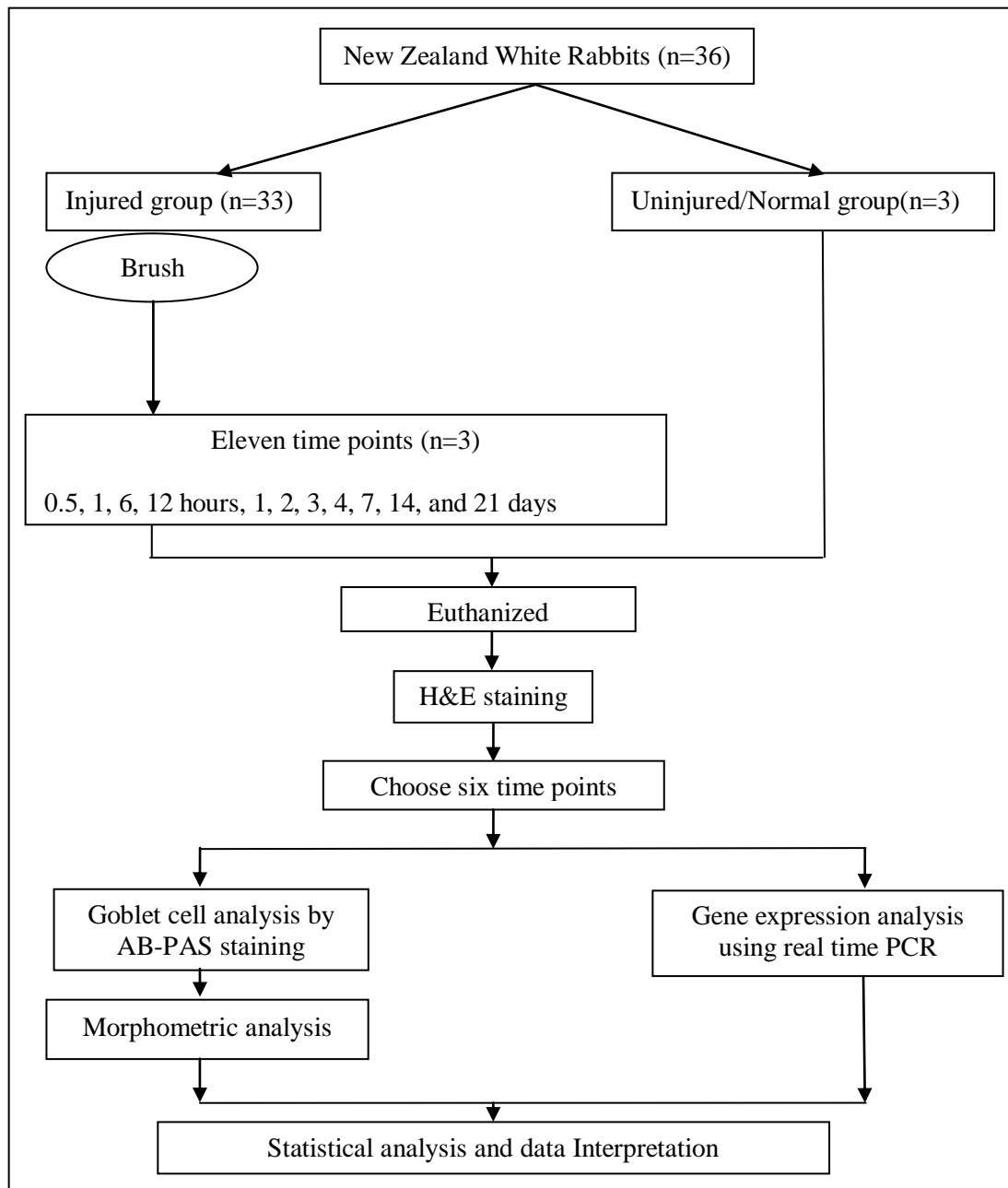


Figure 1.1: Flow chart of the study

[H&E: haematoxylin and eosin, AB-PAS: Alcian blue-periodic acid Schiff]