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"An Investigation Of Murine Trachea Cellular Kinetics: Implications For Stem Cell Gene Therapy"

Duncan William Borthwick

Submitted For The Degree Of Doctorate Of Philosophy

The University of Edinburgh

1998

"Whatever You Do Will Be Insignificant

But It Is Very Important That You Do It."

Ghandi

DECLARATION

I declare that the experiments described in this thesis were, unless otherwise stated, undertaken by me.

(Duncan W. Borthwick)

October, 1998

Acknowledgements

This page, as one of many in this thesis, is unique. Not only is this page devoid of any intellectual input from my Supervisors and learned Colleagues (inadvisable many would say) but this page is written in the *first person*. It is also significant in that it will undoubtedly be the most read page in the entire thesis (reference: personal experience!) But since brevity is the soul of photocopying and rambling is the impediment of clarity, I shall continue.

There are many, many people who have contributed to the existence of this piece of work. Many have encouraged, many have advised and many have had the wisdom to tell me when I've strayed from the paths of common sense and logical interpretation. In all of these categories, Julia Dorin, John West, David Porteous and Scott Randell have been of paramount importance. To them I send my grateful thanks and my admiration.

I am very grateful to the inhabitants of the labs in which I have worked: in Edinburgh (MRC Human Genetics Unit, the Centre for Reproductive Biology, the Department of Pathology) and also in the US, the CF Center (that's how they spell it) in Chapel Hill, North Carolina. Of special mention are those who have been involved in some of the experiments and in the production of this thesis; Donald Davidson, Duncan Davidson, Norman Davidson, Brendan Innes, Margaret Keighren, Jean Flockhart, Lorna Mitchell, Paul Perry, Alison Ross and Sheila Webb.

I would especially like to thank the staff of the Biomedical Research Facility and the Transgenic Unit for their care of my experimental animals and their unlimited supply of wisdom, good humour and advice.

On a more domestic front, I thank those who have made the last 3 years an altogether bearable and yeah, pleasant experience. To Kate, my Folks and flat mates old and new, my humble thanks and gratitude. Also deserving special mention are my climbing partners, biking chums and all those people who have helped to retain some level of sanity, mental clarity and concentration in my life.

And that's it really.

Hope you enjoy the next few hundred pages.

Adieu,

Duncan

Abstract

Cystic fibrosis (CF) is a highly prevalent autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The main phenotype of clinical significance is the development of chronic lung infections which is the cause of morbidity and mortality in 95% of patients. The processes by which CF lung disease occurs are however, poorly understood.

In the last 5 years there has been considerable interest in gene therapy as a form of treatment for CF. Gene therapy is a method of therapeutic intervention in which a functional copy of a gene is inserted into a cell to replace or complement a non-functional gene. One area of growing importance in this field is that of the kinetics of target tissues; in the case of CF gene therapy, the lung epithelium. By understanding the kinetics of the lung epithelium more efficient methods of gene therapy can be envisaged, especially if epithelial progenitor cells or cells expressing *CFTR* could be targeted.

This thesis aimed to investigate the cellular kinetics of the trachea including that of the submucosal glands. Submucosal glands produce mucus and antimicrobial agents onto the epithelial surface and are thought to be implicated in the occurrence of the abnormal mucus and pathology, which are characteristics of the CF lung. In addition, this thesis aimed to explore methods of liposome somatic gene therapy, which may prove, in the future, to be useful in the targeting of progenitor cell populations.

The suitability of the mouse to act as the animal model for these studies was examined. The mouse showed high similarity to the human with respect to pulmonary pathology and anatomy implicated in CF pathophysiology. This included epithelial cell types and the structure and functionality of submucosal glands. Some differences were also observed including epithelial cell type frequency and a restricted submucosal gland distribution.

The spatial distribution of murine submucosal glands was found to be significantly affected by both genetic background and cftr expression. These data suggested that *cftr* may play a developmental role and that genes which act as modifiers of submucosal gland distribution may exist. This study led to the development of a model which outlined a possible misnomer in the traditional interpretation of single point electrophysiology measurements as a means to measuring CF phenotype in the mouse.

Submucosal glands were found to be the major site of cftr expression in the mouse trachea (an observation similar to that previously reported in humans). Reduced *cftr* expression in this region may have the consequence of raising chloride concentrations in the airway surface fluid. In this thesis, data are presented showing that both the human and mouse homologue of the β-defensin-1 peptide shows a selective salt dependent antimicrobial activity. This may in part explain the higher susceptibility to colonisation by certain bacteria in the CF lung.

In the search for possible progenitor cell populations in the mouse trachea, studies were conducted into the development of the submucosal glands and the kinetics of the tracheal epithelium in steady state and after damage. Through the study of aggregation chimaeric mice, evidence is presented to suggest that mouse submucosal glands are clonally derived from a single progenitor cell.

In the mouse tracheal epithelium, basal cells were found to be the epithelial cell type with greatest proliferative capacity. Furthermore, a discrete niche of cells exhibiting stem cell characteristics were found in the submucosal gland ciliated duct. These cells were observed to be proliferative in times of stress to produce migratory transit amplifying cells which act to repopulate and maintain the local epithelium.

Experiments were conducted using liposome mediated gene therapy to approach the issue of targeting the tracheal progenitor cell populations. Although positive results for tissue transfection were obtained, the levels were disappointingly low.

This thesis concludes that firstly, the mouse provides a useful animal model in which to study human tracheal kinetics and secondly, cells of high proliferative capacity displaying stem cell characteristics exist in the tracheal epithelium. The targeting of these progenitor cells may be of considerable importance in the future development of CF gene therapy.

Abbreviations

μ	micro ($x10^{-6}$)
β-gal	beta galactosidase
A	adenine
AAV	adeno-associated virus
AB	alcian blue
ABC	ATP binding cassette
ABC	avidin biotin complex
AP	alkaline phosphatase
ASF	airway surface fluid
ATP	adenosine triphosphate
BrdU	bromo deoxyuridine
BRF	Biomedical Research Facility
BSA	bovine serum albumin
C	cytosine
cat	chloramphenicol acetyltransferase
CF	cystic fibrosis
cftr	mouse cystic fibrosis transmembrane conductance regulator gene
cftr	mouse cystic fibrosis transmembrane conductance regulator protein
CFTR	human cystic fibrosis transmembrane conductance regulator gene
CFTR	human cystic fibrosis transmembrane conductance regulator protein
CMV	cyto meglo virus
CPI	cell proliferation index
cAMP	cyclic adenosine monophosphate
DAB	diaminobenzidine
dH_2O	Distilled water
DNA	deoxyribose nucleic acid
DOGS	dictoadecylamido-glyvylspermine
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N,-trimethylammonium methyl sulphate
E	embryonic day
EDTA	Ethylene diamine tetra acetic acid
ELISA	enzyme linked immuno substrate assay
ENaC	Epithelial sodium channel
fcb	ciliary beat frequency
g	gram
G	guanine
G6PD	glucose-6-phosphate dehydrogenase
GT	gene therapy
H&E	haematoxlin and eosin
H_20	water
H_20_2	hydrogen peroxide
HBD	human beta-defensin
HBECs	human bronchial epithelial cells
IP	Interperitoneal
IRAP	interleukin receptor antagonist protein
l	litre
LEF-1	Lymphoid Enhancer Factor-1
LRC	label retaining cell

m	metre
M	molar
mABs	monoclonal antibodies
MBD	mouse beta-defensin
MRC	Medical Research Council
MRCs	Mitochondrial-rich cells
MRNA	messenger RNA
MtRC	mitochondrial rich cell
MTV	Mucociliary transport velocity
n	nano ($x10^{-12}$)
NBD	nucleotide binding domain
NBF	neutral buffered saline
p	pico ($x10^{-9}$)
PAS	periodic acid schiff
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
p.d.	potential difference
pH	logarithm of hydrogen ion concentration (base 10)
PI	Pancreatic Insufficiency
PKA	Phospho Kinase A
PT	particle transport
RNA	ribose nucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SMG	submucosal gland
SPF	specific pathogen free
SSC	sterilised sodium citrate buffer
SV	simian virus
T	thymine
Taq	*Thermus aquinus*
TAC	transit amplifying cell
TEM	transmission electron microscopy
TM	transmembrane
TPA	12-*O*-tetra-decanolyphorbol-13-acetate
U	uridine

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Statement Of Collaboration

A number of the studies reported in this thesis were undertaken as part of a collaborative effort by other scientists and myself. Full details of the level of collaboration can be found in the appropriate sections:-

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4 : Defensin Study	Dr. Donald Davidson	97
5.2: Study of Aggregation Chimaeras	The Laboratory of Dr. John West	128
7.3: Study of Label Retaining Cells	Tod Krantz	242

Aim

The aim of this thesis was to investigate the kinetics of the mouse trachea with a view towards developing novel strategies for gene therapy treatment of cystic fibrosis in humans.

m	metre
M	molar
mABs	monoclonal antibodies
MBD	mouse beta-defensin
MRC	Medical Research Council
MRCs	Mitochondrial-rich cells
MRNA	messenger RNA
MtRC	mitochondrial rich cell
MTV	Mucociliary transport velocity
n	nano ($x10^{-12}$)
NBD	nucleotide binding domain
NBF	neutral buffered saline
p	pico ($x10^{-9}$)
PAS	periodic acid schiff
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
p.d.	potential difference
pH	logarithm of hydrogen ion concentration (base 10)
PI	Pancreatic Insufficiency
PKA	Phospho Kinase A
PT	particle transport
RNA	ribose nucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SMG	submucosal gland
SPF	specific pathogen free
SSC	sterilised sodium citrate buffer
SV	simian virus
T	thymine
Taq	*Thermus aquinus*
TAC	transit amplifying cell
TEM	transmission electron microscopy
TM	transmembrane
TPA	12-*O*-tetra-decanolyphorbol-13-acetate
U	uridine

Chapter 1

Introduction

A Short Guide for the Reader

This thesis investigates the kinetics of the tissues of the mouse trachea and begins to explore possible means by which progenitor cell populations could be targeted for phenotypic correction by gene therapy.

At this time, the technology of aerosolised somatic gene therapy, as a method of treatment of the lung phenotypes associated with cystic fibrosis, is still in its infancy. Yet even once the technology is improved so that it can be marketed as a useful therapeutic drug, this particular strategy of therapy may still be considered imperfect. Due to the steady turnover of cells in the lung such treatment would require frequent dose delivery and furthermore, pulmonary tissues considered to be important in the development of CF lung disease may not be accessible to aerosolised methods of gene therapy delivery.

The submucosal glands are the prime example for this argument. There are many pieces of albeit circumstantial evidence which links these glands with the development of the abnormal pathology observed in CF patients yet their complex structure, and mucus efflux makes them inaccessible to aerosolised gene therapy drugs. One way to overcome these issues (and indeed, the aim of this thesis) is to target pulmonary progenitor cell populations. Genotypic correction of these cells could perhaps lead to the correction of cell types beyond those accessible by current aerosolisation techniques and reduce the requirement for frequent dosing.

A considerable amount of data has been published concerning the kinetics of the smaller airway epithelium and the alveoli. The method by which cellular renewal and repair occurs in the larger airways is still unclear. Therefore, this thesis focused on two different, though structurally connected, tissues: the tracheal epithelium and submucosal glands.

The mouse was chosen as a candidate for use as the animal model for this research primarily for the in-house expertise concerning this animal and the availability of the many different transgenically produced mice which are deficient in the normal expression of the CF gene.

In particular, the biology of murine submucosal glands, with their strong link with the development of CF lung disease in humans was examined. This work is presented in chapter 2. Chapter 3 extends and broadens this work by investigating variation in submucosal gland biology and distribution in mice of varying genotype and genetic background.

Chapter 4 examines the currently popular theory of β-defensin inactivation. This theory describes the presence of abnormal pulmonary secretions arising from the submucosal glands. This leads to the inactivation of one of the many anti-microbial proteins present in the human and mouse lung and may explain (in part at least) why the CF lung is more susceptible to bacterial colonisation than non-CF lungs.

The submucosal gland story is continued in chapter 5 but with an eye towards the developmental kinetics of the gland. These experiments aim to explain the developmental kinetics of the submucosal glands with view to identifying and developing systems by which progenitor cell populations could be corrected by gene therapy.

Chapter 6 leaves the world of submucosal glands briefly and is involved in the current methods for gene therapy delivery to the mature mouse trachea. Chapter 7 returns to the big question of whether there are progenitor cell populations in the tissues of the trachea? If there are, in what direction should research continue to fulfil the original aim of targeting these cells for gene therapy correction?

Each chapter is preceded with an introduction that describes the pertinent research and conclusions in the particular field and also describes the specific experimental aims of the studies undertaken. To begin with, however, a general introduction to CF.

Historical Perspectives of Cystic Fibrosis

Prior to the 1930's it was not known why children who had died of lung disease also commonly presented with intestinal blockages and fibrosis of the pancreatic acini. However, since its original classification (and naming as a consequence of pancreatic pathology) by Dorothy Anderson in the late 1930's (Anderson, 1938) much has been learnt about cystic fibrosis (CF). By the end of the 1940's, CF was known to be was caused by the inheritance of two copies of a dysfunctional gene but it was not until 1989 that the CF gene, the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*), was identified (Riordan, 1989). CFTR is a chloride channel, which primarily operates in regulating the ionic composition of cellular secretions and the cell's neighbouring environment. When CFTR does not function normally, the disease CF occurs and a wide range of tissues are affected. The complications that arise lead, most commonly, to the death of the individual by pulmonary failure at a median age of 29 years.

The chances of a cystic fibrosis patient being born to white parents in the UK are about 1 in 2000. In Black populations, this figure is approximately 1 in 17000 and in Oriental populations about 1 in 90000 (Stern, 1986). CF is caused by the inheritance of two copies of a recessive mutation of the CFTR gene. Thus, the frequency of 1 in 22 Caucasians in the UK being "carriers" (heterozygous) of a CFTR mutation puts CF into the unwanted position of the most common autosomal recessive disorder in Northern Europe. The high prevalence of the disease if reflected in the number of CF researchers, scientists and clinicians. CF patients would perhaps be comforted to

know that the ratio of US CF patients to delegates at the 1997 North American CF Conference was approximately 30:1.

Improving techniques for the care of CF patients and the treatment of their symptoms has led to an incredible increase in the median survival age of CF patients from about 1 year in 1938 to 12 years in 1970 and 29 years in 1998 (Davis, 1996). As this survival curve altered its position, the patients' requirements for the care and treatment have also changed. Care of CF patients has grown beyond the exclusively speciality of paediatric doctors into the requirement of practical care and support for adult patients.

Although CF diagnosis is now possible through molecular identification of *CFTR* mutations in the new-born patient, diagnosis still occurs most commonly through more traditional means. The clinical features go to make the classic CF "diagnostic triad" are a high chloride concentration in the sweat (as determined by Gibson and Coke's method from the 1940's (Gibson and Coke, 1959)), chronic pulmonary disease of appropriate character and/or pancreatic insufficiency and/or a sibling or first cousin with CF (Davis, 1984).

There can be considerable variation in the symptoms presented. About 80% of CF patients present with pancreatic insufficiency although nearly all present with abnormal chloride concentrations in the sweat. It is estimated that only about 0.1% of patients with typical CF lung disease have normal sweat chloride concentrations (Davis, 1980).However when the complete triad is present, CF exists as a unique disease.

The Search For The CF Gene

The discovery of the gene whose dysfunction causes CF was only made possible through the development of a technique called "positional cloning". This technique is based on chromosomal position of the gene rather than knowledge of the defective protein. This technique was especially useful since searches for chromosomal alterations in CF patients (which would have provided some structure to the hunt) proved unsuccessful. Linkage studies took the search onto Chromosome 7 and then further to the region of 7cen-q22 (White, 1985). CpG islands were used as markers to reduce the search site and to enable the methods of "chromosome walking" and "chromosome jumping" to be used. These provide the means for covering large areas of a candidate region for markers. The Riordan group eventually identified the CF locus and found a gene which was evolutionary conserved between several mammalian species (Riordan, 1989). This gene was able to fulfil the three key criteria for the "CF" gene: firstly, its locus is situated on the correct chromosomal region; secondly, the expression pattern was found to be consistent with the tissues affected in the disease and thirdly, a mutation in this gene was found that only occurs in CF patients (Rommens, 1989). As a consequence of preliminary cDNA data (and not for ease of acronym pronunciation) the gene was called the Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR). The CFTR gene is composed 27 exons encompasses about 250 kilobases of DNA in addition to the CFTR promoter region of which little is understood (Vuillaumier, 1997, Verlingue, 1998, Denamur and Chehab, 1995).

The Mutations Of CFTR

At this time over 800 different mutations in CFTR have been identified. Of these, one mutation stands out from the rest as a result of its high prevalence. The ΔF508

mutation is caused by a 3 pair deletion of a phenyalanine codon on exon 10. Over 68% of CF patients have this mutation (Kerem, 1989, Tsui, 1992). The remaining mutations represent the complete range of possible genetic mutations although appear to be more highly localised to particular genomic regions including the two nucleotide binding domains. Furthermore, these mutations occur in the regions conserved across species suggesting evolutionary functional homology.

The different mutations bring about dysfunction of CFTR activity in a number of different ways and a corresponding selection of severity of phenotype. Mutations in CFTR can be categorised as those resulting in :

1: Defective CFTR translation: unstable CFTR mRNA (usually caused by the presence of new stop codons) is produced which results in the production of shortened protein which quickly degrades in the cytoplasm.

2: Alternate splice sites: stable but shortened mRNA leading to the production of shortened protein

3: Defective post translation processing of CFTR: CFTR protein is produced but is not properly glycosylated and as a consequence is not correctly localised in the apical membrane. However, often the CFTR is potentially functional if only it could reach the membrane. Such is the case in ΔF508 (Cheng, 1990). By reducing the temperature of ΔF508 expressing cells *in vitro*, some normal CFTR activity can be rescued (Drumm, 1991). This finding has led to considerable amount of research which aims to correctly localise ΔF508 CFTR as a means of therapeutic correction.

4: Defective ion channel activity: CFTR is correctly localised to the apical membrane but does not function correctly. Mutations resulting in only partial

reduction of CFTR activity are often of this class and are associated with mild CF phenotypes as those who will not go to develop fatal lung disease.

Prenatal and postnatal screening

The discovery of the CF gene opened the doors for the development of genetic screening for CF offering informed reproductive decisions to be made by those at risk. With the possibility of prenatal diagnosis, parents would have the knowledge to prepare for having a CF child or infact, give them the right to terminate that pregnancy. Additionally, the screening of new born babies could result in treatments starting sooner, leading to improved health.

It is now possible to test for CF at the pre-implantation stage. Although this technique carries with it great financial costs as well as only a 10% success rate for implantation, it can provide parents with the opportunity to choose against having a CF child without the ordeal of a possible termination later in term. At this time, the procedure of pre-implantation selection is not in general practice (Davis, 1996).

It should be noted that the high number of possible CFTR mutations increases the complexities of screening. Screening is most commonly conducted using PCR for specific mutations so some degree of rationalisation must take place as it would be both costly and inefficient to test for all 800+ mutations (see http://www.uwcm.ac.uk/uwcm/mg/search/120584.html). What most commonly occurs is that an individual is screened for the 5 most common mutations affecting individuals from that particular ethnic background. Such tests have an accuracy level of around 98%. High as this may seem, it also means that about 1 in 50 screens results in false results. This accuracy level is further decreased in multi-racial communities in which the frequencies of CFTR mutations are unknown. It is hoped

that with the advent of the DNA-chip that all possible mutations could be screened for and thereby increase the accuracy of such tests.

Heterozygote Advantage And Population Genetics

CF is highly prevalent in Caucasian populations and less so in African or Asian populations. In such genetic scenarios the question often posed is,"Why is there such a high frequency of a gene which when inherited homozygously causes a fatal condition?". Basic evolutionary arguments point the finger at there being a personal selectable advantage in being a heterozygote ("carrier"). Sickle Cell anaemia is the classic example of such a scenario in which heterozygotes have a reduced susceptibility to malarial infection whilst homozygotes suffer a potentially from potentially lethal anaemia.

Numerous arguments of such structure have been made for CF carriers. It has been suggested that CF carriers have a resistance to the effects of diarrhoea, of asthma, of salmonella infection and even breast cancer. Of these choices, there is most evidence to support the diarrhoea argument. In a study into this theory, cholera toxin was administered to "CF" mice (mice with reduced *cftr* expression), CF heterozygous mice and wildtype mice and the chloride and water secretions from the gut epithelium measured. Mice lacking cftr protein did not secrete fluid in response to this stimulus but heterozygotes secreted about 50% of the normal fluid and chloride ion (Gabriel, 1994). This finding is in contrast with the study by the Cuthbert group (1995) who examined the hypothesis by measuring electrogenic chloride secretion in gut epithelia of normal and heterozygous CF mice. They found no evidence to support the genetic advantage hypothesis in ileal or colonic epithelia of the null CF mouse has been found, at least for acute responses. The Gabriel model is surprising as CF acts as a recessive condition. This reduction in fluid loss, it is argued, may protect them from death from cholera. It is therefore surprising that there is not a

proportional link between CF prevalence in certain ethnic groups and the history of cholera.

Does the high prevalence of these mutations and in particular ΔF508 provide pointers, in any way, towards the historical origins of CF? By just taking ΔF508 as the model, it is possible to generate a map of ΔF508 frequency through Europe (The CF Consortium, 1990). The results is a map resembling a gradient of frequency from low values (30%) in the Southeast rising to higher values (88%) in the Northwest. The pattern suggests that the spread of the CF gene may have accompanied the early migrating farmers from the Middle East and the diffusion of the gene being favoured by whatever selective advantage exists.

CFTR Is A Chloride Channel: The Biochemistry Of CFTR

The *CFTR* gene encodes a protein ion channel which functions in controlling the passage of chloride ions through the membranes of epithelial cells. In order for CFTR to exist as a channel which is both selective for chloride ions and which is also regulatory, a number of distinct protein domains must exist. Indeed, on the basis of amino acid sequence, CFTR was predicted to be composed of two membrane spanning domains (called TM1 and TM2) which are each composed of 6 transmembrane segments. Each domain is associated with a nucleotide binding domains (NBD-1 and NBD-2). See figure 1.0.1. In addition, CFTR protein contains a intracellular hydrophilic regulatory or "R" domain.

The structure of CFTR is similar to members of the ABC (ATP-binding cassette) family of proteins. Family members are found to be highly conserved through evolution acting in the transportation of a wide variety of compounds (Davis, 1996). One significant difference in CFTR is that other ABC proteins lack the R domain. The similarities between CFTR and the ABC family suggested to researchers that CFTR may also resemble members of this family structurally and also in behaviour.

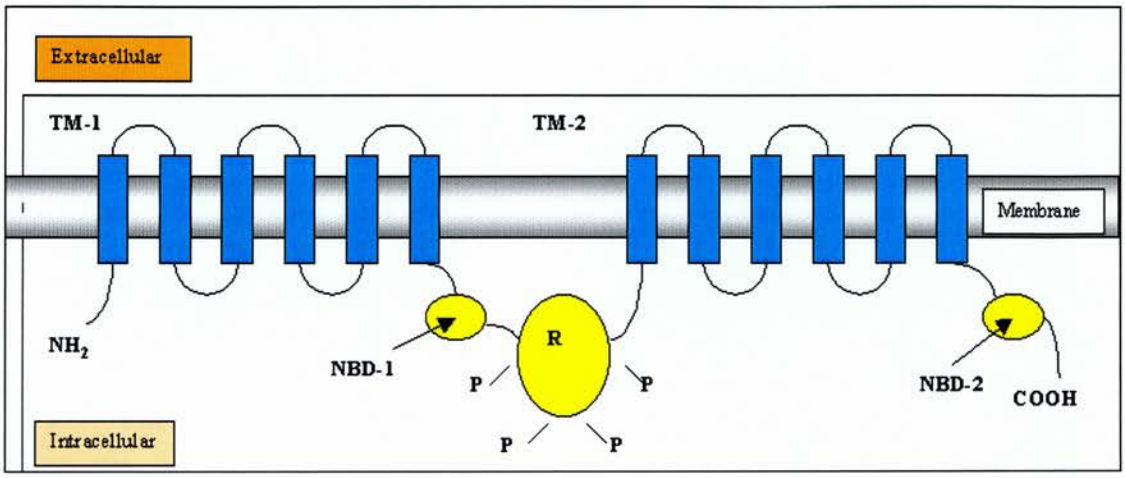
The first, sixth and twelfth transmembrane segments have been identified as forming the core of the channel (Anderson, 1991). The regulation of CFTR activity is regulated by means of the two NBD domains and the "R" domain. CFTR channels in a membrane patch open in response to applications of PKA and ATP to the intracellular side. The NBD-1 domains act as the lock through which hydrolysis of ATP can open the CFTR channel (Anderson, 1991). However, the "lock" will only work if the "R" domain is sufficiently phosphorylated by PKA. Deletion of the R domain renders the channel unable to close (Rich, 1991) and the addition of excess un-phosphorylated R domain peptides to the intracellular side of the channel results

in the closure of the channel (Ma, 1996). The process of closing the channel is regulated by hydrolysis of ATP at NBD-2 and the de-phosphorylation of the R domain.

It is important to comment that CFTR may have other roles beyond the channelling of chloride ions. CFTR is likely to also transport ATP, water and possibly small solutes (Reisin, 1994). The implications of this may be far reaching as the extracellular transportation of ATP stimulates the activity of another channel called the Outwardly Rectifying Chloride Channel (ORCC) which has many of the physiological properties of CFTR (Egan, 1992). The observation of increased sodium reabsorption in the CF airways led to the discovery of the Epithelial Sodium Channel (ENaC)(Stutts, 1995). When CFTR was expressed *in vitro*, ENaC channel activity is reduced and its regulation by cAMP is altered from stimulation in the absence of CFTR to inhibition in its presence. These finding have been found to have profound implications on the understanding of electrophysiology data and in therapeutic concepts for the treatment of CF. It is therefore puzzling that when the defective *CFTR* is replaced through methods of gene therapy in CF tissues that the sodium defect is not corrected (Boucher, 1996). Furthermore in diseases associated with defective ENaC expression, abnormal mucus rheology is noted without the other pathological defects associated with CF (Quinton, 1990).

Figure 1.0.1

Predicted Secondary Structure Of The CFTR Protein



CFTR is composed of two membrane spanning domains (TM-1 and TM-2) each composed of 6 transmembrane segments. Regulation of CFTR channel activity is controlled through ATP hydrolysis by the Nucleotide Binding Domains (NBD-1 and NBD-2) and the phosphorylation of the Regulatory "R" domain .

Clinical Aspects Of CF

CF manifests itself in the development of a wide range of symptoms affecting a number of different organs. The severity of symptoms can be highly variable depending on the CFTR mutation, health care, environment and also on the individuals genetic makeup. The success in treating the different symptoms of each organ is variable.

Sweat Duct Abnormality

During a particularly hot New York summer in 1949, the diSant' Agnese group (1953) observed that an abnormally large number of infants presenting symptoms of heat prostration (occurrence of salt crystals on the skin) also had CF. It was subsequently discovered that mutations in CFTR has the effects of causing a 5 fold increase in the normal chloride concentrations in the sweat ducts. This is due to an inability of the salt duct to properly reabsorb NaCl from the sweat before it is released through the duct (Quinton, 1996).

Beyond the increased susceptibility to heat prostration, this defect does not apparently lead to any further clinical complication.

Reduced Fertility In CF Patients

CF leads to a reduced fertility in women and a high frequency of infertility in men. In the women, the lack of functional CFTR leads to a reduction in the water content of cervical mucus in the uterine cervix (Kopito, 1973). The abnormality would appear to be highly localised to the region of the cervix and this is reflected in the success of artificial insemination procedures which place sperm beyond this region. CF Male infertility results not from a lack of mature and fertile sperm but due to the blocking or absence of the vas deferens. Male infertility may be bypassed through the removal of sperm from the testes and artificial insemination of the partner

(Raeymaekers, 1995). Any artificial insemination treatment using CF sperm or egg obviously results in the passing of copies of the faulty gene to the child. However, there is little increased risk to the women with CF during pregnancy (assuming good pulmonary health)(Huang, 1987). With screening of both partners now available and the possibility of pre-implantation screening, there is a good prognosis for CF patients who want to start a family.

Pancreatic Insufficiency In CF

Many patients present with pancreatic insufficiency (PI) at the time of diagnosis (80%) or go on to develop PI (95%) later in their lives (Davis, 1980). Either way, PI leads to an reduced ability to digest food. The physiology of this defect is discussed in chapter 4. If left unchecked, PI results in the failure to put on weight (although CF patients have a characteristic voracious appetite), greasy stools and colicky pain after eating (Davis, 1996). CF patients taking up to 100 pancreatic enzyme pills a day to compensate for PI are not unheard of. Further complicating this issue is the high rate of anorexia in patients with active lung inflammation. About 1% of patients with CF additionally require insulin injections with the frequency of such incidences increase with age of the CF population.

Improvements in the field of nutrition and the welfare of CF patients have also contributed to the increased survival of CF patients. The CF diet is now liberal, high in calories and often with added vitamins and salt (to replace that loss through sweating) (Ramsey, 1992).

Blockages In The Intestines

The exchange of water across the membranes of the intestine is associated with the exchange of chloride ions. Therefore, in CF, when the CFTR chloride channel is inoperative there is a significant reduction in the secretion of water into the gut

(Raeymaekers, 1995). This leads to the sludging of intestinal contents and the development of intestinal blockages ("meconium ileus equivalent" which occurs in neonates or "distal intestinal obstruction syndrome" which can occur postnatally (Park, 1981)). These blockages occur in between 10 and 15% of new-born babies and can be treated surgically (Stern, 1986).

Liver Disease

Liver disease is the second most common cause of mortality in CF patients although its occurrence is not inevitable (FitzSimmons, 1993). CFTR is normally expressed in the epithelial lining of the biliary ductules where it acts to maintain the water and electrolyte content of bile. In the non CF ductules, the secretion of chloride leads to the passive secretion of water (Davis, 1996). When CFTR is dysfunctional, the biliary ductules can become blocked which can lead to obstructive cirrhosis and bleeding. The most common method of treatment for this situation is liver transplantation.

Related to the abnormal secretions, CF patients are more susceptible to the development of gallstones (a prevalence of about 15% in young CF adults)(Stern, 1986). Treatment for CF gallstones is similar to that for non-CF patients although there is a stronger argument for surgery of asymptomatic gallstone in CF patients, as surgical risk will increase as the pulmonary health of the individual decreases.

Lung Disease In CF Patients

Although lung disease is the major cause of death in CF patients, the precise aetiology of CF lung disease is still poorly understood (Davidson, 1998). The development of CF lung disease begins shortly after birth and is characterised in the chronic bacterial colonisation of the lung and the presence of excessive and

abnormal mucus which leads on to the development of bronchial and glandular obstruction. An ongoing massive inflammatory response ultimately results in the severe bronchiectasis and finally respiratory failure (Davis, 1996).

It is likely that CF lung disease develops through a multi-step cascade. Though even this statement is subject to argument since the logical progression of abnormal mucus to bacterial infection to inflammatory response to abnormal histology does not seem to operate in the CF patient. Instead, the first signs of CF lung disease are histological (dilation of submucosal glands) (Madden, 1991) and indeed studies in CF infants detected raised levels of inflammatory markers (IL-8 and neutrophils) without any corresponding evidence for infection (Armstrong, 1995).

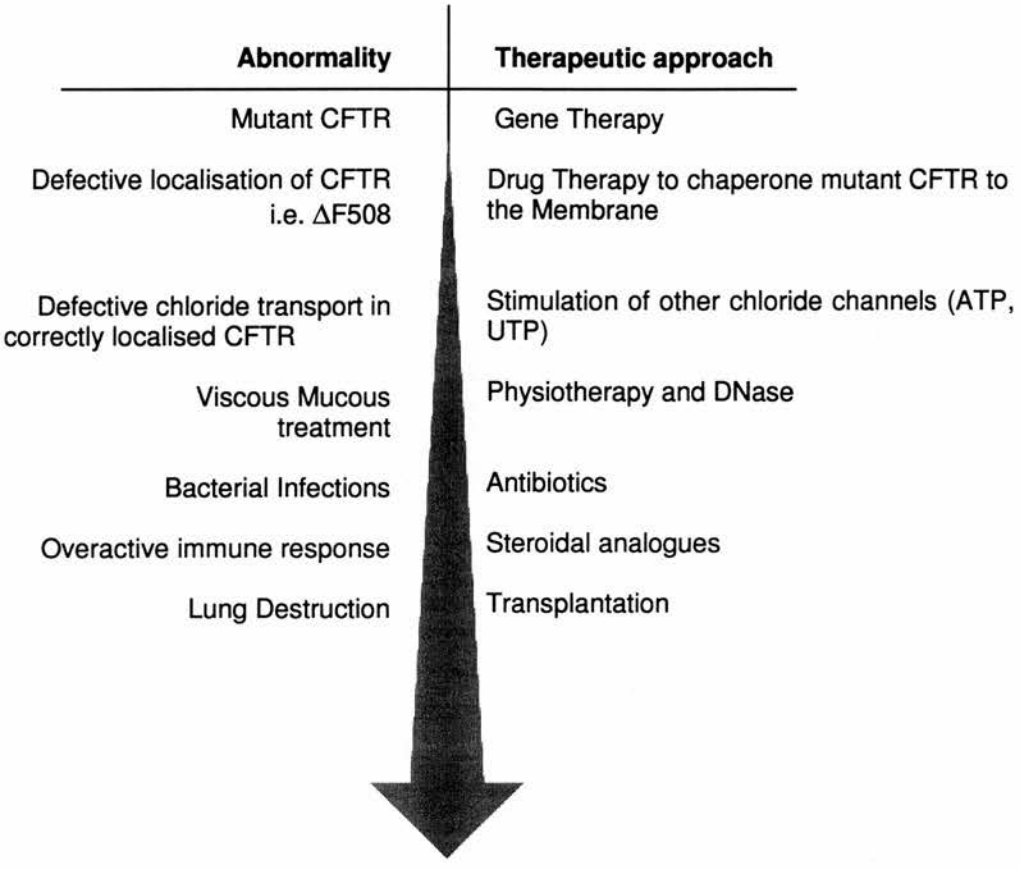
Treatment For CF Lung Disease

The treatment of CF lung disease is largely based on interrupting the disease-forming cascade which eventually manifests in the destruction of the lung. Such a framework for clinical intervention is displayed in figure 1.0.2.

Figure 1.0.2

Therapeutic Strategies For Treating CF Lung Disease:
Breaking The Chain Of Events

CF gene therapy is discussed more fully in chapter 6.



Clearance Of Excessive Mucous Build Up

Treatment of CF lung disease has improved steadily over the last 50 years with methods of therapy now available to tackle each part of the lung disease jigsaw, albeit to varying degrees of success. The build up of excess mucus is most commonly treated by extensive programmes of chest physiotherapy or more mechanical methods such as the Flutter devise which has been shown to raise four times as much mucus as traditional physiotherapy and does not require a partner (Konstan, 1995). Associated with these methods is the use of bronchiodilators such as β-adrenergic agonists and cholinergic blockers that act to dilate the small airways.

In tandem to these methods, mucolytics are often used. Mucolytics are chemicals which aim to digest the DNA and/or the protein components of the CF lung mucus which are largely to blame for the mucus viscoelasticity. Early mucolytics, such as bovine pancreatic DNase, were found to be bronchial irritants and have unpleasant side effects (Raskin, 1968). More recently a large double-blind trial was undertaken to study the effect of recombinant human DNase ("dornase alpha") (Shak, 1990). The results were very positive and in the first year of commercial release of dornase alpha, more than 40% of the CF population in the US (over the age of 5) received the drug (Davis, 1996).

Controlling CF Lung Infections

The battle against bacterial colonisation in the CF lung has been largely waged through the use of antibiotics. Although the antibiotics often fail to completely clear bacterial infections (especially *P. aeruginosa* which appears virtually impossible to eradicate) there is little doubt that their use has contributed to the increased survival of CF patients. Courses of antibiotics are usually administered in intermittently in response to a sudden decrease in lung activity or health.

As an alternative to antibiotics, the concept of immunisation against specific bacteria through vaccination has been explored. Clinical trials, such as those using a vaccine directed against *P. aeruginosa*, have been largely disappointing and may even have predisposed some patients to more severe disease once *P. aeruginosa* infection occurred (Langford, 1984, Pennington, 1975).

Controlling Lung Inflammation

Inflammation is a significant cause of damage to the CF lung. Raised levels of neutrophils are noted in the CF lung even in infancy thus the structure of any programme of anti-inflammatory therapy would most likely begin in infancy and continue indefinitely. There are a number of ant-inflammatory drugs available used in the treatment of CF.

The use of corticosteroids in the treatment of CF patients has had a controversial history. In two large scale trials, recruiting over 300 CF patients, corticosteroids were found to lead to an immediate increase in pulmonary function and fewer admissions into hospital (Donati, 1987, Auerbach, 1985). However, in both cases, follow up studies (4-6 years) showed strong evidence that the treatment led to side effects which included growth retardation, glucose abnormalities, osteoporosis and the formation of cataracts (Rosenstein, 1991).

Non steroidal inflammatory drugs (such as ibuprofen) generally have fewer side effects than their steroidal counterparts. Used in high concentrations, they were shown to have specific activity against neutrophils making them a possible candidate for use in CF (Konstan, 1990). This hunch was shown to be correct in a 4 year trial conducted on CF patients over 5 years of age with mild lung disease (Konstan, 1991). Test patients (as compared to those who received placebo) had significantly

less decline in pulmonary function, maintained their normal body weight and had fewer hospitalisation. Most importantly, there was no development of side effects as noted in the corticosteroid trials.

Lung Transplantation

CF patients with end-stage lung disease often attempt to find a donor lung. This procedure is fraught with uncertainty and is over shadowed by a low long term survival rate : 15% death rate in the first year and 33% in the second year (Davis, 1996). Deaths during the first few months after transplantation are predominantly as a consequence of bacterial infection probably resulting from native bacteria residing in the upper trachea (Egan, 1992). However, for many CF patients, the increased quality of life resulting from a lung transplantation can be considerable. Unfortunately, at this time a global sparcity of donor organs. Some patients have to wait for over 2 years for a donor organ during which time their pulmonary health may decrease in such a manner as to make the surgical procedure even more dangerous.

Chapter 2

The Biology Of Murine Submucosal Glands

Introduction

The aim of this chapter was to examine the biology of the murine submucosal gland. Submucosal glands are thought to be important in the development of CF lung disease in humans but have been up to now, poorly characterised in the mouse. The experiments in this chapter address the suitability of the mouse as a model for human pulmonary airways.

Mouse Models Of Cystic Fibrosis

There are many technological and practical reasons why the mouse, as opposed to other mammals, should be used as an experimental model for genetic and clinical studies. Mice are small in size and have a short reproductive cycle thus making them economical to house and quick to generate data. As a consequence, they have been used for over a century for experimentation (Erickson, 1996).

Traditionally mouse mutants have been generated through radiation treatment of animals or by breeding naturally found mutants. These techniques have however, always relied on chance or randomness. With the advent of molecular genetics, the possibility of generating mice with mutations of choice has become reality and in the last decade the field of mouse genetics has seen a rapid change with the production of transgenic mice now commonplace.

The extrapolation of data from *Mus musculus* to *Homo sapiens* (which is often the purpose of clinically orientated genetic studies) must not be blind to anatomical, development or pathological differences between species. Yet, reporting the pathological variation arising from the species difference is often of great importance in highlighting critical steps in the disease pathogenesis. The margin of similarity between what we perceive to be important in the pathogenesis of a

particular disease is likely to be an ever changing one as research both in the human condition and in the mouse grows and becomes more comprehensive. The study of the CF mouse is such an example.

There is no known naturally occurring animal model for CF (Dorin, 1996b) however a number of murine models of transgenic CF have now been generated. (Snouwaert, 1992, Dorin, 1992a, Rozmahel, 1996, O'Neal, 1993, Zeiher, 1995). A CF mouse is defined as a mouse with reduced CFTR expression and not necessarily a reflection on its phenotype. There are essentially two ways to generate the CF mice. Firstly, Insertional Gene Targeting which aims to introduce a fragment of foreign DNA which will disrupt the normal functioning of the target gene. Secondly, gene replacement, which relies on a double reciprocal recombination which will delete a vital segment of the target gene (Dorin, 1996b). One of the first CF mice to be reported was that of the Dorin group (1992a). This mouse was developed at the Medical Research Council's Human Genetics Unit in Edinburgh and is therefore gains the designated abbreviation of *Cftr*^{tm1HGU} ("tm1" refers to, "transgenic mouse number 1"). The strategy devised was to disrupt the normal read through of exon 10 of the mouse *CFTR* homologue in embryonic stem cells. A 3.5kb fragment of DNA which was isogenic with areas of intron 9 and exon 10 was cloned into a vector carrying the neomycin resistant gene. The vector was linearised and electroporated into an E14 embryonal stem cell line. Correctly targeted clones were determined by antibiotic resistance and Southern blot and cells were injected from a clone into C57BL/6 blastocysts, which were transferred to pseudopregnant albino female mice. Male chimaeric offspring were bred with MF1 females to test for germline transmission. Offspring from these matings were genotyped by southern blot for presence of the transgene. As the transgene was successfully shown to be present in chimaeric germ cells, the chimaeric mice were further bred to produce wildtype animals and heterozygous and homozygous animals which carried the transgene (Dorin, 1992b, Dorin 1992a).

The *Cftr*^{tm1HGU} mouse, unlike many of the other CF mice, is not however, a complete cftr "null" ("null" meaning that 0% of wildtype *cftr* is expressed). Animals homozygous for the *cftr* transgene were found to express between 5 and 10% of wildtype levels of *cftr* as a results of aberrant splicing and exon skipping (Dorin, 1994). Interestingly, the *Cftr*^{tm1Bay} mouse which was also generated by Insertional targeting but this time targeting exon 3 does not show this same "leaky" expression of *cftr* (O'Neal, 1993). It is considered that the efficiency of aberrant splicing in the exon 3 region of the *cftr* gene may be low compared to the exon 10 region (Dorin, 1996b). A summary of CF mouse models is given in figure 2.0.1.

CF affects the normal functioning of many organs in the human patient including the sweat gland duct, the pancreas, the lung, the gut and the reproductive tract. The phenotypes of these tissues in the CF mouse models have been discussed extensively elsewhere (Dorin, 1996b, Dickinson, 1995). The most significant phenotype affecting survival in the CF mouse "null" models (see figure 2.0.1) is the occurrence of gut blockages which cause the majority of mice to die perinatally (Dorin, 1994). This phenotype is similar to meconium ileus which occurs in ~20% of new-born CF patients. This high perinatal death rate was originally feared to hamper further experimentation until the finding that by feeding the mice a liquid diet could prolong their life. Interestingly, it was found that the positioning of CFTR in its normal physiological crypts position was not entirely necessary for the correct functioning of the gut. When CFTR was expressed on the villi of the mouse intestine (CFTR is normally expressed in the crypts) it was found to be sufficient to prevent the mouses' reduced survival rates (Zhou, 1994). The *Cftr*^{tm1HGU} mouse also shows overt histological changes in the gut, but less than 10% die of meconium ileus or equivalent. In addition to the phenotypes seen in the human, the *Cftr*^{tm1UNC} null mouse was found to show abnormal incisor enamel (Wright, 1996).

Table 2.0.1

Transgenic Mouse models for CF

CF Mouse	Reference	Gene Mutation	CFTR activity
Cftr^{tm1HGU}	(Dorin, 1992a)	Partial Duplication of Exon 10	5-10%
Cftr^{tm1UNC}	(Snouwaert, 1992)	Disruption of Exon 10	Null
Cftr^{tm1CAM}	(Ratcliff, 1993)	Disruption of Exon 10	Null
Cftr^{tm1BAY}	(O'Neal, 1993)	Duplication of Exon 3	Null
Cftr^{tm1HSC}	(Rozmahel, 1996)	Disruption of Exon 1	Null
Cftr^{G551D}	(Delaney, 1996	Disruption of Exon 11	53%
Cftr$_{\Delta}^{F508Cam}$	(Colledge , 1995)	Deletion of Phenylalanine at position 508 (exon 10 replacement)	15%
Cftr$_{\Delta}^{F508Rot}$	(van Doornick, 1995)	Deletion of Phenylalanine at position 508 (exon 10 insertion)	100%
Cftr $_{\Delta}^{F508Uta}$	(Zeiher, 1995)	Deletion of Phenylalanine at position 508 (exon 10 replacement)	100% though lower in gut

One of the most fundamental phenotypes which can be studied in the CF mouse is that of chloride ion transport activity. Dorin (1992a) reported electrophysiological dysfunction in the respiratory and gastrointestinal tracts in the *Cftr*^{tm1HGU} mouse. A variety of tissues were incubated with forskolin and zardaverine in the presence of amiloride and the potential difference (p.d.) across the tissues measured. P.d. measurements were found to be significantly to be raised in the nasal epithelium, trachea, caecum/colon and rectum (Dorin, 1992a). Similar results have been found in the other mouse models though the degree of difference in p.d. measurements is less in the *cftr* "nulls" than in the *Cftr*^{tm1HGU} mouse (Dorin, 1996b).These channels were found to be largely inhibited by bumetemide and therefore likely to represent chloride secretions (Smith, 1992).

Despite the electrophysiological profiles of the CF mice, there are no reports of spontaneous development of lung disease in any CF mouse models (Davidson, 1998). However, However in an earlier paper, Davidson (1995) demonstrated that the *Cftr*^{tm1HGU} mouse shows an increased *susceptibility* to developing lung disease after repeated nebulised exposure to *Staphylococcus aureus* and *Burkholderia cepacia*, as compared to non CF littermates. These bacteria have been previously demonstrated to be of clinical significance in CF. The spectrum of pathology was similar to that observed in CF patients including goblet cell hyperplasia and metaplasia, mucus retention, bronchiolitis, pneumonia and inflammatory infiltration (Davidson, 1995). Interestingly, as with human CF patients, pneumonia was only noted in animals treated with *B. cepacia* and not *S. aureus*.

More recently, *P. aeruginosa* coated beads were used to deliver a high dose of bacteria to the lungs of CF "null" and wildtype mice. After treatment, a higher rate of morbidity in CF mice (82%) than wildtype mice (23%) within 10 days of infection was recorded (Kent, 1997). The increased mortality in the CF group was associated

with a markedly elevated inflammatory response and weight loss in comparison to the wildtype animals. No CF or wildtype mice in a control group receiving sterile beads died within 10 days of the procedure.

Using a similar method of infection, the Cowley group examined mucociliary clearance in CF and non CF animals after infection by *Pseudomonas aeruginosa* (Cowley, 1997). Two parameters were measured using an *in vitro* lung explant preparation, that of ciliary beat frequency (fcb) and particle transport (PT). No statistical difference was found between the fcb between CFTR -/- mice (*Cftr*^{tm1UNC} on C57BL/6 inbred genetic background) and their +/+ controls either in the presence or absence of *Pseudomonas aeruginosa*. However, PT rates (measured by rate of movement of finely ground charcoal across a microscope field of view) were seen to be dependent on both CFTR genotype and infection status. Uninfected CF mice demonstrated higher rates of PT than wildtype littermates while Pseudomonas aeruginosa infected CF mice showed lower PT rates than infected wildtype controls($p<0.05$).

This report is in contrast with that of Zahm (1997) who studied the mucociliary transport velocity (rate of mucus clearance from the lower part of the trachea) (MTV) in homozygous *Cftr*^{tm1HGU} mice (on MF1 outbred genetic background) as compared to wildtype littermates. MTV was significantly lower in the CF mice (14.2±4.4 μm/mm) than in the wildtype controls (30.6±5.9μm/mm)($P<0.04$). These animals were housed in germ-free conditions in an isolator unit and were found to be free of bacteria when screened for 15 common pathogenic strains. The CF mice were found to have higher number of inflammatory cells in than in the wildtype controls. These data are particularly interesting in the light of data from CF infants who have shown increased levels of neutrophils and increased levels of interleukin-8 despite the absence of CF related pathogens (Khan, 1995).

CF mouse versus CF Man

The use of animal models can be perceived in two, non-exclusive, ways. Firstly, as a substitute human. That is to say a non-human species to receive often prototype forms of therapeutic intervention. Secondly, differences between humans and an animal model can, often by serendipity, elucidate critical steps of a disease pathogenesis.

In the context of CF, what could explain the differences in lung disease pathogenesis between the CF mouse and the human CF patient? Here are some possibilities.

1: Temporal: It can be argued that as human CF patients do not exhibit full lung disease phenotypes until late infancy. Does the much shorter life span of the mouse not permit the development of CF lung pathogenesis?

2: Size: An observation which may appear starkly obvious but yet is important to consider further, is the difference in size between the Mouse and Man. Does the size and therefore surface area of the lung in the CF mouse somehow, make it less susceptible to developing CF lung disease?

3: Environment: By philosophising on the question, "What is an *actual* cause of CF?", one can argue that if CF patients were kept in an isolated environment away from pathogenic bacteria they would not go onto develop lung disease. In this case, it is likely that the housing environment of the mice will be crucial to the development of, or lack of, CF related lung disease (as recognised in the *Cftr*^{tm1HGU} mouse when housed in non-SPF conditions (Dorin, 1992a)).

4: Method of Breathing: The mouse predominantly breathes through its nose (the human breathes more through the mouth). Does nasal breathing reduce the chances of infection?

5: Lung Structure: Is there adequate homology between the cell types, lung structure and the ion channels between mouse and man to expect a similar pattern of lung pathogenesis to develop?

Chapter 2 aims to investigate further the issues raised in point 5. That is, does the mouse possess the tissues and cell types which are thought to be associated with the development of CF pathogenesis. In other words, should we expect the CF mouse to develop CF lung disease and if not, why? Furthermore, can we use the mouse as a suitable animal model for the human lung in studies of CF lung pathogenesis and cellular kinetics?

The Role Of Submucosal Glands In The Development Of CF Lung Disease

There is considerable evidence to suggest that SMGs may play a significant role in the development of CF lung disease.

- SMGs are the predominant site of CFTR expression in the human bronchus (Engelhardt, 1992). Engelhardt *et al.* localised expression of CFTR to the serous cells and to a subpopulation of "flask like" cells in the ciliated ducts.
- When chloride transport in SMGs is blocked *in vitro*, mucus production is severely reduced (Inglis, 1997a) and the mucous obstructions resembling those observed in CF arise (Widdicombe, 1994).
- The first observable phenotype in CF infants is the dilation, hyperplasia and obstruction of SMGs. Interestingly, this can occur prior to and independent of other signs of lung disease or infection (Madden, 1991, Oppenheimer, 1975).

It is therefore important, in the development of the mouse as a model for CF, to examine the presence and nature of SMGs in the mouse.

Mice have been previously been reported to "lack" (Widdicombe, 1994)or at best, be "essentially void" (Engelhardt, 1992)of SMGs. The "absence" (Widdicombe, 1997) of murine SMGs has been reported as to be a likely cause of the weak CF mouse lung phenotype.

Submucosal Glands in Man

In Man, submucosal glands (SMGs) consist of a network of secretory tubules and associated ducts which act in the production and delivery of mucous and antimicrobial agents to the epithelium of the cartilaginous airways (Basbaum, 1990, Engelhardt, 1992, Meyrick, 1969).

SMGs are found in the trachea, bronchi and proximal bronchioles of the human lung, lying most commonly between the epithelium and plates of supporting cartilage though occasionally they can lie external to the cartilage (Tos, 1966). Serial reconstruction and cell type analysis of the gland structure has revealed the presence of 4 different tubule types and a number of cell types, summarised in figure 2.0.2. (Meyrick, 1970, Engelhardt, 1992).

The SMG ciliated duct connects the surface epithelium to the collecting duct. It is lined mainly with ciliated cells but also with cells with numerous large mitochondria and a well developed Golgi apparatus (Meyrick, 1970, Meyrick, 1969). These cells may be the "flask like" cells observed by Engelhardt *et al.* (1992). A role in fluid transport has been suggested for the mitochondrial rich cells (MRCs) (Meyrick, 1970). However, their physiological significance is unclear as in humans they

constitute only an average of 6% of all the duct cells (maximum frequency ~23%) (Matsuba, 1972). Furthermore, a significant positive correlation between numbers of these cells and the age of the sample, perhaps suggesting that these cells are products of cellular degeneration. Goblet cells may also be present (Meyrick, 1970).

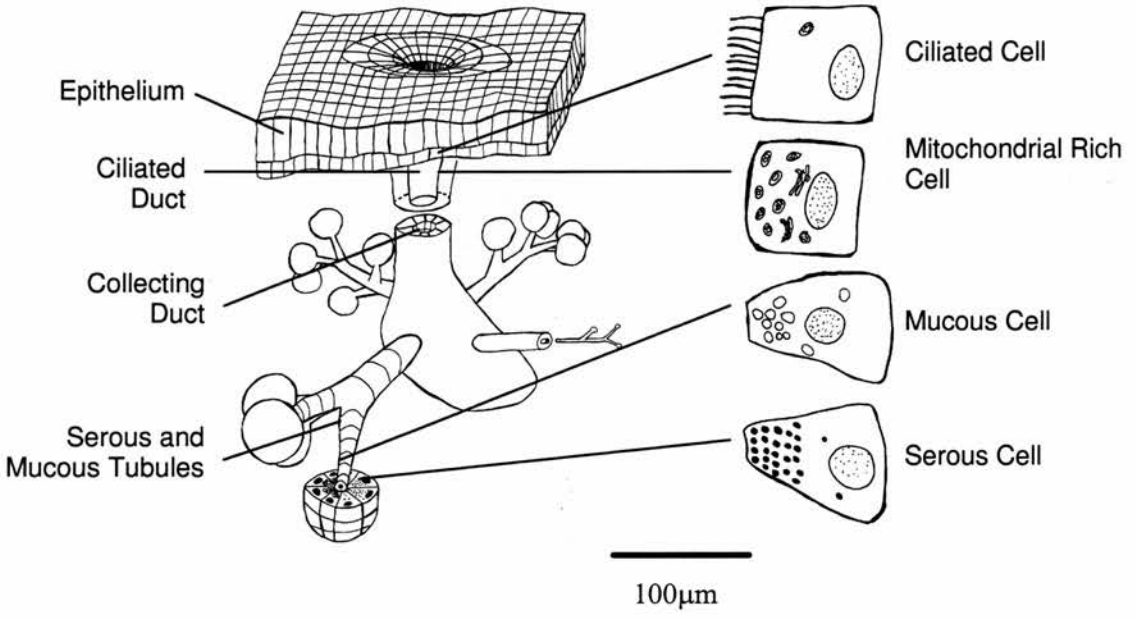
The transition from ciliated duct to the collecting duct is abrupt (Meyrick, 1969).The collecting duct has a larger diameter than the ciliated duct and is associated with the presence of tall non ciliated columnar cells densely packed with mitochondria and Golgi (Meyrick, 1970).

The junctions between the collecting duct and the secretory tubules also show an abrupt change in epithelial cell type to one composed entirely of mucous cells and serous cells distinguished primarily by differences in the ultrastructure and histochemistry of the granules (Widdicombe, 1994). The secretory tubules end in spherical acini which are composed of one or both cell types (Meyrick, 1969).

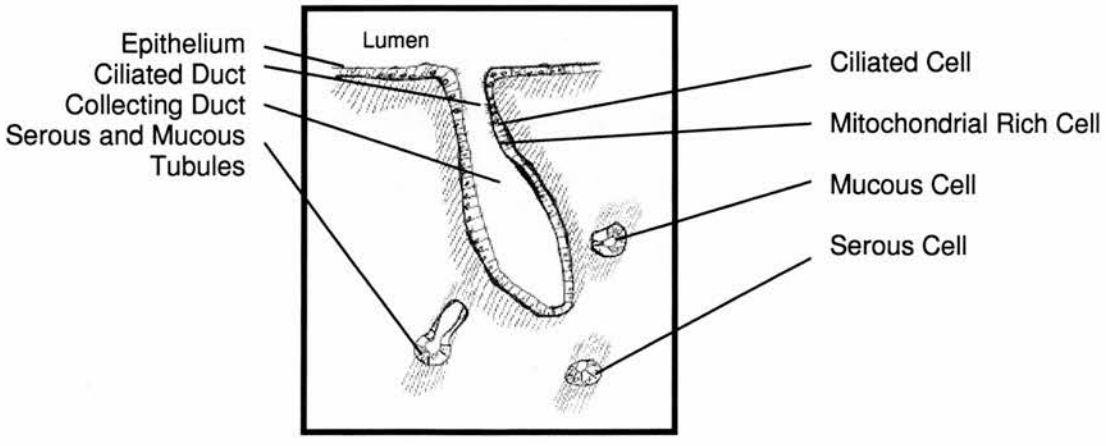
Figure 2.0.2

Structure And Cell Types Of The Human SMG.

A: 3d dimensional image of a human submucosal gland based on the observations of Meyrick (1969).



B: Submucosal gland in cross section.



Mucous cells contain a basally located nucleus and an extensive Golgi apparatus. The Golgi generate electron lucent secretory granules which increase in diameter towards the apical membrane (Meyrick, 1970). These granules vary in diameter from 300 to 1800 nm, the larger ones having a tendency to fuse.

By contrast, serous cells have electron dense membrane bound granules generally measuring between 300 and 1000 nm but occasionally up to 2000 nm (Widdicombe, 1994, Bowes, 1977). The cells have a pyramidal shape and a basally located nucleus and are extremely rich in rough endoplasmic reticula and mitochondria (Basbaum, 1990).

The great abundance of serous cells (61% by volume of the human submucosal gland (Basbaum, 1990)) suggests their role in human lung physiology as one of great importance. Indeed, serous cells are reported to act as one the main lines of defence in the lung through the production of bactericidal compounds including lysozyme and lactoferrin (Basbaum, 1990).

This chapter aims to re-examine the question of murine SMGs in relation to their presence or absence in the mouse, their structural homology to those of the human and expression of CFTR.

2.1

Characterisation and localisation of Murine SMGs

Reported below are the results from a qualitative and structural analysis of murine SMGs.

Materials and Methods

Six 6-week-old wildtype mice on a mixed (MF1/129) genetic background from conventional (non-specific pathogen free) housing were sacrificed through IP injection of 0.4ml IP injection of Euthatal (Rhone Merieoux, UK). Their lungs were inflated with 4% paraformaldehyde and the lung and trachea then dissected out whole. The tissue was fixed in 4% paraformaldehyde at +4°C for 6 hours then stored in 70% alcohol at +4°C until being processed into wax blocks using a VIP wax processor (Tissue-Tek, UK). 6μm sections were cut from the trachea and lung lobes and these were floated out on distilled water at 58°C and transferred onto glass slides coated with Vectabond (Vector laboratories, Peterborough UK). Slides were dried for 2 hours at 60°C. Wax was removed and tissues rehydrated by transferring the slides through baths of xylene (2x 10 minutes) and a decreasing gradient of alcohol (100%, 90%, 70%, 50%, 30%) to water.

Sections were counterstained with haematoxylin by incubating the slides in haematoxylin for 5 minutes, washing in tap water for 5 minutes, incubating in eosin solution for 3 minutes, washing in Scott's Water and acid alcohol (5 seconds each) before washing 3 x 5 minutes in water.

Slides were left to dry at room temperature overnight and viewed with bright field microscopy (Axioplan 2, Zeiss, UK). The identity of the SMGs was examined through comparative histological analysis to those found in the human as discussed above.

Results

Histological examination of the lung of the MF1/129 wildtype mouse revealed the presence of SMGs in the proximal regions of the trachea. See figure 2.1.1.

Discussion

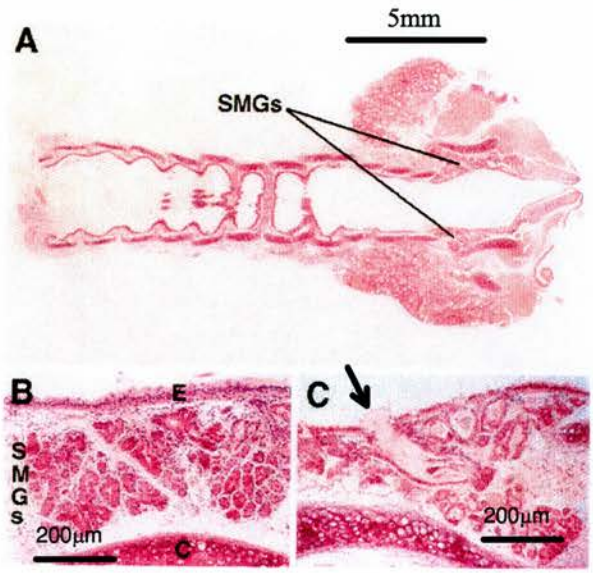
The cartilaginous airways of the mouse lung were examined for the presence of SMGs. Murine SMGs were found in all mice studied and their identity was confirmed through comparative histological analysis of mouse SMGs to human glands described in the literature. Murine SMGs in wildtype mice on an outbred (MF1/129 mixed) genetic background were located in the proximal regions of the trachea. The main concentration of glands occurred between cartilage rings 0 and 2. Gland duct openings occurred at a density of ~1mm^{-2} as determined by gross planimetry (data not shown).

These preliminary data do not however demonstrate that the mouse SMGs do indeed function or have the same cell types as in the human. Furthermore, it does not answer the question of whether the SMG elements thought to be pertinent in the development of CF lung disease, namely the presence of serous cells, mucus production and *cftr* expression, are present.

Figure 2.1.1

Presence Of SMGs In The Mouse Trachea As Identified By Light Microscopy.

See figure 2.0.2. for comparative histology.



2.1.1A H&E counterstained longitudinal section of the trachea of the MF1/129 mouse. Proximal (towards the mouth) is to the right of the figure whilst distal (towards the bronchi) is to the left of the figure. Submucosal glands can be seen towards the proximal end of the trachea.
2.1.1B SMGs at higher power (x 40) showing the presence of tracheal epithelium (labelled "E"), SMG region and cartilage ("C").
2.1.1C SMG (x40) showing prominent ciliated duct (labelled) producing mucus material out onto the epithelial surface.

Figure 2.1.1

Presence Of SMGs In The Mouse Trachea

2.2

Cell Type Analysis By Transmission Electron Microscopy

Introduction

Of the cell types found in the human SMGs, the serous cells are thought to be most importantly connected with the development of CF due to their reported expression of CFTR and multiple antimicrobial proteins (Basbaum, 1990 ,Engelhardt, 1992). It was therefore important to examine the cell types found in the murine SMGs. To that end, a study using transmission electron microscopy was performed to identify cell types present in the distal portions of murine SMGs.

Materials and Methods

Two 6 week old homozygous *Cftr*^{tm1HGU} mice and two 6 week old wild type littermate mice (MF1/129 mixed genetic background) were sacrificed with a 0.4ml IP injection of Euthatal (2mg/ml pentobarbitone)(Rhone Merieux, UK). Tracheas from these mice were excised and 5mm^2 blocks of tracheal ring removed from the proximal end of the trachea adjacent to the thyroid gland. Tissues were fixed in freshly prepared gluteraldehyde fix (2.5% gluteraldehyde in 0.1M cacodylate buffer pH 7.3 (25mls 4.28% sodium cacodylate + 1.6mls 0.2M HCl + 10 mls H_2O) + 0.1M sucrose) overnight at +4°C. Tissues were rinsed in 0.1M sucrose in cacodylate buffer for 2 x 10 minutes and post fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 45 minutes. After 2x10 minutes of rinsing in cacodylate buffer, the tissues were dehydrated in increasing concentrations of ethanol. Tissues were transferred to a thin layer of araldite overnight and heated at 60°C for 35 minutes. Immediately

afterwards, the tissues were transferred to embedding araldite and baked at 60°C for 3 days.

Sections of 1μm nominal thickness were cut from tissues blocks and counterstained with toluidine blue. From observation on these slides, sections of 90nm were cut for TEM. Sections were transferred to 200 mesh copper grids and stained with 4 % uranyl acetate and Reynold's lead citrate. The ultrathin sections were examined with the Phillips CM120 Biotwin transmission electron microscope from which photomicrographs were taken.

Results

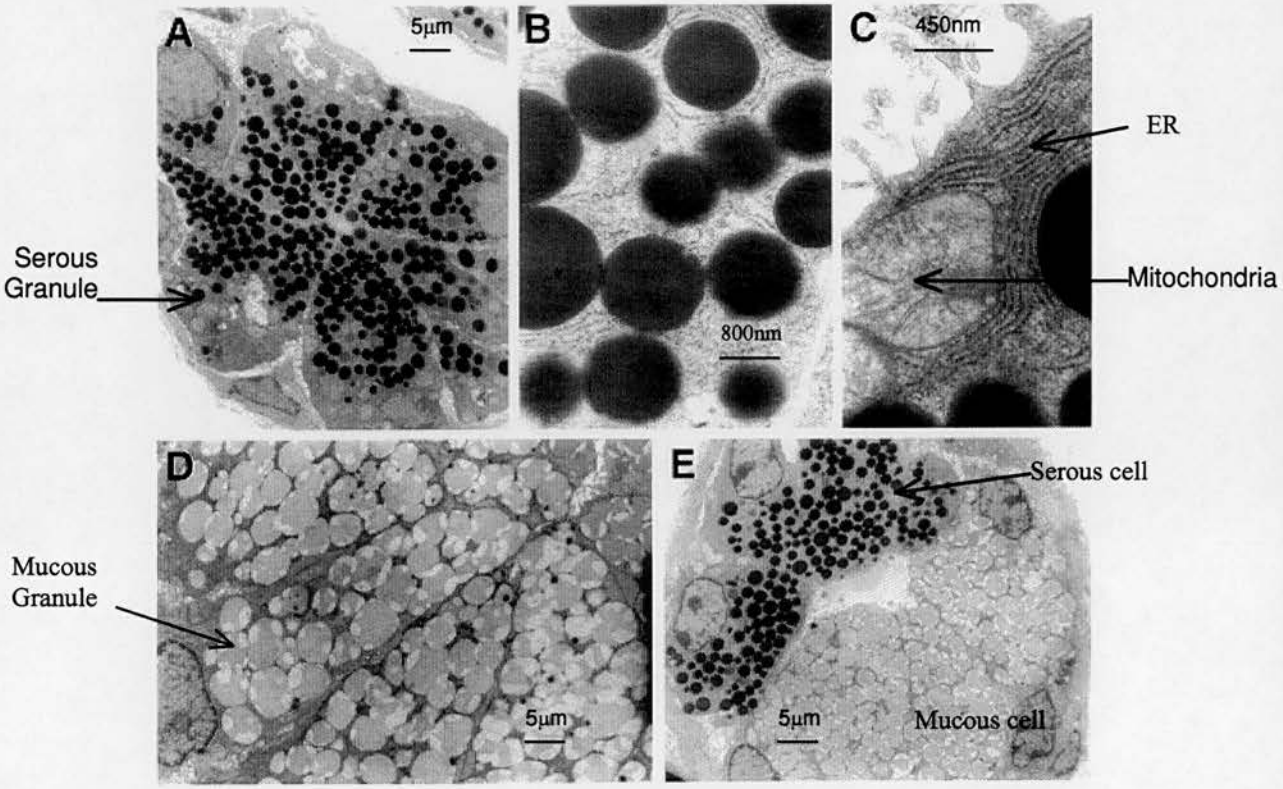
See figure 2.2.1.

Figure 2.2.1

Identification Of Serous And Mucous Cell Types In Murine Submucosal Glands By TEM

Serous cells were recognised by cell shape and ultrastructural content namely, the presence of electron dense granules, a high frequency of endoplasmic reticulum, being of pyramidal structure and having basally located nucleus. Mucous cells were identified by having electron lucent granules of variable shape.

The images are taken from wildtype mice. No statistical difference was noted in comparison to *cftr* deficient mice (see text for details).



2.2.1A Serous cell acini in a wild type mouse showing characteristic 6 cell structure. Note presence of dark, electron dense granules (see arrows) and basally located nuclei.
2.2.1B Serous cells contained electron dense granules which were heterogeneous in size (550nm to 1900nm) and appearance.
2.2.1C Serous cells were found to be extremely rich in endoplasmic reticulum (arrowed and labelled ER) and mitochondria (arrowed and labelled).
2.2.1D Mucous acini. Note irregular shaped mucous granules (marked with arrow).
2.2.1E Mixed serous/mucous acini.

Identification of Serous and Mucous cell type in murine SMGs.

In the most distal portions of the human SMGs are small cups of cells referred to as a gland acini. The presence and character of serous and mucous cell types in these regions of murine SMGs was confirmed by the use of transmission electron microscopy (TEM). Serous cells were identified through the presence of electron dense granules and an extremely high concentration of rough endoplasmic reticulum and mitochondria (figure 2.2.1a, 2.2.1b, 2.2.1c). Although homogenous in shape, the electron dense granules varied considerably in appearance and size. The larger granules appeared to be membrane bound and varied in diameter from 500 to 730nm in the homozygote *Cftr*^{tm1HGU} mice and 555 to 1500 nm in the wildtype mice. Smaller granules had an indistinct membranous boundary or diffuse edge. These smaller granules varied from 400 to 500 nm size in the homozygote *Cftr*^{tm1HGU} (n=18, median = 470nm) and 450 to 800 in the wildtype (n=20, median = 700nm). The ranges of sizes in these groupings do not present a statistically significant difference between genotypes (P>0.05 by Student's T-Test). A 3rd group of vesicles were classified according to their electron lucent appearance after staining with osmium tetroxide. These varied in size from 480nm to 550nm in size in the homozygote *Cftr*^{tm1HGU} (n=20, median = 540 nm) and 550nm to 830nm in the wildtype (n=20, median = 700nm). and were judged to be lysozomes.

Mucous cells were identified by the prevalence of irregularly shaped electron lucent vesicles (figure 2.2.1d). In both genotypes the granules ranged from approximately 550nm to 1900 nm in diameter (n=20, median = 1400nm).

The most common type of acini (~95% by area of vision) consisted of serous cells only as seen in figure 2.2.1a. In this figure, the characteristic pyramidal shape of the cells is clearly seen. Mucous only, or rarely, mucous and serous were also observed (figure 2.2.1d, 2.2.1e). These mixed acini may be undergoing transdifferentiation

from being composed of serous cells to one of mucous cells.

Discussion

Murine SMGs were found to be rich in serous cells, which displayed high similarity to that of the human cell type. Mucous cells were also present though less frequent in number. This experiment provided confirmation that these cell types existed in the murine SMGs providing a strengthen platform from which to argue the similarity of the murine SMGs to that of the human and thus, the suitability of the mouse as a model animal for use in human CF studies.

In following experiments when light microscopy was used, serous and mucous cells were identified by their differential appearance when viewed with Nomarski optics after Sharma (Sharma, 1997). The mucous cell acini appeared very flat whilst membranes and nucleus of the serous cells appeared rugged and rough.

2.3

Lysozyme expression in the Murine SMGs

Introduction

Human Serous cells are reported to produce, amongst other proteins, lysozyme. Lysozyme is an important antimicrobial agent in the lung and may be involved in CF lung pathogenesis through its synergistic relationship with the β-defensin family (Basbaum, 1990) (see chapter 4). An immunohistochemical study of lysozyme production in the murine SMGs was undertaken.

Materials and methods

Ten 6μm sections of 4% neutral buffered formalin fixed trachea from five 8 weeks old wildtype MF1 mice were cut and floated out onto Vectabond coated slides. The slides were dried and the sections de-waxed and rehydrated to water as before. Using Vectastain Anti-rabbit IgG kit (Vector laboratories, Peterborough, UK) the tissues were blocked for 20 minutes with goat serum. Serum was blotted off and 100μl of 1:200 anti-lysozyme (DAKO, High Wycombe, UK) antibody was added and the slides incubated for 30 minutes at room temperature. The slides were washed 3 times in PBS before addition of the anti-rabbit secondary antibody for 30 minutes. A further 3 washes in PBS followed before the addition of ABC solution (avidin-biotinylated enzyme complex) and incubation for 30 minutes at room temperature. Slides were washed 3 times in PBS and the antibody complex recognised with the addition of a solution of diaminobenzidine (DAB) reagent (one 10mg DAB tablet (Sigma-Aldrich, Poole, UK) in 10ml 0.05M Tris-HCl buffer pH7.4 activated with hydrogen peroxide) for 15 seconds. The slides were washed with PBS counterstained

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with haematoxylin for 10 seconds before dehydration through alcohols and xylene to final mounting in DPX. Slides were viewed with bright field microscopy (Zeiss Axioplan 2). Negative controls with no primary antibody were run on concurrent serial sections.

Results

See figure 2.3.1.

A sub-population of serous cells of the SMG were seen to express lysozyme protein through the positive signal with anti-lysozyme antibody. Within this population there was considerable heterogeneity of signal between cells. The identity of the cells was recognised through the use of Nomarski optics. Mucous cells and negative control slides with no primary antibody showed no signal.

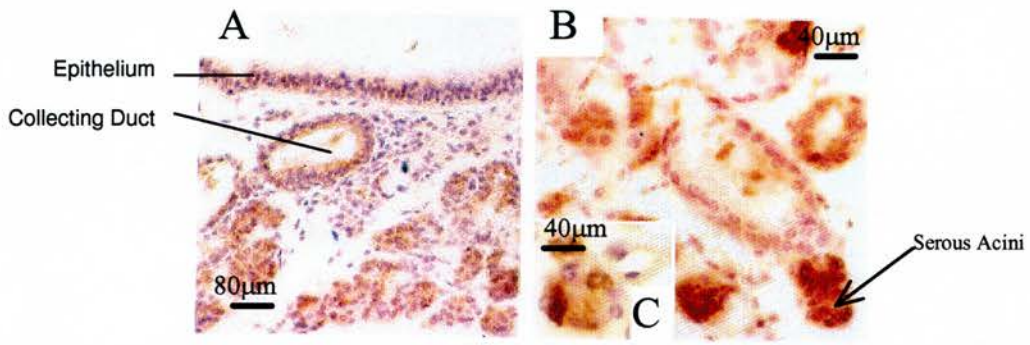
Conclusion

Serous cells lying within murine SMGs were observed to produce lysozyme protein. The pattern of production was observed to be highly heterogeneous between individual cells and glands.

Figure 2.3.1

Heterogeneous Pattern Of Lysozyme In The Serous Cells Of Murine SMGs

Murine SMGs were observed to produce lysozyme from the distal serous cells. Production was observed to be highly heterogeneous both between tubules (figure 2.3.1b) and cells (figure 2.3.1c). Negative control slides of no primary antibody and staining of non lysozyme expressing tissue, resulted in no brown cellular staining (data not shown).



2.3.1a Presence of Lysozyme protein in murine SMGs using anti-Lysozyme immuno peroxidase histology (DAKO) (positive signal appearing as brown stain). Tissue was further counterstained with haematoxlin.
2.3.1b Lysozyme protein identified to be present in a subset of serous cells by viewing with Nomarski optics.
2.3.1c Non-homogenous staining pattern of Lysozyme suggests that not all serous cells produce Lysozyme in the same quantity.

Figure 2.3.1

2.4

Mucus Production from the Murine SMGs

Introduction

H&E histology of murine SMG revealed the presence of a mucus like material in the ducts of the murine SMGs. The presence and pH of mucin secretions from the SMGs was characterised through the use of Periodic Acid/Schiff (PAS) and /or Alcian Blue (AB) staining on paraffin sections. PAS reacts with neutral mucin and AB with acidic mucins.

Materials and methods

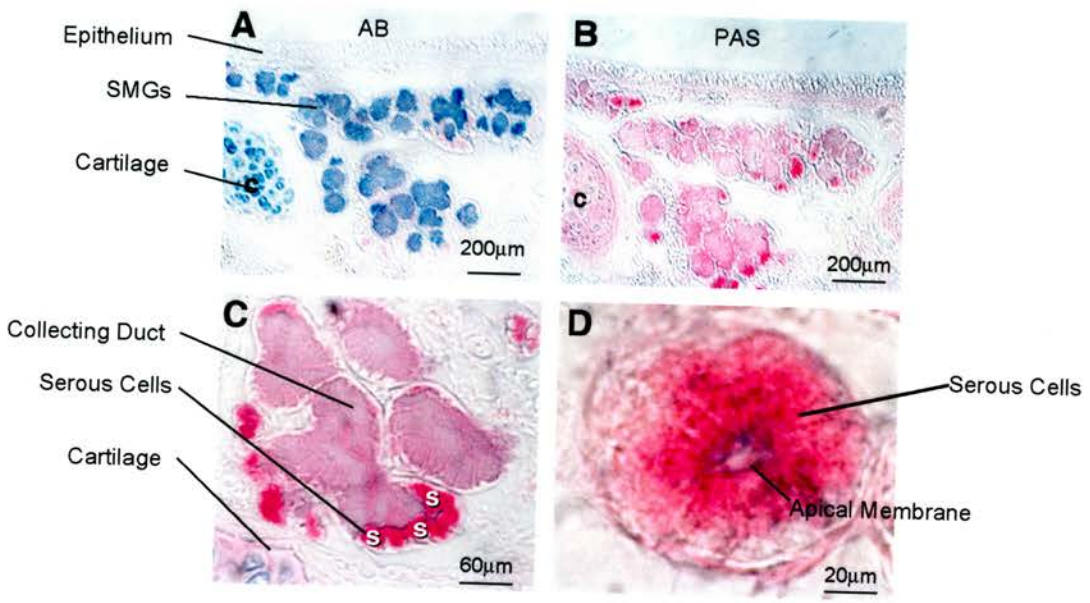
6μm paraffin sections of mouse trachea were de-waxed and rehydrated to PBS as before. Periodic acid stain, Schiff's Reagent (Sigma-Aldrich, Poole, UK) and Alcian Blue stain (1% in dH_2O (Sigma-Aldrich, Poole, UK)) were warmed to room temperature before use. For staining with PAS, slides were washed in a coplin jar for 3 minutes with Periodic acid, washed 3 times with PBS and then washed in a coplin jar for 3 minutes with Schiff's Reagent. For AB staining, slides were washed in AB stain for 3 minutes. When both staining methods were used, methods were run sequentially on the same slides. After staining, slides were dehydrated and mounted with DPX as before. See figure 2.4.1

Results

See figure 2.4.1.

Figure 2.4.1

PAS And Alcian Blue Staining Of The Murine SMG Shows Heterogeneous Pattern Of Mucus pH.



2.4.1a and 2.4.1b Serial sections of murine submucosal tissue stained with Alcian Blue and Periodic Acid /Schiff's respectively. Note weak Alcian Blue and PAS staining in the mucus situated in the ducts of the glands and the intense PAS signal in the distal regions of the gland in the serous and mucous tubules and cells.
2.4.1c and 2.4.1d: Serous cell acini viewed with Nomarski optics staining positive for PAS in the cytoplasm and positive for Alcian Blue on the serous cell apical surface.

Figure 2.4.1

2.5

cftr expression in the murine SMGs

Introduction

CFTR, in the human lung, is predominantly expressed in the serous cells and the mitochondrial rich "flask like" cells of the SMGs (Engelhardt, 1992). In the characterisation of the mouse as a suitable animal model for use in CF research, a study was conducted into the distribution of CFTR in the mouse trachea using an anti-human CFTR antibody.

Materials and Methods

Six C57BL/6 and six BALB/c wildtype mice were sacrificed as before and their tracheas dissected. The tissues were immediately wrapped in aluminium foil and immersed in liquid nitrogen. Tissues were later transferred to a -70°C freezer for storage. The tissues were cryo-supported in polyvinyl alcohol and polyethylene glycol in sucrose solution(O.C.T. compound, Tissue Tek, UK) and 10μm cryosections cut onto Vectabond coated slides (Vector laboratories, Peterborough, UK). Sections were thawed and dried for 2 hours at room temperature before fixation for 15 minutes in 4% paraformaldehyde. Sections were permeabilised with 0.2% Triton-X in PBS and after further rinsing in PBS blocked with 2% Bovine Serum albumin, 2% goat serum (DAKO, High Wycombe, UK), 7% glycerol and 0.2% Tween-20 (Sigma, Poole, UK) in distilled water. A polyclonal antibody, PC-termB (Genzyme Corp., Kent, UK) raised against the C-terminal portion of the human CFTR protein, was used to localise cftr in the mouse trachea. The primary antibody was diluted 1:100 in PBS/1% goat serum and incubated for 1 hour. Slides were washed three times in PBS and incubated with a 1:400 dilution of goat anti-rabbit-

FITC (Jackson ImmunoResearch Laboratories, Luton, UK) for 30 minutes. Sections were further washed with PBS and mounted in 1% DAPI in Vectashield (Vector laboratories, Peterborough UK). Negative controls were conducted using tissue from a C57BL/6 *Cftr*^{tm1UNC} CF "null" mouse and without primary antibody.

Results

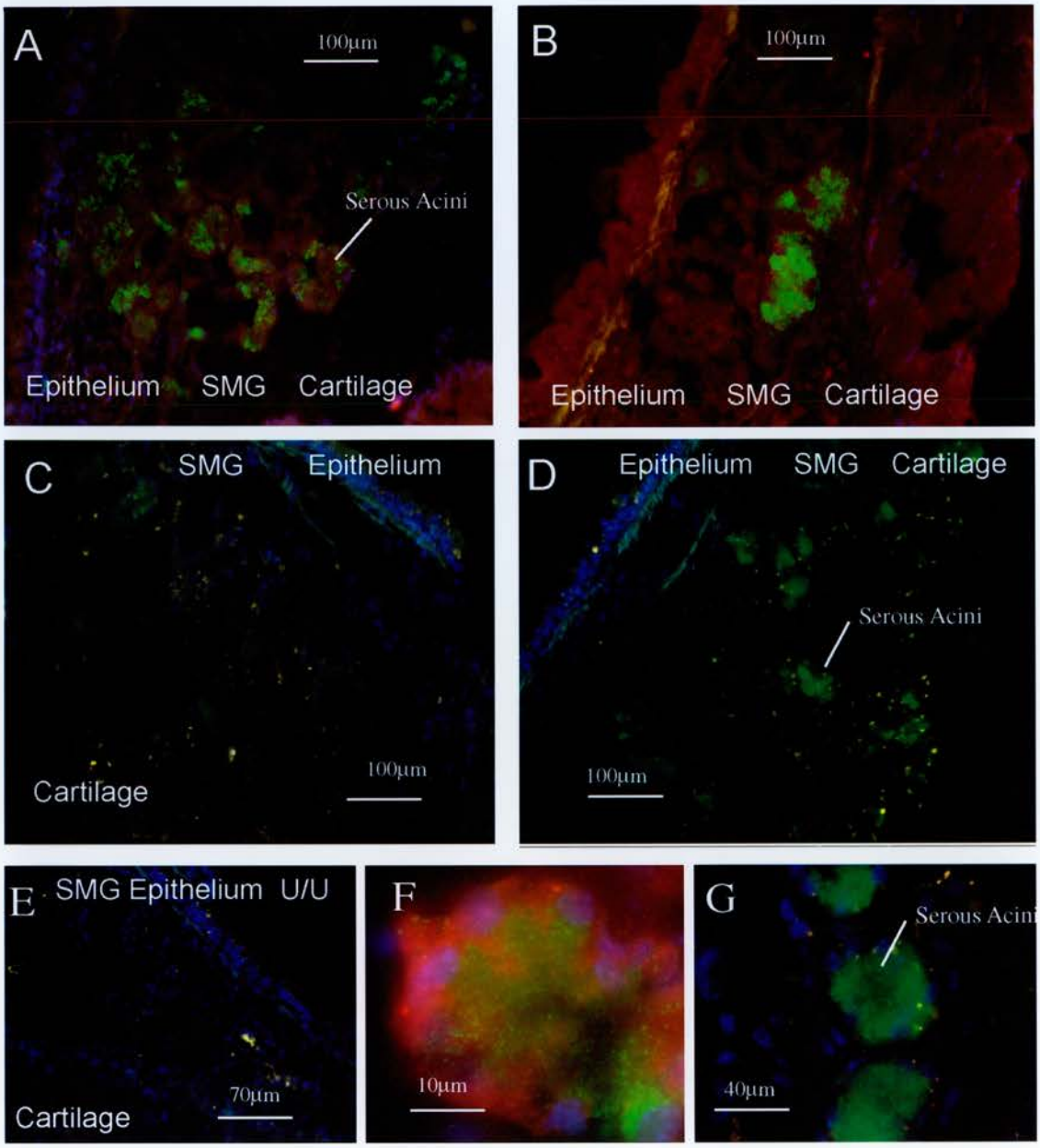
See figure 2.5.1.

Figure 2.5.1

***cftr* Localisation Using Pcterm-B Polyclonal (Anti Human CFTR C-Terminal) Antibody.**

All the images in this figure were obtained using identical exposure parameters. Images are composite RGB fluorescent images (IP-Lab Software) observing reactivity of PCterm-B antibody to the murine SMGs. Blue image represents DAPI nuclear counterstain, red image observes inherent autofluorescence and green image represents *cftr*. Overlap between red and green images would suggest that the green signal may not represent antibody signal rather, a consequence of autofluorescence.

2.5.1A-B PC-termB reactivity to BALB/c SMGs. *Cftr* appears localised in the distal portions of the murine SMGs. A high level of autofluroscence was observed (especially in 2.5.1B) although this was not observed to over lap the *cftr* image (noted by the absence of yellow signalling).
2.5.1C-D PC-termB reactivity to C57BL/6 SMGs. A similar pattern of reactivity was observed as with the BALB/c although the level of autofluorescence was considerably lower. In addition, green signal was observed in the lamina propria filaments.
2.5.1E PC-termB reactivity to a SMG of the homozygous $Cftr^{tm1UNC}$ (CF null) mouse on C57BL/6 background. No green image was observed in the SMGs. Green image was still observed in the lamina propria filaments (as with 2.5.1C-D) suggesting that this signal may be artefact.
2.5.1F High power (x100) image of serous acini of wildtype BALB/C mouse. The pattern of serous cells lying around the acini is readily observable. *cftr* appears to be spread throughout the cytoplasm of the serous cells.
2.5.1G x40 magnification of wildtype C57BL/6 mouse showing high levels of *cftr* protein in serous cells (identified by cellular patterning).


A
100μm
Serous Acini
Epithelium SMG Cartilage
B
100μm
Epithelium SMG Cartilage
C
SMG Epithelium
Cartilage
100μm
D
Epithelium SMG Cartilage
Serous Acini
100μm
E
SMG Epithelium U/U
70μm
Cartilage
F
10μm
G
Serous Acini
40μm

Discussion

cftr was observed to be present in the serous cells of the murine SMGs through the use of an anti-human CFTR antibody. The antibody's specificity to cftr was confirmed through its negative reaction with tissue from the *Cftr*^{tm1UNC} "null" mouse.

2.6

Chapter Discussion

In humans, SMGs are found in the cartilaginous airways of the lung extending from the trachea down into the bronchi and into the larger bronchioles (Widdicombe, 1994). However, mice have been described in previous reports as not having or being essentially devoid of SMGs (Widdicombe, 1994, Engelhardt, 1991, Sehgal, 1996, Widdicombe, 1985). At most, SMGs in the mouse have been reported in the larynx region, and only in the most proximal regions of the trachea (Pack, 1980). By histological examination of wholemount tracheas and paraffin sections we show that SMGs are clearly present in the mouse. Murine SMGs are most common in the proximal regions of the mouse trachea with their frequency rapidly decreasing more distally. Interestingly, the density of murine SMGs in cartilage gaps 0 and 1 was found to be ~1mm^{-2} , this being the same approximate density as glands in the human trachea (see chapter 3 for details of cartilage gap localisation). The existence of murine SMGs may have been overlooked in the past because of their restricted location in the trachea.

On a cellular level, murine SMGs were found to have mucous cells and be rich in serous cells in accordance with findings in the human. The electron dense granules in human serous cells are well characterised (Bowes, 1977) and were found to show great similarity to those in the mouse. The electron dense granules in murine serous

cells were seen to range in sizes (400-1500nm) similar to those observed in humans (Bowes, 1977) and were variable in their membrane integrity. In the larger granules, membranous structures surrounded the granules whilst in smaller granules, the membrane was less distinct and appeared diffuse.

Through the use of Periodic Acid Schiff's and Alcian Blue reagents it was demonstrated that murine SMGs produce mucus which shows a heterogeneous staining pattern reflecting a variation in mucus pH within the gland. Mucous and serous cells both appear to be highly active in the secretion of materials in the gland lumen. The apical membrane of the serous cell also stains positive for Alcian Blue reflecting an discrete alteration in the pH of its secretions (neutral to acidic).

Lysozyme is an antibacterial protein found in secretions protecting most of the body surfaces and was one of the first proteins found to be expressed in human SMGs (Basbaum, 1990). In accordance with this finding, we observe the presence of lysozyme protein in a subset of serous cells in the murine SMGs. This heterogeneity of lysozyme signal may be connected to the heterogeneous nature of MUC7 expression in human serous cells (Sharma, 1997) and variation in serous cell granule composition.

Previously, studies in the human lung have revealed that SMGs are a major site of CFTR expression (Engelhardt, 1992). The expression pattern was found to be localised to the serous cells and a subpopulation (1-3%) of cells in the collecting ducts of the glands (Engelhardt, 1992). It is thought that SMG CFTR may play a role in the maintenance of ionic composition of fluid secretions, similar in role to that found in the sweat glands (Quinton, 1996,Widdicombe, 1994).

In the study of *cftr* expression in the murine trachea, data is presented demonstrating the presence of CFTR protein in the serous cells of the submucosal glands. No

epithelial localised or collecting duct signal was noted. The serous cell staining pattern shows considerable heterogeneity between serous cells and the presence of non-apically localised signal. This pattern may be partially due to the thickness of the sections and the use of permeabilised cells in the protocol or may be the detection of non-apical CFTR in this cell type. In a previous study using anti CFTR antibodies to study ΔF508 mutant CFTR localisation in sweat ducts (Kartner, 1992) such problems were not encountered and indeed, highly apically localised staining patterns were observed. Distinction exist between the Kartner study and those reported in this thesis. Firstly, the Kartner group was studying human tissue (on which the anti-human antibody may be more sensitive) and secondly, the Kartner study used specifically generated monoclonal antibodies on non-permeabilised frozen sections. The possibility that CFTR protein is present in epithelial cells below the level of detection with the polyclonal PC-termB antibody cannot be excluded from the interpretation of these data.

In conclusion, murine SMGs were found to be highly similar to human SMGs as reported in the literature. Mucus is produced in the SMGs and the serous and mucous cells present express CFTR and lysozyme. There is strong evidence to suggest that SMGs play a major role in the development of CF in humans (Inglis, 1997a, Widdicombe, 1994). The characterisation of murine SMGs in this study is thought to further support the argument for the mouse being a useful and suitable animal model for studying CF. One significant difference noted was that murine SMGs were only noted in the proximal regions of the trachea. This difference in distribution is further explored in the next chapter.

Chapter 3

Genetic Factors Affecting SMG distribution

Introduction

This chapter aims to investigate SMG distribution in mice of different genetic strain and phenotypes.

The genotypic-phenotypic relationship of modifier genes

The route from genetic mutation to disease phenotype in CF is not straightforward with a consider degree of phenotypic heterogeneity. This variation can only be partly explained by the diverse spectrum of CFTR mutations (of which there are now over 800 known)(Corey, 1989, Kristidis, 1992) since family members with the same genotype have been observed to show clinical variation (Davis, 1996). It is therefore likely that environmental and other genetic factors modulate the CF phenotype.

Through the use of inbred mice it is quite possible to restrict the *nature* and control the *nurture* of any genetic study with a phenotypic endpoint. Alternatively, it is possible to make comparisons of a specific mutation on different genetic backgrounds and thereupon examine the effects of other genes upon the final phenotype. Such secondary acting genes are known as "modifiers". Examples of phenotypic variation in transgenic mice on different genetic backgrounds is presented in figure 3.0.1.

The assumption of such studies is that the genetic differences in the mouse strain are somehow suppressing or enhancing the expression of genes involved in the patho-physiological disease pathway (Erickson, 1996). These studies can provide the framework and the data to conduct genetic screens for modifier genes.

Figure 3.0.1

Phenotypic variation in transgenic "knock out" mice on different strains (genetic backgrounds).

Targeted gene	Phenotype	Variation by Mouse strain			Reference
Keratin 8	Embryonic lethality	**C57BL/6** 1.6% viable		**FVB/N** 55% viable	**(Baribault, 1994)**
Epidermal growth factor receptor	Time of embryonic lethality	**CF1** preimplantation	**129/SV** mid gestation	**CD1** Survival to 3 weeks	**(Threadgill, 1995)**

The hunt for modifier genes is strongly dependent on there being a definitive and measurable phenotype. Although, the lung disease phenotype in CF mice is considered to be weak, in the complete absence of *cftr*, mice, on a normal solid diet, suffer from severe intestinal obstruction and rupture leading to death before 5 weeks of age (Snouwaert, 1992, O'Neal, 1993, Ratcliff, 1993). This phenotype is similar to the development of meconium ileus in ~20% of CF patients (Oppenheimer, 1975).

The Rozmahel group (1996) used this death by gut blockage as an endpoint in a study of modifier genes for CF in CFTR deficient mice. A CF "null" mouse (named $Cftr^{tm1HSC}$) was established through the disruption of exon 1 of the mouse *cftr* gene by homologous recombination. Severe intestinal blockage was found to occur in the majority (>95%) of homozygous $Cftr^{tm1HSC}/Cftr^{tm1HSC}$ mice.

The founder mouse was on a 129/Sv genetic background. It was observed that the mice died in one of three quite distinct time periods (termed "early", "middle" or "late" with the "late" animals living the longest).

The founder mouse was crossed with females from the outbred CD1 strain and 4 different inbred strains 129/Sv, DBA/2J, BALB/CJ and D57BL/6J and then backcrossed to generate homozygous mice ($Cftr^{tm1HSC}/Cftr^{tm1HSC}$) on 4 different genetic backgrounds.

The inbred strains were found to show quite distinct mortality profiles. F1 crosses were made between the $Cftr^{tm1HSC}/Cftr^{tm1HSC}$ strains and the results shown in figure 3.0.2.

Effect Of Genetic Background On The Survival Of $Cftr^{tm1HSC}/Cftr^{tm1HSC}$ Mice.

On the basis of these studies, a modifier locus for mouse survival conferred by homozygosity of the CD1, C57BL/6J or BALB/cJ alleles but *not* the DBA/2J alleles was found to prolong the survival of the $Cftr^{tm1HSC}/Cftr^{tm1HSC}$ mice.

A genome scan for the modifier locus was conducted by seeking candidate linkage loci which also demonstrated significant deviation from the expected random segregation of CD1 and 129/Sv alleles.

These data identified a candidate region near the centromere of chromosome 7 (Rozmahel, 1996) in which at least 2 independent loci possessing major modifiers exist (Rozmahel, 1997). Characterisation of these loci suggested that their contribution to the CF gut phenotype was both dependent on mouse strain and gender. Furthermore, one of these loci has conserved synteny to a known diabetes modifier in humans lying on 19q13. It has been suggested that the modifier gene may encode for a calcium dependent chloride channel that may be act to compensate for the lack of CFTR activity.

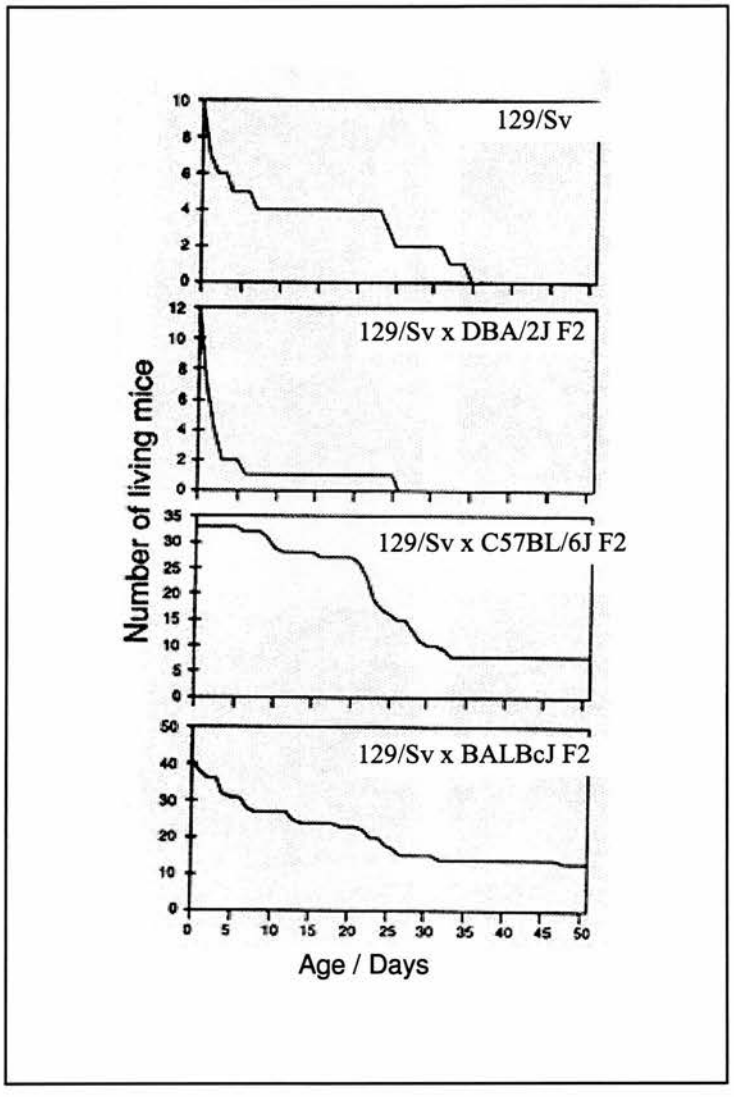
The previous studies focused on using mouse survival (as a result of intestinal blockage) as the basis of their genetic screen. Yet, linkage studies in humans suggest possible correlation between incidences of intestinal disease but no link to the severity of lung disease. With reference to the life threatening phenotypes in human CF disease, it may be of greater significance to use base a genetic screen on some aspect of lung disease phenotype.

One possible measurement of the CF lung phenotype in the mouse is the altered respiratory chloride conductance. Reduction of cAMP-dependent chloride ion conductance (as measured by *in vitro* Ussing chamber) has been demonstrated in all mutant CF mice (Dickinson, 1995) as this is assumed to be a direct reflection on the *cftr* chloride channel activity. This technique has now been used to examine the different electrophysiological profiles of wildtype mice on different mouse strains with the aim of developing a screen for modifier genes.

Provisional data generated in a collaboration between Dr. Dorin and by Prof. Alton (Imperial College London) describes the changes in the short circuit current across the tracheal epithelium in response to the addition of forskolin as measured by single point measurements in a number of mouse strains (CBA, DBA, 129, BALB/c, C57BL/6 and FVB) (for further details of these mouse strains see appendix 1). Of these strains there was a significantly greater chloride channel activity in BALB/c mice compared to all the others with the greatest difference being between the BALB/c mouse and C57BL/6 (57.6$\mu A/cm^2$ (SEM 8.7) and 12.6$\mu A/cm^2$ (SEM 2.2) n=8).

Figure 3.0.2.

Survival Profiles of the *Cftr*^{tm1HSC} homozygous mouse on different genetic backgrounds (from Rozmahel, R. *et al* 1996).



3.1

Interstrain variation in SMG histology

Introduction

CF mice on different mouse strains have been demonstrated to show significant variation in survival rates (Rozmahel, 1996) and wildtype mice on different strains have been reported to exhibit variable tracheal electrophysiological measurements (Dorin, J. Personal communication). It was hypothesised that there may be variation in SMG structure and distribution between the mouse strains that may have the consequence of affecting the CF mouse lung phenotype and tracheal electrophysiological measurements. To begin examination of this hypothesis, a histological comparison of SMG structure in 3 different mouse strains was conducted.

Materials and Methods

BALB/c, C57BL/6 and CD1 mice (n=6 in each strain) were sacrificed by lethal injection of Euthatal. Their tracheas were dissected out and after fixation in 4% paraformaldehyde for 6 hours at $+4^0$C and processed for embedding in wax blocks. 6 μm serial sections were cut and rehydrated to water and counterstained with haematoxylin and eosin. In an attempt to reduce variation arising from histological processing and sectioning, all tissues were processed and were sectioned at the same time. Furthermore, SMG analysis was restricted to longitudinal sections of the area adjacent to the hyaline cartilage as this area is easily recognisable and has the highest concentration of SMGs.

SMG Histological Interstrain Variation

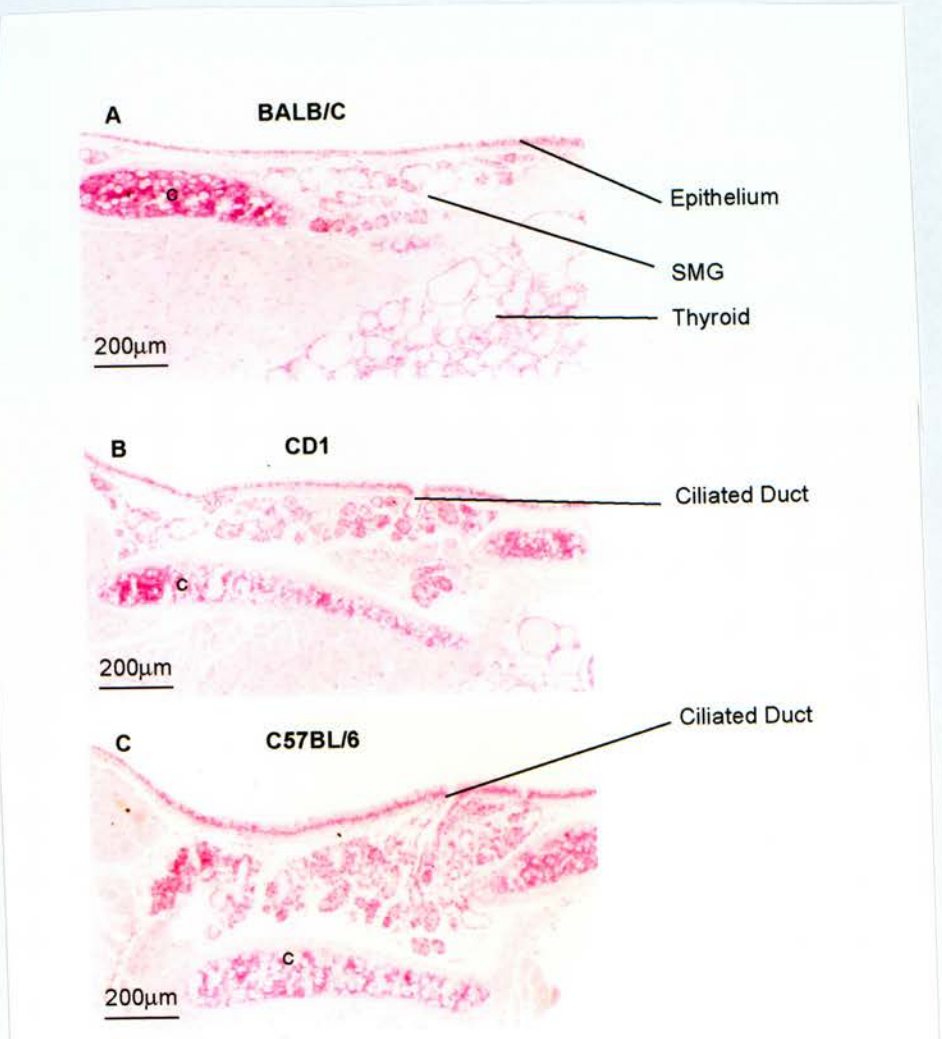
Examination of SMGs from mice of 3 different genetic backgrounds revealed marked histological variation (see figure 3.1.1). Of the 3 strains studied, the SMGs from C57BL/6 mice (3.1.1c) were observed as being larger than those of the CD1's (3.1.1a) and BALB/c's (3.1.1b) occupying a larger area of mesechymal tissue between the lumen and the hyaline cartilage ring. The C57BL/6 glands showed long and narrow ciliated ducts and multiple acini branching from the collecting ducts. It is unclear from this evidence whether the SMGs of the C57BL/6 are larger as a consequence of filling the larger mesechymal space present or do indeed force a larger area to exist.

Attempts to quantify these observations (by planimetry) were unsuccessful due to the complex 3D structure of these glands, their variable distribution within the trachea and the elastic effects of the cartilage on the sections. However, this experiment was repeated and the slides screened blind. The mouse strains were correctly identified 100% of the time (n=8 for each strain).

Figure 3.1.1

Histological Comparison Of SMGs In 3 Mice Strains

3.1.1a H&E histology of SMGs in the CD1 mouse. SMGs were seen to occupy a small tangential area although ducts were observed as considerably dilated.
3.1.1b H&E histology of SMGs in the BALB/c mouse.
3.1.1c H&E histology of SMGs in the C57BL/6 mouse. The area occupied by the C57BL/6 SMGs was the largest of the strain studied. Ducts were noted to be narrow and elongated.



3.2

SMG distribution in mice of varying genetic background

Introduction

Preliminary studies (reported in chapter 2) suggested that murine SMG distribution is restricted to the proximal regions of the mouse trachea. A more comprehensive study of SMG distribution in 4 different mouse strains was undertaken. In both sections 3.2 and 3.3, a method of PAS stained tracheal wholemount was used to overcome the complexities and inaccuracies of scoring for infrequent 3D structures on a 2D plane. This method is adapted from a similar method for human foetal lung as reported by Tos (1966) with the assistance of Duncan Davidson.

Materials and Methods

6 week old mice from 4 different genetic backgrounds (129/MF1 Mixed, C57BL/6, BALB/c and FVB) (n=6-9) were sacrificed by lethal injection with a 0.4ml IP injection of Euthatal (Rhone Merieux, UK). The 129/MF1 background mice were housed in a conventional non-SPF facility and were on an outbred strain whereas the other mice were inbred (>8 generations) and housed in a SPF facility. The tracheal distribution of SMGs within and between the groups was studied using a method of PAS tracheal wholemount. Excised tracheas were fixed in 4% neutral buffered formalin for 6 hours and then transferred to 70% ethanol for storage at +4°C. The tissues were stained in Periodic acid for 3 minutes, washed 3 times in PBS and stained in Schiff's reagent for 3 minutes before washing and storage in glycerol at room temperature.

The stained tracheal wholemounts were pinned out on a flat wax bed using

sharpened tungsten needles and the viewed with a optic fibre dissecting microscope at x80 magnification (Volpi, Switzerland). The SMGs were visible lying between the cartilage rings (this observation was confirmed by post fixation and histological examination of sections). The tracheas were scored for the presence and location of SMGs. As the more distal SMGs lay between cartilage rings, a system of scoring with reference to cartilage ring gaps was designed. The gap distal to the hyaline Cartilage ring was numbered gap 0 running down to gap 14 or 15 at the carina. See figure 3.2.1.

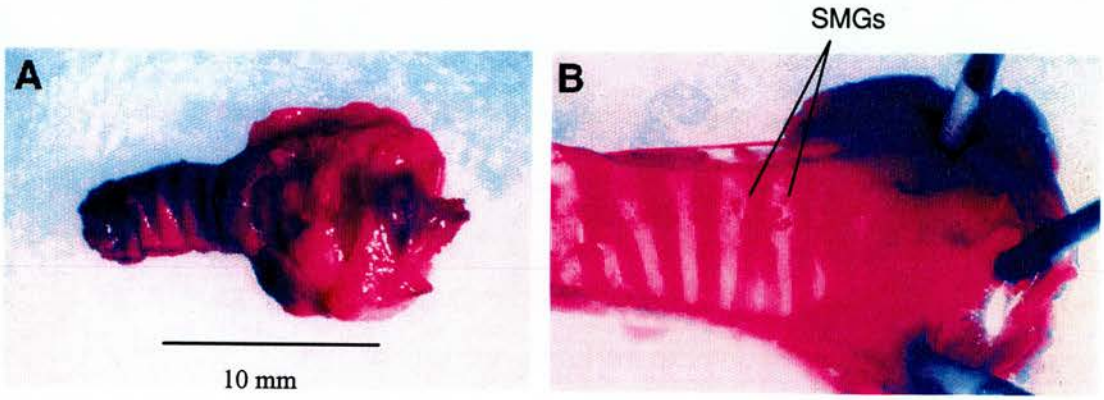
Results

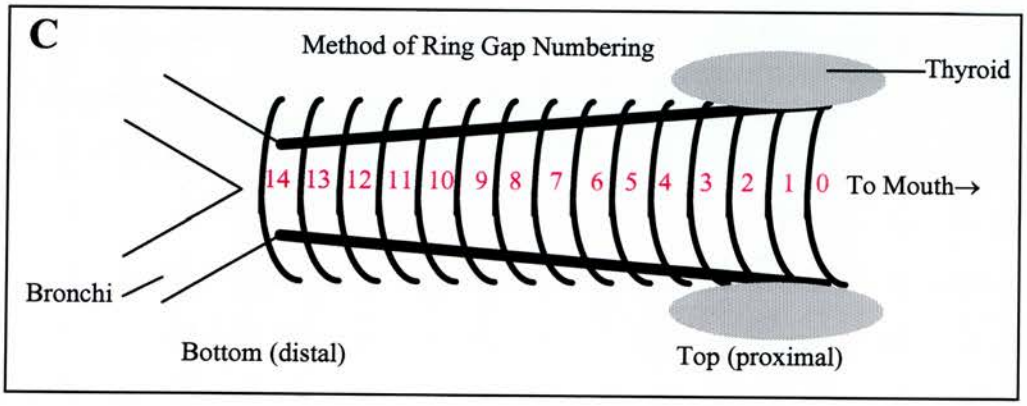
A method of wholemount PAS staining of the mouse trachea was developed to aid whole tracheal screening for SMGs. See figure 3.2.1.

Mice from 3 different mouse strains were studied for SMG distribution. Data is presented in figure 3.2.2. X axis of the figure represents mouse number with score on Y axis corresponding to ring gap number as defined above. The symbol "⊕" represents more than 10 SMGs being present in a single gap. Above 10 SMGs per gap, examination of the individual glands became difficult. If less than 10, numbers are used.

Figure 3.2.1

Method Of Wholemount Analysis Of Murine Trachea For Presence Of SMGs (Adapted From Tos, M. *et al,* 1966)





3.2.1a Trachea was stained in PAS and subsequently pinned out with tungsten needles on a bed of wax.
3.2.1b SMGs can be seen lying between the cartilage rings in the more proximal regions of the trachea
3.2.1c For quantification of the data received through this protocol, the ring gaps were numbed from "ring 0" (most proximal) downwards.

Figure 3.2.2

SMG distribution in wildtype mice of 4 different genetic strains

Mouse number is displayed on the X axis and tracheal ring gap position on the Y axis. Number of glands at each gap is presented on the table (⊕ representing >10).

129/MF1 **BALB/c**

Mouse→

	1	2	3	4	5	6	7		1	2	3	4	5	6	7	8	9
Proximal	⊕	⊕	⊕	⊕	⊕	⊕	⊕	0	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	⊕	⊕	⊕	⊕	⊕	⊕	⊕	1	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
T	5	3	⊕	⊕	⊕	⊕	⊕	2	⊕	1	4	⊕	⊕	6	8	⊕	
r	1	1		1		5		3	8	1	3	3	5	2	5	6	3
a								4	6		3	3	3	2	8	5	2
c								5	7			3	5		4	5	
h								6	2			4	2		2	3	
e								7	3				2		1		
a								8					1		2		
↓																	
Distal																	

C57BL/6 **FVB**

1	2	3	4	5	6		1	2	3	4	5	6
⊕	⊕	⊕	⊕	⊕	⊕	0	⊕	⊕	⊕	⊕	⊕	⊕
⊕	⊕	⊕	⊕		⊕	1	5	3	⊕	⊕	⊕	⊕
1	6					2	2		1	3	2	2
	3					3	1		1	3	2	1
	3					4					1	
	1					5						
						6						

The SMG distribution in relation to the location in the trachea showed considerable variation both within and between groups of varying genetic background. The location of SMGs in the C57BL/6 mice was restricted to cartilage ring gaps 0 and 1 in 4/6 mice. One mouse had one gland present in gap 2 whilst one mouse had SMGs located as far as gap 5. The results from the C57BL/6 are in marked contrast to those observed in the BALB/c mice. All BALB/c mice had SMGs located in cartilage gap 3 (n=9). 5/9 mice had SMGs at gap 6 and 2 mice had glands present at gap 8. Mice have on average, 15 cartilage rings.

Two other mice strains were studied, namely, those of a 129/MF1 mixed genetic background and also FVB mice. The SMG distribution phenotype in these mice lay between the observations as seen in the C57BL/6 and BALB/c strains.

A statistical comparison was made of these data using the unpaired student's t-test. Each mouse was scored for most distal SMG and the groups compared for statistical significance: MF1/129 distribution versus BALB/c $p=0.0001$, C57BL/6 versus BALB/c $p=0.0002$, FVB versus BALB/c $p=0.0071$. No statistical difference was noted in the comparison of the 129/MF1, C57BL/6 and FVB strains.

3.3

SMG distribution in wild type and CF Mutant mice

Introduction

Hyperplasia and hypertrophy (an increase in both volume and number) of SMGs in CF infants as compared to non-CF infants has been reported (Madden, 1991,Oppenheimer, 1975, Yankaskas, Personal communication). A study into the effect of reduced CFTR expression on SMG distribution in the mouse was undertaken.

Materials and Methods

8 week old Mice homozygous for the CFTR insertional mutation *Cftr*^{tm1HGU} (Dorin, 1992a) on all three genetic backgrounds (n=4-7) and mice homozygous for the protein mislocalisation mutation *Cftr*^{tm1G551D} (Delaney, 1996) on the 129/MF1 mixed genetic background (n=6) (all littermates of mice used in section 3.2) were sacrificed by lethal injection with a 0.4ml IP injection of Euthatal (Rhone Merieux, UK) and processed for PAS wholemount as before.

Results

SMGs show a significantly more Distal distribution in *Cftr* deficient mice

In an experiment blinded to genotype, the distribution of PAS stained SMGs was studied by using whole mount preparation of tracheas as described above. See figure 3.3.1. Wild type mice used were littermates to the *Cftr*^{tm1HGU} mice. All but the MF1/129 mice were obtained from a SPF facility. Wildtype mice showed the

presence of more than 10 SMGs in gaps 0 and most commonly, gap 1 and extending distally to gap number 4 in 2/7 animals. SMGs extend more distally in mice with low level or mutant *cftr* expression. Homozygous $Cftr^{tm1HGU}$ and homozygous $Cftr^{tm1G551D}$ mice were studied for SMG distribution. In the homozygote $Cftr^{tm1HGU}$ animals of MF1/129 mixed genetic background, gland masses (where gland number>10 per gap) are present in gaps 0, 1 and 2 in the majority of cases. 6/7 mice had glands present in gap number 4 and in one mouse a SMG was identified as distal as gap number 8. In the homozygote $Cftr^{tm1G551D}$ mice, gland masses were present in all mice in gaps 0 and 1. At gap number 4, 4 /6 mice scored positive for presence of glands. The most distal glands in this group of mice were found in gap number 7 (see figure 6c). The statistical significance of these results (generated as before with unpaired t-test) were as follows: 129MF/1 mice wildtype versus $Cftr^{tm1HGU}$ p=0.002 and versus $Cftr^{tm1G551D}$, p=0.02. C57BL/6 wildtype versus $Cftr^{tm1HGU}$ p=0.002. No statistical significance (p=0.96) was noted between the wild type and $Cftr^{tm1HGU}$ BALB/c mice.

Figure 3.3.1

SMG distribution in CF mice and wildtype mice

Details for the design of this figure is presented in section 3.2.

129/MF1 Mixed Genetic Background (non-SPF)

Wild type								*Cftr*^{tm1HGU}								*Cftr*^{tm1G551D}					
1	**2**	**3**	**4**	**5**	**6**	**7**		**1**	**2**	**3**	**4**	**5**	**6**	**7**		**1**	**2**	**3**	**4**	**5**	**6**
⊕	⊕	⊕	⊕	⊕	⊕	⊕	**0**	⊕	⊕	⊕	⊕	⊕	⊕	⊕	**0**	⊕	⊕	⊕	⊕	⊕	⊕
⊕	⊕	⊕	⊕	⊕	⊕	⊕	**1**	⊕	⊕	⊕	⊕	⊕	⊕	⊕	**1**	⊕	⊕	⊕	⊕	⊕	⊕
5	3	⊕	⊕	⊕	⊕	⊕	**2**	⊕	⊕	⊕	⊕	⊕			**2**		2	5	6	2	5
1	1		1		5		**3**				5	2		1	**3**		3	3	5		5
							4		1	4	1	1	1		**4**		1	1	1		2
							5	4		2					**5**					1	2
							6			4	1		1		**6**				1	1	5
							7								**7**					1	1

C57BL/6: Inbred Genetic Background (SPF housed animal)

Wild type							*Cftr*^{tm1HGU}						
1	**2**	**3**	**4**	**5**	**6**		**1**	**2**	**3**	**4**	**5**	**6**
⊕	⊕	⊕	⊕	⊕	⊕	**0**	⊕	⊕	⊕	⊕	⊕	⊕
⊕	⊕	⊕	⊕		⊕	**1**	⊕	⊕	⊕	⊕	⊕	⊕
1	6					**2**	6	4	5	⊕	2	4
	3					**3**	1	6	4	1	3	
	3					**4**		1	2		1	3
	1					**5**					1	
						6						

BALB/c: Inbred Genetic Background (SPF housed animals)

Wild type										*Cftr*^{tm1HGU}			
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		**1**	**2**	**3**	**4**
⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	**0**	⊕	⊕	⊕	⊕
⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	**1**	⊕	⊕	⊕	⊕
⊕	1	**4**	⊕	⊕	6	8	⊕		**2**	⊕	⊕	⊕	7
8	1	**3**	3	5	2	5	6	3	**3**	7	3	⊕	4
6		**3**	3	3	2	8	5	2	**4**	5	4	7	4
7			3	5		4	5		**5**	3	1	5	2
2			4	2		2	3		**6**	4	2		
3				2		1			**7**	3	1		
				1		2			**8**	4			
										3			

3.4

Evidence for *de novo* SMGs generation in the adult mouse.

The question of whether the more distal distribution of SMGs in the CF mice have arisen as a physiological response to damage was addressed by studying SMG distribution and structure after artificial damage induction by 20μl 1% polidocanol tracheal instillation.

Materials and Methods

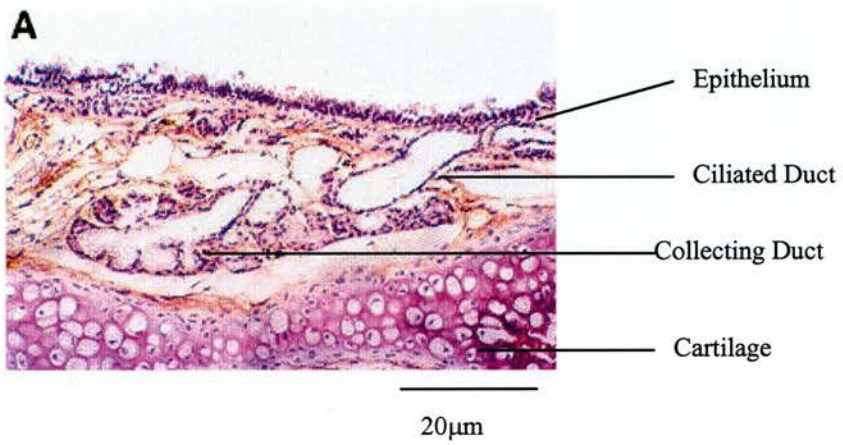
Wild type mice were intraperitoneally injected with 1ml of 10mM Bromodeoxyuridine (BrdU) solution per 100g body weight (approximately 1 mg per mouse) before and after instillation of 20μl 1% (v/v) polidocanol by catheter by a method adapted from Ho and Furst (1973). After 3 to 21 days, the tracheas were removed and processed to wax blocks. Immunohistochemistry for the presence of incorporated was conducted on these sections using the "*In situ* cell proliferation kit-AP" (Boehringer Mannheim, Lewes, UK). Tissues were de-waxed and re-hydrated by transferring slides through 2x xylene for 10 minutes and a decreasing concentration of ethanol (2x 100%, 90%, 70%, 50%, 1 minute each) to PBS. All incubation steps took place in a humidified chamber at room temperature unless otherwise directed. Slides were transferred to a large beaker containing excess PBS and the beaker covered with cling film prior to boiling using a microwave oven at high power for 3 x 5 minute intervals. Slides were left to cool in clean PBS.

Slides were incubated with a 1:4 dilution of anti-BrdU-AP antibody in Incubation buffer (PBS, 1% BSA) for 30 minutes at 37°C in a humid chamber. Slides were washed in PBS for 3x 5 minutes. Substrate solution was prepared by dissolving 1mg Fast Red (2-methyl-4-chlorobenzene-diazonium: tetrachlorizonicate (2:1)(Boehringer Mannheim, Lewes, UK) in 2 ml of 1% BSA in PBS.

Substrate solution was shaken for 5 - 10 minutes until dissolved. Slides were incubated in substrate buffer for 15-30 minutes or until colour develops. Slides were counterstained with haematoxylin and mounted in glycerol mountant (10g gelatine, 60ml dH_2O, 70ml glycerol, 0.25 phenol) and dried overnight at room temperature prior to viewing with bright field Nomarski microscopy (Zeiss Axioplan II) (see figure 3.4.1). Although SMG duct dilation was observed, no results inferring *de novo* gland production was noted.

Figure 3.4.1

Polidocanol damage of the trachea results in gland dilation but no *de novo* gland production.



3.4.1a Mouse SMG counterstained with eosin showing lumenal dilation 7 days after polidocanol instillation. Many glands demonstrated dilation in response to polidocanol damage though no sign of new gland formation (by glandular labelling with BrdU) was noted.

Discussion

From these studies, no evidence was gained which would suggest that new SMGs can be generated in response to tracheal damage. If this is related to a CF lung situation, it would appear to suggest that the more distal pattern of SMG distribution observed in the CF mouse (and in the human) is not related to an increased level and susceptibility to tracheal damage but perhaps due to the lack of *cftr* expression. This argument is further backed up by the observation of interstrain differences in SMG distribution in mice from inbred backgrounds and SPF housing facilities. It would be of interest to examine, if the technology permitted, whether there is stability in the number and distribution of SMGs throughout the life span of a mouse.

3.5

Discussion

The data presented in this section demonstrates that mice with greatly reduced *cftr* expression (5-10% of wildtype expression in case of $Cftr^{tm1HGU}$ (Dorin, 1992a) or mutant *cftr* in $Cftr^{tm1G551D}$ (Delaney, 1996)) SMGs extend more distally than in wild type controls ($P<0.05$). In addition, there was a significant variation in SMG distribution between different mouse strains with the C57BL/6 mouse showing the most restricted distribution and the BALB/c mouse showing the most expansive distribution of SMGs of strains studied. Of further interest was the observation that the distal glands were often found to lie on a very similar longitudinal plane. In such cases, the line of distal glands was most often on the ventral side of the trachea. Data not shown.

The SMG variation through different mouse strains is most likely to be as a consequence of the presence of alternative genes that determine SMG distribution. The reason for the *cftr* specific distribution is not yet certain, but could be explained

either as a consequence of CF lung inflammation (though not evident histologically), response to damage or possibly due to a developmental effect of reduced *cftr* expression.

The MF1/129 mice used in this experiment were the anomaly of the experimental animals as they are of outbred background and were housed in non-SPF facilities. The data obtained from these animals, although reflecting similar patterns observed in the other strains, show greater variability in results suggesting perhaps that other modifier genes or indeed, environmental factors influence SMG distribution. It would be of interest to see if these animals when housed in SPF facilities show a reduced range of variability.

From the data presented, it is considered unlikely that new SMGs are developing in response to epithelial damage. Mice received artificial epithelial damage by polidocanol instillation and although, SMG dilation occurred, no indication of *de novo* gland development was observed.

Do electrophysiology results reflect CF phenotype or SMG distribution?

The correlation between the SMG distribution in different mouse strains and Prof. Alton's single point electrophysiology results was noted. BALB/c mice which showed the most distal pattern of SMG distribution also showed the highest levels of chloride conductance in the trachea by measurement in the Ussing chamber. C57BL/6 mice showed the opposite, with a very restricted pattern of SMG distribution and very low chloride conductance.

A model is presented below which outlines the argument that single point electrophysiology measurements in the trachea of the mouse are not a direct reflection of a CF phenotype but are instead a reflection on SMG distribution. The

relationship between the two is, at this time, unclear. The first model (figure 3.5.1) is based on the assumption that the electrophysiology measurements are taken approximately in the middle of the trachea. Model number two (figure 3.5.2) discusses that if the electrophysiology results were taken from different sites on the trachea, or indeed the trachea orientation was varied, variable electrophysiological measurements would be obtained.

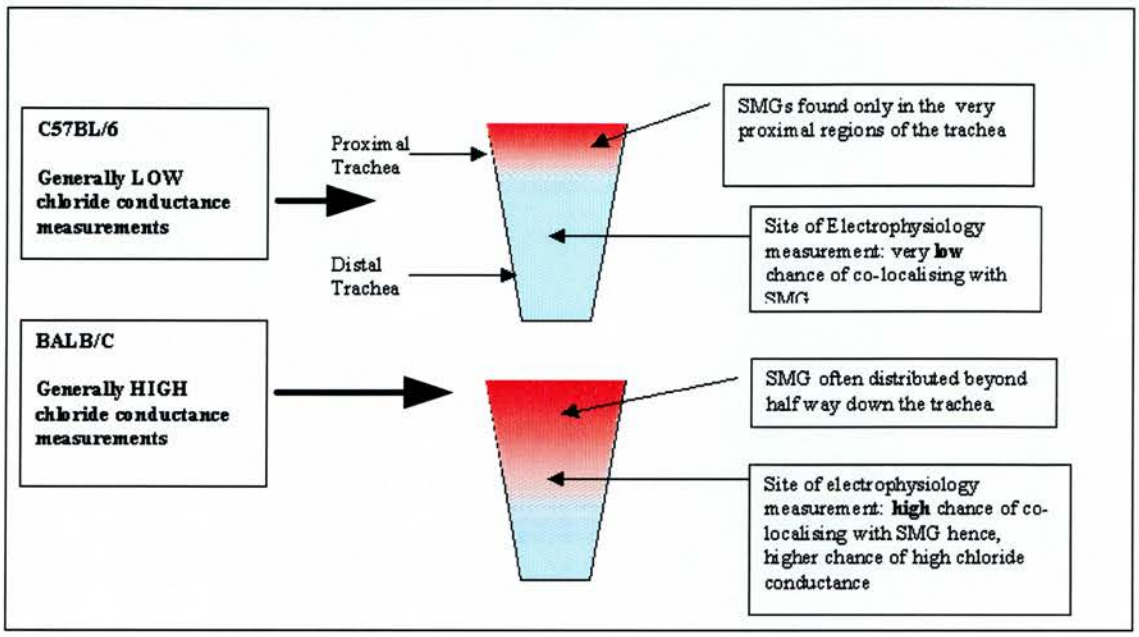
Recent data from Prof. Alton's laboratory would appear to disagree with model 2 (personal communication). In this study, tracheal tissue from the laryngeal end or carinal end of was mounted in a mini-Ussing chamber. Drugs were applied to the tissue to measure the chloride conductance in these areas. In addition, chloride conductance was measured after the artificial stimulation of any SMG lying in that region. A decrease in sodium absorption was noted in laryngeal portion of the tissue as compared to the carinal end but no similar trend in chloride conductance was detected. These data do not wholly contradict the models presented above since neither the mouse strain used in this experiment is revealed nor a more detailed description of what and where the "carinal" or "laryngeal" areas of the trachea actually are.

These models may have strong implications for the use of single point electrophysiological measurements as a method of screening for modifier genes for CF. To test these models experimentally, it would be interesting to correlate the SMG distribution of tracheal tissue used in the actual Ussing chamber measurements. Provisional approaches to conduct this experiment have been made (in collaboration with Dr. B. Innes) however, the method of tissue fixation and quality of the tissue has prevented progress.

3.5.1

Electrophysiology measurements and SMG distribution Model 1:

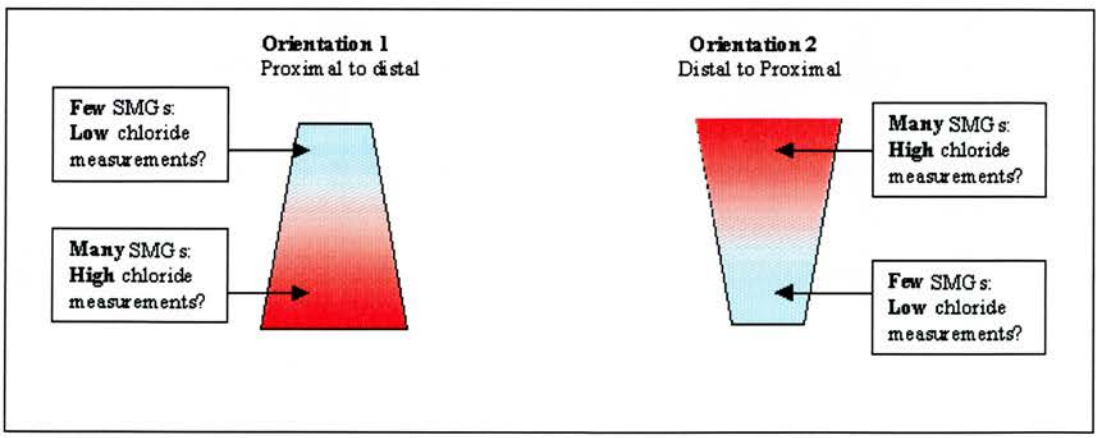
Do single point electrophysiological measurements of wildtype tracheas on different mouse strains reflect a direct CF phenotype or just SMG distribution?



3.5.2

Electrophysiology measurements and SMG distribution Model 2:

Is there any evidence for tracheal orientation (and therefore, SMG distribution) in the Ussing chamber resulting in different electrophysiological results?



Chapter 4

Antimicrobial activity of the human and mouse β-defensin-1 peptide

Introduction

Theories of the development of CF lung disease

CF lung disease is characterised by the development of mucous obstructions leading to bronchiolitis and goblet cell hyperplasia leading to squamous metaplasia, bronchiectasis and possibly pneumonia (Boat, 1989). While mucous obstruction may be the primary pathophysiological event (as seen by early SMG dilation (Widdicombe, 1994)), the persistent and chronic infections by a discrete number of bacterial species including *P. aeruginosa*, *S. aureus* and *B. cepacia* would appear to have the major destructive effect on the lung (Govan, 1996, Grimwood, 1993). Once established, these bacterial infections are nearly impossible to clear.

The questions of why mucous obstruction occurs and why such a discrete group of bacteria should show such tropism for the CF lung still remain unanswered. Furthermore the relationship between the two processes is unclear.

The traditional model for CF lung disease is based on the disease causing processes in the CF pancreas. The pancreas is a branched organ involved in the production of enzymes crucial for the digestion of food. The movement of bicarbonate across the apical membrane of the pancreatic duct is CFTR dependent. In CF patients a reduction in bicarbonate secretion in the ducts can occur which leads directly to an imbalance in the pH of secretions and inactivation of pancreatic enzymes (Lippe, 1977, Kopito, 1965, Quinton, 1996).

It is certainly conceivable that the electrolyte transport defects of the CF airway could result in abnormal airway secretions. The failure of chloride secretion through CFTR or the outwardly rectifying chloride channel (Li, 1988) could produce less salt and therefore less water secretion and subsequently, increase amiloride-sensitive

sodium reabsorption that would also tend to draw sodium with water out of the airways. If this pattern occurred in the lung, it can be presumed that the volume of airway surface fluid (ASF) secreted, under normal conditions, on to the epithelial surface would be abnormally small. The normal processes of cilia clearance of mucous and associated pathogens may be disrupted and lead to increased concentration and colonisation of bacteria in the ASF (Jiang, 1993). This idea has supported the popular belief that CF ASF is dehydrated and "sticky" (Quinton, 1996).

It is technically very difficult to sample and measure electrolyte concentrations in ASF due to both the extremely low volume (the "teaspoon of ASF spread over a tennis court" is a popular analogy; the actual volume of ASF is about 5μl per cm^2 and the lung's hypersecretion reaction to many of the sampling protocols (Joris, 1993).

Models of CF airway epithelium in culture have in part supported the model of ASF being dehydrated in the CF lung (Smith, 1994, Jiang, 1993). However, there is considerable evidence against this model. Firstly, bronchoscopy observation of CF airways suggests that CF ASF is not recognisably dry (Joris, 1993). Secondly, diseases in which cilia action and mucus clearance is known to be impaired (such as Kartagener's syndrome (Kollberg, 1978)) or in which mucus dehydration occurs (asthma (Freed, 1987) or hypohidrtic ectodermal dysplasia (Clarke, 1987)) do not produce the same airway pathology as in CF.

The Role Of SMGs In The Development Of CF Lung Disease

The highest density of CFTR in the human lung is in the serous and ciliated duct cells of the submucosal glands (Engelhardt, 1992). SMGs have a dual defensive role

in the lung; supplying the airway epithelium with both fluid and additionally a mixture of antimicrobial proteins and mucoproteins (Basbaum, 1990, Widdicombe, 1994, Gabay, 1993). SMGs have been reported to show a high degree of plasticity in response to inflammatory challenge altering both in shape and in the degree of acidity and sulphation of secretions (Sturgess, 1972). The Yamaya group (1996) reported that CF SMGs fail to respond to the normal stimulatory β-adrenergic or cholinergic stimulation suggesting that the defensive role of SMGs may be impaired in CF. As interesting as these data are, it is puzzling that disease known to have defective SMG secretions do not show a CF phenotype (Clarke, 1987, Kollberg, 1978). In addition, this theory does not describe the apparent selectivity in bacterial species that colonise the CF lung nor why CF lung disease appears to develop firstly in an area devoid of SMGs.

Bacterial Tropism To The CF Lung

CF patients are particularly susceptible to infection by a small number of bacteria including S. *aureus* (often the first bacteria to colonise), *P. aeruginosa* and *B. cepacia* (Govan, 1996, Grimwood, 1993). This would suggest an enhanced tropism, or reduced resistance in the CF lung for these bacteria. The development of CF lung disease is most likely to be a multi-step process. In this structure, recent experiments have pointed towards the interaction between bacteria and CFTR deficient epithelial cells to be very near the head of this process.

P. aeruginosa has been found to be able to upregulate the expression of mucin genes (Li, 1997). Indeed, there is evidence that the adherence pattern and internalisation of *P. aeruginosa* to CF pulmonary epithelial cells differs from that of wildtype. Details of these arguments are comprehensively covered by Porteous and Davidson (Porteous, 1997a) and Davidson and Porteous (Davidson, 1998).

Theory Of Defensin Inactivation In CF

A currently highly fashionable and exciting theory to explain the occurrence of bacterial infection in the CF lung has developed around the discovery of salt sensitive antibacterial agents in the lung in particular the β-defensin family. β-defensin are members of a family of small antimicrobial peptides which contribute to the innate immunity mechanism of a wide range of invertebrate and vertebrate organisms (Ganz, 1995).

In 1996, Smith *et al.* (1996) reported that ASF from primary CF bronchial epithelial cultures had reduced antimicrobial activity (against *P. aeruginosa* and *S. aureus)* as compared to wild types, indeed, bacteria were found to multiply in CF ASF. When the chloride concentration in nasal ASF was measured in CF and non CF patients, it was found that CF patients had a higher chloride concentration. Interestingly, it was found that by reducing the salt concentration in the CF ASF normal anti microbial activity resumed.

These data suggested the presence of an antimicrobial agent in the ASF that was inactivated at high salt concentrations. Goldman *et al.* (1997) reported a year later that β-defensin-1 was a likely candidate for the antimicrobial agent. Human β-defensin-1 (hBD-1) was found to be expressed throughout the respiratory epithelia and show a salt dependent anti-microbial profile against *P. aeruginosa*. In xenografts (see chapter 7 for details of this system), correction of the CF defect with adenovirus mediated CFTR gene therapy was found to lead to the normalisation of ASF salt concentration and the restoration of its bactericidal activity. Furthermore, down-regulation of hBD-1 RNA by antisense oligonucleotide led to the loss of antimicrobial activity in ASF from non-CF xenografts.

The publication of this paper generated a renewed interest in the anti microbial agents of the lung. A second human β-defensin has been cloned (hBD-2) in addition to a murine homologue of hBD-1 (mBD-1)(Morrison, 1998, Huttner, 1997). The theory is very attractive and accessible in explaining CF lung disease. CFTR dysfunction leads directly to a change in the ASF environment by raising chloride levels and although the components of the lungs defence system it is inactivated. The lung is thereby vulnerable to infection that may precipitate a more pronounced inflammatory response.

Recently, this picture has become more complicated through the reporting that defensins and other respiratory anti microbial agents may operate in a synergistic relationship (Singh, 1997).

Alterations in the ability of cilia to clear sputum and other materials from the airway epithelium in CF patients may be related to the hypothesised change in airway surface fluid sodium chloride concentration. Wills *et al.* (1997) studied the rate at which cilia could transport sputum in CF patients. Using mucus depleted bovine trachea, the transportability of CF and non sputum was found to be slow. Adding sodium chloride to sputum was found to enhance its transportability by possibly altering the sputum's rheology and the ciliary transportability.

Critics of this theory attack the assumption the *CFTR* defect in the lung results in increased chloride levels as seen in the sweat ducts of CF patients (Quinton, 1983). Attempts to measure the Chloride concentration of ASF in the lung is virtually impossible without the use of some invasive technique (Quinton, 1996) giving rise to arguments over whether they do in fact reflect *in vivo* results. One common technique is to apply a piece of "blotting paper" to the lung epithelium in order to soak up ASF. Bronchoscopy examination of this protocol has often observed localised hypersecretion of mucus (Quinton, 1996). At this time, the answer to this criticism is still unclear. Joris *et al.* (1993) reported an increase in chloride

concentrations in CF patients (chloride concentration 84+9mM and 129±5μM). Knowles *et al.* (1997) found no difference in the ion composition of bronchial ASF from *in vitro* culture. Since the airway epithelium is characterised as being "leaky" to the paracellular flow of water and salt, it is not clear to what extent abnormalities in electrolyte concentration can actually exist (Davis, 1996).

Although functional studies on the human β-defensin-1 peptide had been previously reported by Goldman *et al.* (1997) their data failed to present the effect of varying salt concentration on control samples or analysis of error variation. This chapter examines the relation between the antimicrobial activity of synthetically produced hBD-1 and mBD-1 and salt concentration.

This work was done in collaboration with Dr. Donald Davidson. The laboratory work was shared equally between Dr. Davidson and myself. Dr. Davidson arranged the statistical analysis for the data with Mr. Peter Teague.

4.1

Effect Of Salt Concentration On β-Defensin-1 Antibacterial Activity

Introduction

The effect of varying buffer salt concentration on the functional activity of human β defensin-1 (hBD-1) and mouse β-defensin-1 (mBD-1) against 2 bacterial strains of clinical significance was conducted.

Materials and Methods

Mature Human β defensin 1 and mouse β defensin 1 peptide were synthesised by Prof. Ramage (Albachem Ltd., Edinburgh, UK) and correct secondary structure cysteine bond formation and purity was confirmed using High Performance Liquid Chromatography and MALDI TOF MS (mass spectroscopy) analysis.

Functional analysis of synthetic mBD-1 and hBD-1

The functional activity of the synthetic defensins was tested against *P. aeruginosa* (CF clinical isolate K1385) (kindly supplied by Prof. J. Govan). This bacteria was chosen because of its recognised pathogenic involvement in CF lung disease (Govan, 1996). Overnight cultures of bacteria were grown in nutrient broth and then suspended in PBS at a concentration of ~1 x 10^9 colony forming units per ml. These samples were then split, resuspended and diluted in 10mM Phosphate buffer (0.01M KH_2PO_4, 0.01M K_2HPO_4 and 0.1% D-glucose) varying in concentration on NaCl (final concentrations and pH can be seen in table 4.1.1). Two duplicate 500µl reactions were prepared containing either buffer alone or with synthetic peptide rehydrated in buffer in the range of NaCl as seen in figure 4.1.1. Approximately

$5x10^4$ bacteria were added to these solutions. Reaction mixtures were incubated for 20 minutes at 37°C after which duplicate sets of serial dilutions were prepared from each sample, again in appropriate saline buffer, plated out on P. isolation agar (DIFCO , Detroit, USA) and incubated overnight at 37°C for 48 hours. Colony counts were performed. Controls of peptide and buffer and buffer alone were run to test for possible contamination.

Figure 4.1.1

The Effect Of Varying NaCl Concentration On The pH Of Phosphate Buffers.

Salt concentration/mM	pH
0	7.60
30	7.40
60	7.33
90	7.27
120	7.23
150	7.20

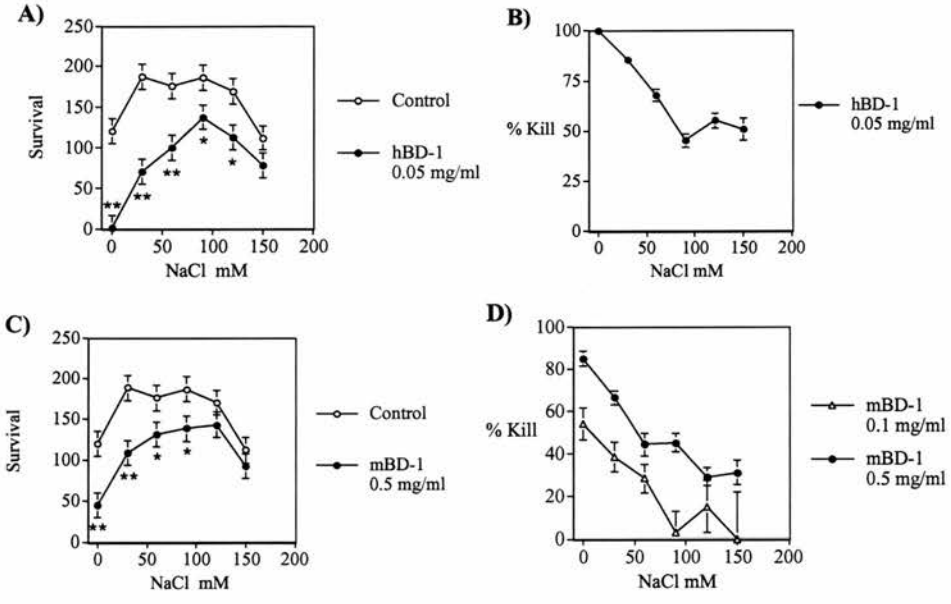
Results

Data are presented in table form in graph form in figure 4.1.2

Statistical Analysis and Data representation was conducted by Peter Teague and Donald Davidson and is reported fully in Morrison paper (1998). Briefly, data were shown either as % killed (percentage of difference between test and control group as compared to control) or % survival (percentage of bacteria in test group as compared to control). The experiment was designed to give duplicate and dilutions of each measurement thus providing four values for each point. The variance between these groups was conducted by calculating the square root transformation of data on a Poisson distribution. Significance was calculated at a 1% level. Analysis of test versus controls as performed by plotting NaCl concentrations against means under square root transformations, with grouped standard errors.

Figure 4.1.2

Functional activity of mouse and human β-defensin-1 synthetic peptides against *Pseudomonas aeruginosa* in variable salt concentrations.



Survival rate (as expressed as the mean of colony forming units under square root transformation) of *Pseudomonas aeruginosa* after 20 minute incubation with 0.05mg/ml human ß-defensin-1 (figure 4.1.2a) or 0.5mg/ml mouse human ß-defensin-1 (figure 4.1.2c) solutions of varying salt concentrations. Results from control samples (no ß-defensin added) are also shown. Two asterisks denote statistical significance to 1% and one asterisk denotes significance to the 5% level. Data is also presented as percentage bacteria killed (as compared to control samples) (figure 4.1.2b and 4.1.2c).

Discussion

Defensin inactivation as a consequence of ionic abnormalities in the CF lung is an attractive model to explain the impaired ability to clear bacterial infection. The data presented in this chapter described a study into the activity of human and mouse β-defensin-1 peptides against two CF clinically relevant bacterial strains in varying sodium chloride concentrations (a possible scenario in the CF lung). Defensins from both species were seen to result in significantly increased killing of *P. aeruginosa* as compared to controls at saline concentrations ≤125 mM. It was interesting to note that the percentage of bacteria killed was seen to be dependent on saline concentration both in test and control groups though more affecting the test defensin group more significantly.

pH variation due to salt concentration was negligible and thought unlikely to contribute to the varied antimicrobial activity observed.

The clinical significance of these data cannot at this time be determined as the ionic composition of the human and mouse ASF in CF and non-CFs is still unclear. However, it would appear likely that altered ionic composition in the CF lung would have the effect of impairing β-defensin activity against *P. aeruginosa.* Furthermore, the biochemical mechanisms by which β-defensins operates are at this time, poorly understood and therefore can not be used to elucidate this observed difference.

Dr. Donald Davidson has expanded on this line of research by investigating the salt dependent antimicrobial profile of the mouse and human β-defensin against other CF related bacteria. Interestingly, when *B. cepacia* was tested, human β-defensin would appear to have to proliferative effect (D. Davidson, Personal Communication). This observation suggests that the predisposition of CF patients to infections by *B.*

cepacia may arise from the impairment of some alternative defence mechanism or alternative defensin molecules.

It would be of great interest to expand this research into studying the salt concentration profile of β-defensin-1 activity against a wider range of bacteria and furthermore compare these findings with those generated by the use of an alternative defensin or other antimicrobial agent. The generation of a β-defensin-1 "knock-out" mouse is currently underway (G. Morrison, personal communication). It will be very interesting to examine whether this mouse exhibits a reduced ability to kill bacteria.

Chapter 5

Approaches towards Genotypic Correction of Submucosal Glands

Introduction

This chapter aims to study the development of murine SMG with a view to developing strategies for delivery of gene therapy vectors to submucosal glands.

Theodore Freidmann wrote in 1996, "Gene therapy is not a failure- it is simply still too immature to deliver yet on its promises"(Friedmann, 1996). This may indeed be so but this should not prevent discussion on what would be an ideal system for CF gene therapy, if the technology existed. Included would be the "where" question and the "when" question. *Where* would be the most advantageous site for gene therapy correction of CF phenotype in the lung and *when* would be the most optimal time (or times) for delivery.

The lack of pulmonary disease in foetal CF patients (Szeifert, 1985) is perhaps surprising since there is robust expression of CFTR in the foetal lung, which diminishes around the time of birth (McCray, 1992, McGrath, 1994). In the postnatal lung, CFTR expression in the human lung becomes restricted to the serous cells and the ciliated ducts of the SMGs (Engelhardt, 1992).

The critical steps leading to classic CF lung pathology are poorly understood. The first signs of CF pathogenesis are noted in infants and include SMG dilation (Oppenheimer, 1975) and increased levels of IL-8 and neutrophils (Khan, 1995). This pathology can occur without indication of bacterial infection suggesting that reduced CFTR may produce pulmonary abnormalities independent of the bacterial stimuli. By 6 months postnatal, CF patients have often developed the first signs of bronchiectasis (Oppenheimer, 1975). As lung disease progresses signs of cell hypertrophy and hyperplasia within the SMGs occurs and the *Reid* index of the gland increases (Oppenheimer, 1975). The Reid index is a measure of glandular area (on

cross section) versus submucosa area. Later, the secretions from CF SMGs may become dehydrated and in extreme cases, the whole gland may become completely blocked by mucus becoming a nidus for bacterial infection (Oppenheimer, 1975).

It has been suggested that the early alterations to the lung pathology can breach a "point of no return" after which a self perpetuating process of lung disease will develop independent of CFTR activity (Drittanti, 1997). On this evidence the "when" question can perhaps be answered by encouraging a programme of CF gene therapy (or whatever form or therapy) as soon after birth as possible.

Targeting SMGs For Gene Therapy Intervention

The targeting of SMGs is likely to be an important strategy for somatic gene therapy. However, due to the relative inaccessibility of SMGs to aerosolised vectors (partially due to the constant efflux of mucous), the question of just where the most optimal site of delivery becomes more complicated. One possibility would be to target the progenitor cells of SMG (which may be more accessible). As long as gene expression continues in the successive generation of daughter cells, total gene correction of SMGs may occur. Before such strategies can become a realistic possibility however, the biology of SMG development and the identity of any progenitor cells must be understood.

The prenatal development of human SMGs has been studied extensively. Human SMGs begin to develop at 10-15 weeks gestation at a time when the trachea and bronchi have already developed and the bronchioles are developing (Tos, 1966). Secreting acini begin to be observed by week 25 (Thurlbeck, 1961). D*e novo* SMG generation occurs until the 25th gestational week after which the glands expand and mature. New-born children have between 3500 and 6000 tracheal glands at a density

of ~1 mm^{-2} (Tos, 1966). It is widely considered that no new glands form after birth (Sturgess, 1982, Tos, 1966).

The first indication of gland formation is the appearance of the epithelial gland buds. This structure is spherical and consists of closely packed cells often being seen to dome slightly (Tos, 1966, Smolich, 1978). Using animal models, this bud was found to be associated with a high level of *CFTR* expression (Sehgal, 1996). The bud rapidly divides to produce irregularly shaped tubules which extend into the submucosa through the lamina propria and the dense basal connective tissue (Smolich, 1978). Prolific formation of the branched network follows (Smolich, 1978) generating the tubule structure and cell type observed in the mature SMG.

It is important to comment that the pattern of SMG development in the human differs, in some respects, from that in other mammals. Ferrets begin to develop submucosal glands postnatally within the first week of life. Gland buds are present at around 3 days leading to simple tubule formation and elongation in the first week. During the second and third week of tracheal development, dichotomous branching of glands occurs within the onset of serous tubule formation. By 5 weeks after birth, serous and mucous tubule is complete although gland mass and volume continues to increase until the animal is in early adulthood (Sehgal, 1996, Leigh, 1986).

In the adult rat, SMGs have been observed at the level of the larynx and in the proximal part of the trachea (Korhonen, 1969) but not at or beyond the origin of the main bronchi as is the case in humans (Sturgess, 1982). As with the ferret, rat SMGs develop in early postnatal life. During the first 3 weeks, the epithelial gland bud forms (between 3 and 6 days postnatal (Smolich, 1978) and divides to produce the network of ducts and tubules that make up the SMG. All cell types are normally present 3 weeks postnatal. Over the following 3 weeks, glands increase in volume

and there is an associated increase in number of ciliated cells in the ciliated duct and an increase number of mucous cells in the acini (Smolich,1978).

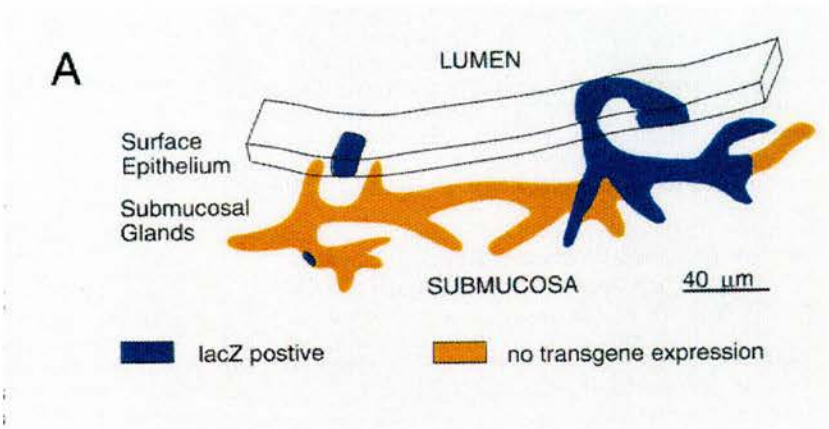
SMGs develop foetally in both the sheep and the dog in a pattern more akin to that of the human than that of the rat or ferret. Further to these similarities, SMGs in the both these species extend to the level of the small bronchi (de Haller, 1969).

Mechanisms of SMG development

In a study based on the xenograft system (see chapter 7 for details), Engelhardt *et al.* (1995) investigated whether SMG are clonally derived i.e. does one single cell in the epithelium or gland bud go to produce a whole gland? As has been highlighted above, if such a progenitor cell existed it would be a very attractive target for gene therapy. In this experiment, cultures of human bronchial cells were partially transfected by recombinant retrovirus prior to seeding in xenografts. These studies demonstrated that there was a high prevalence of non-clonality in the SMGs. This may either be due to more than one airway progenitor cell being involved in the initial stages of gland development or alternatively due to the frequent formation of glands in the xenografts with more than one duct to the surface airway epithelium. These data suggested that the classical model of one duct: one SMG may not operate exclusively in the development of SMGs. Instead, a proportion of glands may interact with each through the joining of glandular lumens. See figure 5.0.1.

Figure 5.0.1.

Polyductal Development Of SMGs As Displayed In The Xenograft Model (After Engelhardt *Et Al.* (Engelhardt, J. F.*et al*, 1995))



5.0.1a Model obtained through transgene expression studies of bronchial xenografts describing the development of SMGs through interaction between multiple glands to produce a polyductal gland network. SMGs of this nature were not observed in the studies presented in this thesis.

Advances towards understanding the molecular control of SMG progenitor cells was made with the discovery of the Ferret HMG transcription factor Lymphoid Enhancer Factor-1 (LEF-1). LEF-1 was found to be expressed specifically in a subset of epithelial cells associated with the development of the gland bud in the ferret, suggesting that LEF-1 may act as a developmental switch for SMG development (Duan, 1998). Using recombinant retroviral vectors, SMG progenitor cells were successfully targeted leading to stable expression of transgene throughout subsequent gland development.

Just how critical and necessary LEF-1 is in SMG development was explored using antisense technology directed against LEF-1 expression in the xenograft model (Duan, 1997).When anti-sense oligonucleotides were applied to newborn tracheal xenografts, a 4-fold decrease in the number and size of SMGs was noted. To the other extreme, an experiment was conducted to over express LEF-1 in the xenograft using AAV. Although that a significant proportion of the epithelium was transfected, there was no significant difference in SMG size or number. This suggests that LEF-1 is necessary but not sufficient to promote SMG development. At this time, a "knock-out" LEF-1 mouse is currently being developed (D. Duan, personal communication).

5.1

Development Of SMGs In The Mouse

Introduction

There would appear to exist a correlation in mammals between the size and the time of SMG development. SMGs in larger mammals (humans, sheep, dogs) (Goco, 1964, Tos, 1966,de Haller, 1969) develop *prenatally* whilst in smaller mammals including the ferret and rat (Leigh, 1986, Engelhardt, 1995) SMG development is *postnatal.* An experiment was conducted to study SMG development in the mouse.

Materials and Methods

Matings between SPF MF1 mice were set up to provide embryonic and perinatal mice at E17.5, E18.5 and post natal days 0.5, 3.5, 6.5 and 10.5 (n=4). Day E0.5 was defined as the day when cervical plugs were found in the mating females. The gestation period of the MF1 mice was around 19.5 days. Tracheas were dissected and processed to produce wax blocks from which semi-serial (approximately one section every 100μl) longitudinal sections and also cross sections (n=4 at each time point). Sections were prepared and counterstained with haematoxylin and eosin before dehydration and mounting with DPX. Slides were viewed with a bright field microscopy.

Results

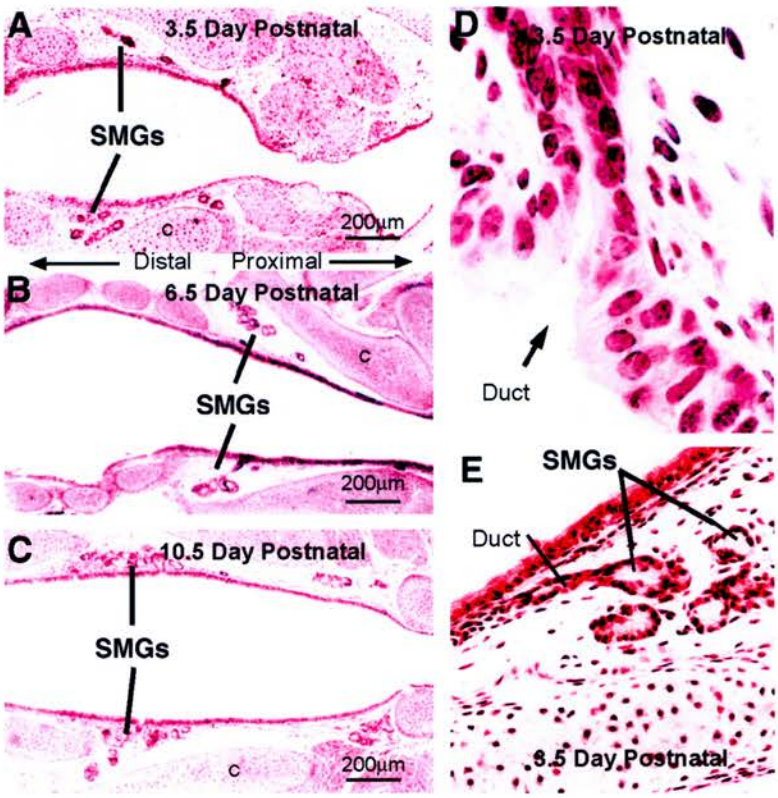
See Figure 5.1.1

Figure 5.1.1

Early Development Of Murine Smgs.

SMGs were observed in the tracheas of mice aged 3.5, 6.5 and 10.5 postnatal. Mice of ages 0.5 postnatal and embryonic days E17.5 and E18.5 showed no signs of SMGs

5.1.1A: Longitudinal section of proximal trachea from 3.5 day old mouse. SMGs were observed to be already present and are highlighted by arrows.
5.1.1B: Proximal trachea of 6.5 day old mice displaying presence of SMGs.
5.1.1C: Proximal trachea of 10.5 day old mice displaying presence of SMGs.
5.1.1D-E Higher power images of SMGs at 3.5 days postnatal. The ciliated and collecting ducts are observed to be present at this time point yet, are very narrow.


A
3.5 Day Postnatal
SMGs
C
200μm
Distal
Proximal
B
6.5 Day Postnatal
C
SMGs
200μm
C
10.5 Day Postnatal
SMGs
C
200μm
D
3.5 Day Postnatal
Duct
E
SMGs
Duct
8.5 Day Postnatal

Discussion

These data suggest that SMGs develop in the mouse between days 1 and 3 postnatal after which time, SMGs develop rapidly increasing in volume and complexity. This pattern is in common with that observe in the ferret and the rat, other potential mammalian models (Leigh, 1986, Engelhardt, 1995).

It is interesting to consider whether the time of SMG development is related to the diameter or physiological environment of the animal. In the context of studying lung physiology and disease, it is as yet unclear whether the prenatal establishment of and the physiological activity of SMGs in an essentially aqueous environment may be important in pathogenesis of CF lung in humans.

As was the argument for using the wholemount method reported in section 3.1, it can not be said conclusively that there are no SMGs in mice in the mice younger than 3.5 days from evidence provided by thin sections. Wholemount preparation proved to be technically unfeasible due to the size of the tracheas at these stages of development so thin sectioning was the only practical alternative. To make this method as effective as possible, semi serial sectioning was made of tracheas in both longitudinal and cross-section orientation was made.

5.2

A Study Of SMG Development Through The Generation And Study Of Aggregation Chimaeric Mice

Introduction

Human SMGs act in the production an efflux of mucous and antimicrobial materials out onto the luminal epithelium of the trachea and the bronchi. Due to the complex structure and the presence of mucous, the SMGs are considered inaccessible to conventional aerosolised delivery systems of gene therapy vectors.

One possible strategy to overcome this problem would be to target and correct the genetic defect in the cell or cells which go to produce SMGs. Correction of the SMG progenitor cell population will, theoretically, give rise to a SMG in which every cell would be corrected.

Although xenograft based studies have been conducted into the mechanisms of SMG development (Engelhardt, 1995), there has been little in the way of *in vivo* studies. To this end, aggregation chimaeric mice were produced to study the development of murine SMGs.

Materials and Methods

Generation of Chimaeras

Two series of adult aggregation chimaeric mice were produced each carrying a contribution of transgenically marked cells. The CA series chimaeric animals carried a contribution of cells carrying a 1000-copy tandem array murine ß-globin insert

detectable by DNA:DNA in situ hybridisation as described by Keighren and West (1993). The CJ series carried a contribution of cells which express a Lac-Z transgene. The Series CA was analysed by DNA *in situ* hybridisation against the β globin transgene and series CJ was analysed by β galactosidase histochemistry to detect expression of a Lac Z transgene. The strain designation are shown in table 5.2.1.

This work was done in collaboration with the lab of Dr. John West. Margaret Keighren and Jean Flockhart generated the large majority of chimaeric animals. The author conducted most of the transgene detection protocol and GPI analysis.

Figure 5.2.1

Details the stocks of mice used in the production of CA and CJ chimaeras.

Stock name	Details	Genotype		
		Albino	GPI-1	Transgene
EXP	(BF_1 female x TGB male)F_1	C/C	b/b	Tg/-
AAF_1	(BALB/c female x A/J male)F_1 hybrid	C/C	a/a	-/-
CMA	Derived from transgenic strain 83	C/C	a/a	Tg/Tg
BF_1	(C57BL/Ola female x CBA/Ca male)F1 hybrid	C/C	b/b	-/-
ROSA	Derived from transgenic strain ROSA26	C/C	b/b	Lac Z/Lac Z
TGB	Derived from transgenic strain 83 and BF_1	C/C	b/b	Tg/Tg

Transgenic abbreviations; Tg = TgN(Hbb-b1)83Clo; *lac Z*=TgR(ROSA26)26Sor

Females were induced to ovulate by interperitoneal injections of 5IU of pregnant mares' serum gonadotrophin at ~12 noon, followed 48 hours later by interperitoneal injection of 5 IU of human chorionic gonadotrophin. Females were caged overnight with males of appropriate stock, and mating was confirmed the following morning by

the presence of a vaginal copulation plug; the day following the vaginal plug was designated 0.5 day postcoitum (d.p.c) On the same day as vaginal plugs were identified, CF_1 females in oestrus were paired overnight with vasectomized CF_1 males and matings were verified the following morning by the presence of a vaginal plug. These provided homozygous, pseudopregnant females differing in GPI status for embryo recipients.

Embryos were flushed from the superovulated females at 2.5 d.p.c. with HEPES-buffered M2 handling medium. After overnight culture, the aggregated embryos were surgically transferred to the uteri of pseudopregnant CF_1 females. Chimaeric mice were born some 18 days later. The chimaeric nature of the mice was confirmed by coat colour and glucose-phosphate isomerase analysis of blood taken from the mouse at 6 months of age and the left kidney, cerebrum, lung and the median lobe of the liver taken at time of sacrifice. Briefly, 50µl blood was dissolved 1:5 in distilled H_2O. An acetate gel plate was pre-washed in TBE buffer and then dried. 0.2µl of diluted bloods were applied to the gel plate and the gel run at 200V for 1 hour (blood applications ran from the anode towards the cathode) to separate the different enzymes according to electric charge and size. Controls of group B, group A and group AB bloods were also run. The gel was removed and dried on a glass heater. Staining solution was freshly prepared (0.75mls glycerol, 0.75ml 0.2% $MgCl_2$, 170µl tris-citrate (pH 8), 170µl 20mg/ml Fluoride-6-phosphate, 170µl NBT, 170µl 2.7 mg/ml NADP, 4 unitsG6PD, 20µl 2.7mg/ml PMS) and poured over the plate and incubated for 3 minutes in the dark before washing in distilled H_2O + 5% ethanoic acid. The GPI bands was visible on the gel plates. Quantitative measurements of band intensities were made by use of the Helena Process 24 plate reader.

The tissues chosen for GPI analysis include derivatives of all three germ layers: endoderm (liver and lung) mesoderm (kidney and blood) and ectoderm (brain).

At 6 months of age, the mice were sacrificed and tracheas dissected as outlined above. Tracheas from the CJ mice were rinsed in PBS and fixed for 6 hours at +4 °C in a solution of 1% formaldehyde, 0.2% gluteraldehyde, 0.02% NP-40 in PBS. Samples were washed in PBS and incubated over night at 37°C in X-gal stain (0.43 mg/ml 5-bromo-4-Chloro-3-indolyl-1-D-Galactosidase in N'-N'-dimethyl-formamide (Melford Laboratories, Chelsworth, UK), 3.1mM $K_3Fe^{III}(CN_6)$, 3.1mM $K_4Fe^{III}(CN_6)$, 1mM $MgCl_2$ in PBS). The next morning the tissues were washed twice in PBS and then fixed into wax blocks as outlined above. Serial 6μm sections were cut and dewaxed and hydrated by passing the slides through 2x xylene (40 seconds each) and a gradient of alcohols (70%, 50%, 30%; 15 seconds each). Sections were counterstained with neutral red then dehydrated to xylene and mounted with DPX.

Generation Of Digoxygenin (DIG) Labelled Probe

Digoxygenin-labelled pMβδ2 (β-globin) probe was prepared by Midi prep of an *E. coli* based vector and plasmid DNA purified using the Quiagen Plasmid Midi Kit (Quiagen, Crawley, UK) and identity confirmed by digestion with HindIII and EcoR1 (see figure 5.2.2). DIG label was incorporated into the plasmid using Boehringer Mannheim DIG labelling kit (Boehringer Mannheim, Lewes, UK). Probe was further purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Lewes, UK). To test the identity of the probe, DNA was extracted from the lung of a mouse homozygous for the β-globin insert and one wildtype mouse by phenol chloroform extraction. The DNA was randomly digested and run on a 0.8% midi gel.

Bacterial Cell Culture

Glycerol stocks of the pMβδ2 β-gal expression vector transformed in *E. coli* was gifted by John West. Glycerol stock were streaked out on L-broth agar plates (2.46g magnesium sulphate, 10g tryptone, 5g yeast extract, 10g sodium chloride, 15 g agar (Oxoid ltd., Sussex, UK). in 1l dH_2O) and incubated overnight at 37°C to give

identifiable single colonies.

5ml of Terrific Broth (12g Bacto-tryptone (DIFCO, Detroit, USA), 24g Yeast extract (DIFCO, Detroit, USA) and 4g glycerol added to 900ml distilled water prior to autoclaving and the addition of 100ml sterile phosphate solution (0.1M potassium dihydrogen phosphate and 0.72M dipotassium hydrogen phosphate)) and was inoculated with a single colony of *E.coli*. Bacteria were grown up for 3 hours in a 37°C shaker prior to transferring the solution to 500ml Terrific broth and overnight incubation at 37°C.

DNA was extracted from the bacteria by the method of alkaline lysis. Bacteria in broth were centrifuged at 5k rpm for 15 minutes. Supernatant was discarded and the pellet resuspended in 20mls solution 1 (50mM glucose, 25mM Tris-HCl, 50mM EDTA, 10mg/ml lysozyme (Sigma)). The solution was incubated for 5 minutes at room 10 minutes. 27.6mls of 1% (w/v) SDS in 0.2M sodium hydroxide was added and the solution gently mixed and left on ice for 15 minutes. 14mls pre-cooled potassium acetate was added and the lysis mixed gently. The mixture was incubated for 15 minutes on ice. Cell debris was removed by centrifugation at 12 krpm (4°C for 30 minutes) retaining the supernatant which was filtered through muslin and mixed with 0.6 volumes isopropanol. The mixture was spun at 5000 rpm for 10 minutes and the supernatant discarded. The pellet was rinsed in ethanol and dissolved in 2.5mls Tris-EDTA buffer and store at –20°C.

DNA was purified using the Quiagen Plasmid Midi Kit according to manufacturers directions. Digestion of pMβδ2 with the restriction endonucleases HindIII and BamH1 will (according to plasmid sequencing data) produce fragments of specific size which will be readily identifiable by mini gel electrophoresis. In this case, Hind III digestion of pMβδ2 produces fragments of 2.43 and 5.38 and the BamH1 digest produces fragments of 5.64 and 3.23 kb. See figure 5.2.1.

Digestion of DNA by restriction enzymes was carried out on 1µg plasmid DNA using one unit of restriction enzyme in a total volume of 20µl. Incubation was conducted overnight at 37°C. 0.8% v/v agar was mixed with 30ml dH_2O and boiled in a microwave for approximately 2 minutes. 2µl ethidium bromide was added and the mixture poured into a gel mould which included a toothed comb. When set, the comb was removed . Agarose gel electrophoresis was used to separate the DNA fragments of different sizes. 10µl of the digested DNA samples was mixed with 1µl x10 stop buffer (20% w/v Ficoll (Pharmacia), 100mM EDTA, Orange C (Sigma)) and pipetted into the comb marks. The gel was submerged in 1xTAE buffer (0.04M Tris-HCl, 1mM EDTA pH8.0, 0.02M Acetic acid) and a voltage of 100V passed across the gel (the DNA being attracted to the anode) for one hour. The ethidium incorporated DNA was visualised using UV light.

The plasmid was linearised with incubation with EcoR1 (1 unit/µg) at 37°C for 90 minutes. DIG labelling of the probe was performed according to and using the Boehringer Mannheim DIG labelling kit.

Isolation of mouse DNA by Quick Lysis method

DNA was isolated from mouse lungs by the quick lysis technique. Lungs were dissected out and the tissue incubated at 37°C overnight in a proteinase K solution (100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% w/v SDS, 200mM Sodium chloride and 100µg proteinase K (Boehringer Mannheim). DNA was precipitated through the addition of an equal volume of isopropanol . Precipitated DNA was spooled out using a glass rod and washed briefly in 70% ethanol. The DNA was left to air dry before incubating at 4°C in 500µl Tris EDTA buffer overnight.

DNA:DNA *In-situ* hybridisation technique for ß-globin transgene detection

The CA series chimaeric animals carried a contribution of cells carrying a β-globin insert detectable by DNA:DNA *in situ* hybridisation as described by Keighren and West (Keighren, 1993). Hybridised Digoxygenin-labelled DNA probe was detected by diaminobenzidine staining for peroxidase-labelled antibody (non-radioactive DNA labelling and detection kit; Boehringer Mannheim, Lewes, UK). Slides were counterstained with eosin and examined by bright-field light microscopy. See below for further details of protocol. Individual SMGs were selected at random and the cells scored for presence of absence of hybridisation signal. The absolute number of cells scored was dependent on the size of the SMG tissue on the section but varied from ~100 to ~300 cells. Cells of the tracheal epithelium were also scored for presence of marker.

Chimaeric and control animals were sacrificed by cervical dislocation and tissues fixed in 3 parts ethanol :1 parts ethanoic acid and processed to wax blocks. 6μm sections were prepared on Vectabond coated slides. Sections were dewaxed in Histoclear (B.S. and S., Edinburgh, UK) for 2 x 10 minutes at room temperature. Slides were washed in 100% ethanol for 2 x 5 minutes, in freshly prepared 3% H_2O_2 in methanol for 30 minutes (to reduce endogenous peroxidase activity) then washed in 70% ethanol for 2 x 5 minutes prior to final washing in PBS for 2 x 5 minutes.

To partially denature the cells, slides were incubated in 1mM NaOH at 70°C for 3 minutes then washed in PBS at +4°C for 2 x 5minutes.

Pre hybridisation (to reduce background signal) was carried out by incubating slides with 2 parts 50 x Denhardt's regent (9 parts deioinised formamide, 6 parts 20 x SSC, 3 parts sonicated salmon sperm and 6 parts distilled dH_2O for 15 minutes at 60°C).

Slides were hybridised overnight in a humidified chamber at 60°C with 40μl of hybridisation mixture which contained 10μl labelled DIG probe (freshly boiled for

10 and sat on ice for 3 minutes), 20µl sonicated salmon sperm, 200µl 20% dextran sulphate, 100µl 20 x SSC and 70µl H_2O.

The next day, slides were washed in 2xSSC, 0.1 triton X for 1x 10 minutes and 1x 5 minutes, washed in 0.1x SSC, 0.1 triton-X for 1 x 5 minutes, washed in 0.1x SSC, 0.1 triton-X for 1 x 5 minutes (at 50°C), then finally 0.1 x SSC, 0.1 triton-X , 5 % BSA (by mass). Washes were carried out at RT unless otherwise noted.

Signal was visualised by washing slides in buffer 1 (0.1M Tris 0.15M NaCl pH 7.5) for 5 minutes at room temperature. Slides were then place in a humidified chamber and tissues flooded with a 1:100 dilution of anti-Digoxygenin HRP antibody (Boehringer Mannheim, Lewes, UK) in buffer 1 for 30 minutes. Slides were washed in buffer 1 (2x 5 minutes at room temperature) then washed in DAB buffer (buffer 1 + 5% BSA) for 5 minutes. Tissues were flooded with developmental reagent for 40 minutes (45µl NBT solution (75mg/ml dimethylformamide 70% v/v), 35 µl X-phosphate (Boehringer Mannheim, Lewes, UK), 10mls 0.1M tris, 0.1M NaCl (pH 9.5), 0.05M $MgCl_2$) in a dark humidified chamber and then placed directly in H_2O to stop the reaction.

To counterstain the tissue, slides were incubated in 1% Eosin for about 20 seconds (the timing varied depending on the freshness of the eosin) and then dehydrated through a increasing concentration gradient of alcohols. Slides were incubated in Histoclear for 2 minutes then rinsed in xylene prior to coverslip mounting with liquid pertex solution (Cellpath plc, Herts, UK).

Hemizygous transgenic (Tg/-) and homozygous non-transgenic (-/-) tissues were used as positive and negative controls for comparison with hemizygous chimaeras (Tg/- ⇔ -/-). In cells carrying the transgenic marker (especially hemizygous transgenic cells) it is possible for the hybridisation signal not to be detectable (Quinn, 1996). This will commonly be due to the process of tissue sectioning; cutting a nucleus in two and removing the transgene from tissue otherwise histologically intact. The uncorrected percentage of cells with a hybridisation signal was categorised on a non-linear scale (see figure 5.2.5).

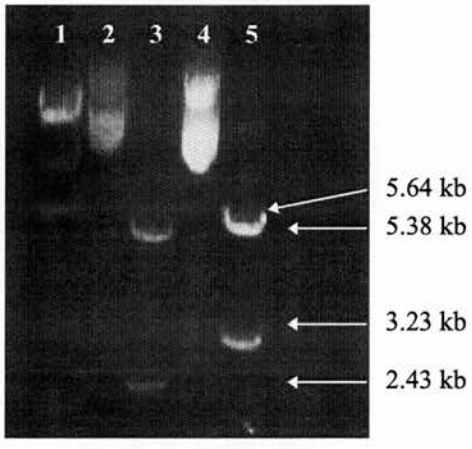
Results

Generation of Probe pMβδ2

Confirmation of probe pMβδ2 production was provided through digestion of the probe with HindIII and BamH1 and running out products and controls on a 0.8% agarose gel (see figure 5.2.2).

Figure 5.2.2

0.8% Agarose gel demonstrating correct generation of the ß-globin probe plasmid.



Lane 1: Marker Ladder

Lane 2 : Undigested probe plasmid

Lane 3 : Plasmid digested with HindIII showing 2.43kb and 5.38kb bands.

Lane 4 : Undigested probe

Lane 5: Probe digested with BamH1 showing 5.64kb and 3.23kb bands

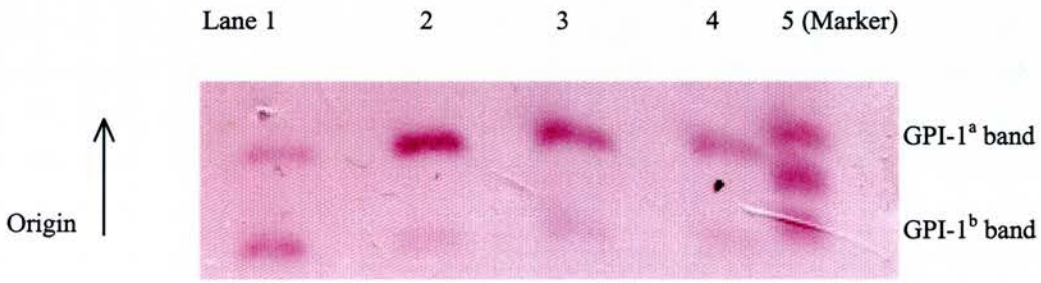
Evidence for generation of chimaeric offspring by GPI assay.

A simple and quantitative technique to measure the contribution of each donor mating to the final chimaera was to select the mice strains so that the different donor pairs have different blood groups. This difference is measurable by the GPI assay which detects different isomers of the enzyme glucose phosphate isomerase 1. The isomers are detectable by a method of acetate gel electrophoresis and staining. Examples of GPI assay results are given in figure 5.2.3.

Figure 5.2.3

Evidence For Chimaerism By GPI Allozyme Assay.

Each band is blood from different mice with the bands demonstrating the varying composition in GPI status of the aggregation chimaeras. The allozyme bands are referred to as GPI-1^a and GPI-1^b due to their occurrence in bloods of particular groupings.



Lanes 1-4: Mice demonstrating evidence for chimaerism by possessing both blood containing the GPI-1^a and GPI-1^b band. The cellular contribution by the donors to the chimaeras can be estimated by quantifying the comparitive level of band intensity.

Lane 5: Marker from known AB blood. Intermediate band shows the presence of a GPI-1^a and GPI-1^b dimer.

Evidence for Clonal development of murine SMGs

The development of murine SMGs was examined through the generation of aggregation chimaeric mice. Two different series of chimaeras were produced (referred to as CJ and CA) and the percentage contribution of transgenically marked cells was confirmed and quantified by analysis of glucose phosphate isomerase allozymes in tail tip blood and other tissues (5-95% transgenic contribution). 120 SMGs from 30 of these mice were studied for transgenic cell contribution.

CJ chimaeras were composed of two different transgenic genotypes. The composition of these chimaeras was determined by the contribution of cells expressing the *Lac Z* transgene. Non chimaeric positive control (either standard *lac Z/-* hemizygotes or mice in the CJ series that proved to be non chimaeric) were stained with X-gal solution resulting in a blue staining pattern in all SMG and epithelial cells type. Negative controls of X-gal stained wildtype mice showed occasional weak background staining. This staining pattern was readily distinguishable from positive control staining on account of signal intensity and variation on serial sections.

By studying the SMGs of the CJ chimaeric animals, the glands were found to be of single ductal structure and composed of either cells all staining positive with X-gal or all negative (figure 5.2.4).

CA series chimaeras (Tg/- ⇔ -/-) carry a contribution of cells which are genomically marked with a large 1000 copy β-globin insert. This marker is detectable not by identification of an expressed gene product as in the case of the CJ chimaeras, but by DNA *in situ* hybridisation. For technical reasons, as discussed in section 5.2, a

proportion of transgenically marked cells may not be identifiable by DNA *in situ* hybridisation.

To analyse the development of SMGs using a non-expressed transgenic marker, chimaeras carrying a contribution of cells heterozygously marked with a β-globin insert were studied. The percentage of cells carrying the transgene in the chimaeric animals was estimated through analysis of coat colour pigmentation in the animal and by analysis of body tissues by the glucose phosphate isomerase assay (transgene carrying cells were homozygous GPI-1^b/GPI-1^b whilst non transgene cells were homozygous GPI-1^a/GPI-1^a). Cell contribution to SMGs and the tracheal epithelium was then estimated by DNA *in situ* hybridisation in CA chimaeric tissue and both positive and negative control tissues containing respectively 100% or 0% of Tg/- cells hemizygotically carrying the ß-globin insert. The results are shown in figure 5.2.5

Figure 5.2.4.

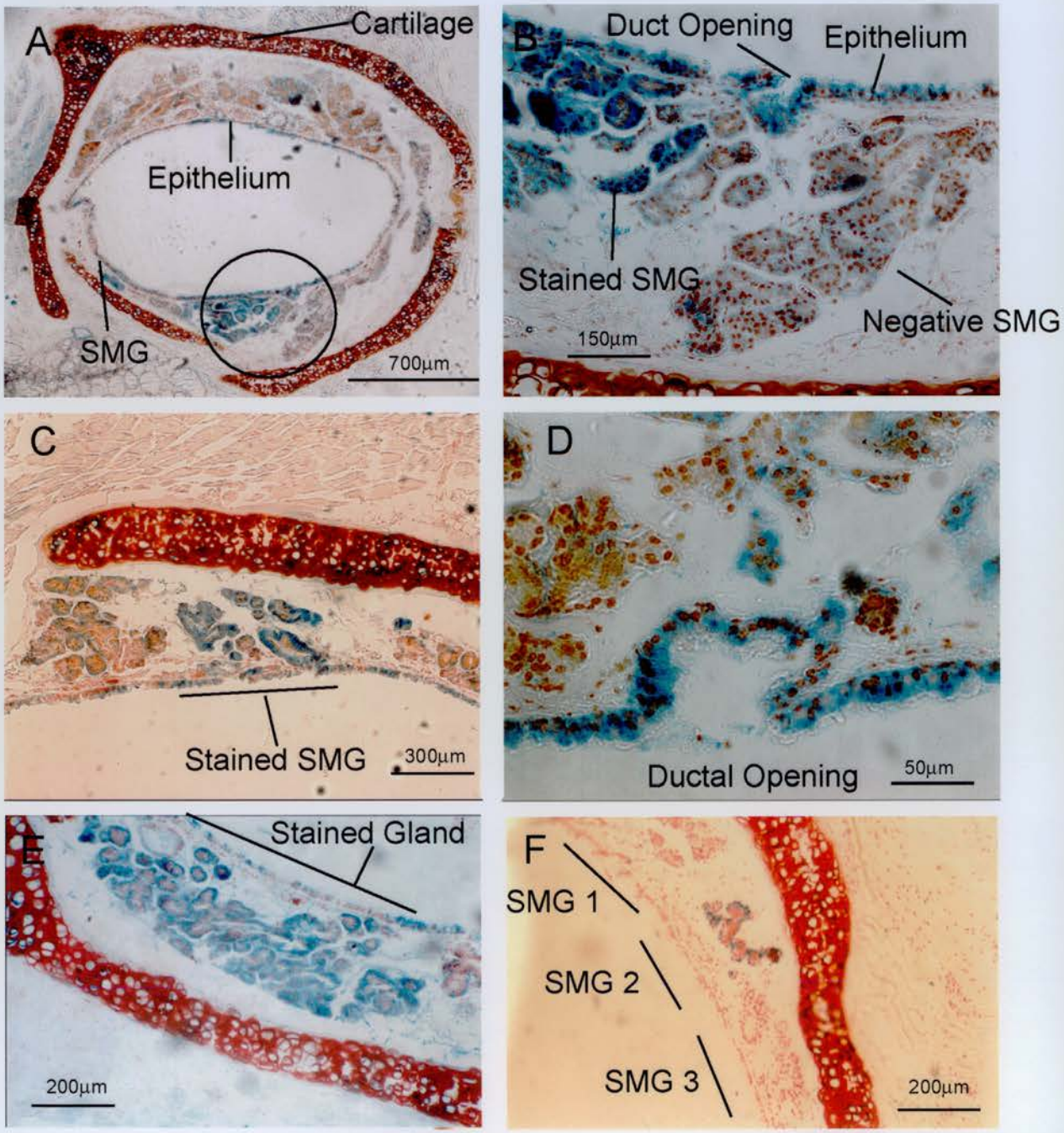
Examples of Chimaeric tissues after staining for transgene visualisation

Tracheal tissues from either the series of Lac-Z aggregation chimaeric mice or those carrying the β-globin transgene. Lac-Z gene product is visualised using X-gal stain (staining blue) and histology visualised with Neutral red nuclear counterstain (red).

5.2.4A The cartilage ring also stains for neutral red and was found to buckle when cut in cross sections. The tissue showed both stained and non-stained SMGs. Area circled is at higher power in figure 5.2.4b.

5.2.4B-C, E-F A positively stained SMG was determined as that whose cells consistently and concurrently stained blue, the staining intensity of which was readily observed to be above that of background. SMGs were found to either completely be composed of cells expressing the transgene or completely devoid of transgene expressing cells. Furthermore, SMGs were found to be only of single ductal type.

5.2.4D The epithelium surrounding the ductal opening was observed to carry the same genotype (with relation to marked gene) as the SMG. This observation is considered further in chapter 7.3.


A
Cartilage
Epithelium
SMG
700µm
B
Duct Opening
Epithelium
Stained SMG
Negative SMG
150µm
C
Stained SMG
300µm
D
Ductal Opening
50µm
E
Stained Gland
200µm
F
SMG 1
SMG 2
SMG 3
200µm

5.2.4G, J Examples of images from CA series chimaeras after transgene visualisation with DNA:DNA *insitu* hybridisation. Cell were counted in each SMG for presence or absence of transgene marker (seen as a brown dot).

5.2.4H SMG acini at higher power showing presence of β-globin transgene. In some cells both β-globin inserts in the homozygous transgene carrying cells can be observed (see arrow on figure H). In some cells, one or both (approximately 20%) the signals are lost due to sectioning.

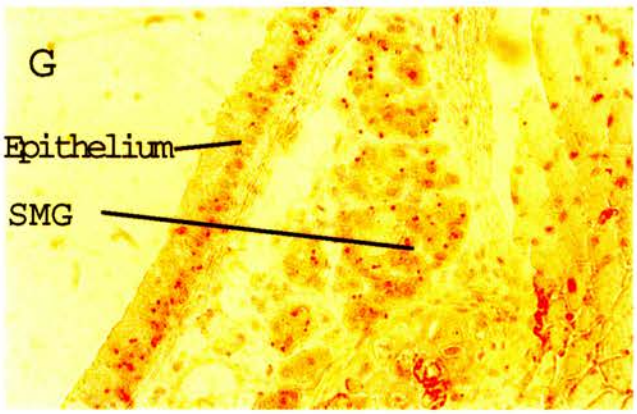
5.2.4I Other tissues showed evidence for chimaeric development including the oesophagus. This tissue showed blocks of epithelial tissue staining positive for Lac-Z.

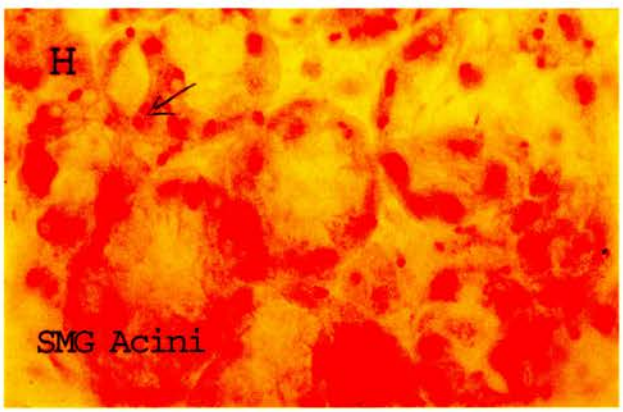
Figure 5.2.5

Analysis Of CA Chimaeras; % Contribution of Tg/- cells in Tg/-⇔-/- chimaeras (CA) and controls

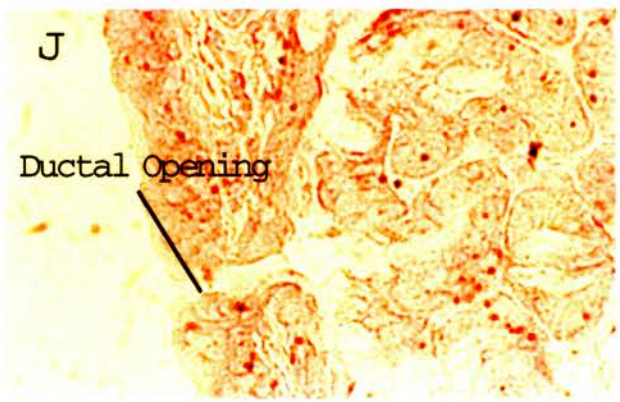
Tissue compositions of adult (Tg/-⇔-/-) chimaeras (Series CA; EXPxEXP⇔AAF_1 x AAF_1) and both positive (Tg/-) and negative (-/-) control animals (n=6). The proportion of transgenic (EXP x EXP) cells was estimated by coat colour and GPI assay on a variety of body tissues. The contribution of transgenic (Tg/-) cells in the tracheal epithelium and three randomly chosen SMGs is shown as the uncorrected percentage of cells with a hybridisation signal after DNA *in situ* hybridisation.

Coat colour hybridisation score was measured by the extent of coat colour pigmentation in the mouse. Brain, kidney, cerebrum, lung, liver and trachea scores were measured by GPI assay. Submucosal glands were scored by *in situ* hybridisation.


G
Epithelium
SMG


H
SMG Acini


I
Oesophagus


J
Ductal Opening

The uncorrected percentage hybridisation score was categorised as follows:
0%-4% ○ ;5-29% ◒ ;30-49% ⊙ ; 50-74% ◎ ; 75-100% ●

Mouse	Co	B	K	Ce	Lu	Li	Tr	1	2	3
CA5	●	●	◎	●	●	●	⊙	●	○	○
CA19	●	●	◎	●	●	●	◎	●	○	○
CA23	●	●	●	●	●	●	⊙	●	○	○
CA22	●	●	◎	◎	◎	●	⊙	●	○	●
CA24	●	●	◎	●	◎	●	◒	●	●	○
CA20	●	●	⊙	◎	◎	⊙	◒	●	●	○
CA26	◎	◎	◒	⊙	◒	◒	◒	○	○	●
CA18	◎	⊙	⊙	⊙	⊙	◎	◎	●	○	○
CA17	◎	⊙	⊙	⊙	⊙	⊙	◒	○	●	●
Positive	●	●	●	●	●	●	●	●	●	●
Negative	○	○	○	○	○	○	○	○	○	○

Co=Coat, B=Blood, K=Kidney, Ce=Cerebrum, Lu=Lung, Li=Liver, T=Trachea, 1=SMG#1, 2=SMG#2, 3,SMG#3, Positive = positive controls, Negative =Negative controls.

Submucosal glands from the CA chimaeras were analysed to the percentage of transgene carrying cells in each gland by way of *in situ* hybridisation. Analysis of the data from CA chimaeras shows distinct groupings of scoring for detection of transgene in the SMGs. The first group contains SMGs in which at least 75% of cells had a hybridisation signal, indicating the presence of β-globin transgene (mean 84.8, SD 4.7) and the second group which contains SMGs in which fewer than 5% of cells had a hybridisation signal (mean 0.58, SD 1.06).

These groupings and levels of detection are similar to those observed in controls (positive control mean 81%, SD 0.42, negative control mean 0.4%, SD 0.12). The failure of this assay system to detect 100% of marked cells in the positive controls will be due in part to the loss of transgene from a part of a nucleus which is cut during histological sectioning. This problem is highlighted by the infrequent observation of the double hybridisation signal in individual cells. The infrequent hybridisation signal seen in negative control tissue may be due to staining artefacts. The level of transgene detection in chimaeric tracheal epithelial tissue was highly variable with a mean of 40.0% (SD 15.3) inferring a low level of clonal development.

The composition of the SMGs by transgenically marked cells in both CA and CJ chimaeras was similar to either the positive or negative controls. This suggests that the each SMG is clonally derived from a single cell.

Discussion

Chimaerism is closely associated with naturally occurring cellular mosaicism in which the inactivation of X-chromosomes occurs in females. In this example, the cellular patterning can be shown histochemistically (Griffiths, 1988). Griffiths *et al.* (1988) observed that the patterning of glucose-6-phosphate dehydrogenase (G6PD) expression in the cells of female mice can either be normal or low. This observation was used as the basis of system of somatic clonal marking for the study of large intestinal development and showed, most eloquently, clonal development of the individual crypts.

Chimaeric mice provide means by which developmental pathways can be studied in a completely *in vivo* and developmentally sensitive manner. 2 series of chimaeric mice were generated; one series (CJ) contained a contribution of cells carrying *lac Z* and another series (CA) contained a contribution of cells carrying a non expressed β -globin transgene. Chimaeric CJ SMGs were found to be either composed of cells all expressing *Lac Z* (noted by blue staining with X-gal) or composed entirely non expressing *Lac Z* cells. In SMGs from the CA chimaeras, the contribution of β-globin carrying cells in the SMGs was estimated using DNA:DNA *in situ* hybridisation. The composition of transgenically marked cells in the chimaeric SMG was similar to that observed in the control animals. These data suggests that the SMGs are clonally derived from a single progenitor cell type.

These observations differ from the findings of Engelhardt *et al.* (1995) who studied the development of SMGs in a partially *ex vivo* xenograft model of the human bronchi and reported the presence of submucosal glands which appeared to be non clonally derived. Two possible explanations are cited: either the glands are derived from more than one progenitor cell or alternatively, some glands contain more than one duct opening and are formed by glands interacting through the formation of joint

lumen. This situation can be seen as somewhat akin to the small intestine where crypts are clonal whilst the villi are polyclonal (Ponder, 1985).

Over 120 chimaeric mouse SMGs were examined by semi-serial sectioning in this experiment and recorded no evidence for glands either being derived from more than one progenitor cell or through poly-gland interaction. Furthermore, no previous studies of SMGs have reported on finding glands with more than one ductal opening. From our findings we conclude that the novel gland morphogenesis (as compared to the "classical" single duct glands) seen in the xenograft model does not operate in mice and perhaps its occurrence is restricted to the xenograft model.

Chapter 6

Liposome Mediated Gene Transfer To The Mouse Tracheal Epithelium

Introduction

Through the rapid advances in molecular genetics and gene transfer technology, gene therapy has become one of the landmarks of 90's technology. In gene therapy, molecular biology has met clinical science head on. However, possibly due to media and scientific hype (Friedmann, 1996), some of the original lustre of the science has been lost. After a decade of gene therapy research no single patient out of over 2000 patients involved in gene therapy trials has definitely improved in health (Friedmann, 1997b). That is not to say however, that the results from such trials and other research have been entirely negative. Examples of successful gene transfer to cell culture, *ex vivo* models and to laboratory animals are now common place (represented in part by in the number of citations to "gene therapy" (Friedmann, 1996)) and gene transfer and expression in clinical trials have been reported for treatment for a variety of diseases. In this introduction, gene therapy as a method of therapeutic intervention to CF will be discussed outlining the theory, the difficulties, the actualities and the plans for the future.

Gene therapy has been defined as any treatment of any disorder or pathophysiologic state which is based upon the transfer of genetic information (Curiel, 1996). This broad statement can be rationalised into two areas of research; that of somatic gene therapy and germ line gene therapy. Germ line gene therapy is based on the manipulation of the embryo or germ cells to change or add to the existing genome resulting in a permanent correction which moreover will be then heritable to future generations. At this time, germ line gene therapy is not permitted on ethical and moral grounds. Somatic gene therapy results in a non heritable form of intervention. That is to say correction or alteration of an individuals genome is likely to be localised and temporary (often dependent on the life time of the target cell). There is of course a scenario where the two areas can overlap when a treatment designed for somatic gene therapy reaches and affects the germ line cells of a patient. This

situation is a part of the design of any gene therapy trial programme (Porteous, 1997b).

CF somatic gene therapy is based on the concept that by replacing the single abnormal gene defect (mutant CFTR) with a functional copy, normal gene expression and protein function could be returned to the epithelium. The assumption being that by correcting CFTR function, the CF lung phenotype will be reverted to that of the non CF patient.

The lung represents an attractive target organ for gene therapy for a number of reasons. This is reflected in the disproportionately large fraction of number of lung orientated gene therapy trials which have been conducted (Sobol, 1996). Firstly, there are a number of relatively common pulmonary diseases whose genetic basis has been determined. CF and α_1-antitrypsin deficiency represent the two most common single gene disorders of Caucasian North Americans (Curiel, 1996). Secondly, current treatments for pulmonary disease although often improving survival rate and symptoms, rarely represent a cure. Thirdly, on a more practical basis, the lung is a highly accessible organ with an extremely large surface area making it likely to be able to receive high doses of the vector.

When CF is considered as a candidate for gene therapy, the concept becomes even more attractive for it is believed that a towards wildtype correction of just 5-10%% may be sufficient to correct the CF lung phenotype (Dorin, 1996a). Other clinical data suggests that some CF individuals which have as little as 8% normally spliced *CFTR* mRNA are phenotypically normal (Chu, 1992). This notion is backed up by studies using the CF mouse models. By crossing mice of different genotype to produce compound heterozygotes, it was found that as little as 5-10% of wildtype *cftr* expression was sufficient to restore gut electrophysiological measurements and

mouse survival to near normal (Dorin, 1996a). As a consequence, CF was held up, by many, as a model disease for correction by gene therapy.

Research into CF lung gene therapy can be seen as belonging to three intertwining sets. The first set (the "How?" set) aims to design an efficient system by which a piece of foreign DNA can be delivered by means of a carrying vector from the air, into a cell and there remain stable and be expressed to produce functional protein. The second set (the "where?" set) asks where would be the most efficient area of the lung (often in reference to cell types and specific tissues) to deliver the gene therapy to. This area can only be fully explored once the first set of questions have been answered. The final third set (the "when" set) considers the timing and frequency of gene therapy dose delivery. Such research is based on the pathogenesis of the disease in the infant and also the life span of target cell types.

Vector Systems for Gene Therapy

One of the requirements of gene therapy for any disease is the development of a vector system by which the DNA is delivered to the target cell. There are a number of different approaches that are available which can be grouped into two general categories- viral and non viral. Each vector system however has its own drawbacks.

Viral based vectors

In the search for a vector which would carry DNA into a cell and there express it, many groups chose to study and use viral systems. It would appear on the outset the obvious choice; for evolution has been fine tuning viruses for this very purpose. Viruses could be adapted to remove genes involved in viral replication and replacing

them with the gene of choice. In principal the altered, tamed gene should transfer the gene into the cell with great efficiency yet not multiply or cause disease (Friedmann, 1997a).

The design of a gene therapy virus vector involves first producing a shuttle DNA construct which may include the transgene, a promoter (often a viral promoter such as CMV or SV-40), genes which allow the packaging of the retroviral genome into viral capsids and sequences to permit the integration of the construct into the viral genome. The site of incorporation is such that the wildtype viral genes necessary for the production of new replication-competent viruses are lost.

Adenoviral Based Vectors

Out of the 3 main types of viruses, recombinant Adenovirus vectors have been traditionally favoured for CF gene therapy (Porteous, 1997b). Their original attraction stemming from the natural tropism of Serotypes 2 and 5 to the human airways (Curiel, 1996). Adenoviruses have a double stranded DNA genome and a naked protein capsid coat. Cell entry can occur in either dividing or non dividing cells and is thought to be through endosomes (Inglis, 1997b). Once in the cell, the viral genome does not become integrated into the cell's genome rather remaining in the cytoplasm. This feature is possibly favourable in gene therapy as it would avoid the possibility that genomic incorporation of the gene therapy cassette may disturb vital cellular genes or indeed cancer inducing genes (Friedmann, 1997a). However, adenoviruses often behave transiently in the cytoplasm which may results in low expression of the transgene (Porteous, 1997b).

In vitro studies using adenoviral vectors mediated gene transduction have been very encouraging: transduction efficiencies to both murine and human cultured airway epithelial cells of 90-100% (Zabner, 1994) and full correction of CF phenotype in

culture by electrophysiological measurement (Johnson, 1992). Although prolonged *in vivo* exposure of Adenovirus vectors to rodents and primates did show evidence of correction (Zabner, 1994, Wilson,1995, Engelhardt, 1993), the level of transduction was disappointing. Hopes that adenovirus may be a suitable vector for CF gene therapy were further dimmed when transduction to CF mice and in human trials (Grubb, 1994, Crystal, 1995) were found to be poor and were associated with a dose dependent inflammatory response (Wagner, 1997). With CF gene therapy likely to be administered on a frequent time course, the problem of adenovirus induced inflammatory response has become a major issue for its continued use as a vector. Modifications of early generation vectors have produced new viruses lacking in or having a reduction in expression of late viral genes, and subsequently, the host immune response (Wilson, 1997).

Progress towards understanding the levels of poor *in vivo* transduction have been made. Pickles *et al.* (1996) with the reporting that adenovirus is poorly trophic to airway columnar cells though highly trophic to basal cells and poorly differentiated epithelium. In one experiment, the tracheas of the mice were mechanically damaged prior to infection with non-replicating adenovirus carrying the type CMV driven *LacZ*. High levels of *LacZ* expression were found in basal cells exposed by mechanical damage. The pattern of transduction efficiency was seen to remain high during the process of epithelial repair. Adenovirus entry into cells involves both high affinity fiber attachment and integrin-mediated internalisation (Wickham, 1993). The authors argue that the difference in transduction efficiencies may be due to differences in integrin receptor populations in the basal and poorly differentiated cell types and mature columnar cells (Pickles, 1996). Cultured airway epithelial cells often exhibit a poorly differentiated basal cell-like phenotype (Grubb, 1994) and this may explain the high transduction efficiency of Adenovirus *in vitro*. It hoped that by incorporating regions antigenic against columnar cell integrins onto the adenovirus

fibres the adenovirus may become more trophic to columnar cells (RJ Pickles, personal communication).

Retrovirus Based Vectors

Retroviruses are single strand RNA viruses which can only transduce dividing cells (Inglis, 1997b) with a lifecycle which involves integration into the host chromosome (Curiel, 1996). This may be thought to exclude retroviruses from gene therapy to the lung, a largely quiescent organ, from the outset. However, the extremely high rate of gene transfer in dividing cell in culture and long term expression has made the retrovirus the most commonly used viral vector in gene therapy trials (Inglis, 1997b, Curiel, 1996). Possible problems arising from integration into the host genome must also be treated with caution for the reasons outlined above.

Adeno-Associated Virus (AAV)

One final viral vector type, which may theoretically bypass the problems of both the retrovirus and the Adenovirus, is the adeno-associated virus (AAV). They can infect a wide range of host cells in a wide range of mammalian species (including man) but have never been shown to be pathogenic or lead to an inflammatory response, suggesting a significant safety margin for use in gene therapy (Curiel, 1996). Unlike adenoviruses and retroviruses which have comparatively large genomes, the AAV's genome is very small and consequently is limited in its capacity to carry a transgenic insert (Flotte, 1993). This is partially overcome by using the nascent AAV promoter regions. AAV is a naturally replication deficient single stranded DNA virus which relies on co-infection by a helper virus (such as adenovirus) for replication. Normally, without the presence of a helper virus transgenic AAV will integrate into the host genome, perhaps site specifically and remain quiescent in the cell until

permissive condition arise. However, when AAV carrying a transgene is used, the virus most commonly exists extra-chromosomally (Kotin, 1990).

Non Viral Vector Systems.

Viral vectors continue to show promise for use in CF gene therapy however the possible problems of associated inflammatory responses, restricted transgenic loads, possible hazards of transgene integration and restricted cell type transduction has remained. It is still unclear whether theses disabled viruses could mutate *in vivo* to return to a pathogenic form (Friedmann, 1997a). Consideration of these factors have led some groups to develop other non viral methods of gene delivery. Non viral vectors come in two main areas: liposome-DNA complexes and molecular conjugates. Like the viral vectors, these systems aim to condense the DNA and encourage safe delivery of a transgene package into the cell.

Liposome-DNA complexes

The negative charge of DNA in solution tends to cause it to be repelled from the negatively charged membranes of cells. Cationic liposomes are self assembling positively charged lipids resembling small portions of eukaryotic cell membranes. When mixed with DNA, liposomes encapsulate the DNA thus hiding its electrical identity. The liposome-DNA complex is thought to either fuse with the target cell membrane or enter the cell by endocytosis where it releases its DNA load into the cell (Stamatatos, 1988).

Liposome based vectors do not face the same size constraints as seen with viral vectors. However, there do face their own selection of draw backs. Although, reports for successful systemic delivery of liposome-DNA it is widely considered that such complexes have are prone to rapid degradation in plasma (Crook, 1996).

Liposome complexes may face the same fate when passing from the airway lumen through the layer of airway surface fluid and mucus into the cells of the airway epithelium (Crook, 1996). This problem can be only magnified in CF patients whose mucus may exhibit abnormal rheological properties (Crook, 1997). A number of different cationic liposomes are now available in the market place. By varying the type and mixture ratio of liposome and plasmid, a wide range of results have been reported (Puchelle, 1985). The addition of cholesterol to the DNA liposome mix was found to both increase the efficiency of transfection and stability of complexes in serum (Crook, 1996).

Liposomes lack tropism for airway epithelium. One way envisaged to counteract this problem is to attach a receptor, cell surface protein, ligand or antibody into the lipid layer of the liposome and deliver the liposome construct either through aerosolisation or perhaps systemically. Such approaches have been successful in increasing transfection efficiencies for other cell types. Transferrin molecules coupled to liposomes were shown to significantly increase transfection efficiencies to bone marrow erythroblasts (Stavridis, 1986) as did glioma cell specific antibodies coupled to liposomes (Mizuno, 1990).

With reference to CF gene therapy, liposome-mediated gene transfer has been reported to be effective in a variety of both *in vitro* and *in vivo systems*. MacVinish *et al.* (1997) reported the normalisation of calcium dependent chloride ion secretion in homozygous $Cft^{rtm1CAM}$ mice after liposome mediated gene transfer of CFTR. McLachlan *et al.* (1995) reported delivery and expression of reporter genes to the lungs of mice which furthermore showed no signs of toxicity. Progress towards conducting human trials on cationic liposome-DNA complexes for CF was made when a number of groups reported complete or partial correction to the electrophysiological defect in CF mice (Hyde, 1993, McLachlan, 1996, Alton, 1993). Recently, Goddard *et al.* (1997) reported that a second delivery of cDNA-liposome complex resulted in a similar level of functional CFTR expression to a single

delivery. These data represents a fundamental stepping stone towards the concept of repeated liposome gene therapy delivery in the treatment of human CF patients.

Three cationic gene therapy human trials have been conducted in the UK (Caplen, 1994, Porteous, 1997b, Gill, 1997. These studies were all preliminary phase I trials delivering a single dose of DNA: cationic liposome complex to the nasal epithelium of CF patients. Phase I trials are primarily designed to both investigate the efficiency of transduction as well as the safety of the protocol before phase II trials delivering a single gene therapy dose to the full lung are planned. The Caplan trial (1994) delivered a CFTR construct under SV40 promoter control complexed with DC-cholesterol:DOPE. Variable and transient expression (until 4 days after treatment) as well as a modest functional restoration, as measured by electrophysiological correction was observed. However, a false positive result in the placebo group limited the complete interpretation of this study. The Porteous phase I trial (Porteous, 1997b) chose a different DNA-liposome complex. Human *CFTR* was driven by a CMV promoter which had been previously been showed to show sustained *in vivo* expression to at least 17 days after transfection to mice by aerosolisation (McLachlan, 1996). The cationic liposome DOTAP had been previously shown to be non toxic after repeated doses to mice (McLachlan, 1995) and when complexed to CMV driven CFTR, correct up to 80% of the electrophysiological deficit in CF mutant mice at up to 9 days after administration (McLachlan, 1996). The trial was successful in mediating gene transfer and for a longer period as compared to the Caplan trial (Porteous, 1997b). No false positives were noted instead, presence of the transgene was noted in 7/8 patients 3 days after administration and in 2 patients at day 28 after administration. Expression of the transgene as measure by RT-PCR showed a positive result in one patient at day +3 and one at day +7. The results of the trial are comparable to adenovirus trial results (Knowles, 1995, Crystal, 1994, Zabner, 1996, Zabner, 1993) and pave the way to future liposome trials.

Molecular Conjugates

Molecular conjugates are a non viral system of gene delivery which may overcome the problem of the lack of airway tropism as found in liposome based vectors. One successful example of this approach was by the development of a complex including adenovirus virions, polylysine and a DNA plasmid. The polylysine condenses the DNA whereas the adenovirus particles augment tropism by binding to transferrin receptors. (Curiel, 1991, Cotton, 1987). Encouraging as these *in vitro* results were, there is as yet, no evidence for *in vivo* transfection in laboratory animals.

In this chapter, liposome mediated gene therapy approaches to the delivery of a marker transgene to the steady state and injured mouse lung are investigated.

6.1

Liposome Mediated Gene Transfer To The Steady State Mouse Trachea

Introduction

A study was made into the efficiency of gene delivery to the tracheal epithelium by liposome gene therapy

Materials And Methods

14 female CD1 mice (approximately 6 weeks old) were separated into a test group of 9 and a control group of 5 animals. Animals received a 0.75ml IP injection of hypnoval:hypnorm anaesthetic and placed in a frame as described by Ho and Furst (Ho, 1973). 20µl of β-gal reporter gene:DOTAP construct (10µl 12mg/ml. DOTAP, 10µl CMV-β-gal) delivered to the trachea of test animal just distal to the vocal folds. Control animals received a 20µl dose of sterile PBS by the same method.

Animals were left for 5 days before sacrifice by cervical dislocation. Lungs of the animals were removed and further dissected to provide separate samples of the trachea, upper lobes and lower lobes. Tissues were processed for DNA extraction by proteinase K digestion and purification by phenol-alcohol extraction. Polymerase chain reaction (PCR) analysis was conducted to test for the presence of the transgene in the tissue. PCR primers were designed against the β-gal transgene. These were for the 5' end TTG GCG TAA GTG AAG C and for the 3' end AGC GGC TGA TGT TGA ACT G. PCR reactions using the enzyme *Thermus aquinus* (Taq) were run on test and control samples using a 30 cycle protocol: 1 cycle at denaturing at 94°C for 3 minutes, annealing at 50 °C for 1 minute and elongation of strands at 75 °C for 1

minute and 29 cycles at denaturing at 94°C for 30 seconds, annealing at 50 °C for 1 minute and elongation of strands at 75 °C for 1 minute. If the transgene was present, a band of 350 base pairs would be produced. Agarose gels bathed in buffer and placed between a cathode and an anode were used to separate the DNA samples. DNA is negatively charged and will be attracted to the anode through the gel. Sections of DNA can, through this method, be separated according to size or state of condensation. Results from the PCR reaction were run on a 0.8% agarose gel with ethidium bromide in TBE buffer for 1 hour at 100V. The gel was illuminated in ultraviolet light and a photograph taken (see figure 6.1.1). Internal controls of two negative control lanes (no Taq) and known concentrations of β-Gal plasmid DNA (100fg, 10 fg, 1fg, 100pg) were also run to validate this assay. It should be noted that no experimental confirmation of presence of DNA in the samples was made prior to PCR amplification.

Tissue samples were also removed for analysis by reverse transcriptase PCR (RT-PCR). This method is used to measure the presence of transgenically expressed RNA. The RT-PCR protocol was conducted by Hazel Davidson-Smith. No positive results for test or negative control samples were observed.

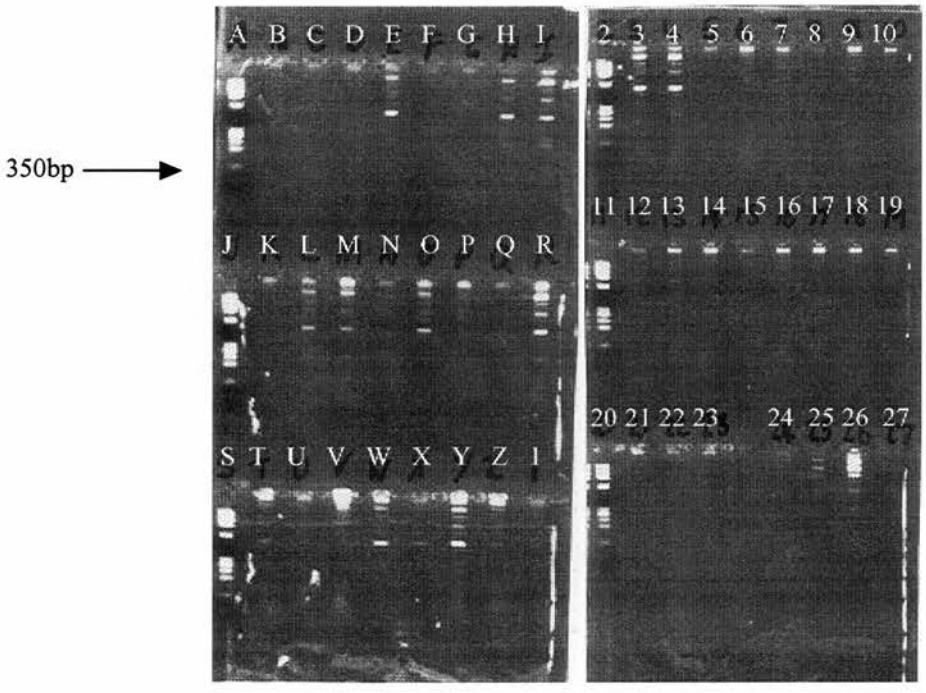
Results

Gel data is presented in figure 6.1.1 and summarised in figure 6.1.2.

Figure 6.1.1

Detection Of Transgenic DNA by PCR In The Pulmonary Tissue Of Mice After Liposome Mediated Gene Transfer

3 tissues (trachea, proximal left lob, distal left lobe) were removed from each test (DNA:DOTAP (10μl, 1mgml^{-1} CMV-β-gal, 10μl x12mgml^{-1} DOTAP) and control (just DOTAP) mouse. Tissues were assayed for presence of transgene by DNA PCR. Amplified PCR products were separated using a agarose gel plate lying within a voltage gradient. The keys for each lane is as follows (lanes not referred to are of ϕx molecular marker ladder):



Mouse	Trachea	Proximal	Distal	Mouse	Trachea	Proximal	Distal
1	-	Lane B	Lane C	8	Lane X	Lane Y	Lane Z
2	Lane D	Lane E	Lane F	9	Lane 1	Lane 3	Lane 4
3	Lane G	Lane H	Lane I	10	Lane 5	Lane 6	Lane 7
4	Lane K	Lane L	LaneM	11	Lane 8	Lane 9	Lane 10
5	Lane N	Lane O	Lane P	12	Lane 12	Lane 13	Lane 14
6	Lane Q	Lane R	Lane T	13	Lane 15	Lane 16	Lane 17
7	Lane U	Lane V	LaneW	14	Lane 18	Lane 19	Lane 20

Figure 6.1.2

Summary of data from liposome mediated gene transfer to the mouse lung experiment

Mouse	Treatment	DNA-PCR Result			
		Presence of β-gal plasmid	Trachea	Upper lobes	Lower lobes
1	Test		-	-	-
2	Test		-	+	-
3	Test		-	+	+
4	Test		-	+	+
5	Test		-	+	-
6	Test		-	+	+
7	Test		-	-	+
8	Test		-	+	-
9	Test		-	+	+
10	Control (100fg)	+			
11	Control (10fg)	+			
12	Control (1fg)	+			
13	Control (100pg)	+			
14	Control (H_2O)	-			

Positive controls of known concentrations of the β-gal plasmid (100fg, 10fg, 1fg, 100pg) were used to confirm protocol efficiency. All positive control samples resulted in a positive result. Negative control of water only was used to test for contamination.

Discussion

Presence of transgenic DNA was detectable in 8/9 test animals by PCR. RT-PCR studies revealed that there was however, no detectable level of transgene expression. The design of the experiment permitted a profile of the areas transfected to be generated which provided some interesting data. A higher level of transgenic DNA was detected in the upper lobes of the lung as opposed to the lower lobes. This was largely to be expected, due to the relative distance from the sight of dose delivery. One exception was animal 7 which showed presence of transgenic DNA in the lower lobes but not the upper lobes. No transgenic DNA was detected in the mouse trachea. These data were surprising as the delivery catheter was positioned in the proximal regions of the trachea.

6.2

Liposome Mediated Gene Transfer To The Polidocanol Damaged Mouse Trachea

Introduction

It was of interest whether the mouse trachea demonstrates a reduced tropism to the liposome vectors. A hypothesis was tested along the lines of the adenoviral studies conducted by Grubb *et al.* (1994) (as discussed above) namely whether the liposome vector would demonstrate increased tropism and transfection efficiency to areas of damaged trachea.

Damage to the trachea was produced through the tracheal instillation of polidocanol solution. Polidocanol is a detergent that acts to remove the columnar cells of the pseudostratified epithelium of the trachea (see section 7.3 for further details).

Materials and Methods

24 female CD1 mice (approximately 25g in weight) were separated into 4 groups. Group 1 contained negative controls and received a 20µl dose of sterile PBS by the tracheal instillation after anaesthetic as described above. Group 2 animals received tracheal instillations of 25µl 1% polidocanol solution to just beneath the vocal cords to partially denude the tracheal epithelium. Group 3 animals received instillations of 20µl 1:12 CMV-β-gal:DOTAP. Group 4 animals received both tracheal damage by polidocanol instillation and 20µl 1:12 CMV-β-gal:DOTAP.

The animals were left for 5 days (two group 4 animals died during this period of unknown causes) prior to sacrifice by cervical dislocation. Tracheas were dissected out. Transfection by the β-gal construct was assayed for by PCR using two tracheas from each group. The tracheas from the remaining animals were assayed for β-gal presence by PCR or for expression by staining for *Lac*-Z activity with X-gal stain. These protocols are described either in section 6.1 (PCR) or in chapter 5 (*Lac*-Z staining). In the PCR analysis, a series of positive and negative control samples were also used as described in figure 6.2.1. Thyroid tissue endogenously expresses *Lac*-Z and was retained during the tracheal dissection protocol to act as an internal control.

Results

Presence of β-gal transgene construct was detectable by PCR as described in section 6.1 generating a PCR product of 350bp. Expression of β-gal was assayed for by examining for X-gal blue staining in tissue by bright field microscopy of 6μm tissue sections.

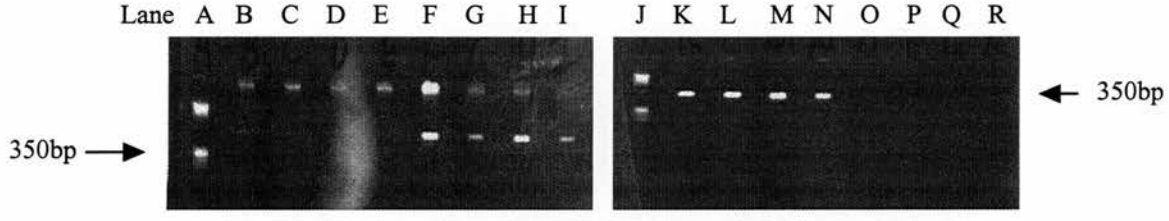
Polidocanol was seen to denude the tracheal epithelium of columnar cells exposing basal cells to the air. For a fuller description of this damage and recovery process please see section 7.3.

PCR results are shown in figure 6.2.1. Examples of X-gal stained tissues are shown in figure 6.2.2 and the data from section 6.2 is summarised in table 6.2.3.

Figure 6.2.1

PCR Data From Gene Therapy Experiment To Steady State And Damaged Tracheal Tissue

The tracheas from test and control mice were assayed for presence of transgenic DNA (ß-Gal) by PCR. Tracheas were excised from test and control animals and DNA extracted and purified. PCR was used to amplify and transgenic DNA which may be present. The amplified products were examined using a 0.8% agarose gel (see text for details.)

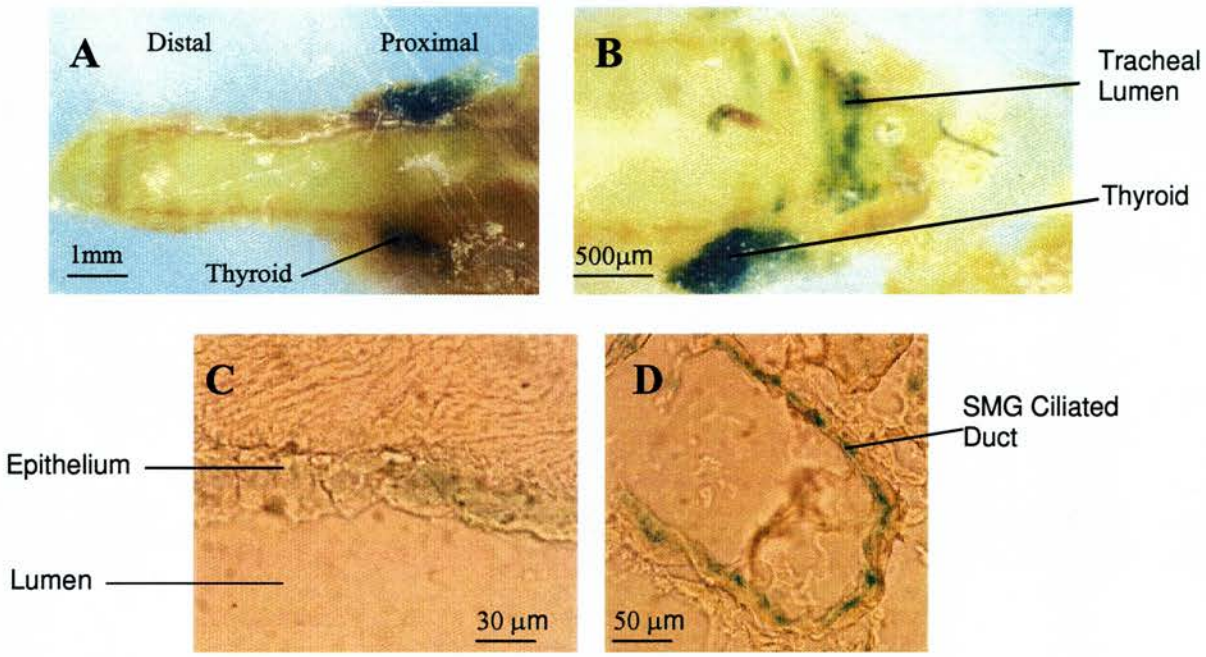


Lane A: Standard Marker Ladder
Lanes B-E: Mouse controls #1-4
Lanes F-I: Mouse tests #5-8
Lane J: Standard Marker Ladder
Lane K: 100fg control
Lane L: 10fg control
Lane M: 1 fg control
Lane N: 100A
Lanes O-R Negative control lanes

6.2.2

Detection of transgene expression by X-Gal staining

Test and control tissues were stained with X-Gal stain to assay for the presence of β-galactosidase, the protein product of Lac-Z expression.



6.2.2a Stained mouse trachea (mouse number 7) embedded in wax. Note heavily stained thyroid glands adjacent to the proximal end of the trachea. No staining observed in the tracheal lumen.
6.2.2b Mouse trachea 13 after staining and prior to sectioning. When positive Lac-Z staining was noted in the trachea, it was most commonly concentrated to the proximal end of the trachea. This coincided with the site of liposome construct delivery. This image shows a heavily stained thyroid gland and bands of blue staining in the tracheal lumen.
6.2.2c On thin section, intermittent weak staining was observed in the tracheal epithelium (mouse number 19).
6.2.2d Staining also occurred rarely (only in mouse number 19) in the top region of the ciliated ducts of SMGs.

Figure 6.2.3

Data Summary From Liposome Gene Therapy Delivery And Construct Expression To Injured And Uninjured Lung Experiment

Ref.	Group	Polidocanol	DNA:DOTAP	Method of assay		Signal detection
				PCR	X-gal	
1	1	-	-	+	-	-
2		-	-	+	-	-
3		-	-	-	+	-
4		-	-	-	+	-
5		-	-	-	+	-
6		-	-	-	+	-
7	2	+	-	+	-	-
8		+	-	+	-	-
9		+	-	-	+	-
10		+	-	-	+	N/a #
11		+	-	-	+	-
12		+	-	-	+	-
13	3	-	+	+	-	++
14		-	+	+	-	-
15		-	+	-	+	-
16		-	+	-	+	+
17		-	+	-	+	+
18		-	+	-	+	-
19	4	+	+	+	-	++
20		+	+	+	-	-
21		+	+	-	+	+
22		+	+	-	+	-

Positive control in this sample did not work.

Discussion

In section 6.1 an experiment was conducted to investigate liposome mediated gene transfer to pulmonary tissues in steady state. One of the conclusions from this experiment was that although lung lobes showed evidence of transfection, there was no detectable level of transgene DNA in the trachea. Section 6.2 aimed to study whether the levels of transfection to damaged tracheal epithelium would be increased in comparison to "steady state" or undamaged epithelial tissue.

It is therefore surprising to note in experiment 6.2, data is presented showing a positive results for transgene detection in the undamaged trachea after a single liposome gene therapy dose. In this test group 3/6 animals show positive evidence for transfection. There were no observable difference in protocols in sections 6.1 and 6.2. Indeed, every effort was made to make the tracheal instillation protocol reproducible. No negative control samples produced positive results.

5 days after tracheal damage by polidocanol, the tracheal tissue demonstrates full recovery to normal histology (see chapter 7 for more details). There is no evidence for an increased efficiency rate of transgene expression or transfection to the damaged tracheal tissue as compared to the undamaged tissue. On analysis of the X-gal stained tissue sections (figure 6.2.2), X-gal staining was observed in the epithelial lining of the trachea and also in the ciliated SMG duct. Staining was most localised to the proximal region of the trachea, where delivery of the construct was made, which may infer that distant aerosol delivery, as is envisaged in the treatment of the human lung, may be problematic. There is no evidence from these images to suggest that transfection levels to the basal cells are increased in comparison to columnar cells.

One possibility to explain why an increased transfection efficiency is observed is that the residual polidocanol in the trachea is acting as an emulsifier and interfering with the liposome:DNA interaction. One possible way to alter the protocol to avoid this issue would be to put in a time delay between damaging and dose delivery. However, the hours after polidocanol injury are characterised by rapid tissue renewal (as demonstrated in chapter 7). It is perceived that mucus and inflammatory cells already affect liposome transfection efficiencies. Therefore, establishing a time delay in the protocol may infact result in an even greater barrier to transfection.

Such issues were not faced in the Grubb paper (1994) as a surgical method of tracheal damage was utilised. It would be of interest to repeat this study using this surgical procedure.

From the evidence presented in this section, evidence is presented showing successful gene transfer using liposome vectors to the pulmonary tissues of mice. There was no observable difference in transfection efficiency to damaged tracheal versus steady state tissue. Disappointing as this is, it was interesting to observe gene delivery to the top region of the SMG ciliated ducts and to the tracheal epithelium: an area believed to be highly relevant to CF gene therapy.

Chapter 7

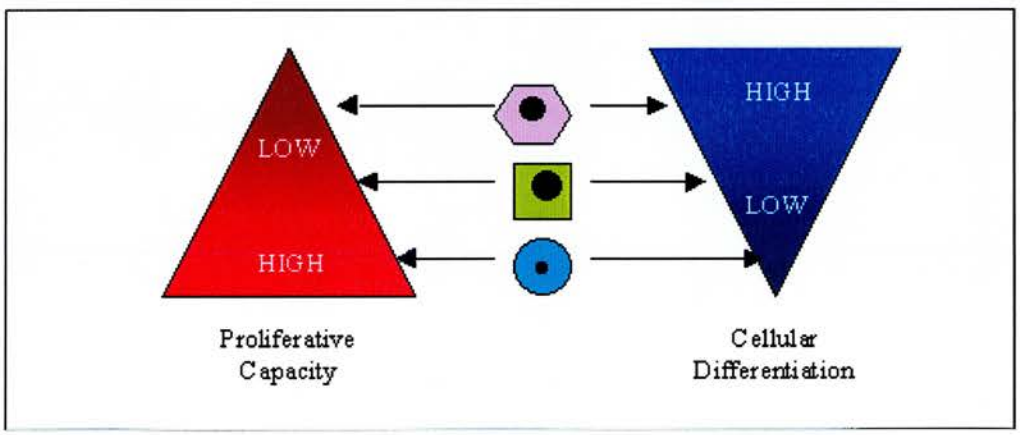
The Kinetics Of The Murine Tracheal Epithelium

Introduction

Many epithelial tissues act as physical (though dynamic) barriers between two opposing environments and as such, are prone to damage therein requiring the means for self renewal and repair. Stem cell theory describes the role of the different epithelial cell types in terms of occupying different locations on a hierarchy of cellular differentiation and proliferative capacity. The primary assumption of this model is that the processes of proliferation and differentiation can be perceived as individual entities (Potten, 1990). At one end of this system lies the stem cell which is characterised by poor morphological differentiation but seemingly infinite proliferative capacity while at the other end of the model exists mature and functional end point cells. See figure 7.0.1.

Figure 7.0.1.

Different Epithelial Cells Types Can Be Perceived As Existing At Different Positions On A Model Of Proliferative Capacity And Differentiation.



The stem cells are not the only the only cells in an epithelial model which have proliferative capacity. Transit amplifying cells (TACs) are a transition population of proliferating cells which occupy the area between stem cells and the mature functional cells. According to the stem cell model, they will retain some stem cell properties including proliferative capacity yet also have some properties of the mature cells. A primary feature of TACs is that they are predestined to move towards functional status (Potten, 1990). TACs at different positions of the model can therefore be termed either as *Early* TACs or *Late* TACs.

The identification of stem cells can be fraught with difficulties as they can only be truly identified by merit of their future potential. The situation is not indifferent to the Uncertainty Principle which describes the difficulties in measuring the position of a particle. Light (or some other form of radiation) is used to illuminate the particle. This radiation has momentum and therefore distorts the position of the particle we are trying to see. Examples of such a predicament can be seen in everyday life; i.e. a thermometer used to measure the temperature of a bath will absorb some heat energy in that process thus changing the original temperature of the bath water. So with the stem cell; experiments to test their functional capabilities may, by merit of the assay procedure, may alter the cell's characteristics.

There is at this time no universal molecular or any other kind of marker for the virtue of *stemness*. So how can stem cells be identified? Most research has been conducted by considering some definitions of what characteristics a stem cell should exhibit and then comparing and contrasting these against what is observed *in vivo*. Potten and Loeffler (1990) set out such a frame work which would hopefully help in the identification of stem cells. They reported that stem cells should have 4 main features(Potten and Loeffler, 1990, Mason, 1997):

①: Stem cells are poorly differentiated (i.e. morphologically simple)

②: The Stem cells' capacity for proliferation extends throughout the life span of an individual dividing asymmetrically as to maintain its own population.

③: Progeny of stem cells will give rise to functional progeny

④: Stem cells should be able to regenerate an entire tissue which has been damaged and is therefore likely to be spatially restricted to a specific compartment.

From these definitions, a perceived image of stem kinetics can be sought. Stem cells are likely to be slow cycling morphologically simple cells being compartmentalised away from the most common sites of epithelial damage (Potten, 1988). It is thought that the slow turnover of stem cells may be advantageous as to avoid potential mutations arising through DNA replication.

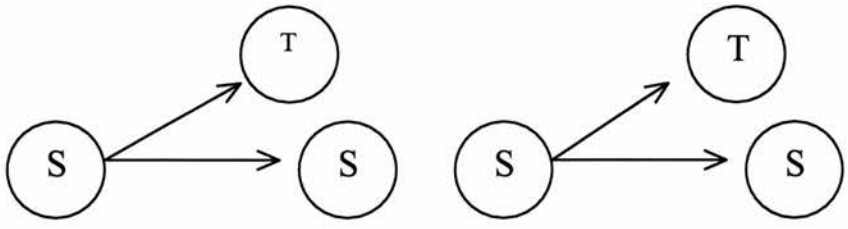
There is a number of possible systems of cellular division by which the stem cells can maintain their own population whilst also giving rise to TACs. These are displayed in figure 7.0.2.

Figure 7.0.2

Figure 7.0.2

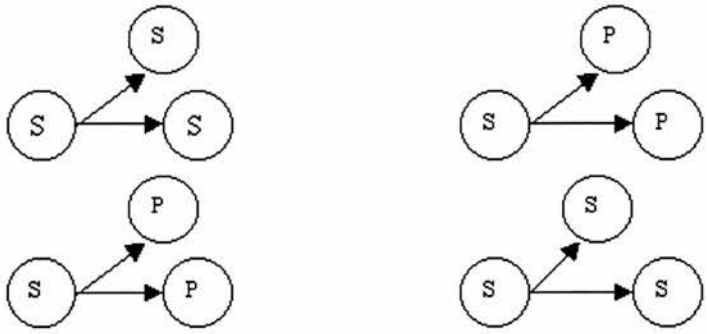
Possible patterns of Cell Division in a Stem Cell driven lineage

"S" refers to Stem Cell, "T" refers to a transit amplifying cell (a cell with restricted proliferative capacity and committed to deriving mature cell types), "P" refers to physiologically functional end point cell.



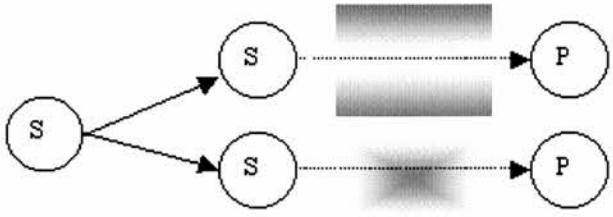
In model A, the stem cells is dividing in an asymmetric pattern thus maintaining its own population but incapable of expanding it.

B



Model B describes the action of four different stem cells. Their division is symmetrical but half the time they are self-renewing.

C



In this model, the cell lineage is asymmetric yet is not intrinsically so. The behaviour of the daughter Stem cells can differ owing to local environmental or cell adhesion factors (depicted by the shaded boxes)(Morrison, S. J.*et al*, 1997).

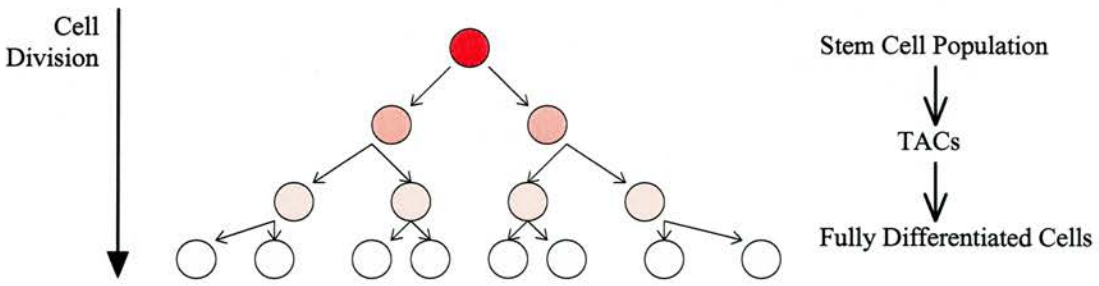
It must also be appreciated that the nature of stem cells, the virtue of *stemness*, can at times be invisible to the features outlined above. If a stem cell was to stop replicating, i.e. become quiescent, it is fair to appreciate that the cell has not lost any of it potential qualities of stemness since it could re-enter the cycle at any time. Therefore, the concept of "potential stem cells" (Potten, 1990) must be introduced. Stem cells which are active in division as thus termed "actual stem cells".

The most readily identifiable trait of stem cells is their long life time and slow cellular turnover. The life time of a particular cell type can be quantified through the use of nucleotide analogue labels such as bromodeoxyuridine (BrdU) and tritiated thymidine. These chemicals are incorporated into a cell's DNA at the time of mitosis and will remain incorporated in the cell until cell death occurs or the cell divides in which case in which case, both daughter cells will also retain label. After repeated cell division, the faster cycling cells will dilute their label dose. Eventually, this reduction in dose will result in the cell marker content becoming so low that labelled cells will become invisible to the sensitivity of antibody (immunohistochemistry) or autoradiographic techniques. Dilution of label is depicted in figure 7.0.3.

Figure 7.0.3

Decrease in BrdU dose after repeated cellular divisions.

This model describes the dilution of BrdU labelling after repeated cellular division. The rate of BrdU dilution being directly proportional to the number of cell divisions that have occurred.



By a cell's BrdU labelling profile it is therefore possible to deduce the approximate life span and kinetics of a cell. Here are two examples. Firstly, a cell labelled at mitosis with a long life time will retain the nucleotide analogue for an extended time period in comparison to a cell with a short life span. Secondly, a marked cell with a high proliferative capacity will give rise to a high number of marked daughter cells whereas a marked cell with low proliferative capacity may not give rise to any further marked cells. Cells will eventually loose their detectable signal depending on the number of cell division that have occurred and the sensitivity of the label detection method.

Label retaining profile of stem cell

A BrdU labelled stem cell, which is, as proposed by the model, inherently slow cycling and has a huge proliferative capacity, will retain its label for a longer time than its progeny. Cell's displaying such characteristics are termed Label Retaining Cells (LRCs). This theoretical label retaining property has been used widely in the search for stem cells (Bickenbach, 1984, Bickenbach 1981, Bickenbach, 1984, Cotsarelis, 1989, Cotsarelis, 1990, Graeter, 1996, Mackenzie, 1985).

One example is in the skin and oral mucosa of both hamsters and mice where cells retaining tritiated thymidine labelling characteristics have been recognised. These two tissues are similar in histology both possessing a thick layer of stratified epithelium. Cells retaining tritiated thymidine label (LRCs) some 69 days after dosing were recognised in the deepest part of the epithelial papillae in both tissues and in both hamsters and mice. This observation suggests that retention of a nuclear label is a reproducible phenomenon across different tissues and species (Bickenbach, 1981, Bickenbach, 1984).

Seeking LRCs in tissues which are, usually, largely quiescent by nature can lead to an important practical problem namely, a single pulse of label, such as of BrdU or tritiated thymidine, is not likely to results in the labelling of stem cells. The epithelium of the bronchus is such an example. Using immunohistochemistry techniques on human bronchial tissue, it was found that less than 0.2% of epithelial cells were marked as undergoing cell division in the steady state(Leigh, 1995).

The epithelium of the trachea is another example of a largely quiescent tissue. With a low cell proliferation index in trachea of rodents (Breuer, 1990, Donnelly, 1982, Randell, 1992) it is unlikely that if a stem cell was present that a single pulse would result in its marking. It is much more likely that the TAC population would be labelled. One way around this problem would be to design an experiment in which the cell proliferation is first raised prior to BrdU injection. A common way to do this is by artificially damaging the epithelium with the hope that this will increase the recruitment of stem cells into dividing and therefore increase the possibility of actually labelling them.

This approach was used in a study into the progenitor-progeny relationship in the cornea of rabbits (Cotsarelis, 1989). After physically damaging the cornea, tritiated thymidine was delivered to the rabbits for a 14 day period. By sampling groups of animals at different time points after damaging and dosing, cells in the limbal basal epithelium were observed to have been stimulated into division leading to the migration of new cells to the wound site in the cornea epithelium. These limbal basal cells show 2 functional traits which suggests stem cell behaviour. Firstly, the limbal basal epithelium contains the least differentiated cells of the corneal epithelium. Second, the limbal basal epithelium contains cells which exhibit the proliferative characteristics of tritiated thymidine retention(Cotsarelis, 1989). At 4 weeks a subpopulation of the limbal basal cells still retained labelling.

To establish further that these LRCs possessed stem cell properties, topical application of the tumour promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate) was used to induce terminal differentiation of corneal and limbal cells *in vitro*. Treatment of corneal cells with TPA resulted in rapid maturation of all cells whilst a fraction of cells from the limbal epithelium retained their undifferentiated phenotype and continued to proliferate despite the presence of TPA. These TPA-resistance cells are the least differentiated cells in the epithelium and thus, the authors argue, are stem cells (Kruse, 1994).

Compartmentalisation of LRCs

The variability in the label retaining property of cells that are otherwise morphologically similar, can be used to the further categorise cells types. In the case of rat caecal enterochromaffin cells, two kinetically distinct populations of cells were identified by their BrdU retaining properties (de Bruine, 1992). One population, comprising of 60-65% of cells, has a high turnover time of approximately 16 days, whilst 35-40% of cells have a turnover time of approximately 150 days. These data suggest the presence of two distinct cell populations occupying different position of the scale of proliferative potential.

The LRCs of the cornea also show the property in common with LRCs from other tissue, that of being spatially restricted to specific tissue compartments. Similar compartmentalisation of stem cells is seen in a variety of tissues including palm skin (Lavker, 1982), the dorsal tongue (Bickenbach, 1981) and in the small intestine (Potten, 1990).

Some epithelial tissues, such as the dorsal skin of the mouse, exist as a stratified structure composed of flat basal layers and columns of keratinocytes arranged like stacks of coins (Hall, 1989). Can such compartmentalisation still exist in such a

tissue? Potten proposed the concept of Epidermal Proliferative Units (EPU) (Potten, 1974). The theory describes that each column of cells corresponds to a single EPU which is in turn composed of a single stem cell surrounded by 5-6 transit amplifying cells. This work suggests that even in an epithelial layer, compartmentalisation can still occur.

The small intestine has become one of the model tissues for stem cell research possibly due to its rapid turnover of tissue and well established histology. The lining of the small intestine is an epithelial monolayer divided histologically into crypts and finger like projections called villi. Small intestine stem cells are found lying either proximal to the paneth cells in the base of the crypts (Potten, 1982, Potten, 1990). The stem cells proliferate to produce TACs migrating downwards to produce paneth cells and upwards to produce the cells of the crypt and the villi. Migration of the cells towards the tip of the villi (where they are eventually shed off) takes between 2 to 3 days and involves the differentiation of stem cells into four mature cell types which have no further proliferative capacity. The proliferative capacity of the gut stem cells is remarkable. It is estimated that each crypt produces approximately 300 new villi cells a day and over 2 x 10^8 villi cells are shed off every day (Potten and Loeffler, 1990). It is now thought that small intestinal crypts contain between 4-16 *actual* stem cells producing clonally derived crypts yet polyclonal villi (i.e. only one stem cell is active in each crypt at any one time)(Potten and Loeffler, 1990). In the hair follicles, LRCs are located in close proximity with the follicular papillae and associated vasculature (Cotsarelis, 1989).This spatial localisation greatly adds to the conceptual understanding of stem cells.

Regulation of stem cell behaviour

Stem cell research for many tissues has now progressed beyond the stage of stem cell identification to investigating the methods of stem cell control. This has become a

large and complex field in itself pulling fate map technology from *C. elegans* and *Drosophila melanogster* (Morrison, 1997) as well as *in vitro* studies designed to identify the signals that regulate stem cell differentiation (Johe, 1996). The brain has provided much scope for study due to its cellular diversity and the presence of easily identifiable cell types. The Johe group (1996) studied the differentiation mechanism of embryonic cells of the central nervous system into 3 main cell types: neurons, astrocytes and oligodendrocytes. He showed that both proliferation and differentiation of the neural stem cells could be controlled *in vivo* through the addition of different extracellular signals. Platelet derived growth factor supported neuronal differentiation, ciliary neurotrophic factor acts on stem cells to generate astrocytes and addition thyroid hormone T3 results in the production of oligodendrocytes.

Growth factors have also been found to be able to regulate the proliferation of cells in the small intestinal crypts *in vivo*(Potten, 1995). Three different assays were developed to measure the effect of *in vivo* delivery of growth factors on the position of tritiated thymidine labelled cells and mitotic activity in the gut. The effect of 8 different growth factors was assayed using this method. Epidermal growth factor was found to be the most effective in stimulation proliferation on the complete gut whereas Insulin-like-growth-factor showed a proliferative effect on only the upper crypt and transforming growth factor β-1 was shown to have a proliferative effect in the lower (stem cell) regions of the crypt.

Recent evidence from transgenic mice suggests that cell to cell interactions may have a profound effect on stem cell behaviour. When cell-cell adhesion molecule E cadherin was over expressed in both the crypts and villi of mouse small intestine under the control of a fatty acid promoter, cell proliferation was seen to decrease and the rate of cellular migration was reduced (Hermiston, 1995). In the opposite situation, when an N cadherin mutant that functions as a dominant negative for E

cadherin is over expressed in the small intestine, the rate of proliferation and migration increases (Hermiston, 1996, Hermiston, 1995). It is believed that then phenotype of these transgenics may be as a consequence of a disruption of cell to cell adhesion or a disruption to in the intracellular distribution of β-catenin (Jones, 1997, Peifer, 1992).

This situation is thought to be akin to the *Drosophila* mutation *Wingless*. β-catenin has a *Drosophila* homologue called Armadillo which is a member of the *Wingless* signal transduction pathway proteins (Peifer, 1992). The *Wingless* proteins are short range secreted molecules which act over a range of several cell diameters. In *Drosophila* the *Wingless* proteins specify the local fate of epithelial cells whose cellular distribution has been shown to be disrupted by misexpression of cadherins (Jones, 1997).

This research suggests that cell to cell adhesion is crucial for stem cell activity is likely to have implications on *in vitro* or *ex vivo* study into stem cells. It can be envisaged that disturbing cell to cell adhesion through experimental manipulation (i.e. protease methods of digestion) may have a considerable effect on the activity of stem cells.

The kinetics of the epithelium of the upper respiratory airways

The mammalian respiratory system is a structurally complex arrangement of organs whose primary function is in the intake of oxygen into the blood and the elimination of carbon dioxide. The respiratory system is lined with epithelial tissue which in addition to the processes of gas exchange, forms a physical barrier to prevent penetration of noxious substances, and also serves an essential role in bacterial and pathogenic defence through the production of mucous and anti microbial agents (Pack,, 1981,Basbaum, 1990,Widdicombe, 1994).

Efficient gas exchange in mammals is based upon the respiratory system having a huge surface area of thin simple epithelium. To this evolutionary end, the structure of a respiratory system is structured in a huge branching system of tubes of every decreasing lumen. The trachea is the largest of the branches and connects the lung towards the mouth. The trachea splits into two bronchi at the carina thereby forming the two sides of the lung (the left lung is slightly smaller than the right to make space for the heart). The trachea and the bronchi are structurally supported against collapse by cartilage rings connected to a layer of muscle. The bronchi split to form the basic structure of the lung lobes giving rise to the non-cartilaginous bronchioles. Bronchioles further split to eventually form the alveoli.

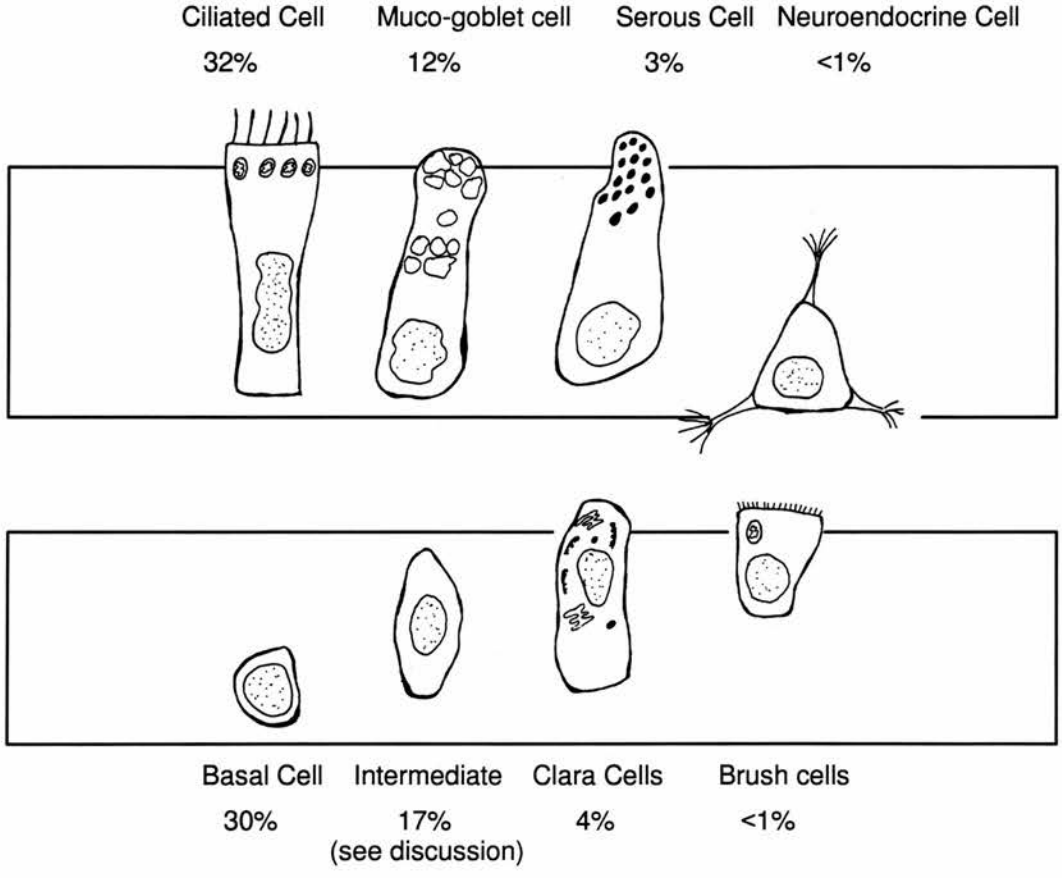
There are three quite discrete epithelial types in the human adult lung: those of the trachea and the bronchi (the cartilaginous airways), the bronchioles and those of the alveoli. The role of these tissues vary widely as does complexity in respect to the number of different cell type and kinetic models. At least eight principal types of epithelial cells have been identified in the linings of the conducting airways of mammals (Breeze, 1977, Pack, 1980, Boren, 1978) and a further 3 cell types in the alveolar regions (these cell types are introduced in figure 7.0.4). The primary cell types are the ciliated cells, the mucous goblet cells, the Clara cells, the basal cells, neuroendocrine cells, the serous cells, brush cells and K cells. There is yet unclear whether a cell type referred to as the "Intermediate" or "indeterminate" is a discrete cell type or a histological artefact (Mercer, 1994, Houck, 1985, Breuer, 1990), The possible implications of this argument are more fully discussed later in this chapter. In the alveoli there are type 1 cells , type 2 and type 3 cells. The cell types found in the submucosal glands are discussed in chapter 2.

Figure 7.0.4

Figure 7.0.4

The Primary Cell Types Of The Human Trachea And Bronchus

Approximate percentage contribution of the cell types are provided after Mercer *et al.* (1994). These figures are derived from analysis of non smoking adults who had normal lung function. It should be noted that the percentages would be seen to alter considerably in diseased or inflamed tissues.



The difficulties of studying the kinetics of the upper airway epithelium.

The kinetics of the human terminal bronchioles and the alveoli are largely understood (and appear to be well conserved through a number of different species) (Breeze, 1977, Randell, 1992, Evans, 1976b, Evans, 1976a, Evans, 1975) and will therefore not be investigated directly in this thesis. Briefly, in the alveoli, type 1 cells are large squamous cells that cover 93-97% of the alveolar surface. Type 2 cells are smaller and more cuboidal in shape and cover most of the remaining alveolar surface. A type 3 cell, albeit very rare, does exist though its physiological and proliferative role is poorly understood. The kinetics of this system is simple. Type 1 cells are terminally differentiated and type 2 cells are capable of cell division, acting as the progenitor cells for the alveolar epithelium.

The kinetics of the human terminal bronchioles are also straightforward. Only two cell types are present; the Clara cell and the ciliated cell. The Clara cells are non ciliated columnar cells and are believed to be the progenitor cells in this tissue, being capable of cell division to produce terminally differentiated ciliated cells (Evans, 1976b).

The mechanism by which the tracheo-bronchial epithelium undergoes self-repair has still to be resolved despite considerable research into this area being conducted over the last 40 years. There are a number of technical reasons why this may be so.

Cell identification

Unlike many of the well characterised epithelial cells types, identification of the cells of the tracheo-bronchial epithelium is often difficult due to the lack of strong and easily applicable cell-type-specific biochemical markers. Additionally,

ambiguous histology has often lead to the use of phrases such as, "poorly differentiated" (Randell, 1992) or "intermediate" (Breuer, 1990, Engelhardt, 1995) to describe cell types. To aid identification, Shimizu *et al.* (1992) reported the development of "cell type specific" monoclonal antibodies. The expression of specific keratins and lectins has been previously shown to be useful in the identification of cell types in a number of different epithelial tissues (Purkis, 1990). By raising antibodies against a range of keratins and lectins, identification of 5 main mature cell types in regenerating *in vitro* rat trachea was possible (Shimizu, 1992). Poorly differentiated cells were not seen to react with any mABS used. Such approaches, though restricted to mature epithelium and restricted at this time by immunological reactivity to rat tissue (Randell, personal communication), may pave the way for better experimental approaches to the study of tracheal epithelium.

The identity of the "intermediate" cell type has instilled a degree of uncertainty into many quantitative attempts to understand the tracheobronchial epithelial kinetics. "Intermediate" cells are classified as cells either lacking sufficient cytoplasmic characteristics for proper categorisation or cells which had the morphological characteristics of basal cells but lacked basement membranes (Mercer, 1994). It is yet unclear whether further classification of the intermediate cells is a problem associated directly with histology (for it is easily possible to generate a cell lacking basement or apical membrane by making a tangential cut through a columnar cell) or whether cells are transforming from one cell type to another or indeed, they exist as a discrete cell type. Whichever way the argument is considered the problems arising from this cell type are considerable.

The Breuer study (1990) of hamster bronchial epithelial kinetics is victim of the lack of sound epithelial cell identification. Breuer *et al.* reported that 15% of epithelial cells are of "intermediate" phenotype. Further more, in their classification of basal cells they "assume contamination by non basal cells of as much as 50% of the B_2

(taller basal cell) category." The "corrected" B_2 population increased from 7.6% to 11.4% and this figure is used throughout their calculations. Mercer *et al.* (1994) identified 17% of cells to be of "indeterminate" identity in their EM study of the human trachea and 9.5% in the rat. Such studies would appear to be inherently hazardous due to the weak statistics they generate. However, it is a weakness that must be faced as any study into the kinetics of the lung epithelium will ultimately rely on histological examination and cell identification until such time as reliable cell markers can be found.

It is therefore interesting to examine the workings on the Engelhardt study on epithelial kinetics using the xenograft system (Engelhardt, 1995). See chapter 7 for details. The model generated describes among other things, the requirement of goblet cells to pass through an intermediate cell type to become ciliated cells. The main basis for this claim is that there were no clones found with just ciliated and goblet cell types but always had intermediate cells present. *If* however, a single intermediate cell was misidentified in the process and a clone of just the ciliated and goblet cells types existed, the whole model would collapse.

Factors Affecting Cell Type Distribution

There are a number of factors which affect the cell types found, their frequency and their location in the tracheobronchial tree. In response to lung disease (such as CF) and pollutant damage, there can be considerable cellular plasticity ranging from hyperplasia and hypertrophy of goblet cells and a proportional reduction in ciliated cells to squamous metaplasia and neoplasia (Davidson, 1995, Pack, 1981, Thurlbeck, 1963,Kruse, 1994, Breeze, 1977, Randell, 1992). Subtle changes such as conditions for housing of laboratory animals can result in considerable change in epithelial composition, especially in transgenic mice prone to lung disease such as a the CF mouse (Dorin, 1992a, Davidson, personal communication).

Many different animal species have been used experimentally to study lung kinetics. These animals usually being used on the basis that they will model the dynamics of the human lung. Many of the cell types in the mouse resemble those of other species including humans (Pack, 1981). Goblet cells are rare (<0.5%) in mice from SPF conditions though more common in the CF mice and mice kept in non SPF conditions (Dorin, 1992a, Davidson, , 1995). Ciliated cells only appear in scattered patches (Pack, 1981) at a frequency of ~38%. Basal cells are present in both humans and the mouse. In humans, they contribute to 30% of the cells of the bronchi (Mercer, 1994) and in the mouse, about 10% (Pack, 1981). Clara cells are non-ciliated columnar cells that show considerable variation in ultrastructural characteristics between species. In many non human species (e.g. horse, sheep, rabbit, mouse, rat) more than 40% of the cytoplasm is occupied by endoplasmic reticulum whereas in the human and other primates, this is restricted to the apical side of the nucleus (Plopper, 1983, 1989). There are also species differences in the distribution of the Clara cells. The Clara cell is the most common cell type in the mouse trachea and shows a slight variation in frequency through the length of the trachea (Pack, 1981). At the top of the trachea, 53.2% of cells are Clara cells, reducing to 49.4% in the lower trachea but again rising to be 57.5% of cells at the carina (Pack, 1981). These data are starkly different from observations in the human bronchi where only 3.2% of cells are Clara cells but more similar to the human bronchiole where 35% of cells are Clara cells (Mercer, 1994, Pack, 1981). The human bronchiole however lacks basal cells (Mercer, 1994).

A Review Of Kinetic Studies Of The Epithelium Of The Upper Airways

Understanding the kinetics of the lung is fundamentally important to understanding pulmonary diseases in which epithelial remodelling occurs and to the development of gene therapy targeted to progenitor cell populations in the lung. Probably due to the

difficulties of studying the kinetics of the lung epithelium as outlined above there have been numerous reports published which propose nearly as many different mechanisms for the kinetics of the lung. They can be separated into two groups: those studying the steady-state lung and those on diseased, damaged or disturbed lung.

Kinetic Role Of The Ciliated Cell

It is widely considered that ciliated cells are terminally differentiated and have a very low proliferative capacity (Johnson, 1990a). Under steady state conditions, the ciliated cells are thought to be incapable of division and only under exceptional conditions (hamster tracheal epithelium in vitamin A deprived culture medium (McDowell, 1985a)) have ciliated cells been seen to divide and synthesise new DNA.

Kinetic Role Of The Basal Cell

Traditionally, basal cells have been perceived as the progenitor cell type in the lung. This is possibly due to their positioning in the epithelium being in accordance with progenitor cells in other tissue types (for instance, the in the skin (Potten, 1988). Indeed, there is some evidence that this may be the case for basal cells in both injured and steady state tissues *in vivo* have been shown to be capable of DNA synthesis (Boren, 1978, Donnelly, 1982, Lane, 1974).

Inayama *et al.* (1989) tested the proliferative capacity of basal cells by purifying basal cells from rabbit trachea by centrifugal elutriation and inoculating tracheal xenografts. The purified cell solutions were found to rapidly give rise to epithelial tissue composed of basal, ciliated, goblet and secretory cells. The conclusion being that since basal cells could give rise to all the major tracheal cell types, they were the

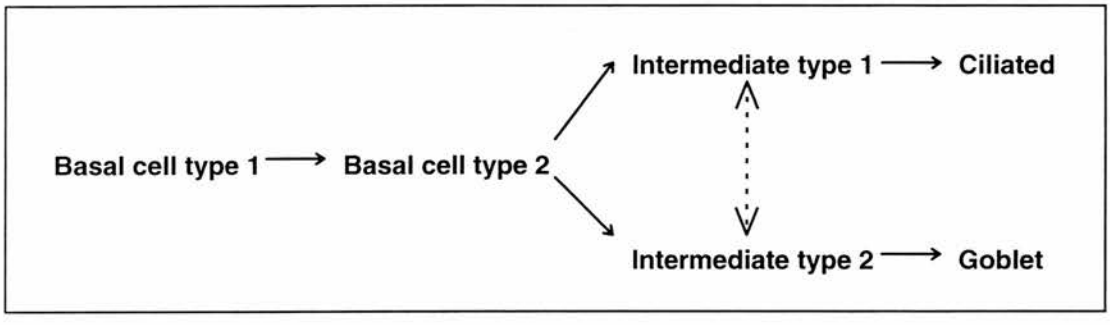
"main stem cell". However, it must be noted that firstly, the basal cell suspensions were only between 83% and 94% pure (as determined by EM) or alternatively, between 6% and 17% contaminated with other cell types. These other cell types were of ciliated, mucous, Clara and "undetermined" morphology. It is therefore, possible to consider an alternative conclusion to this experiment that of the "main stem cell" being one of the other cell types and of being of sufficient proliferative capacity to produce the mature epithelium.

Both Zepeda *et al.* (1995) and Engelhardt *et al.* (1995) used a system of clonal analysis in xenografts to explore the kinetics of the human bronchial epithelium. Primary isolates of human bronchial epithelial cells (HBECs) were partially infected with retroviruses carrying marker constructs (*Lac*Z and alkaline phosphatase in both the studies). This infection resulted in the partial retroviral transfection of dividing cells. These cells integrated the transgene into their own genome from where they were expressed. Cultures were seeded into denuded rat tracheas and implanted subcutaneously in mice where they repopulate the tracheas and produce epithelial tissue indistinguishable in terms of morphology from human bronchus at the light and electron microscopic level (Engelhardt, 1991, Zepeda, 1995, Engelhardt, 1995).

Engelhardt *et al.* studied the epithelial regions marked by the transgene expression. The study was based on the premise that clusters of transgene – expressing epithelial cells, which express a single histochemical marker, reflect the expansion of a single progenitor cell which was genetically tagged *in vitro* (Engelhardt, 1995). The authors predicted a possible error in clonal assignment of less than 2.0% though concede that they cannot rule out the seeded clones are derived from smaller clumps of cells which expanded clonally *in vitro*. 4 cell types were considered (basal, intermediate, goblet and ciliated) however, analysis of the clones revealed that not all possible clone compositions were present. No clones of just ciliated and goblet cells were found. Furthermore, there was correlation between the number of different cell types

and the clone size. By far the largest clones were those containing the four cell types and the smallest, those of just basal cells. From these data a model was produced to describe the lineage of the airway epithelium differentiation. See figure 7.0.5.

Figure 7.0.5.
Engelhardt Lineage model of airway epithelial differentiation (after Engelhardt (1995))



This model describes the presence of two types of basal cells and two types of intermediate cells. Basal cell type 1 possible acts as a stem cell. Both ciliated and goblet cells are mature and have no proliferative capacity.

Zepeda *et al.* described a similar experiment though declared that the overlapping cells in the 5μm sections in addition to the diffusion of the histochemical reagent products made the identification of cell type by light microscopy "difficult" (Zepeda, 1995). The possible problems arising from false identification of the "intermediate" cell type in the Engelhardt paper are discussed above. Therefore, anti keratin 14 and anti keratin 18 antibodies were used to differentiate between basal and columnar cells respectively (Lane, 1974). The data from these experiments describe the

presence of a significant number of cytokeratin 14 and cytokeratin 18 restricted clones (termed "simple" clones) (ranging from 6.1% to 40.0% of total clones in individual xenografts) as well as mixed ("complex") expression clones. These data support a model of epithelial reconstruction through a restricted number of highly proliferative stem cells from which all the cell types in the human lung can develop although the nature of the proliferative cells cannot be determined.

There are now several lines of evidence to challenge the concept of the progenitor role of basal cells. During development, ciliated and secretory cells appear in the hamster bronchial epithelium prior to basal cells (McDowell, 1985b). Furthermore, intense proliferation of secretory cells appear prior to extensive mitotic activity in basal cells in Rhesus monkeys (Plopper, 1989) where pre-ciliated cells were seen to contain both secretory granules and basal bodies suggesting a secretory to ciliated cell pathway.

One of the traditional attractions of considering the basal cells as the main progenitor type in the epithelium relies on the notion of stem cells being poorly differentiated. Indeed, out of the main cell types, the basal cell does appear, morphologically, the simplest. This observation may be misleading as a complex system of junction adhesion structures (including keratin filaments, hemisdesmosomes and desmosomes) have been identified in the basal cells of a variety of mammalian species (Evans, 1989, 1990). The structure and composition of the junction adhesion molecules were found to vary according to animal age and cell height. These data therefore suggest that the basal cells are indeed differentiated and have functionality in the attachment of columnar cells to the basal lamina.

Kinetic Role Of Clara Cells

The ability of a cell to synthesise its own DNA is not necessarily an indication of proliferative capacity. Instead, it may just be that basal cells are maintaining their basal cell population. Evans *et al.* (1976b) studied the dividing cells of the rat bronchi using tritiated thymidine and identified about 28% of the labelled cells as basal cells and 45% were Clara cells and the rest unidentifiable. After a mild NO_2 exposure, the proportion of labelled cells that were Clara cells rapidly increased suggesting that the Clara cells played a more major role in the repair of epithelial tissue.

Kinetic Role of Secretory Cells

In vitro culturing of purified rat tracheal basal and secretory cells gave data suggesting that the secretory cell and not the basal cell was primarily involved in the maintenance of the normal tracheal epithelium (Johnson, 1990b). Rat tracheal cells were isolated by DNase treatment (over 95% of cells were found to be viable). The proliferative nature and capacity of the basal versus secretory cell types was analysed by measuring the proportion of each cell type undergoing cell proliferation and the colony forming capacity of the different cell types. 10% of the original cell suspension was found to be in S, G_2 or M phase (according to their increased per cell DNA content). Cells were separated by Flow cytometry using flow cytometric analysis of the cell suspensions involving 90° light scatter versus 2° (forward) light scatter. Two major cell populations were revealed showing distinct morphological and ultrastructural characteristics of basal cell type and secretory cell type. The

purity of the basal cell and the secretory cell type was 94±1% and 91±3% respectively. The remaining cells could not be identified as they showed no definitive characteristics (Johnson, 1990b). Of the cycling cells, >85% were found to be secretory cells. When the separate cell type groups were grown in serum free culture conditions, the secretory cell group were found to have a higher colony-forming efficiency (3.4%) than basal cells (0.6%). The authors suggest that as the secretory cells show a higher proliferative activity than the basal cells, secretory cells are the main cell type involved in epithelial maintenance.

A similar method and outcome was published by Johnson and Hubbs (Johnson, 1990a). Rat Basal cells purified to in excess of 94% and secretory (muco-goblet and Clara) cells purified to in excess of 92% (separated by forward angle and side scatter cytometry) were seeded into rat tracheal xenografts. 4 weeks after subcutaneous implantation, the xenografts were removed and the epithelium studied for cell type (by TEM) and epithelial height. Tracheal grafts inoculated with sorted basal cells resulted in the re-establishment of epithelial linings in 5 out of 10 experiments. The epithelium was composed of low cuboidal cells (76%) and prominent basal cells (24%). Less than 1% were secretory cells. This was in comparison to grafts re-seeded with purified secretory cells which re-established epithelium showing basal, secretory and ciliated cells (17.1%, 44.3%, 38.5% respectively). These results are slightly different to those in control xenografts which had been seeded with unsorted tracheal cells (21.0%, 28.8%, 50.2% respectively). Although the same problems of non-100% purity are encountered in this experiment, these data provide convincing evidence that in the rat trachea, the secretory cell is the main progenitor cell type and the basal cell has only limited progenitor capacity.

7.1

A Study Into The Cell Proliferation Index In Mouse Trachea

Introduction

A number of transgenic mice deficient in or completely without *cftr* expression have now been generated. Although these animals display the electrophysiological defect in the gastrointestinal and respiratory tract as seen in humans, the mice do not *spontaneously* develop lung disease (Porteous, 1993, Davidson, 1995, Davidson, 1998). In humans, Leigh *et al.* (1995) observed that the percentage of bronchial epithelial cell undergoing cell division (as determined by proliferating cell nuclear antigen (PCNA) nuclei immunostaining) was significantly higher in CF patients (17.0±4.6%) as compared to non CF patients (<0.2%). Infrequently, in both test groups, PCNA positive cells were recognised in the ciliated ducts of the submucosal glands. This increased cell proliferation index in CF patients is most likely to be a response to increased damage in of CF epithelial tissue.

An experiment was designed to determine whether a similar increased cell proliferation index (CPI) would be found in the CF mice as compared to wildtype animals. The Leigh study used tissues from patients who had either died as a consequence of lung disease or from patients receiving lung transplantation (Leigh, 1995). As CF mice do not die of lung disease, a direct comparison of tissue pathology was not possible. CF mice do however, show varying degrees of lung pathology according to their housing conditions and after receiving repeated doses of bacteria (Davidson,1995, Dorin, 1996b, Dorin, 1992a). Therefore, 3 different groups mice were used; those housed in a non Specific Pathogen Free facility (Biomedical

Research Facility (BRF)), those from a Specific Pathogen Free facility (Evan's Building) and those who had received either one month or two months repeated nebulisation with *S. aureus.*

An immunohistochemistry procedure for identifying proliferating cells was utilised. Proliferating cell nuclear antigen (PCNA) is a 36-kD nuclear protein that peaks in nuclear concentration during S phase of the cell cycle (Morris, 1989, Jonsson, 1997). Several commercially available monoclonal antibodies are now available against this antigen. Detection of PCNA does not depend on the incorporation of labelled nucleotides such as BrdU or tritiated thymidine.

Materials and Methods

Animals

Mice of 4 different genotypes, two different methods of housing and groups of mice which had received 2 different repeated nebulisation treatments with *S. aureus* (30 or 60 days: kind gift from Dr. Donald Davidson) were used in this experiment. Of the different genotypes, mice homozygous for the *cftr* insertional mutant $Cftr^{tm1HGU}$, heterozygous $Cftr^{tm1HGU}$ mice and wildtype littermates on a outbred mixed MF1/129 genetic background were used. In addition, compound heterozygote mice between the Edinburgh insertional mutant mouse and University of North Carolina CF "null" ($Cftr^{tm1HGU}/Cftr^{tm1UNC}$) also on the mixed MF1/129 genetic background were used.

This experiment aimed to directly compare the cell proliferation index of mice of different genotypes and environments. The mice were all between 5 and 6 weeks old. The mouse is a fully developed adult by week 4 of life. Due to a limitation in the availability of the transgenic animals direct comparisons between all these variable

cannot be made. Furthermore, the experiment used animals of both sexes. Full details of the animals and results are given in figures 7.1.1., 7.1.2 and 7.1.3.

PCNA immunohistochemistry

Mice were sacrificed by cervical dislocation and tracheas dissected out. Tissues were fixed in 4% paraformaldehyde and dehydrated and processed to wax blocks using the VIP tissue-tek processor. 6mm sections were cut and floated out on a 56°C water bath before transferring to a Vectabond coated slide.

Slides were dried out at room temperature overnight. Tissues were de-waxed and re-hydrated by transferring slides through 2x xylene for 10 minutes and a decreasing concentration of ethanol (2x 100%, 90%, 70%, 50%, 1 minute each) to PBS. All further incubation steps took place in a humidified chamber at room temperature. Tissues were immunologically blocked against non specific binding of the secondary antibody by incubating with donkey blocking solution (5% donkey serum (DAKO, Bucks, UK) , 1% fish gelatin (DAKO, Bucks, UK) 1% BSA (Sigma-Aldrich, Poole, UK) in PBST). Mouse monoclonal PC10 anti PCNA (Santa Crux biotechnology, CA, USA) was incubated at a concentration of 1:50 in blocking solution (1% fish gelatin (DAKO, Bucks, UK) 1% BSA (Sigma-Aldrich, Poole, UK) in PBST) for an hour. Tissues received 3 x 5 minute washes in PBST and further blocked for 20 minutes in donkey serum blocking solution. Tissues were incubated with 1:50 dilution donkey serum, 1:500 dilution biotin-SP-conjugated affinity purified donkey anti-mouse IgG (Jackson Immunological Research, ME, USA) in PBS for 1 hour prior to washing 3 times in PBST. Peroxidase conjugated streptavidin (Jackson Immunological Research, ME, USA) was incubated at a 1:500 concentration in PBS for one hour which binds with great affinity to the conjugated biotin of the secondary antibody. After 3 further 5 minute PBST washes, DAB solution (10mg DAB (Sigma, St. Louis, NJ) in 50 ml 0.0.5M, pH 7.6 TRIS-HCl activated with 30ml 30% H_2O_2 for

15 minutes) provided a substrate with a visual product. The DAB peroxidase reaction was stopped by washing slides in H_2O

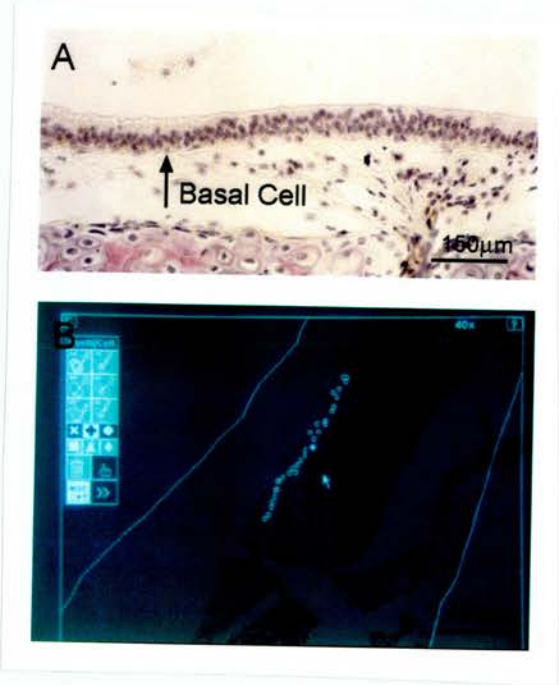
Tissues were de-hydrated by transferring slides through a increasing ethanol gradient to 100%. Tissues were dehydrated in xylene (2x10 minutes) and mounted with a coverslip using DPX. Slides were examined by brightfield microscopy in conjunction with the High Optimised Microscopy Environment (HOME) for presence of marked cells (Brugal, 1992). Basal cells were identified by having adherence to the basal lamina propria only, intermediate cells having cytoplasm which did not reach either the luminal surface of the epithelium or the basal layer and columnar cells whose cytoplasm reached the luminal surface only. Cell counting was conducted on the pseudostratified epithelium of the trachea. Cells were counted on one side of the trachea only (chosen at random). Cells were not counted where poor histology was encountered.

Results

See figures 7.1.1 for examples of immunohistochemistry and HOME counting system.
See figures 7.1.2 and 7.1.3 for numerical data and data analysis.

Figure 7.1.1

Examples of HOME Cell Counting System



7.1.1a Example of PCNA immunohistochemistry showing the presence of one labelled basal cell (labelled brown) in a section of tracheal epithelium.

7.1.1b Cells labelled positive for PCNA were counted using the HOME microscope system. This system interfaced a standard brightfield microscope through a computer interface permitting accumulation of data on computer files. The green filter was found to aid identification of the brown antibody signal against blue nuclear counterstain.

Figure 7.1.2

Description of mice used and PCNA immunological count data.

B=Basal cells, I=Intermediate cells, C=Columnar cells.

Ref	Geno-Type	Housing	Sex	Marked B Cells	Marked I Cells	Marked C Cells	Total Positive	Total Negative	Marked/ %	Average Marked / %	Error	Mean Error
1	+/+	BRF	f	18	0	0	18	699	2.5		1.1	
2	+/+	BRF	f	13	0	0	13	846	1.5		0.1	
3	+/+	BRF	f	7	0	0	7	706	1.0		0.8	
4	+/+	BRF	f	8	0	0	8	947	0.8	1.4	0.6	0.7
5	H/H	BRF	f	24	0	1	23	586	4.0		0.2	
6	H/H	BRF	m	33	0	0	33	574	5.4		1.2	
7	H/H	BRF	f	29	0	0	29	830	3.1	4.2	0	0.5
8	U/H	BRF	f	13	0	0	13	677	1.7		0.7	
9	U/H	BRF	f	3	0	0	3	469	0.6		0.4	
10	U/H	BRF	f	4	0	0	4	478	0.7	1.0	0.3	0.5
11	H/+	30 d	f	21	0	0	21	394	5.2		0.0	
12	H/+	30 d	f	45	0	0	45	515	3.1		2.1	
13	H/+	30 d	f	18	0	0	18	441	4.1		1.1	
14	H/+	30 d	f	72	0	0	72	795	8.3	5.2	3.1	1.6
15	H/H	30 d	f	41	0	0	41	484	7.8		4.3	
16	H/H	30 d	f	9	0	0	9	635	1.3		2.2	
17	H/H	30 d	f	10	0	0	10	683	1.4		2.1	
18	H/H	30 d	f	17	0	0	17	584	2.8		0.7	
19	H/H	30 d	f	32	0	0	32	687	4.4	3.5	0.9	2.0
20	+/+	60 d	f	10	0	0	10	194	4.9		1.4	
21	+/+	60 d	f	20	0	0	20	447	4.2		0.7	

22	+/+	60 d	f	9	0	0	9	678	1.3	3.5	2.2	1.4
23	H/+	60 d	f	26	0	0	26	777	3.2		7.7	
24	H/+	60 d	f	97	0	0	97	394	18.5	10.9	7.6	7.7
25	H/H	60 d	m	34	0	0	34	891	3.7		0.2	
26	H/H	60 d	m	16	0	0	16	581	2.6		0.9	
27	H/H	60 d	m	13	0	0	13	640	1.9		1.6	
28	H/H	60 d	m	53	0	0	53	891	5.6	3.5	2.1	1.2
29	+/+	SPF	m	13	0	0	13	582	2.2		0.0	
30	+/+	SPF	m	26	0	0	26	766	3.2		1.0	
31	+/+	SPF	m	4	0	0	4	483	1.0		1.2	
32	+/+	SPF	m	11	0	0	11	396	2.7	2.2	1.5	1.2
33	H/+	SPF	m	11	0	0	11	489	2.2		0.4	
34	H/+	SPF	m	7	0	0	7	466	1.4		0.4	
35	H/+	SPF	m	10	0	0	10	528	1.9	1.8	0.1	0.3
36	U/H	SPF	f	5	0	0	5	223	2.2		0.9	
37	U/H	SPF	m	26	0	0	26	579	4.3	3.1	1.2	1.1
38	H/H	SPF	m	23	0	0	23	596	3.7	3.7	N/a	N/a

Housing notation: SPF = Specific pathogen free facility (Evan's Building)

BRF = Biomedical research facility (non Specific Pathogen free facility)

30 d= 30 day repeated nebulisation with *S. aureus*. 60 d= 60 day repeated nebulisation with *S. aureus* .

Genotype nomenclature: H/H = mouse homozygous for the insertional CFTR mutation *Cftr*^{tm1HGU} on a mixed MF1/129 background. H/+ = mouse heterozygous for the insertional CFTR mutation *Cftr*^{tm1HGU} on a mixed MF1/129 background. +/+ = mixed MF1/120 genetic background littermate of the above transgenic animals

Figure 7.1.3

CPI data and error analysis summary

Percentage of PCNA nuclei marked cells presented in each test group with mean error (in brackets). Number of animals in each group (N) is also shown.

	+/+ / %	H/+ / %	H/H / %	U/H / %
non SPF	1.4 (0.7) N=4	/	4.2 (0.5) N=3	1.0 (0.5) N=3
SPF	2.2 (1.2) N=4	1.8 (0.3) N=3	3.7 (-)N=1	3.1 (1.1)N=2
30 day	/	5.2 (1.6)N=4	3.5 (2.0) N=5	/
60 day	3.5 (1.4) N=3	10.9 (7.7)N=2	3.5 (1.2) N=4	/

Statistical analysis between test groups

Data from each test group was compared to each other group using the unpaired t-test method.

The only differences of statistical significance ($p<0.05$) were:

Non SPF H/H versus Non SPF wildtype: p=0.0128
Non SPF H/H versus Non SPF U/H : p=0.0138

Discussion

The results from this experiment can be discussed from two different viewpoints: that of the relationship between genotype and environmental conditions on tracheal CPI of mice and secondly, what cell types were observed to be undergoing division.

Great variation in the CPI was found within each test group. This, in addition to the non comprehensive N number cover of some groups makes direct comparison between the groups difficult.

It was noted that the CPI of the $Cftr^{tm1HGU}$ / $Cftr^{tm1HGU}$ non SPF group is significantly higher (p=0.0128) than the wildtype animals in the same group. As exciting as this results is, the possible relationship between *cftr* expression and CPI is not maintained. The $Cftr^{tm1UNC}$/ $Cftr^{tm1HGU}$ animals should have approximately half the *cftr* expression of the $Cftr^{tm1HGU}$ animals ($Cftr^{tm1UNC}$ animals are CF nulls and $Cftr^{tm1HGU}$ animals express between 5-10% of wildtype signal (Dorin, 1992a, Snouwaert, 1992)). However, there is no significant difference between wildtype animals and $Cftr^{tm1UNC}$ / $Cftr^{tm1HGU}$ compound heterozygotes. Indeed, the $Cftr^{tm1UNC}$ / $Cftr^{tm1HGU}$ non-SPF animals were shown to have a significantly higher CPI than their $Cftr^{tm1UNC}$ / $Cftr^{tm1HGU}$ counterparts. Furthermore, in the groups that had received bacterial instillations, the heterozygote animals showed higher CPIs than either CF homozygotes or wildtypes. Therefore, it is difficult to argue a credible case for a genotypic effect on mouse CPI.

The CPI of the mice were found generally to be low with only 4 mice out of 38 showing a CPI of >5%. These mice were all found in the *S. aureus* groups suggesting greater cellular turnover hence, a greater degree of epithelial damage in these animals. It is interesting to note that these animals are deficient in *cftr* (2 are

Cftr^{tm1HGU} /+ and 2 are *Cftr*^{tm1HGU} / *Cftr*^{tm1HGU}) perhaps suggesting an increased susceptibility to progression of lung damage as observed in Davidson *et al.* (1995). However, it can be equally argued that mice in the same group showed lower CPIs than wildtype mice in the SPF group. It would be of obvious interest to repeat this study at a time when sufficient mice stocks existed, to remove the areas of possible error as outlined above.

Although that this experiment does not show a relationship between CFTR deficiency and raised CPI in the mouse as was seen in the Leigh paper (1995) it is worthwhile repeating that the CF tissue used in that study was obtainable only because the patients had either died as a consequence of lung disease or had received a lung transplant. The high CPI in these subjects was therefore to be expected and probably represent CPI towards the extremity of possible results.

Beyond the original aims of this experiment, it is very interesting to note that of the cells marked as positive for reaction with PCNA over 99% of them are basal cells. These data do not directly relate to the question of epithelial kinetics as it portrays a single steady state measurement. The role of PCNA in DNA synthesis is still largely not known (Jonsson, 1997) but is known to show a 2-3 time increase in concentration during DNA synthesis (Morris, 1989). The decrease of which, in the G2 phase, has been reported to take up to 18 hours (Prelich,1987). It would seem likely then that both daughter cells should show a positive reaction with PCNA for some time. No data backing up this hypothesis was produced in this study.

It is interesting (and perhaps surprising) to note that the positively marked basal cells do not occur adjacent to positively marked columnar cells. This may infer that basal cells do not divide to produce any other cell type but more basal cells as suggested by McDowell (1985b).

To further study the kinetic relationship between cell types in the mouse trachea, a more long term BrdU labelling experiment was designed.

7.2

Steady State Kinetics Of The Murine Tracheal Epithelium

Introduction

There is, at this time, no *in vivo* method for following the fate of individual cells through time. Instead, it is possible to make a projection of the fate of individual cell types by following the spread of an incorporated nuclear label. Breuer *et al.* (1990) used such a method to study the cell kinetics of the adult hamster bronchial epithelium. 7 groups of hamsters were sacrificed at 1 hour and 1,2,3,4,7 and 14 days after an intraperitoneal injection of [^{3}H] thymidine. Autoradiograms of bronchial tissue was prepared. Cells were classified into 7 categories and the number of labelled and unlabelled cells counted. The cell categorisation was as follows: basal type 1 (B_1) and basal type 2 (B_2), depending on nuclear height; secretory cells which were described as S type 1 (with zero to four secretory granules: possibly Clara cells), S type 2 (5 or more granules with intervening cytoplasm) or S type 3 (where the cytoplasm was completely filled with granules); ciliated cells and indeterminate cells. At 1 hour after injection, labelling (as a percentage of unmarked cells of the same type) was highest for the B_1 and B_2 type cells while the most commonly stained cell (as a percentage of all unmarked cell types) was the S type 1 cell. With time, the fraction of labelled B_1 and B_2 cells decreased whereas the percentage of S type 2, ciliated and intermediate cells increased. The fraction of S types 1 and 3 did not change. These results suggests that the basal cells and the secretory type 1 cell contribute the most to the processes of self renewal in the steady state hamster bronchus.

The potential errors and statistical instability arising from cell identification in this paper must be born in mind when regarding the paper's conclusions. The potential

errors and statistical instability are described above (e.g. the authors are "assuming contamination" of the identification of the basal cell type by 50%). The paper does not show any figures of the autoradiographs. Furthermore, regression projections are made through the 14 day period for all their results which does not take into consideration non linearity. This is particular hazardous considering the very low levels of labelled cells which are being considered.

An experiment was designed to repeat the study done by Breuer *et al.* (1990) but trying to avoid the areas of potential uncertainty as outlined above. At the upper level of safe cell type identification, only 4 cell type groupings were identified; basal, ciliated, non-ciliated columnar, intermediate. Linear regression of data was not conducted.

Materials And Methods

Thirty five 6 week old male mice on an outbred MF1 genetic background weighing approximately 25g were obtained from Charles Rivers Ltd and separated randomly into 7 groups of 5 animals. BrdU labelling and immunohistochemistry was conducted according to and using the contents of the Boehringer Mannheim "*In situ* Cell Proliferation kit, AP". Mice in 6 of the groups received a 1ml IP injection of 10mM BrdU in PBS per 100g of mouse (approximately 1mg dose per mouse) whilst group 7 was left as a negative control. Mouse groups were left for between 1 hour and 14 days (as shown in figure 7.2.1) prior to sacrifice by 0.5ml Euthatal (Rhone Merieoux, UK) IP injection. Trachea and duodenum tissue was dissected out and fixed in 10% buffered formalin for 6hrs at +4°C prior to processing to wax blocks. 4 µm sections were cut and floated out on a 56°C water bath onto Vectabond coated slides. Slides were dried over night at 37 °C.

Tissues were de-waxed and re-hydrated by transferring slides through 2 x xylene for 10 minutes and a decreasing concentration of ethanol (2x 100%, 90%, 70%, 50%, 1 minute each) to PBS. All incubation steps took place in a humidified chamber at room temperature unless otherwise directed. Slides were transferred to a large beaker containing excess PBS and the beaker covered with cling film prior to heating using a microwave oven at high power for 3 x 5 minute intervals. Slides were left to cool in clean PBS.

Slides were incubated with a 1:4 dilution of anti-BrdU-AP antibody in incubation buffer (Boehringer Mannheim) for 30 minutes at 37°C in a humid chamber. Slides were washed in PBS for 3x 5 minutes. Substrate solution was prepared by dissolving one substrate table(bottle 4) in 2 ml of substrate buffer (bottle 5) and shaken for 5 - 10 minutes until dissolved. Slides were incubated in substrate buffer for 15-30 minutes or until colour develops. Slides were mounted in glycerol mountant (10g gelatine, 60ml dH_2O, 70ml glycerol, 0.25 phenol) and dried overnight at room temperature prior to viewing with bright field Nomarski microscopy with a Zeiss Axioplan microscope.

The number of labelled and unlabelled cells of each cell type was counted. One side of the trachea was chosen at random an counting was started at the proximal end of the trachea where the pseudostratified epithelium begins. Cells were not counted where poor histology was encountered. Cell types were defined as: Basal, cuboidal cells with basal membrane attachment only; intermediate cells, cells with neither basal or apical membranes; ciliated cells, columnar cells with cilia visible on apical surface; non ciliated cells, columnar cells reaching apical surface but with no cilia present.

Figure 7.2.1

Design Of Single Pulse Chasing Experiment. Times Of Sacrifice After BrdU Injection.

Groups number	BrdU dose	Time between injection and sacrifice
1	1mg IP	1hr
2	1mg IP	3hr
3	1mg IP	24hr
4	1mg IP	72hr
5	1mg IP	168hr
6	1mg IP	336hr
7	-ve control	n/a

	336.2	645	807	11	1.7			1NC	0.1		
	336.3	592	677	10	1.6			0	0		
	336.4	125	155	2	1.5			0	0		
	336.5	208	341	4	1.9	1.6	0.2	0	0	0.1	0.1
7	c.1	465	866	0	0			0	0		
	c.2	481	621	0	0			0	0		
	c.3	384	597	0	0			0	0		
	c.4	166	245	0	0			0	0		
	c.5	277	366	0	0	0.0	0.0	0	0	0.0	0.0

Table Abbreviations:

B= Basal Cell Type

B^M= Marked (labelled) Basal Cell Type

I = Intermediate Cell Type

NC= Non Ciliated Columnar Cell Type

C= Ciliated Columnar Cell Type

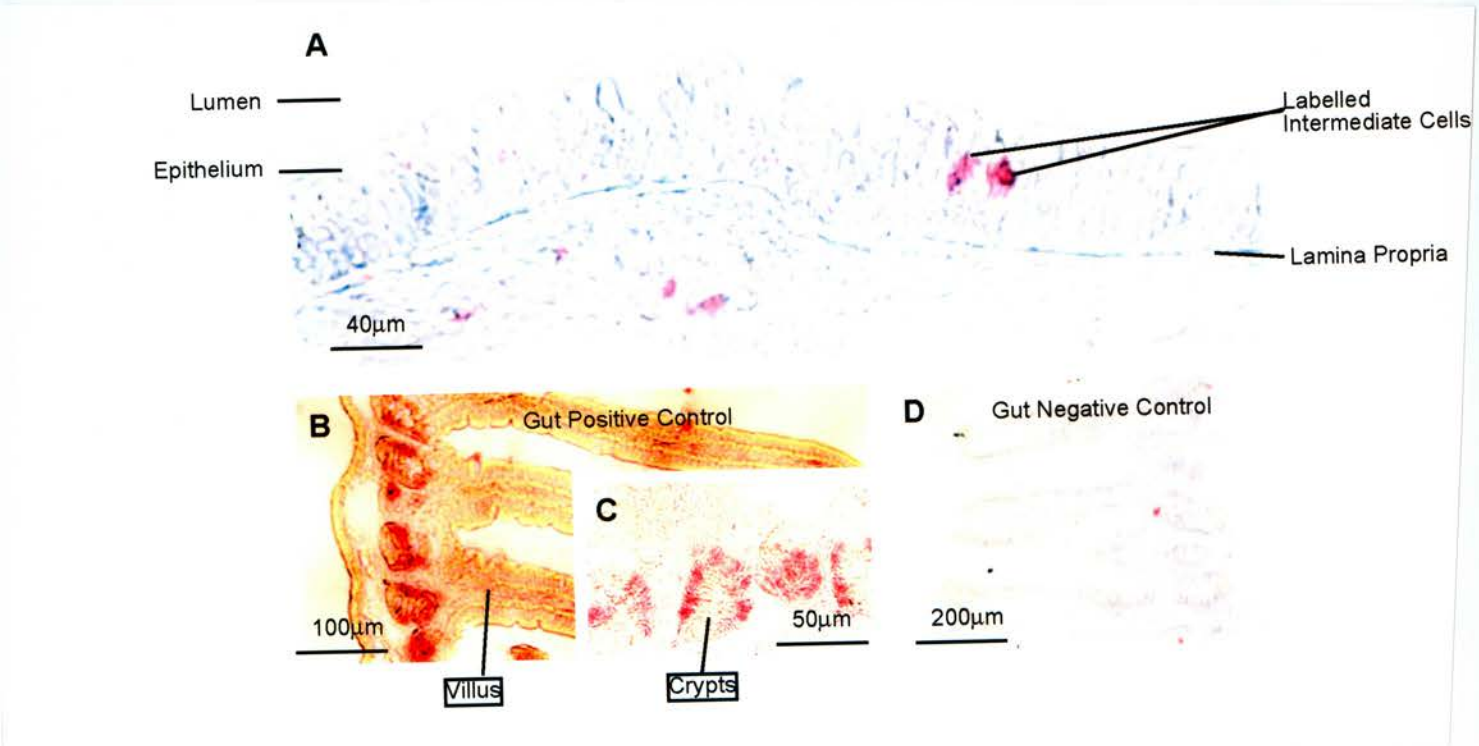
Results

Examples of immunohistochemistry data are displayed in figure 7.2.2. Numerical data is presented in figures 7.2.3 and 7.2.4.

Figure 7.2.2

Incorporated BrdU detection by anti-BrdU-AP Immunohistochemistry.

An Alkaline phosphatase bound anti BrdU antibody and a neutral-red endpoint was used to visualise nuclear incorporated BrdU.



7.2.2A Presence of 2 labelled intermediate cells 336 hours after mouse BrdU dosing.
7.2.2B + C Positive control slide (small intestine 1 hours after BrdU dosing) showing presence of labelling in the small intestine crypts but not on the villi.
7.2.2D Negative control slide showing anti-BrdU immunohistochemistry on small intestine tissue which had not received BrdU dosing. Occasional non cellular neutral red crystals were noted.

Figure 7.2.3

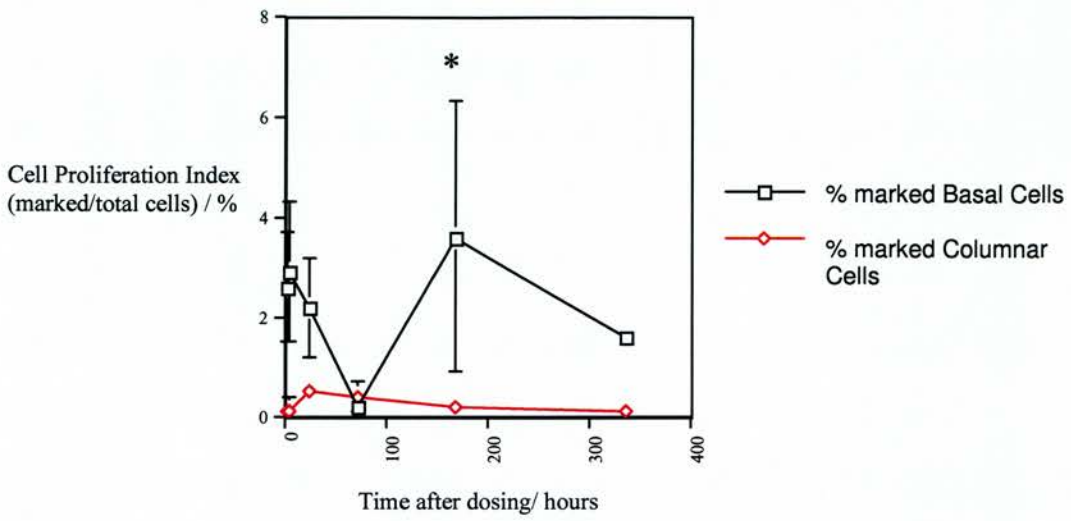
Number Of Marked Basal And Non Basal Cells As A Percentage Of Total Cells At Different Times After IP BrdU Pulse Injection.

See base of table for abbreviations.

Gp.	Mouse	B	Non B	B^M	B^M / B (%)	Mean B	Mean Error	Marked Non B Cells	B^M/ Non B / %	Mean Non B	Mean Error
1	1.1	534	619	14	2.2			0	0		
	1.2	369	691	8	2.2			0	0		
	1.3	321	390	17	5			0	0		
	1.4	419	612	5	1.1	2.6	1.1	1NC	0.2	0.1	0.1
2	3.1	259	334	4	1.5			0	0		
	3.2	250	329	3	1.1			0	0		
	3.3	562	731	15	2.6			0	0		
	3.4	845	972	25	2.8			2x C	0.2		
	3.5	145	201	10	6.4	2.9	1.4	2x C	1	0.1	0.3
3	24.1	621	795	24	3.7			7x NC	0.9		
	24.2	431	519	7	1.5			1x NC	0.2		
	24.5	459	624	7	1.5	2.2	1.0	2x NC	0.3	0.5	0.2
4	72.1	560	817	0	0			0	0		
	72.2	376	451	2	0.5			1C.	0.4		
	72.3	290	341	3	0.1	0.2	0.2	1 C	0.8	0.4	0.3
5	168.1	449	581	4	0.8			0	0.0		
	168.2	450	672	27	5.9			1 C,1 I, 1 NC	0.4		
	168.3	311	371	7	2.2			1I	0.2		
	168.4	684	792	12	1.7			2C	0.3		
	168.5	37	120	3	7.5	3.6	2.7	0	0	0.2	0.1
6	336.1	974	1213	11	1.1			3x I	0.2		

Figure 7.2.3

Data from BrdU single dose experiment



Graph displays data obtained from the single pulse BrdU tagging experiment. Data at different time points was obtained from mean CPI from groups of animals (n=3-5) from cell counts of >1000 cells per animal. Error bars show mean standard error. Asterisk denotes statistical difference between time point and the preceding time point to <0.05 probability by unpaired student's t-test.

Discussion

At 14 days (336 hours) after the initial labelling, a subpopulation of epithelial cells were observed to retain their BrdU labelling characteristics. This included 1.6% (mean error 0.2%) of basal cells and 0.1% (mean error 0.1%) of non basal cells (these cells were identified to be of non ciliated or intermediate cell type). The label retaining property of the non basal cells is not statistically valid however, the presence of labelled basal cells 336 hours infers that (when viewed as a static time point) at the very least, the basal cells have a slow rate of cellular turnover.

Basal cells show a higher frequency of BrdU labelling than non basal cells at all time points. The data presented in graph 7.2.3 shows a two phase cycling of levels of BrdU labelling peaking firstly at 1 hour after labelling (mean basal cell labelling = 2.9% mean error = 1.4%), dropping to a mean of 0.2% (mean error =0.2%) at 72 hours then peaking again at the next time point, 168 hours at 3.6% (mean error 2.7%) before dropping to 1.6% labelling (mean error 0.2%) at 365 hours.

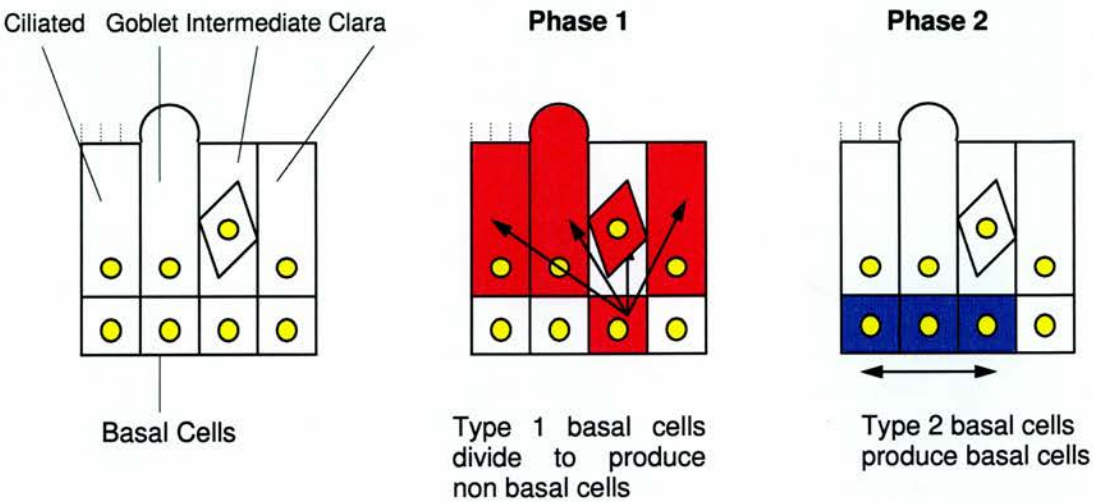
An increase in the labelled percentage of cells can only occur if previously labelled cells divide and thereby, give rise to more labelled daughter cells. Therefore, the second phase of increased labelling is likely to be as a consequence of basal cells marked in the first phase undergoing cell division. Only one non basal cell was labelled in the 1 hour time point. The infrequency of dividing non basal cells suggests that these cells are either end point cells or have an extremely slow rate of cellular turnover. It is more likely that the first theory is true as the non basal cells would have to have an near infinitely long cell cycle for only 1 cell out of 2312 cells counted to be dividing at the time of BrdU injection. Furthermore, it is interesting to note that this bi-phasic pattern of basal cell labelling is associated with an slight increase in the number of labelled non basal cells at 24 hours. This time point coincides with the trough of basal cell labelling. The apparent discrepancy between

the number of dividing basal cells and the number of non basal cells produced is compensated for differences in the cellular frequency (basal cells only contribute between 5 and 10% of cell types in the mouse epithelium (Pack,, 1981) and cell death of columnar cells during the experimental duration. This increase in non basal labelling suggests that new non basal cells are being produced from the labelled basal cells.

A kinetic model for this pattern is presented in figure 7.2.5. Two types of basal cells exist in this model. Type 1 basal cells act as progenitor cells to new non basal cells. Type 2 cells have a longer cell cycle than type 1 and act to maintain the population of basal cells.

Figure 7.2.5

Bi-phasic Model Of Epithelial Kinetics In The Steady State



Although data analysis on results of small quantitative size is always inherently fragile there is external evidence that firstly, two types of basal cells exist and secondly, bi-phasic patterning of the epithelial kinetics occurs.

The evidence for there being two types of basal cells is provided by the morphological evidence of basal cells in cell suspension as reported by Inayama *et al.* (Inayama, 1989). Cells suspensions of basal cells were produced by the pronase digestion of rabbit tracheas and subsequent purification by centrifugal elutriation. This method resulted in basal cell purity of between 92 and 94%. TEM analysis of purified basal cells revealed two morphologically difference types of basal cells. The most common type (91% of cells) was small (6.7±0.6μm) and exhibited a high nucleus to cytoplasm ratio with electron dense cytoplasm, irregular shaped nucleus and conspicuous perinuclear tonofilaments. The second type of basal cell (9%) was slightly larger (8.1±0.4μm), had a round nucleus with few indentations and less abundant heterochromatin and the cytoplasm was less electron dense.

This experiment was modelled on the study conducted by Breuer *et al.* (1990). Data on percentage labelled cells against time was presented as mean value and regression lines (see figure 7.2.6a). Re-examination of these data show that a very similar bi-phasic pattern of basal cell labelling is present (see figure 7.2.6b). However, discussion of this pattern was not presented in the paper.

These data presented in this section suggests that in the steady state mouse tracheal epithelium, basal cells acts as the primary progenitor cell population. Basal cells are of two types, the first a fast cycling population which divide to produce new non basal cells and a second population which cycle slower and divide to maintain the basal cell population. This second population of basal cells show BrdU labelling characteristics of stem cells.

Figure 7.2.6

Evidence for Bi-phasic model after Breuer *et al.* (1990)

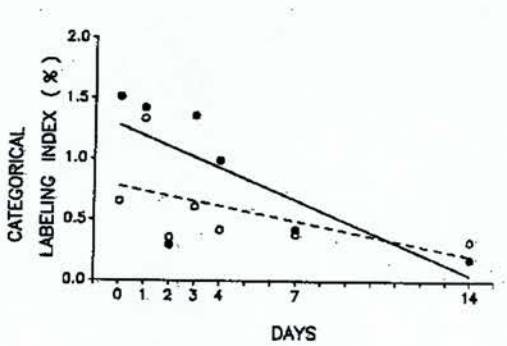


Figure A

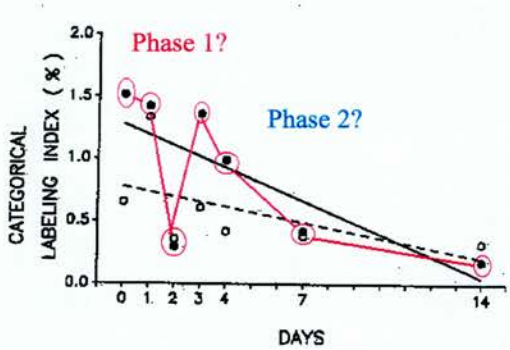


Figure B

Labelling index analysed over time for "B-1" cells (solid line and circles) and "B-2" cells (dotted line and open circles) after a single injection of tritiated thymidine. B-1 and B-2 cells were categorised as cells having nuclei adjacent to basement membranes with B-2 cells being larger than B-1 cells. No histological examples for these cell types were presented in the paper. Compare figure B to the pattern observed in figure 7.2.3.

7.3

A Study Of Label Retaining Cells In The Mouse Tracheal Epithelium

Introduction

According to the Potten and Smith model of stem cell behaviour (Potten, 1988) if a population of epithelial cells are labelled at the time of cellular division, the stem cells will retain their label for a longer duration than other cell types. This non-functional assay has been used in a number of different tissues to identify stem cells (Lavker, 1982, Potten, 1988, Bickenbach, 1981, Potten and Loeffler, 1990). An experiment was designed to examine whether cells could be found in the mouse trachea that exhibited BrdU label retaining characteristics. Due to the slow rate of cellular turnover in the mouse trachea it was unlikely that a single pulse dose of BrdU would results in any stem cells being labelled for reasons discussed above. Therefore, to make such an experiment practically possible, mice received repeated tracheal damage (to increase stem cell recruitment) and repeated BrdU delivery.

Materials and Methods

Animals

Male CD1 mice (22-25g at start each experiment) from Charles Rivers Laboratories were used throughout these experiments.

Damage by poloxethylene 9 lauryl ether (polidocanol) instillation.

Polidocanol (Sigma, St. Louis, MO) was diluted in sterile PBS to 2 % w/w. Mice were anaesthetised with 70µl IP injection of 100mg/ml Ketamine: 100mg/ml Xylazine in PBS (2:3:5 ratio v/v) and placed in a tracheal instillation frame as

described in Ho and Furst (1973).10 µl polidocanol solution was slowly instilled by 24Gx3/4" catheter to the proximal region of the trachea. This protocol had a 100% survival rate.

Damage by sulphur dioxide inhalation.

Mice were received 3, 3.5, 4 or 4.5 hours doses of sulphur dioxide at a concentration of 500ppm. This protocol was performed under guidance and assistance by the Environmental Protection Agency facility in Research Triangle Park, NC 27711, USA.

Assay Of Tracheal Damage By Polidocanol Or Sulphur Dioxide.

The effect of damage to the mouse trachea by polidocanol instillation or sulphur dioxide inhalation was examined. 9 mice received polidocanol damage and 6 mice received sulphur dioxide damage (3 hours at 500ppm). Of these animals, 3 of the polidocanol animals were sacrificed 10 minutes after damage, 3 from each damage group were sacrificed 2 hours after cessation of damage protocol whilst remaining animals were sacrificed 23 hours after damage. Tracheas were dissected out, fixed in omnifix and examined by H&E counterstained histology as outline above. Controls of air inhalation animals and PBS instillation animals were run in tandem.

Results

Pathology Of Damage By Polidocanol And Sulphur Dioxide

Damage to the mouse trachea by polidocanol instillation and sulphur dioxide inhalation was assayed by H&E histology. The results are shown in figure 7.3.1.

Figure 7.3.1

Mouse Trachea After Damage By Polidocanol Instillation Or Sulphur Dioxide Inhalation.

Polidocanol Damage

10 minutes after Polidocanol damage, the columnar cells are seen to have been partially stripped from their adhesion to basal cells (figure 7.3.1A).

2 hours after polidocanol damage the entire layer of columnar cells was observed to have been removed from the mouse trachea from just beneath the larynx to as far as the carina. This damage was observed from just beneath the top of the trachea (figure 7.3.1B complete trachea and at higher power 7.3.1C) to the base of the trachea. In the regions affected, basal cells and the lamina propria were still present and had been exposed to the lumenal air (figure 7.3.1D).

24 hours after polidocanol damage a thick layer of nucleated material existing in the previous epithelial location (figure 7.3.1E)

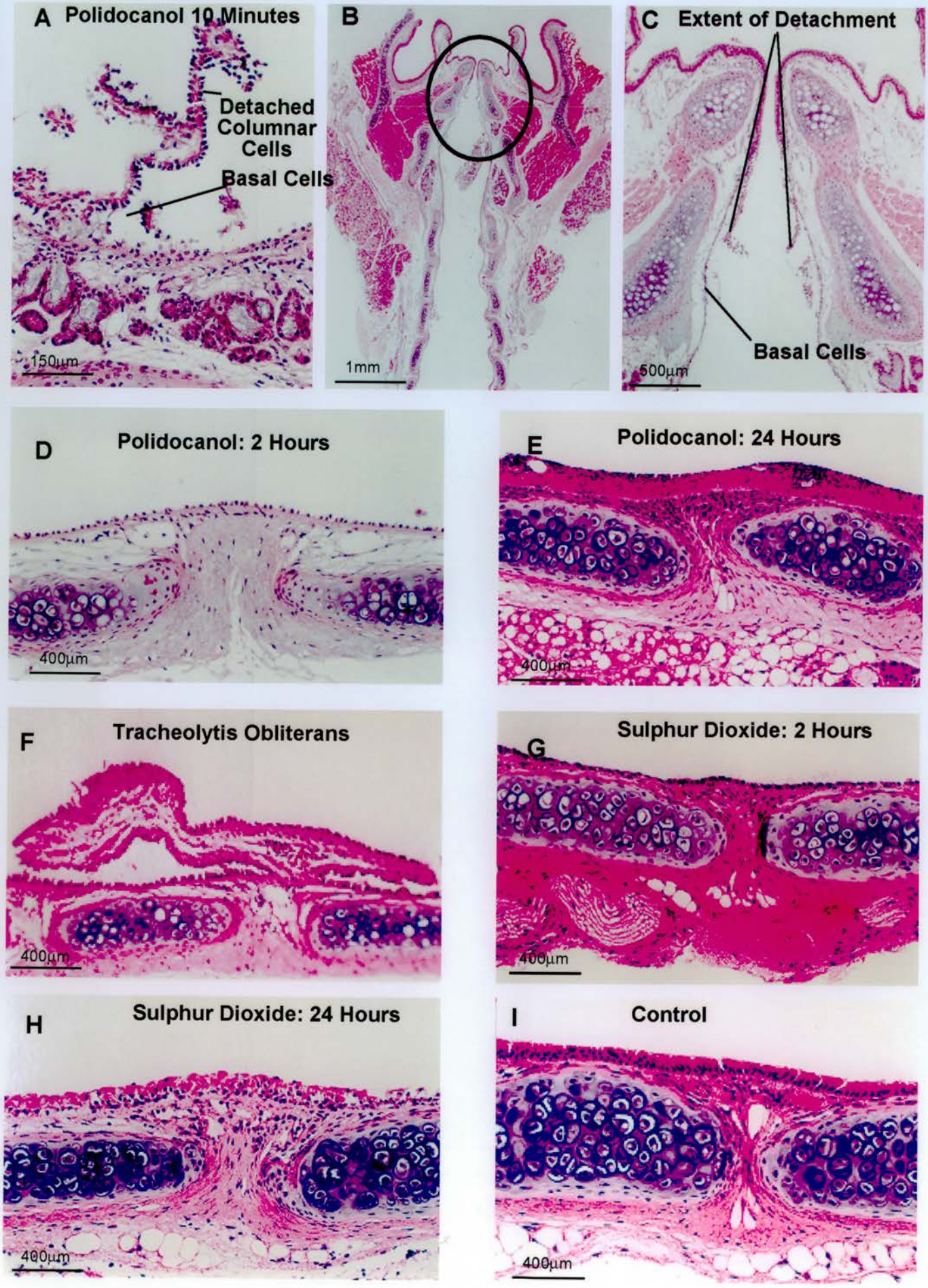
At later time points (>7 days), epithelial architecture was seen to have been completely restored. In a low number of cases, extreme tracheolytis obliterans (gross metplasia of the epithelial tissue produces an outgrowth of epithelial and mesenchymal tissue) pathology was also noted (figure 7.3.1F showing extreme epithelial pathology 12 days after polidocanol damage).

Sulphur Dioxide Damage

2 hours after the cessation of damage by Sulphur dioxide inhalation, columnar cells of the mouse tracheal epithelium appear diffuse and irregular in shape (figure 7.3.1G).

24 hours after sulphur dioxide damage the epithelium appears to be composed of poorly differentiated cells containing a high nuclear:cytoplasmic ratio (figure 7.3.1H).

Figure 7.3.1I Control slide showing tracheal epithelium from undamaged mouse.


A Polidocanol 10 Minutes
Detached Columnar Cells
Basal Cells
150µm
B
1mm
C Extent of Detachment
Basal Cells
500µm
D Polidocanol: 2 Hours
400µm
E Polidocanol: 24 Hours
400µm
F Tracheolytis Obliterans
400µm
G Sulphur Dioxide: 2 Hours
400µm
H Sulphur Dioxide: 24 Hours
400µm
I Control
400µm

Effect of Damage To The SMGs

7.3.1J Control SMG from undamaged mouse

7.3.1K SMG 36 hours after Polidocanol damage. Note dilation of the collecting and secretory ducts and the narrowing of the ciliated duct on reaching the lumen. This constriction proved to be a difficulty in obtaining histological sections showing the top of the ciliated duct (narrowing of the duct reduces the ductal opening area and therefore the chance of cutting a thin section through this area is reduced).

7.3.1L SMG 36 hours after Sulphur dioxide damage. Gross dilation of the ciliated and collecting duct were seen to occur with a widening of the ciliated neck.


J
Control
150µm
150µm
Polidocanol Damage
K
Epithelium
Ciliated Duct
Collecting Duct
Cartilage
Serous and Mucous Cells
150µm
Sulphur Dioxide Damage
L

An Investigation into Using Osmotic Pumps to deliver BrdU

Material and Methods

BrdU has a short unincorporated half-life in an *in vivo* environment. This property results in single injections producing a spiked dose rather than a steady bathing effect. Although this fast rate of *in vivo* degradation is highly desirable for single time point experiments it is less so for long term dosing experiment. To overcome the potential problem of spike dosing the mice with BrdU, the use of osmotic pumps were investigated.

Alzet osmotic pumps (Alzet, Palo Alto, CA). were used to provide continuous dose delivery of BrdU to mice. Each pump was loaded with 200µl 50 mg/ml BrdU solution (distilled H_2O, 1% NH_4OH, pH=8.0), immersed in saline solution at 37°C for 4 hours and implanted subcutaneously to Ketamine/Xylazine anaethnetised mice according to the suppliers guidelines.

To assess the rate of BrdU delivery versus detection by immunohistochemistry, 4 different types of pumps were used delivering doses of 12.5µg/hr (pump model 2004), 27.0µg/hr (pump model 2002), 47.5µg/hr (pump 2001) and 372.6µg/hr (pump 2001d) BrdU over a 24 hour period. After this time, the mice were sacrificed with lethal IP injection of "Sleepaway" (Fort Dodge Labs, Iowaalthough).

The gut was used to assay the level of BrdU incorporation due to its well-characterised pattern of cellular division and easily recognisable histology. 20mm duodenum adjacent the stomach sphincter was dissected out, flushed out with NBF of omnifix (Fisher, Fair Lawn, NJ.) and fixed overnight in NBF or omnifix at +4°C

before processing to wax blocks. 6μm sections were cut and incorporation of BrdU assayed by immunohistochemistry.

BrdU Immunohistochemistry

6μm paraffin sections of control or test tissues were mounted on pre-coated microscope slides.

Sections were dewaxed with xylene and rehydrated through a decreasing concentration gradient of ethanol. Incorporated BrdU was antigenically exposed by incubating the slides in 4M HCl for 20 minutes and trypsin: EDTA solution (0.1% trypsin type III (Sigma T-0646) 1mM EDTA in Dulbeccos PBS). Two different sets of antibodies were used:

Set 1:

Tissues were immuno-blocked in 5% Goat serum, 1 % fish gelatin, 1% BSA in PBST (Sigma, St. Louis, NJ.) and incubated with 1:10 dilution of FITC anti BrdU (Boehringer Mannheim, Lewes, UK) Antibody binding was visualised using a 1:2000 dilution of Rabbit anti FITC (Boehringer Mannheim, Lewes, UK) in PBS and a 1:200 dilution of peroxidase goat anti rabbit (Jackson laboratories, ME, USA). See below for visualisation protocol.

Set 2:

Tissues were immuno-blocked in 5% rabbit serum, 1 % fish gelatin, 1% BSA in PBST (Sigma, St. Louis, NJ.) overnight at 4°C and incubated with 1:200 dilution of sheep anti BrdU Antibody (Fitzgerald labs, FL, USA) binding was visualised using a 1:200 dilution of rabbit anti streptavidin (Jackson laboratories, ME, USA) and a

1:1000 dilution of horse radish peroxidase- streptavidin (Jackson Laboratories, ME, USA)

To visualise the signal, slides were incubated with DAB solution (10mg DAB (Sigma, St. Louis, NJ) in 50 ml 0.0.5M, pH 7.6 Tris-HCl activated with 30ml 30% H_2O_2 for 15 minutes. Slides were washed in dH_2O and counterstained in 1% methyl green in water for 15 minutes. Slides were dehydrated back to xylene and mounted in DPX.

Results

Quantification Of BrdU Immunohistochemistry (Pumps And Fixation Methods)

Alzet osmotic pumps can be used *in vivo* to provide a continuous dose of chemicals into laboratory animals. The potential for using these pumps to deliver a continuous dose rate of BrdU over a 22 day period was investigated. Additionally, two methods of tissue fixation (omnifix or NBF) were assayed. 4 different models of pumps were investigated, as outlined in figure 7.3.2 using detection of incorporated BrdU in the duodenum as an end point.

Figure 7.3.2.

Dose Rate And Duration Of Activity Of Varying Models Of The Alzet Osmotic Pumps.

Model	Pump life time/ days	Mean pump rate / $\mu l\ hr^{-1}$	SD	BrdU / $mg\ ml^{-1}$	Dose/ $\mu g\ hr^{-1}$	Dose per 24hrs/ μg
2001D	1	7.45	0.25	50.0	372.5	8940
2001	3	0.98	0.04	50.0	47.5	1140
2002	14	0.54	0.02	50.0	27.0	648
2004	28	0.25	0.02	50.0	12.5	300

Pumps were prepared and loaded as described above and implanted for 24 hours prior to sacrifice. Duodenum were fixed in either 10% NBF or omnifix and prepared for BrdU visualisation by immunohistochemistry. Set 1 of antibodies was used in this experiment. Pumps and detection methods were assayed for staining visualisation by light microscopy. See figures 7.3.3 and 7.3.4.

Figure 7.3.3

Immunohistochemistry Results For BrdU Detection After Pump Dosing For 24 Hours Using Different Pump Models And Different Fixation Methods.

7.3.3A Set 1 antibody used on mouse small intestine which was sacrificed and fixed in Neutral Buffered Formalin (NBF) 1 hr after the injection of 2mg BrdU in PBS, 1% NH_4OH. Cells in the crypts observed react with DAB (brown colour). Tissue counterstained in methyl green (green/blue colour).

7.3.3B As with 7.3.3A but tissue fixed in Omnifix. Note higher clarity and intensity of signal yet slightly higher cytoplasmic background.

7.3.3C Omnifixed small intestine from a mouse which had carried a 2001d Alzet osmotic pump filled with BrdU (200µl 50mg/ml) for 24 hours. High intensity of staining in the crypts.

7.3.3D As with 7.3.3C but using pump model 2001. Fainter signal than with pump model 2001d.

7.3.3E As with 7.3.3C but using pump model 2002. The results from using this protocol, as with pump 2004 (result not shown) resulted in no measurable sign of BrdU.

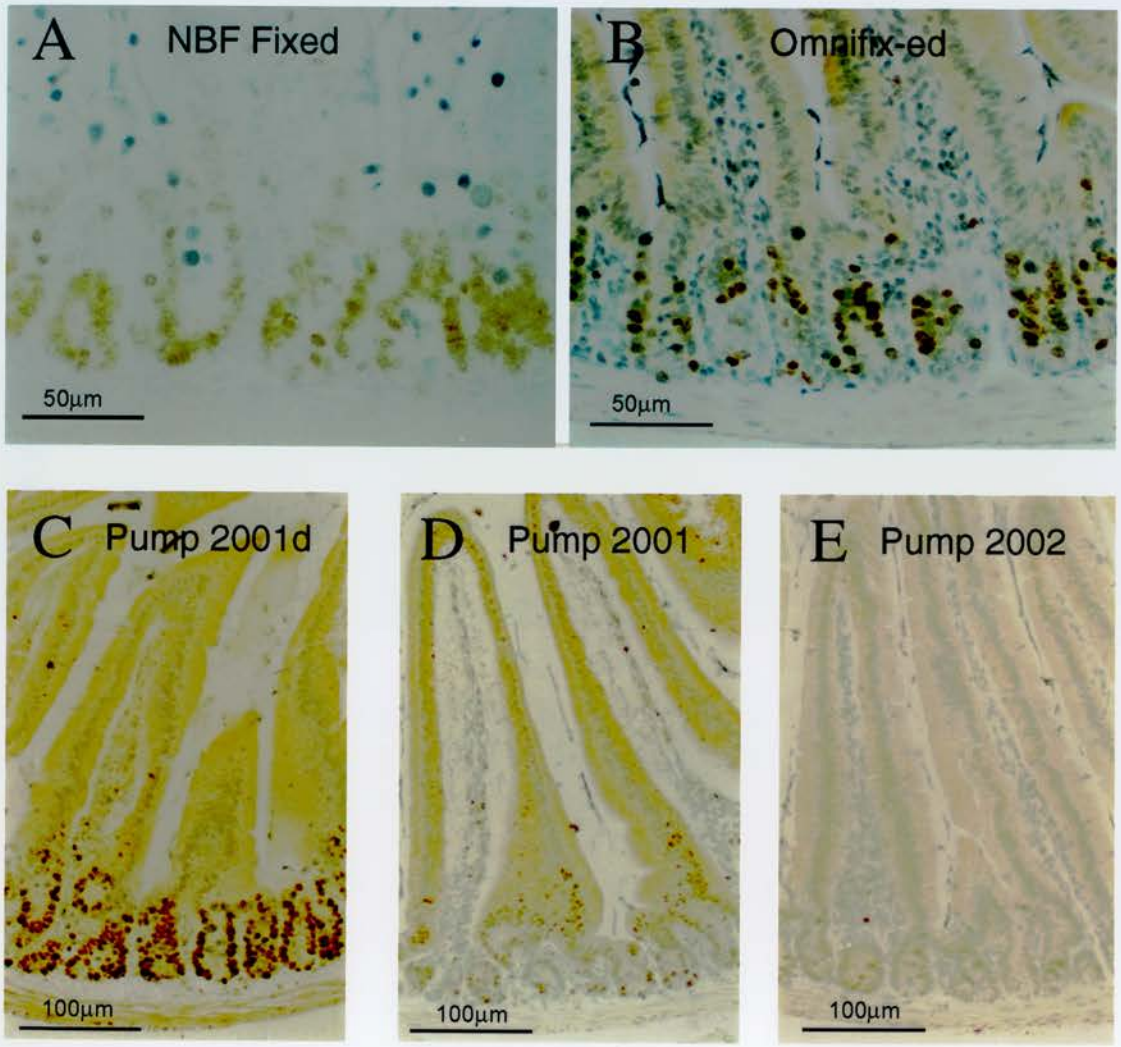

A
NBF Fixed
50µm
B
Omnifix-ed
50µm
C
Pump 2001d
100µm
D
Pump 2001
100µm
E
Pump 2002
100µm

Figure 7.3.4

Summary Of Incorporated BrdU visualisation by Immunohistochemistry After BrdU pump Dosing for 24 hours. N=4

Pump model	10% NBF fixation	Omnifix
2001D	++	++++
2001	+	++
2002	-	-
2004	-	-
-ve control (no pump)	-	-

Discussion

In both fixation methods, BrdU was detectable using pumps 2001d and 2001 only. The working duration of these pumps was however, unsuitable for use in a 22 day experiment. Omnifixed tissue was found to give a much stronger immunohistochemistry signal (as seen by intensity of brown cellular signal) than with NBF fixed tissue. The omnifixed tissue also showed a higher endogenous background (faint brown background colour) and slightly poorer histology (especially in the villi).

Omnifix was found to be the most suitable fixation method for viewing BrdU. As the osmotic pumps proved unsuitable for this experiment, the use of repeated single dose injections were investigated

Study of BrdU injection frequency versus cellular labelling in the mouse small intestine.

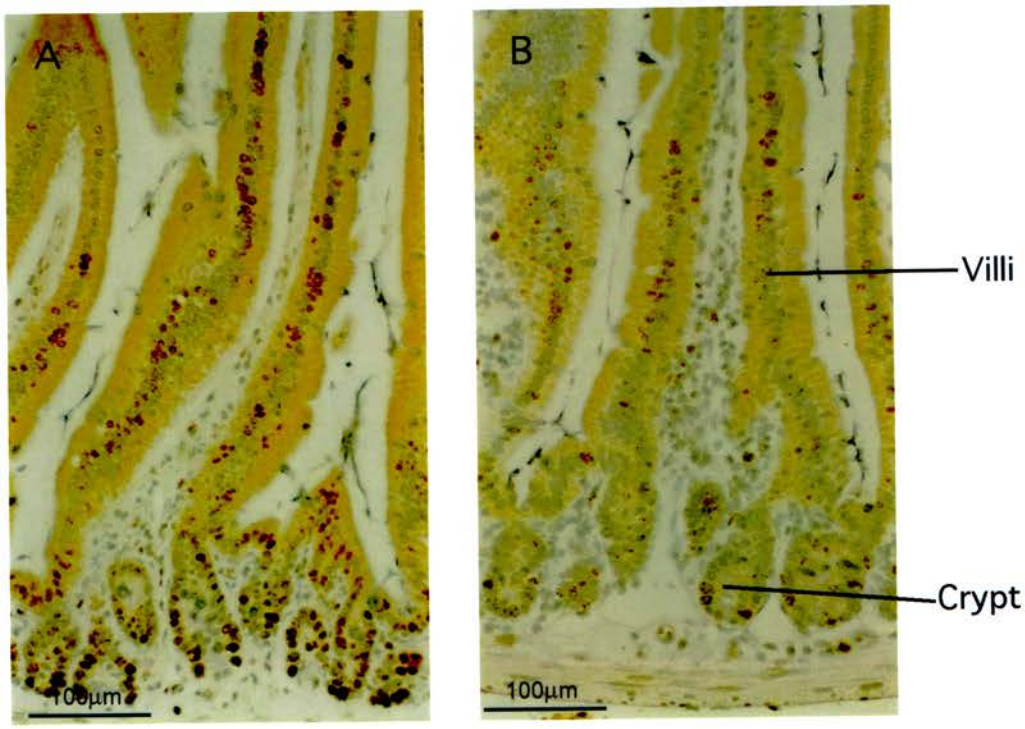
The osmotic pumps were found not to be useful for the purposes of a prolonged labelling experiment. An alternative was to deliver frequent IP injections of BrdU to the animals. One possible draw back from such an approach would be that repeated pulse delivery of BrdU would be likely to produce peaks and troughs of BrdU serum levels and thereby conceivably not labelling every cell undergoing division. An experiment was conducted to examine whether a rate of injections one every two days would be able to provide a sufficient and useful dose for this experiment.

Localisation of BrdU labelled cells in the small intestine was examined after the delivery of two 2mg BrdU IP injections 48 hours apart. 6 mice received a 2mg IP injection of BrdU (50mg/ml in H_20/ 1%NH_4OH). These mice were split into 3 groups. Group 1 animals were left for 48 hours and then sacrificed and tissue from the small intestine fixed in omnifix and processed for BrdU immunohistochemistry as before. Group 2 animals received another BrdU injection after 24 hours and were sacrificed 24 hours later and tissues processed as with group 1. Group 3 animals received addition injections at 24 hours and 47 hours after the original injection. At 48 hours after the start of the experiment, these animals were sacrificed. Negative control animals which did not receive BrdU injections were also processed for BrdU immunohistochemistry.

The results are shown in figure 7.3.5.

Figure 7.3.5.

Evaluation Of BrdU Labelling In Gut After Multiple BrdU Pulses



7.3.5A Group 3. Small intestinal tissue fixed with omnifix and stained for BrdU using set 1 antibody. This animal had received three 2mg BrdU doses at 48hours, 24hours and 1 hour prior to sacrifice. BrdU labelled cells (as identified by nuclear DAB stain) were distributed from the base of the crypts to the central region of the villi.

7.3.5B Group 1. Small intestinal tissue fixed with omnifix and stained for BrdU using set 1 antibody. This animal had received one 2mg BrdU doses at 48 hours prior to sacrifice. Labelling was considerably weaker than in the group 3 animals and observed mainly to be localised in the central region of the villi and in the base of the crypt.

Discussion

Low frequency pulse injections of BrdU are likely to results in varying levels of BrdU in the serum. In this study of BrdU labelling in the duodenum it was shown that by providing a high dose of BrdU at 48 hour intervals produced a high degree of staining homogeneity. This suggests that repeating pulse injections at 48 hour intervals would be suitable to maintain a satisfactory degree of continuity in the labelling of dividing cells. Labelling was planned to be used in conjunction with stem cell recruiting tracheal damage. As it was unknown when stem cells (if present) are recruited after tracheal damage, the schedule of BrdU injections was made asynchronous and an addition BrdU injection was made on the day of tracheal injury.

Study of LRCs in the mouse trachea

70 age matched CD1 male mice were randomly separated into 13 groups. These groups were further randomly chosen to give 5 test polidocanol damage groups, 5 test sulphur dioxide groups and three control groups (either receiving no damage or no damage but BrdU injection).

Test mice repeatedly received *in vivo* tracheal damage by polidocanol instillation or SO_2 inhalation (SO_2 incubations were performed by T. Krantz) as outlined below in figure 7.3.6. In addition, the mice received 200µl 10 mg/ml IP injections of BrdU solution every two days over the 22 day period of the experiment plus an additional dose approximately one hour after tracheal damage.

At different time points after the 22 day period , groups of mice and control animals were sacrificed by lethal IP injection of Euthatal (see figure 7.3.6). Tracheas and duodenum were excised, fixed in omnifix and processed to paraffin blocks. 6µm

sections of tissues were cut. On each slide, one sections was examined by immunohistochemistry for cellular incorporation of BrdU with set 2 antibodies, while a serial section was run as a negative control (no primary antibody added.)

Figure 7.3.6.

Schedule For Experiment: Tracheal Damage, BrdU Injections And Sacrifice times. Control Groups Included Mice Receiving No Damage And No BrdU Injections And Mice Receiving BrdU Injections Only.

Day	0	7	14	21	22	25	29	57	117
Shorthand						+3	+6	+21	+95
Damage[a]	*	*	*	*					
BrdU[b]	--	--	---	---	→				
Sacrifice						◆	◆	◆	◆
n						4	4	4	9

[a] either 10µl 2% polidocanol solution or 500ppm SO_2 inhalation (the duration of SO_2 damage increased from 3hrs to 4.5hrs at 0.5hr steps at the different time points)

[b] 200µl 50mg/ml BrdU in 1% NH_4OH in PBS

The degree of cellular labelling was measured at the different time points. Approximately 100 cells of both basal and columnar type at each of 3 different regions of the trachea were examined for cell type and presence of nuclear immunohistochemistry signal. Signal was confirmed as being positive if the signal was nuclear localised and the brown staining above that detected as background and on control slide. These 3 regions were at cartilage ring number O, number 5 and

number 9 (see chapter 3 for description of cartilage ring positioning). Cellular staining on sections were compared to serial negative control sections or control tissue. Stained and unstained cells were identified as either being basal cells (B) (small cuboidal cells with basolateral membrane attachment), intermediate cell (I) (cells with neither basolateral membrane or luminal interface), ciliated cells (C) (columnar cells with visible cilia) or non ciliated columnar cell (NC) (cells with luminal interface but no visible cilia). Areas of distorted histology or bronchiolitis obliteran pathology were removed from the counting process. Small intestine tissue from the same test animals and positive control animals were used to test the validity of antibody runs.

Results

The percentage of BrdU labelled cells at different time points was measured at three different sections of the trachea. The full data is presented in appendix 2 and is summarised below in figure 7.3.7a and sulphur dioxide damage only in 7.3.7b.

Figure 7.3.7.

Summary of Data From Label Retaining Experiment

Data are displayed in two tables; each one referring to the data gained through the use of a specific antibody set (see above). The "positions" refer to the sections on the trachea where the counts were made: position 1 = cartilage ring 0, position 2 = cartilage ring 5, position 3= cartilage ring 9.

Data are presented as mean percentage marked cells and standard error of the mean in brackets.

The abbreviations to the methods of damage are "SO_2" = damage by sulphur dioxide, "polidoc" = damage by polidocanol solution and "-" = no damage.

Antibody Set 1

	Damage method	Position 1 / %		Position 2 / %		Position 3 / %	
		B	C	B	C	B	C
+3 days	SO_2	47.3 (13.1)	18.9 (6.5)	32.6 3.3)	8.8 (4.6)	35.8 (9.6)	13.6 (5.3)
	Polidoc	1.6 (1.1)	0.3 (0.4)	3.3 (0.9)	1 (1.3)	3.0 (1.0)	1.0 (0.0)
	-	4.0	0.0	2.0	2.0	3.0	4.0
+ 6 days	SO_2	32.8 (6.6)	14.5 (4.5)	33.8 (4.7)	12.5 (2.8)	34.3 (4.8)	5.3 (2.0)
	Polidoc	8.0 (5.0)	0.8 (1.2)	7.0 (4.0)	2.0 (2.5)	3.3 (4.0)	0.5 (0.8)
	-	2.0	0.0	0.0	0.0	0.0	0.0
+ 21 days	SO_2	29.5 (7.5)	18.0 (8.5)	0.5 (0.5)	0.5 (0.5)	0.8 (0.8)	0.0 (0.0)
	Polidoc	0.8 (1.0)	0.3 (0.4)	0.0 (0.0)	0.3 (0.4)	0.5 (0.5)	0.0 (0.0)
	-	3.0	2.0	0.0	0.0	0.0	0.0

Antibody Set 2

	Damage method	Position 1 / %		Position 2 / %		Position 3 / %	
		B	C	B	C	B	C
+3 days	SO_2	37.0 (8.0)	39.0 (9.3)	33.5 (6.3)	31.0 (11.0)	28.8 (4.8)	22.0 (5.5)
	Polidoc	13.0 (6.0)	3.5 (1.5)	7.5 (1.5)	4.5 (1.5)	3.0 (2.0)	1.0 (0.0)
	-	3.0	0.0	2.0	0.0	4.0	0.0
+ 6 days	SO_2	24.5 (6.1)	15.5 (4.8)	17.2 (8.8)	8.8 (1.4)	18.2 (3.4)	10.5 (1.0)
	Polidoc	5.8 (3.1)	2.5 (2.2)	1.8 (1.3)	1.3 (1.4)	2.8 (2.7)	1.3 (1.3)
	-	6.0	1.0	4.0	1.0	0.0	1.0
+21 days	SO_2	21.8 (7.3)	18.3 (8.3)	24.8 (10.3)	17.8 (10.6)	18.0 (8.5)	16.0 98.5)
	Polidoc	13.6 (5.8)	3.7 (2.6)	11.3 (6.2)	5.3 (2.4)	15.3 (5.3)	5.3 (3.3)
	-	11.0	2.0	4.0	2.0	5.0	1.0
+95 days	SO_2	9.3 (3.8)	3.0 (1.0)	1.8 (0.8)	1.3 (1.3)	1.3 (0.6)	1.3 (0.4)
	Polidoc	4.0 (3.2)	1.8 (1.3)	3.4 (2.6)	1.4 (0.9)	1.4 (1.0)	0.6 (0.4)

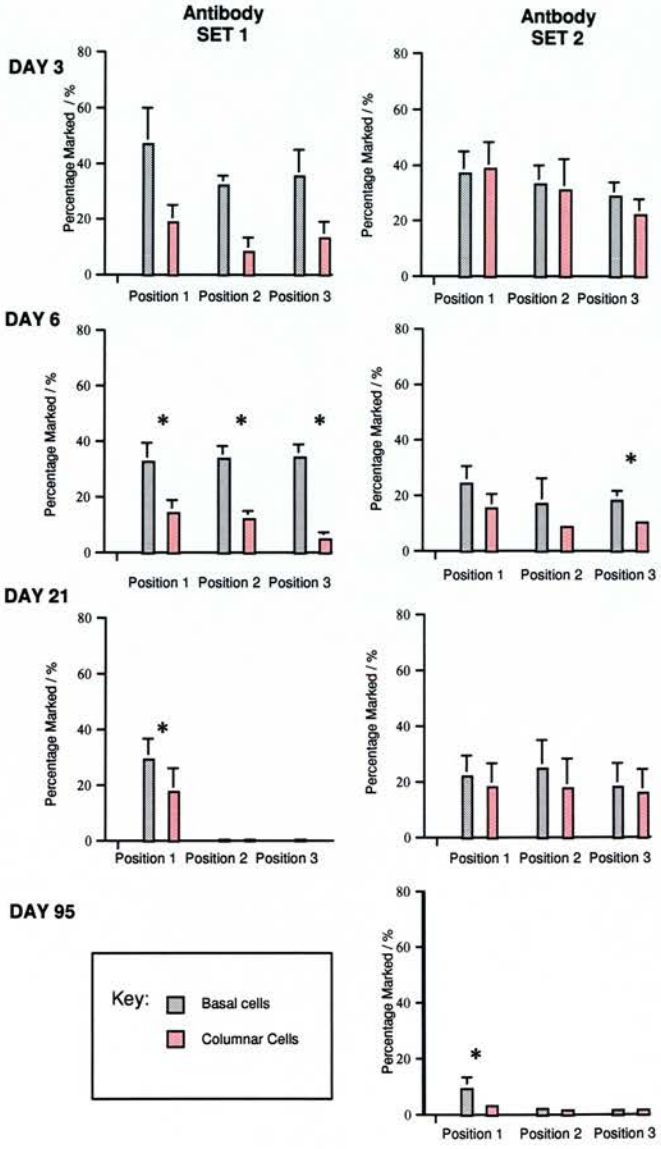
Figure 7.3.7b

Summary of BrdU Profiles- SO_2 Damage

Summary of BrdU positive cell counts. Position 1 is adjacent to cartilage ring 0, position 2 is adjacent to cartilage ring 5, and position 3 is adjacent to cartilage ring 9.

Figure 7.3.7b

Summary of Data from figure 7.3.8. Immunohistochemistry results from SO_2 Damaged, BrdU Labelled Mouse Tracheas.



* Denotes statistical significance of $P<0.05$

graph

Figure 7.3.8

Examples of BrdU immunohistochemistry images from sulphur dioxide damaged mice

For 4 weeks, mice received repeated tracheal damage by sulphur dioxide inhalation and repeated BrdU injections. After this time, groups of mice were sacrificed at 3 days, 6 days, 21 days and 95 days. Tracheal and gut tissues were processed for BrdU visualisation by immunohistochemistry using one of two possible sets of antibodies. Examples of these images are presented below.

+3 days

7.3.8A Set 2 antibody visualising a SMG ciliated duct and neighbouring epithelium (x40). Antibody labelling appears as brown nuclear staining.

7.3.8B Set 2 antibody negative control (no primary antibody)on serial section of 7.3.8A

7.3.8C Set 1 antibody on SMG region (mucus staining grey after omnifixation). Note high frequency of labelled cells

7.3.8D High power view of 7.3.8C ciliated duct. Note presence of heavily stained cells in the base of the ciliated duct.

7.3.8E Control section from mouse (no primary antibody).

7.3.8F: Set 1 antibody on small intestine sample from a +day 3 animal showing cellular staining in the base of the crypt- an area associated with gut stem cells.

+6 days

7.3.8G Set 2 antibody visualising a SMG ciliated duct and neighbouring epithelium (x40).

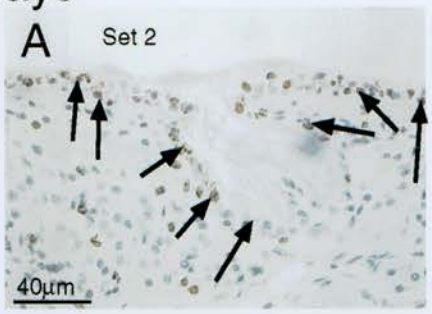
7.3.8H Negative control (no primary antibody) on serial section of 7.3.8G.

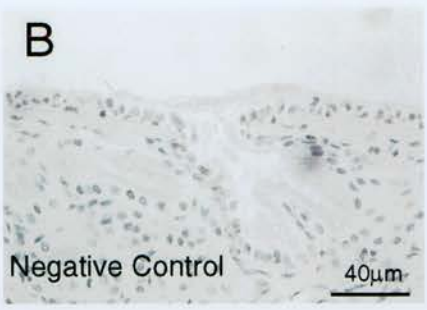
7.3.8I Set 1 antibody. High frequency of epithelial cells are still labelled.

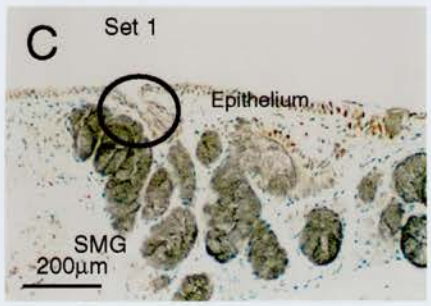
7.3.8J Higher power of ciliated duct of 7.3.8I shows presence of heavily stained cells.

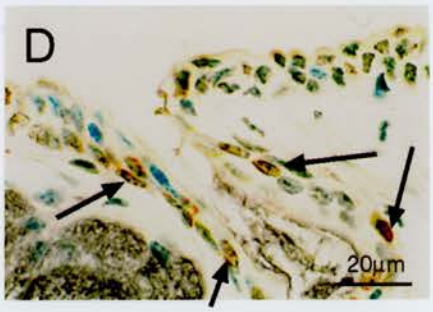
7.3.8I Set 1 antibody on small intestine sample from a +day 6 animal showing similar staining pattern to that observed in 7.3.8F demonstrating retention of BrdU labelling characteristics of stem cell populations

+3 Days

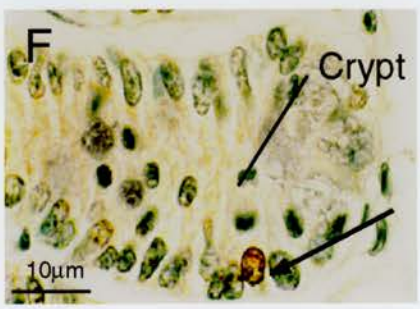

A
Set 2
40µm


B
Negative Control
40µm

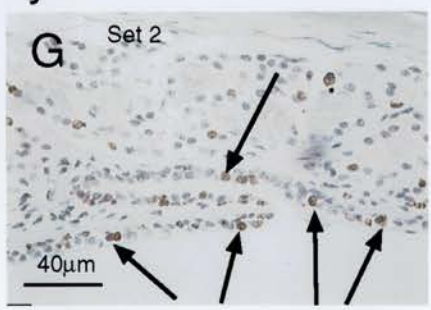

C
Set 1
Epithelium
SMG
200µm

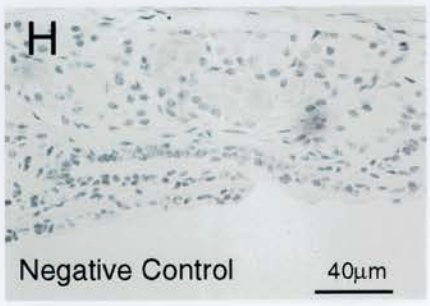

D
20µm

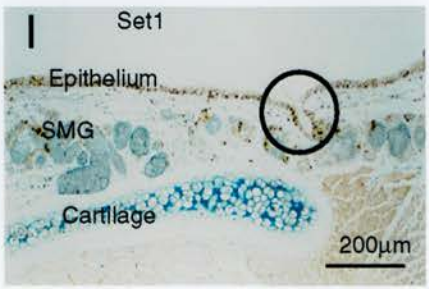

E
BrdU Injections-no primary antibody
200µm

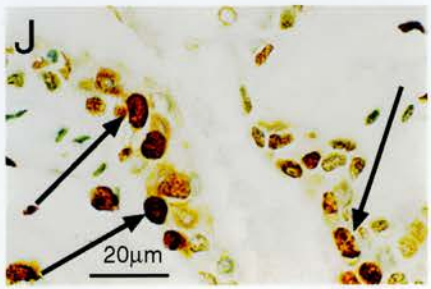

F
Crypt
10µm

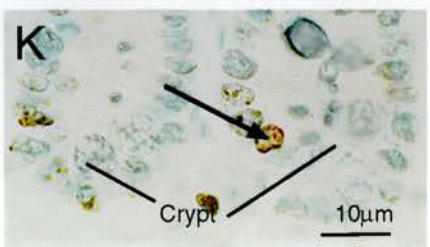
+6 Days


G
Set 2
40µm


H
Negative Control
40µm


I
Set1
Epithelium
SMG
Cartilage
200µm


J
20µm


K
Crypt
10µm

+21 day

7.3.8L Set 2 antibody visualising a SMG ciliated duct and neighbouring epithelium (x40).

7.3.8M Control (no primary antibody) on serial section of 7.3.8L.

7.3.8N, O and P: Set 1 antibody on SMG ciliated duct region. 3 different cell types are detectable by BrdU immunohistochemistry in this image, each existing in a discrete spatial niche: labelled round cells in the ciliated ducts, labelled basal cells adjacent to the ductal openings and labelled intermediate cells lying out-with this region.

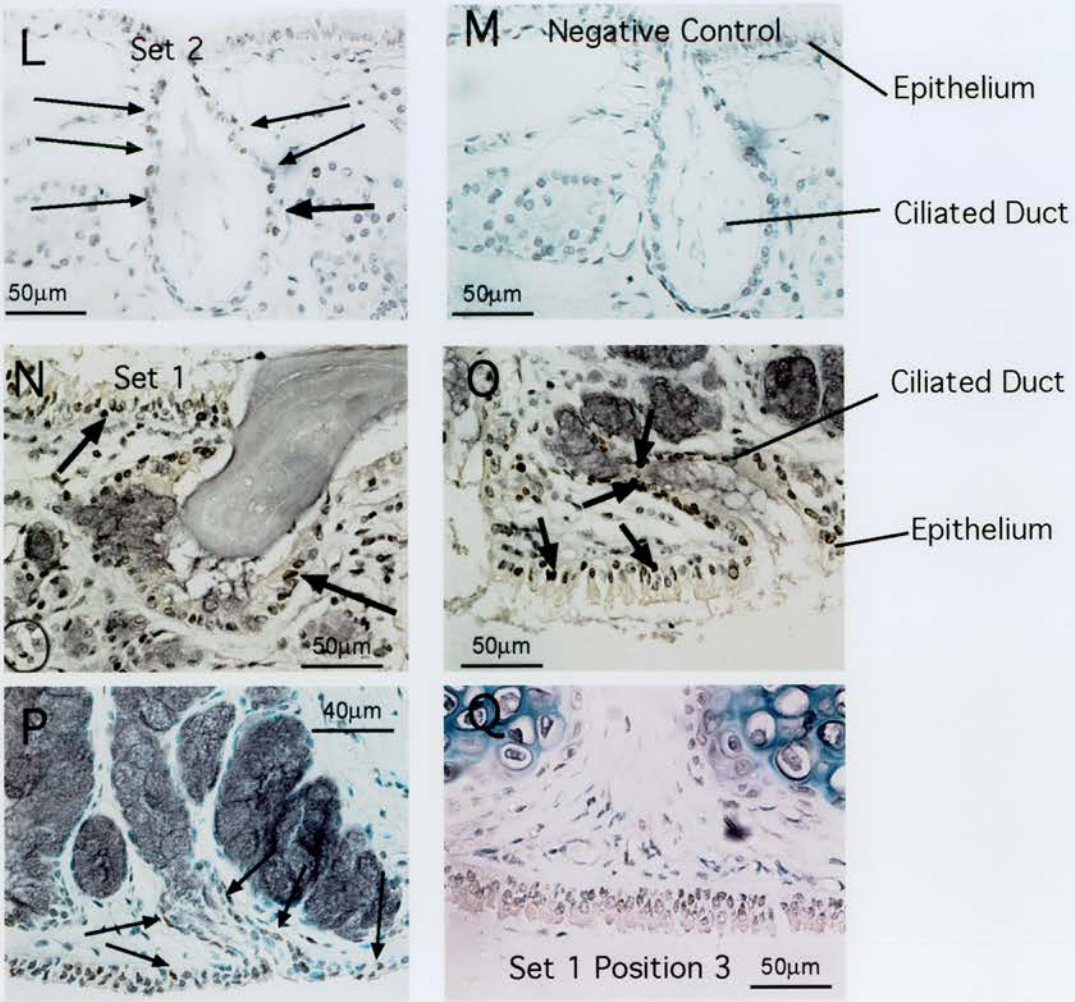
7.3.8 Q: More distal epithelium shows very low levels of labelling (as detected by set 1 antibodies). This is in contrast to the previous time points and the results gained by using set 2 antibodies.

+95 days

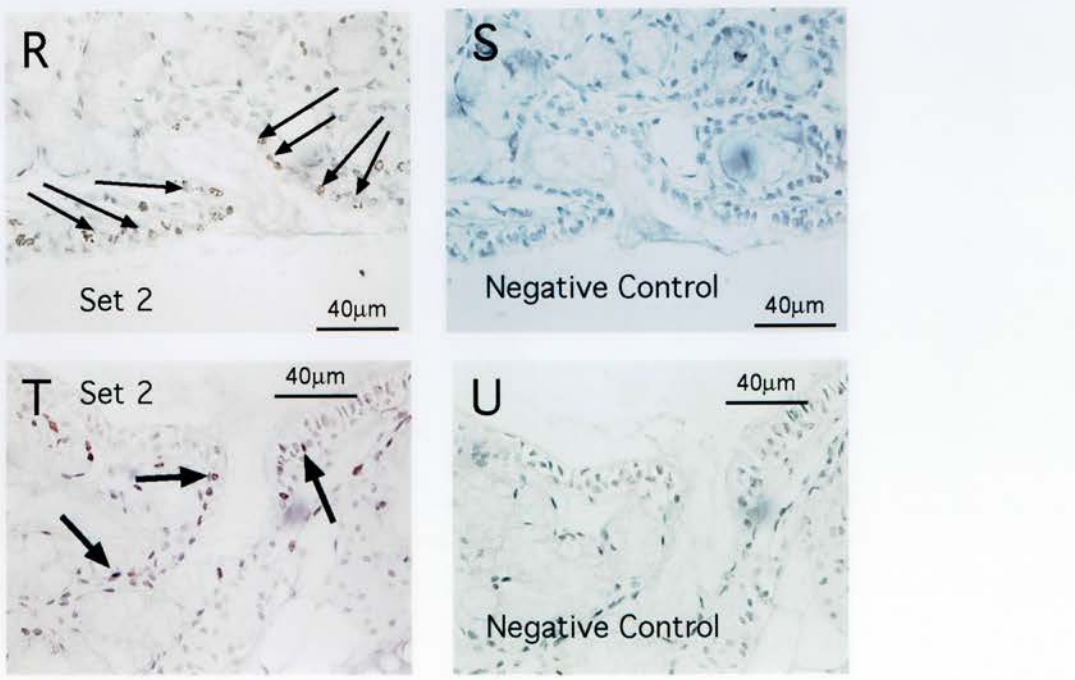
7.3.8 R and T: Set 2 antibodies demonstrating very similar pattern of labelling to that observed with set 1 antibodies at day +21 namely, heavy label retention in the ciliated duct and labelling of basal cells adjacent to the ductal opening and intermediate cells further beyond this zone.

7.3.8 S and U: Control serial sections of slides 7.3.8 R and 7.3.8 T.

+21 Days



+95 Days



BrdU labelling profiles in Sulphur dioxide damaged tracheas

At 3 and 6 days after the cessation of damage and injections, a very high number of cells were marked for presence of BrdU. This was to be expected due to the stimulation of cellular proliferation by SO_2 damage. At this time there was a trend towards higher labelling indexes in position 1 of the trachea although this was not statistically significant. By cell type, a higher percentage of basal cells were labelled with BrdU than were columnar cells. It should be noted however, that basal cells occur with lower frequency in the mouse trachea than do columnar cells (approximately 5% of epithelial cells are of basal type (Wright, 1996, Pack, 1980)) so if any comparison of cell type labelling indexes was to be made, the data should be weighed for cell type frequency.

In day +21 SO_2 damaged tissues, a stark change in the pattern of labelling was noted using the antibody set 1. In these samples, the percentage of cells labelled in regions 2 and 3 was seen to drop to near control (no damage) levels. However, a high number (29.5% (7.5% mean error)) of cells in region 1 were seen to still be labelled.

Closer examination of this area revealed this these labelled cells were only found in the proximity of submucosal gland duct openings. Associated with this observation was the presence of heavily labelled cells within the epithelial lining of the submucosal gland ciliated duct. See figure 7.3.9.

This pattern is not observed when using the 2nd antibody set at day +21. At day +95 however, a similar profile (though with a lower labelling index) is seen as with set 1, day 21 samples. It is thought that this time shift in the observation of the pattern may be due to differences in the sensitivity of the antibody methods. Although there is no direct evidence to prove this a marked similarity between the labelling patterns of set 1 samples and set 2 at one time point later. Antibody set 2 can be envisaged to be

able to detect BrdU levels at lower concentrations than with set 1. If this was the case, then the labelling profile of daughter cells would be seen to change.

BrdU labelling profile in Polidocanol damaged tracheas

Although significantly higher than the labelling profile of control tissues, the polidocanol damaged animals showed much lower levels of labelling in comparison to that observed in the SO_2 damaged animals. This was at first surprising considering that polidocanol was observed to completely remove columnar cells from the epithelium. A theory of why this may occur is presented later.

The results from the two different sets of antibodies show considerable variation in the results. With set 1, basal cell labelling is seen to peak at +6 days at all three sampling positions. With set 2, labelling is seen to peak at day +21. This again is most likely as a consequence of differences in assay sensitivity.

Despite these differences, cells retaining the label to +21 days of +95 days were observed most commonly in tracheal position 1. In addition, the niche of strongly labelled cells in the submucosal gland ciliated ducts were observed.

Further analysis of the region of label retention

At day +21 using antibody set 1 and day +95 using antibody set 2, cells within the ciliated duct and on adjacent luminal epithelial tissue were observed to retain their BrdU labelling properties. The labelled cells were observed to be of 3 types and to exist in 3 quite discrete morphological regions or zones (see figure 7.3.10).

Zone 1: Heavily labelled cells were observed to lie in a discrete niche in the base of the submucosal gland ciliated duct. These cells were morphologically simple in structure (by bright field microscopy).

Zone 2: In a region spanning from the top of the ciliated duct out onto the adjacent epithelium, labelled basal cells only were observed.

Zone 3: External to the previous region, labelled cells of intermediate morphology (i.e. showing no cytoplasmic adherence to either the luminal or apical surface of the epithelium) were observed.

The three zones also displayed a hierarchy of staining intensity with cells in zone 1 showing the strongest level of staining and those in zone 3 the weakest level of staining. This pattern was consistently observed in all slides examined. The number of SMG ducts examined for this experiment was: set 1 day 21 polidocanol damage n= 3, set 1 day 21 SO_2 damage n=8, set 2 day 95 polidocanol damage n=8, set 2 day 95 SO_2 damage n=13. A lower number of polidocanol damaged SMG ducts of suitable histology were observed than those from the SO_2 animals. It is not clear whether this is a purely coincidental sectioning artefact or indeed, chronic polidocanol treatment constricts the gland duct volume or alternatively, SO_2 damage increases gland volume or even gland number.

These data were used to construct a three zone model for label retention as seen in figure 7.3.9.

Figure 7.3.9

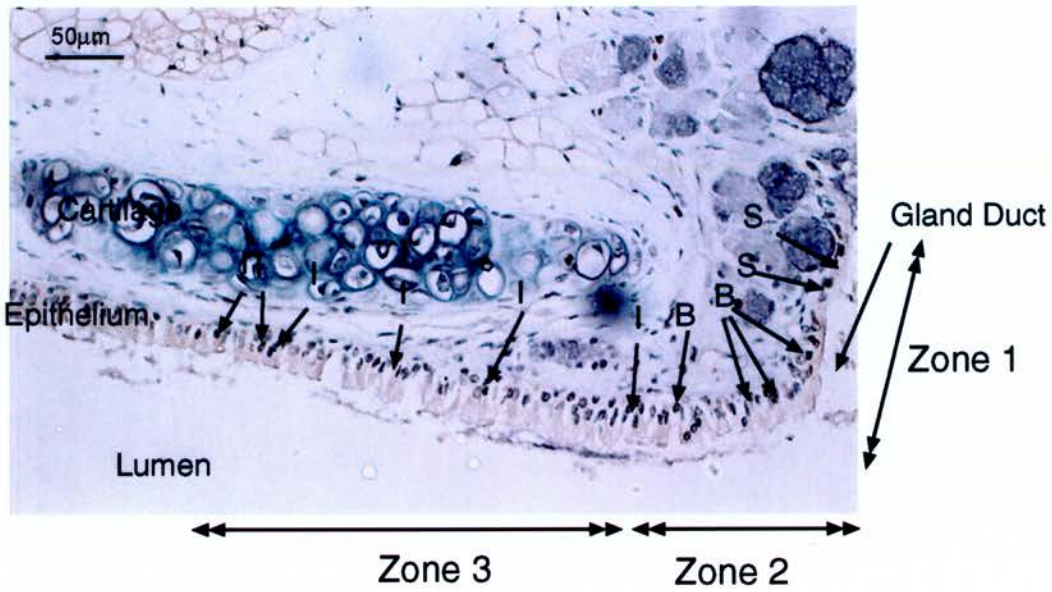
3 Zone Model for Label Retention in the Mouse Trachea.

LRCs exist in the mouse trachea in 3 distinct zones. In each spatial zone, only one cell type is observed as being labelled. The three zones also displayed a hierarchy of staining intensity with cells in zone 1 showing the strongest level of staining and those in zone 3 the weakest level of staining.

Zone 1: In the SMG ciliated duct: poorly differentiated
Zone 2: epithelium adjacent to the ducts: basal cells
Zone 3: epithelium adjacent to zone 2: intermediate cells

This pattern of label retention is consistent with there being a population of cells exhibiting stem cell like features in the ciliated duct of murine submucosal glands.

Figure 7.3.~~10~~ 9



Discussion

This experiment was designed to examine whether BrdU LRCs, which may represent stem cells, existed in the mouse trachea. In addition, it was hoped that the labelling profiles of the basal and columnar cells derived from the experiment would lead to a better understanding of the cellular kinetics of this tissue.

A number of variables were introduced into this experiment to make possible the comparison of data. Two different sets of antibodies were used and two different methods of tracheal damage. Indeed, there was considerable variation in the data derived from the different methods.

Firstly, there were differences between the results gained from the different sets of antibodies. Profiles of labelling peaks and patterns observed through the use of antibody set 1 were seen to occur at a later time point with antibody set 2. For instance, polidocanol damaged tissue labelling peaked at day 6 with antibody set 1 but at day 21 with set 2 and the reduction in position 2 and 3 labelling was observed with antibody set 1 at day 21 but not until day 95 with antibody set 2.

Considerable effort was made into providing both damage and immunohistochemical controls for this experiment. In addition to the damaged animals, tissues from animals which had received BrdU injections but no damage (termed "BND") and animals which had received polidocanol damage but no BrdU (termed "PC") were used to run along side the damaged tissues in the immunohistochemistry runs. The BND tissues showed generally low levels of labelling (<4.0% with set 1 antibodies and <11% in set 2 antibodies). Basal cells were observed most commonly to be stained in this group. The PC controls showed no false positive nuclear staining with set number 1 antibody. In addition to these controls, a negative control serial section was run when using antibody set number 2. Set 2 was found to be more sensitive

than set 1, which sometimes resulted in high background runs. These slides were not examined further. In addition, sporadic areas of very high nuclear and cytoplasmic staining occurred. The pattern of this staining suggested that the secondary antibody had bound without specificity to random areas of tissues. Again, these portions of tissues were not counted.

This experiment fundamentally operates through the detection of BrdU labelled cells and of equal importance, the failure to detect cells when the cellular BrdU content drops as a consequence of cellular division. In this respect, it was important that the method of detection had a finite degree of sensitivity. Moreover, this finite degree would suit the time frame of the experiment.

Other possible sources of variation which may affect the data were restricted through the use of similarly cut sections, the same areas for cell counting and examining the slides in a blinded fashion.

Variation in labelling profiles arising from method of tracheal damage

The two methods of tracheal damage were observed to result in a different degree of cellular labelling in the day +3 and day +6 animals. Taking average figures from all three tracheal positions, the labelling index measured in SO_2 damaged animals was up to 16 times larger than that measured in the polidocanol treated animals. This difference may be perceived as being unexpected since both methods cause considerable damage to the tracheal epithelium. To elucidate this observation the pathology of damage and the conclusions presented from earlier in this chapter must be further explored.

The pattern of damage caused by polidocanol was seen to differ from that of the sulphur dioxide. Polidocanol was observed to remove columnar cells from the

tracheal epithelium exposing the basal cells to the air. The effect of sulphur dioxide was considerably more subtle. The pattern of damage appeared to be one of slower cellular disruption. The consequence of which was that both columnar and basal cells were observed to have received damage. It is additionally interesting to note the development of tracheolytis obliterans in many of the polidocanol treated animals.

Can the difference in labelling index between the two methods of damage therefore be explained as a consequence of the difference in cellular damage? Earlier in this chapter, a theory was proposed that a subpopulation of basal cells were the main cells of proliferative capacity in the mouse tracheal epithelium. On this platform, it is possible to explain the differences in labelling indexes. In the polidocanol animals, basal cells appear to resist damage. If a subpopulation of these cells have a strong proliferative capacity they will be able to rebuild the tracheal epithelium very quickly and with efficiency. When observed through the use of BrdU, this will result in a generally low index since the window for cellular division would probably be short and a small number of cells would be involved in division.

In the case of the SO_2 damaged animals, damage has occurred to both basal cells and columnar cells. It is therefore likely that some of the highly proliferative basal cells would be damaged through this process. Thus the system of epithelial regeneration would be altered. The scenario can be envisaged that for epithelial repair to occur a higher number of cells would have to divide and over a longer period of time than in the polidocanol animals. This model would result in the SO_2 animals demonstrating a higher labelling index than the polidocanol animals.

In conclusion, the differences observed in the labelling index of the SO_2 and the polidocanol damaged tracheas is most likely as a consequence of the differences in the cell types damaged. In the polidocanol animals, basal cells are retained. It is proposed that as the basal cells are the main progenitor cell type in the mouse

trachea, the profile of tissue repair differs between the two damage methods. Polidocanol treated animals can undergo tissue repair much more quickly and without such a high frequency of cell division than with the SO_2 treated animals. This profile has the consequence of a lower degree of cellular labelling in the polidocanol treated animals.

The 3 Zone Model Of Label Retaining Cells In The Mouse Trachea

At the later time points of this experiment (day 21 with antibody set 1 and day 95 with antibody set 2) LRCs were restricted to within or around the submucosal gland ciliated ducts. The LRCs were observed to be of 3 different cell types and to occupy three discrete zones. Zone 1 is a discrete niche of heavily labelled cells of poor morphology situated towards the base of the ciliated duct. Zone 2 is from the upper reaches of the ciliated duct out onto the neighbouring epithelium and contains labelled basal cells. Zone 3 contains weakly labelled intermediate cells in an area adjacent to zone 2 but further from the ductal opening.

The presence of labelled cells in this localised region while labelling in other regions of the trachea is largely lost is of considerable kinetic interest. These data infer that these cells are either derived from or are themselves cells of high proliferative capacity and slow cellular turnover for the reasons described in the introduction to this experiment. Such characteristics are associated with the properties of stem cells.

It is possible to define which of the LRCs may be stem cells and which are transit amplifying cells (TACs) by property of their staining intensity. The higher up the proliferative capacity hierarchy the cell is, the more heavily stained the cell should be. This being due to the cell having a slower rate of cellular turnover.

On this basis, cells in zone 1 are likely to be higher up a proliferative hierarchy than those in zone 3. It is therefore likely, although no functional data is available at this time, that the cells in zone 1 have the properties and perhaps are indeed stem cells. These cells upon division, will produce the early TACs which are represented as the cells in zone 2. The early TACs migrate from zone 1 out onto the epithelial surface where they divide to produce zone 3 cells or late TACs.

This pattern of stem cell activity is in many ways similar to that observed in the cornea (Cotsarelis, 1989). The stem cell niche is situated distant from where the daughter cells will eventually become physiologically active. It is interesting to note however, that zone 1, 2 and zone 3 all contain cell types which do not possess the labelling retaining characteristics. This infers the route from stem cell through TACs to mature cells is likely to include frequent asymmetric divisions as to produce physiological functional cells as well as maintaining the population of migratory proliferative cells.

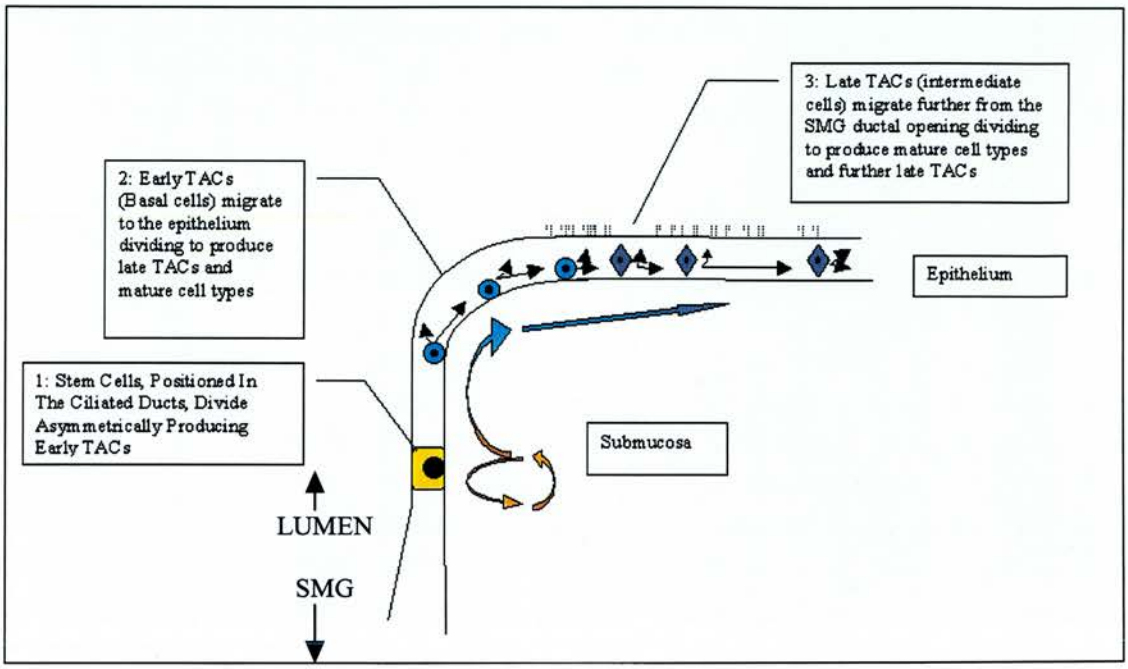
Little is known about the identity of the labelled cells in zone 1. Their location and structure show close resemblance to the "flask like cells" or "mitochondrial rich cells" which have been observed in the human SMG (Meyrick, 1970, Engelhardt, 1992). It is exciting to note also that the flask-like cell have been observed in the human to express *CFTR* (Engelhardt, 1992).

From these observations it is possible to generate a model which describes the role of the LRCs in maintenance and repair of the epithelial tissue. This model is presented in figure 7.3.10.

Figure 7.3.10

Figure 7.3.10

3 Zone Model For Area Of Label Retention



This figure presents the concept that the label retaining cells in the mouse trachea exist in a region within and surrounding SMG ducts. The labelled cells occupy three zones, each zone consisting of a different cell type and a different level of label intensity.

Evidence For Stem Cell Model In The "Steady State" Trachea

One possible way in which an experiment of this design could fail would be when the damage induction results in a situation which does not represent the kinetics of the steady state. Such a design obviously produces an artificial situation in as much as the extent of epithelial damage and thus, recruitment of proliferative cells into cellular division. However, the question will still remain whether the kinetics are disturbed resulting in an "artificial" situation.

There are four main areas of evidence from steady state experiments which can be seen to back up this model. Firstly, the cells from all three zones have been observed to undergo cellular division in the steady state human bronchial SMGs (Leigh, 1995) and from the control slides from this experiment. This suggests that the stem cells, as proposed by this model, are not completely dormant during steady state.

The second piece of evidence is derived from the original definitions of what a stem cell should like. As described in the introduction, stem cells are likely to be slow cycling cells with very high proliferative potential. Furthermore, the stem cells are likely to be poorly differentiated and occupy a cellular niche lying distant from the most common sites of cellular damage (Cotsarelis, 1989). The zone 1 cells closely match this description both having undifferentiated morphology (by light microscopy) and lying in a pocket away from but closely connected to the tracheal epithelium.

The third and most interesting piece of steady state evidence to back up this model is from the aggregation chimaera studies of section 5.2. In that study, clonality of SMGs was shown by studying reporter gene expression in the murine SMGs. From the very same images (figure 5.2.5) it is interesting to note that the epithelium surrounding a SMG which is expressing a reporter gene SMGs also express the

reporter gene. The expression of transgene in the epithelium furthermore appears to be restricted to a distance from the SMG duct similar to that of the extent of zone 3. This provides interesting data to back up the notion that the stem cell activity as observed in this experiment also occurs in steady state.

Lastly, an observation, which may be of importance in this argument, is that cultured pulmonary epithelial cells from CF patients grow quicker than those from non-CF patients (Randell, personal communication). An intriguing explanation for this may be that as CF patients have more SMGs than non-CF patients (Yankaskas, personal communication), there will be a higher frequency of zone 2 and zone 3 TACs in any biopsy. In this scenario, the CF cultures will then have more cells of high proliferative capacity than in non CF cultures and therefore, grow faster.

Spatially Restrictive role of tracheal stem cells in tissue maintenance

The finite nature of zone 3 cells suggests that the TACs derived from the tracheal stem cells may not contribute to the kinetics of the entire tracheal epithelium. Indeed the aggregation chimaera data supports the notion that the new cells arising from the SMG positioned stem cell can only repopulate or maintain a relatively small area of tissue. The question then arises of what of the rest of the remaining tracheal tissue. If SMGs distribution is restricted in mice, how is the kinetics of the tissue regulated when a SMG is not in the vicinity?

There are a number of possible solutions to this problem. Firstly, that as outlined in section 7.2, the basal cells act as the main progenitor cell type in areas with few or no SMGs. This explanation would seem to suggest that there are no cells possessing classic stem cell features beyond the confines of the SMGs. A complementary observation is the slight increase in the number of Clara cells in the distal trachea as opposed to the proximal trachea (Pack, 1981). The proliferative capacity of Clara

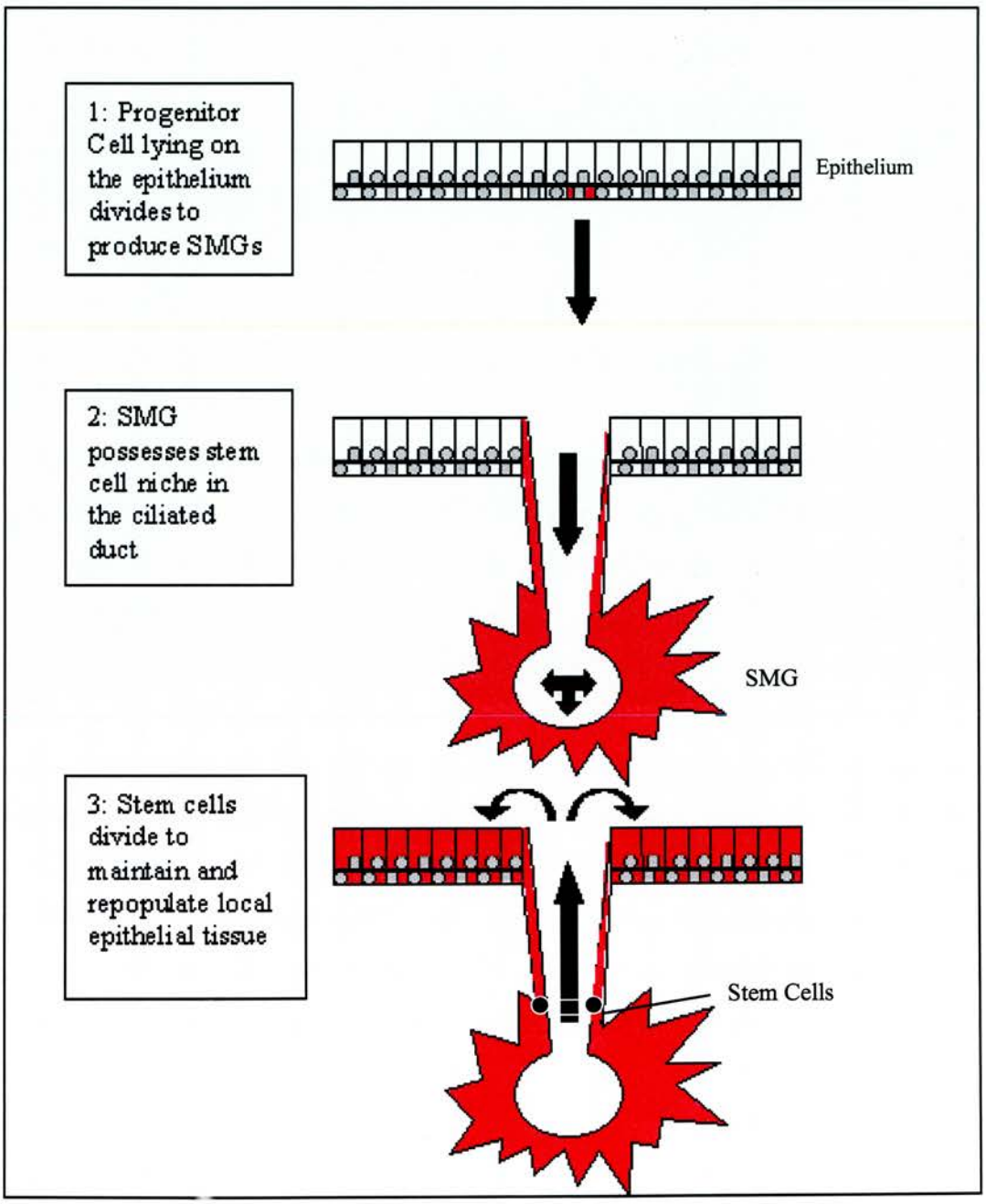
cells in the human bronchiole has been well characterised (Evans, 1976b) and perhaps they too play a role in regulating cellular kinetics in the mouse trachea and therefore are more common in areas with fewer SMGs. There is at this time no data for this last notion. It would be very interesting to make an EM study of the epithelial cell types lying between SMG ducts to see if there is an increase in Clara cells beyond zone 3.

A Model For The Development And Kinetic Role Of SMGs

From the evidence presented in sections 7.3 and 5.2 can be used to present a model of the development and kinetic role of SMGs in the mouse. This model is particularly interesting in that two progenitor cell populations are present. Firstly, the progenitor cell which goes to clonally produce SMGs and secondly the stem cells which feed the local epithelium with new cells. Such a model presents much scope for further examination of the regulation and control of phenotypic differentiation and branching. This model is presented in figure 7.3.11.

Figure 7.3.11

A Model Of Stem Cells And TAC Kinetics In And Surrounding Mouse Submucosal Glands



7.3.11

Implications For Gene Therapy: The Targeting Of The Stem Cell?

This chapter has described the existence of possible stem cells lying within the ciliated ducts of murine SMGs. As were the arguments voiced in chapter 5, the targeting of the tracheal stem cell for gene therapy would hopefully result in the long term expression of the transgene in multiple cells (the stem cell's progeny).

Before the technical aspects of targeted gene therapy for the tracheal stem cell are considered, it is worthwhile examining once more the fundamental assumption of gene therapy for CF. That assumption being that genotypic correction of pulmonary epithelial cells will result in a down stream reduction in disease phenotype in the CF patient. The area of weakness in this argument is that the epithelium appears to express *CFTR* at very low levels (Engelhardt, 1994) so may no necessarily be important in the maintenance of the healthy lung. Targeting gene therapy at the tracheal stem cell would also potentially suffer from this weakness although not to the same extent as the MRC or "flask like cells" within zone 1 which are known to express *CFTR*. Although the tracheal stem cells appear to lie in the same region as these cells, their identity cannot be confirmed at this time.

Targeted gene therapy relies on their being a recognisable molecular marker on the target cell. The most attractive candidate for such a marker for the tracheal stem cell would most probably lie in the cytokeratin family. The cytokeratins are a family of structural proteins whose members are often expressed in a cell type specific manner (Randell, 1992). For instance, basal cells express cytokeratin 18 whilst columnar cells express cytokeratin 14 (Shimizu, 1992). Recently Scott Randell's group, University of North Carolina, generated a transgenic mouse in which the cytokeratin 5 promoter has been linked to the green fluorescent protein gene. Tissues normally expressing cytokeratin 5 are seen to fluoresce. Preliminary data has shown that the pattern of fluorescence in the tracheal epithelium is one of expression in a sub

population of basal cells and also in the ciliated duct of the SMGs. This work is at present unpublished but is very exciting since cytokeratin 5 could prove to be a useful and possible marker for the tracheal stem cell population.

In conclusion, a population of heavily stained BrdU label retaining cells were observed to lie in a discrete niche in the ciliated ducts of murine SMGs. Further LRCs of weaker staining profile were observed higher in the ciliated ducts and on the surrounding epithelium. These data when modelled against current stem cell theory suggest that the LRCs in the ciliated ducts may have stem cell properties. It is hoped that functional studies of isolated populations of these cells in the future may provide the necessary evidence to finally call these cells, "stem cells".

Chapter 8

Concluding Remarks

Chapter 8.1

Thesis Summary

A Summary Of The Findings Of This Thesis Is Presented Below

Chapter 2:

- SMGs are present in the mouse
- Murine SMGs show great similarity to those in the human with regard to *cftr* expression, lysozyme production and cell types.
- Murine SMGs, unlike in humans, are spatially restricted to the more proximal end of the trachea

Chapter 3:

- SMG distribution in SPF inbred mice can be significantly affected by the genetic background of the mouse
- SMG distribution in SPF inbred mice can be significantly affected by CFTR expression in the mouse
- The differences in SMG distribution led to the generation of a model which may undermine the traditional conclusions derived from single point electrophysiology measurements in the mouse trachea.

Chapter 4:

- Both human the mouse β-defensin-1 molecules show a salt sensitive pattern of anti microbial activity with respect to the bacteria *P. aeruginosa*
- The clinical significance of this finding is unclear as the there have not been any definitive results regarding chloride concentrations in the CF and non CF lung.

Chapter 5:

- Murine SMG develop postnatally.
- Murine SMG are clonally derived from a single cell.

Chapter 6:

- Liposome mediated delivery of transgene to the mouse tracheal epithelium resulted in transfection of epithelial cells and low levels of transgene expression.
- Liposome mediated gene therapy, unlike adenovirus mediated, does not show increased tropism to damaged epithelium.

Chapter 7:

- The mouse tracheal epithelium is largely quiescent in steady state with respect to cellular turnover.
- Little difference of statistical significance was observed in the rate of cell proliferation in CF versus non CF mice and in mice from different housing conditions suggesting that spontaneous lung disease in CF mice is limited.
- Basal cells are most likely to be the cell type of major proliferative capacity in the mouse trachea.
- Cells with the characteristics of stem cells exist in the ciliated ducts of mouse SMGs producing transit amplifying populations which migrate out and maintain the cellular population on the surrounding epithelium.

8.2
Thesis Discussion

Modern CF research draws upon many different fields of science and clinical care. Starting from the fundamentals, Pathologists observe what is abnormal in CF tissues. In the molecular corner of the field, there is the research into the genetics of the *CFTR* gene and the elusive promoter. The physiology researcher asks how CFTR functions in the body, how it is regulated and how it interacts with other proteins. As we approach more clinically orientated areas of research, the fields of bacteriology becomes more prominent: what bacteria affect CF patients and why. Further up this ladder, the patients are encountered and with this, clinical and nursing staff in addition to physiotherapists, nutritionists and councillors become of greater importance.

The paths to a possible cure are most commonly aimed at interrupting a specific step of disease progression. However, such research most commonly draws upon the wisdom from a many fields and often is seen to jump across the full extent of the spectrum. It may not therefore be altogether surprising that the original notion that gene therapy could bypass the prerequisite of understanding the underlying pathophysiology of CF has been largely disproved.

Gene therapy research has now become spread through a wide range of fields one of which is the kinetics of the target organ, the lung airway. This thesis aimed to further the understanding of the kinetics of the tissues in the trachea with a view to making possible more efficient systems of gene therapy. The mouse was chosen as a candidate animal model for use throughout this experiment for both practical reasons and also for the expertise and resources with respect to the transgenic CF mouse models.

In preparation for the more kinetic based experiments it was important to have first established that the mouse would provide an adequate model for studying tracheal kinetics. Although a number of previous studies had shown that the same cell types were present in the mouse and the human trachea, they had also shown that these cell types were present in different frequency. The largest number of uncertainties regarding the suitability of the mouse as model were in connection to the SMGs. Very little had been previously published on mouse SMGs and what there was appeared to discount their physiological relevance on grounds of the sparcity of SMGs in the mouse. It is widely thought that SMGs are considered important in the pathophysiology of CF and therefore, it was of primary importance to examine for the presence and nature of murine SMGs.

Mouse SMGs were found largely localised to the proximal end of the trachea of the C57BL/6 and MF1/129 mice. This was in contrast to humans in which SMGs are found distributed throughout the trachea and the bronchi but interestingly reflects SMG distribution in human airway of similar diameter as the mouse (proximal bronchioles). The mouse SMGs were observed to be similar to the human glands in many important ways. Firstly, structurally, murine SMGs possess the same pattern of ductal system and of cell types as in humans. Secondly, they too secrete mucus out on the airway lumen and express lysozyme and CFTR from the serous cells. From this evidence it was concluded that the mouse trachea could be used to adequately model the pulmonary airways of the human for these studies.

It was interesting to re-examine the previous evidence that had led to the negative reports concerning the mouse's suitability as a model for the human CF lung (Widdicombe, 1994). It is argued that this is most likely due to sampling errors of the mouse trachea as it is can be quite possible, through inadequate dissection, to miss

the glandular region. It is hoped that the work presented in this thesis will act to revitalise interest in the mouse as a model for CF lung disease.

Since early studies in the 1960's and 70's, it had been suggested that SMG hyperplasia (increase in gland size) or hypertrophy (increase in number) may occur in CF patients. This pattern has only recently been adequately numerically demonstrated and confirmed (Yankaskas, unpublished). An investigation was undertaken to analyse the differences, if any, in SMG biology in mice on different genetic backgrounds. The restricted pattern of SMGs in the mouse provided a unique opportunity to study the factors affecting SMG distribution. With respect to these studies, it was possible to control the environmental influence on the data by using mouse which were housed in a SPF facility. Furthermore, interfamilial variation was restricted through the use of inbred mouse strains.

By a method of wholemount staining, SMG distribution was found to be significantly affected by both mouse strain and genotype. Reduced CFTR expression was observed to lead to more distal SMG distribution in the C57BL/6 and FVB mice (the expansive wildtype SMG distribution of the BALB/c mice made the observation in this animal difficult). This is an interesting observation that may suggest that CFTR has in some way a developmental role to play in SMG development.

The exception to the protocol was the MF1/129 strain that was studied as it was the original strain of the *Cftr*^{Im1HGU} mouse. This mouse is not housed under SPF conditions and is on an outbred strain. However, this mouse showed the same pattern as with the other mouse strains albeit in a pattern demonstrating greater variability and less statistical significance. These data suggest that other modifier genes of indeed environmental factors may also affect SMG distribution.

It was quickly noted that the SMG distribution data closely reflected published measurable differences in apparent CF phenotype in the different mouse strains. Thus the screen for SMG distribution enlarged the scope of this line of investigation from the examination of murine SMGs into the area of CF modifier genes. It can be postulated, that the difference in gland number could in some way affect the physiology of the trachea leading to the difference in CF phenotype. Yet, on further investigation, these data led to the development of a model which suggested that the method by which the differences in CF phenotype are measured (by single point electrophysiology measurements) may be flawed. The model argues that the electrophysiology technique is purely a reflection on SMG distribution. It has not been adequately proven in this thesis that CFTR does have a direct developmental influence on SMG therefore it cannot be said whether or not the SMG distribution, which the electrophysiology measurements are likely to be measuring, is indeed a measurement of CF phenotype.

In a collaborative effort with Dr. Donald Davidson, a study was made into one possible theory of why bacteria so vigorously colonise the CF lung. This theory describes the inactivation of antimicrobial defensin molecules in the CF lung as a consequence of raised chloride levels in the ASF. This study may be seen as an aside to the through strain of thought. However, as SMGs are the predominant site of CFTR expression in the lung and therefore likely to contribute significantly to the electrolyte maintenance of the ASF, this was considered an appropriate study to make.

Raised chloride concentration was found to statistically limit the antimicrobial activity of both the human β-defensin-1 peptide and its murine homologue against the clinically relevant bacteria *P. aeruginosa*. However, the relevance of this theory as one of major significance in the development of CF lung disease is under question. From the further studies of this system conducted by Dr. Donald Davidson,

of salt dependent activity of the defensins were not found to be applicable to all bacterial types. Indeed, *B. cepacia* appeared to positively prefer the environment found hostile to *P. aeruginosa* suggesting that the defensin inactivation is not likely to be the only reason for increased bacterial tropism. The variations in salt concentration was also seen to affect bacterial survival in control solutions. Overlying these doubts is the unproved primary assumption that CF airway surface fluid is more salty than non CF. This issue will be brought to a head in the 1998 North American CF conference where two prominent CF scientists are speaking back to back on this very issue each with opposing points of view.

The finding that murine SMGs expressed CFTR and produced mucus led onto the question of whether SMGs can be targeted for genotypic correction with gene therapy. It was however considered unlikely that the traditional system of aerosol delivered gene therapy would accomplish this due to the efflux of mucus from the glands. One alternative approach was to investigate the development of SMGs, through which, it was hoped, that a more accessible progenitor cell population could be identified.

In order to study *in vivo* SMG development, aggregation chimaeric mice were generated. The transgenic cell contribution to SMGs was studied which led to the development of a model which suggested that murine SMGs are clonally derived from a single cell type. This cell is most likely, on the basis of previous human and ferret studies, to be located on the airway epithelium. Murine SMGs develop in early postnatal life, this being in contrast to human SMGs which develop in the foetus. In either species, the timing of SMG development presents an additional technical problem. Although it would appear that the SMG progenitor cell population exists on the airway epithelium, these cells would have to be targeted prior to SMG formation, i.e. in the foetus. Although protocols for *in utero* gene therapy have been previously

reported, attempts as repeat these results in this thesis using liposome vectors proved largely unsuccessful.

Gene therapy delivery of a measurable transgene to the mature airway epithelium was also conducted. This study was performed to firstly make a basic study of the efficiency of liposome mediated *in vivo* gene therapy to the mouse lung and secondly to investigate whether liposomes exhibited any particular tropism to cell types which may prove useful in future cell targeting experiments. Liposomes were found to successfully deliver transgenic DNA into the cells of the tracheal epithelium however, levels of expression were low. After polidocanol induced damage of the trachea, a protocol which removed the layer of columnar cells from the epithelium but leaves basal cells, no increased levels of transfection of expression were measured.

In a comprehensive study of the kinetics of the mouse trachea, data was produced which described the varying proliferative roles of some of the main cell types found in the mouse tracheal epithelium. In the steady state trachea in areas where SMGs are not present, basal cells were found to be of greatest proliferative significance. Evidence was provided to suggest that infact 2 types of basal cells are present. Type 1 basal cells produce only basal cells whilst type 2 basal cells produce the columnar cell types. It was acknowledged that such studies are inherently difficult and prone to statistical misinterpretation due to the quiescent nature of the tissue but efforts were made to avoid the main areas of error, as highlighted by previous publications.

In collaboration with the CF Center in UNC-Chapel Hill, NC, USA, a study was made to investigate whether cells possessing classic "stem-cell" features were present in the mouse trachea. Stem cell are defined as cells of poor physiological functionality but infinite proliferative capacity and have been identified in many tissues including the skin, tongue, gut and the cornea. A classic way in which to

study the presence of these cells is to examine a diverse population of cells *in vivo* with respect to the way in which they retain nuclear labelling through detection methods such as immunohistochemistry or autoradiography. The theory behind such experiments is that if stem cells are nuclear labelled (BrdU or tritiated thymidine) at the time of division, they will retain their labelling characteristics longer than other labelled cells as stem cells divide or die less frequently. This experimental design has been used successfully to identify stem cell populations in a variety of other tissues but had not ever been used for studying the trachea. The main reason for this is that the lung has a very slow rate of cellular turnover making it unlikely that a stem cell population would not be dividing and thus, not marked by nuclear labelling. This may seem to remove the trachea from the scope of this experimental design but as a study on the cornea of rabbits demonstrated, the stem cell population can be induced into cellular division by artificially damaging the tissue.

An experiment was therefore designed in which the trachea of mice were repeatedly damaged over a 4 week period by either polidocanol solution of SO_2 inhalation. Over this same period, mice repeatedly received injections of BrdU. Once this period had ended, groups of mice were sacrificed at different time periods and the labelling profiles of the trachea were examined.

This experiment provided data which suggested that a stem cell population existed in a discrete niche in the base of the ciliated duct of submucosal glands. These stem cells divide to produce populations of transit amplifying cells which migrate up onto the epithelium thereby repopulating or maintaining the cellular population of the tracheal epithelium. The area over which the stem cells were observed to function was however finite and restricted to areas surrounding ciliated ducts. It may be that in other regions, the basal cells may provide the source for new epithelial cells. Evidence is also provided that this same system occurs in the steady state trachea.

8.3

Thoughts for the Future

It is hoped that the work presented in this thesis will help to both re-vitalise some pre-existing areas of research and generate some new areas of research.

With regard to pre-existing areas of research, the data reported with reference to the SMGs of the mouse will hopefully regenerate interest in the mouse as a useful model for studying the human lung and to reinforce the credibility of research using the CF mouse models.

One areas of particular interest for future research is the observations regarding the genetic factors affecting SMG distribution. It is very exciting to suggest that this phenotype may demonstrate a novel link into the field of CF modifier genes. Firstly though, the hypothesised link between single point electrophysiology results and SMG distribution requires to be clarified. This will hopefully take place soon once protocols are aligned so that the tracheal tissue can be used for both purposes.

Directly or independently to this, the role of SMG distribution in CF phenotype requires to be addressed which will hopefully strengthen the link between SMG biology and the study of CF. This work would further validate the case for beginning to explore the modifier genes for SMG distribution question. It would exciting to study whether the CF modifier genes as proposed by Rozmahel *et al.* (1996)were associated with the SMG modifier genes.

The suggestion from these data that CFTR is in somehow operating as a gene with developmental influence suggests an exciting new area of research. This question could be explored by developing CF aggregation chimaeras (as described in chapter 3) if mouse resources permitted.

It is interesting to now return to the original question of why do CF mice not spontaneously develop CF lung disease? In the introduction, 5 possible scenarios were presented which though not mutually exclusive, could be used as a the basis for explaining the difference in murine-human CF pathophysiology. The evidence presented in this thesis may in part answer the question of suitable homology between SMGs but has highlighted the differences in SMG distribution. It is puzzling to consider just how the mucus produced in the murine SMGs act in the defence of the lung for it would appear that the mucus would only be present high up in the trachea and not be present lower down where bacteria must also reach. If the basis for this argument is true it would appear more likely that the mice with fewer SMGs would be normally more susceptible to lung infections that those with more distal SMG distribution although there is no experimental evidence to back this up.

It is not yet known from which cells the β-defensin molecules are produced in the mouse lung. It is very possible that if they are produced from epithelial cells and the main cause of possible chloride imbalance in the lung is as a results of defective *cftr* in the mouse SMGs that mice with few SMGs may not suffer from the same degree of defensin inactivation as mice with many SMGs. This argument is in itself reliant on the understanding of the movement of mucus through the lung airways: a field which is also under much review at this time.

Therefore, just why the mouse deficient of *cftr* appears to develop lung disease in a different fashion to that of the human still remains unclear. Indeed, the answer may

lie far from this field, lying perhaps with the variation in alternative pulmonary chloride channels observed between humans and mice.

It is hoped that through the data presented in this thesis, that the whole question of the kinetics of the cartilaginous airway is slightly closer to answering. The data regarding the significant proliferative role of basal cells in the mouse trachea may lead directly to our further kinetic understanding of the tissue although reinforces the need for studying the possible existence of 2 types of basal cells.

The proposed model of stem cell activity in the mouse trachea opens many far more doors than it itself shut. Firstly, this experiment requires to be repeated, perhaps with tritiated thymidine instead of BrdU to remove any doubt of the label retaining characteristics of these cells. Then the question arises, what of these cells. What do these cells look like? Can we characterise them further? Do molecular markers exist for these cells? On the last point, there are studies underway which suggest that cytokeratin 5 may be a reliable marker. It would also be very interesting to make a closer study into the area of epithelium which is under stem cell control for it was observed that the stem cell appears to only have the ability to control cellular population over a small finite area of epithelium. It would be an interesting, if technically difficult, study to examine local variations in cell type frequency between SMG ducts to examine whether the stem cell regulated "islands" vary in cell type than compared to the "water" between them. It is certainly true that a more functional approach will eventually be required to classify these cells truly as stem cells.

Isolation of the two types of progenitor cells found in the SMGs (tracheal stem cells and SMG progenitor cells) would be an obvious extension to this study. This could lead to the possibilities of *ex vivo* gene therapy approaches (as seen using the gut) or

developmental studies into the regulation of SMG growth and branching as well as studies into factor affection hypertrophy and hyperplasia.

Returning to the original aims of this thesis, the possibility of targeting progenitor cell populations in the upper airways for gene therapy correction is slightly closer to reality. Attempts were made (though not reported in this thesis) to deliver gene therapy vectors to E12-E16 mouse embryos with the hope that transfection at this early stage would target the developing submucosal gland precursor cells. The experiments were largely unsuccessful showing only very low levels of transfection.

Progenitor cell populations have been at least shown to exist in the mouse trachea although we still have many long paths to explore before we can turn this knowledge into a practical system through which we may, at last, find an adequate treatment for CF lung disease.

Appendix 1

Inbred Mouse Strains

The inbred mouse strains used in this thesis are a few of the 447 strains currently recorded. Details of the inbred mice strains used are presented below.

CBA

Colour: Agouti

History: Developed in 1920 from a cross between a Bagg albino female and a DBA male. Inbred at source for between 90-179 generations depending on substrain.

Phenotypes: High incidence of tumours, resistant to *Salmonella t.* and susceptible to X-irradiation.

BALB/c

Colour: Albino

History: 3 major substrains (HeAn, J, R1) trace back to before 1940 probably diverging due to mutation or residual heterozygosity rather than genetic contamination.

Phenotypes: Good breeding and long reproductive life-span .Low tumour incidence. Corpus callosum (white matter found to bridge cerebral hemispheres) is absent in 39% of animals.

C57BL/6

Colour: Black

History: Founded 1937 as a substrain of the C57BL (founded 1921). Inbred for more than 150 generations.

Phenotypes: High preference to alcohol, susceptible to aortic lesions, low plasma cholesterol and high incidence of tumours

DBA

Colour: Grey

History: Oldest of all inbred mice. Founded 1909 from stock segregated on coat colour.

Phenotypes: High sensitivity to seizures.

FVB

Colour: albino

History: Originated in 1935 from outbred stocks and further selected for resistance to histamine in 1966 prior to inbreeding.

Phenotypes: High reproductive performance producing large litters. Fertilised eggs used widely for DNA microinjection.

Appendix 2

Numerical Data From Chapter 7.3

Ref	set	day	position 1				position 2				position 3			
			b	bm	c	cm	b	bm	c	cm	b	bm	c	cm
S5	1	3	-		-		-		-		-		-	
S6	1	3	43	62	21	106	39	104	3	78	54	143	16	100
S7	1	3	62	142	46	126	30	100	15	95	60	123	19	100
S8	1	3	34	104	16	66	35	115	6	86	16	76	5	85
P5	1	3	2	100	0	100	2	100	0	100	4	100	1	100
P6	1	3	3	100	1	100	4	100	0	100	1	100	1	100
P7	1	3	0	100	0	100	4	100	3	100	4	100	1	100
Pc2	1	3	0	100	0	100	0	100	0	100	0	100	0	100
Bnd2	1	3	4	100	0	100	2	100	2	100	3	100	4	100
S5	2	3	36	100	34	100	46	100	41	100	31	100	17	100
S6	2	3	42	100	46	100	31	100	26	100	27	100	24	100
S7	2	3	44	100	46	100	39	100	43	100	36	100	31	100
S8	2	3	26	100	30	100	18	100	14	100	21	100	16	100
P6	2	3	19	100	5	100	6	100	3	100	5	100	1	100
P7	2	3	7	100	2	100	9	100	7	100	1	100	1	100
Pc2	2	3	0	100	0	100	0	100	0	100	0	100	0	100
Bnd2	2	3	3	100	0	100	2	100	0	100	4	100	0	100
S9	1	6	32	100	17	100	35	100	13	100	29	100	1	100
S10	1	6	24	100	9	100	34	100	14	100	41	100	6	100
S11	1	6	40	100	21	100	42	100	16	100	37	100	7	100
S12	1	6	35	100	11	100	24	100	7	100	30	100	4	100
P9	1	6	3	100	0	100	5	100	1	100	1	100	0	100
P10	1	6	4	100	0	100	5	100	0	100	1	100	0	100
P11	1	6	7	100	0	100	3	100	0	100	0	100	0	100
P12	1	6	18	100	3	100	15	100	7	100	11	100	2	100
Pc3	1	6	0	100	0	100	0	100	0	100	0	100	0	100
Bnd3	1	6	2	100	0	100	0	100	0	100	0	100	0	100
S9	2	6	15	100	20	100	14	100	9	100	16	100	10	100
S10	2	6	31	100	17	100	27	100	10	100	25	100	11	100
S11	2	6	22	100	9	100	14	100	10	100	16	100	9	100

S12	2	6	30	100	16	100	14	100	6	100	16	100	12	100
P9	2	6	3	100	0	100	0	100	0	100	0	100	0	100
P10	2	6	4	100	1	100	1	100	0	100	2	100	0	100
P11	2	6	4	100	2	100	4	100	4	100	1	100	2	100
P12	2	6	12	100	7	100	2	100	1	100	8	100	3	100
Pc3	2	6	0	100	0	100	0	100	0	100	0	100	0	100
Bnd3	2	6	6	100	1	100	4	100	1	100	0	100	1	100
S13	1	21	25	100	14	100	0	100	1	100	2	100	0	100
S14	1	21	31	100	24	100	0	100	0	100	1	100	0	100
S15	1	21	43	100	29	100	2	100	1	100	0	100	0	100
S16	1	21	19	100	5	100	0	100	0	100	0	100	0	100
P13	1	21	0	100	0	100	0	100	0	100	0	100	0	100
P14	1	21	3	100	0	100	0	100	0	100	2	100	0	100
P16	1	21	0	100	1	100	0	100	1	100	0	100	0	100
Pc4	1	21	0	100	0	100	0	100	0	100	0	100	0	100
Bnd4	1	21	3	100	2	100	0	100	0	100	0	100	0	100
S13	2	21	31	100	34	100	42	100	27	100	35	100	30	100
S14	2	21	15	100	7	100	9	100	4	100	7	100	2	100
S15	2	21	27	100	13	100	21	100	11	100	16	100	12	100
S16	2	21	14	100	19	100	27	100	29	100	14	100	20	100
P13	2	21	15	100	3	100	17	100	9	100	12	100	5	100
P14	2	21	21	100	7	100	15	100	5	100	23	100	7	100
P16	2	21	5	100	1	100	2	100	2	100	11	100	4	100
Pc4	2	21	0	100	0	100	0	100	0	100	0	100	0	100
Bnd4	2	21	11	100	2	100	4	100	2	100	5	100	1	100
S17	2	95	15	100	4	100	4	100	3	100	1	100	2	100
S18	2	95	7	100	3	100	1	100	1	100	1	100	2	100
S19	2	95	11	100	4	100	2	100	1	100	3	100	1	100
S20	2	95	4	100	1	100	0	100	0	100	0	100	0	100
P18	2	95	12	100	6	100	11	100	5	100	6	100	2	100
P19	2	95	3	100	1	100	4	100	1	100	1	100	1	100
P20	2	95	2	100	1	100	1	100	0	100	0	100	0	100
P21	2	95	1	100	0	100	1	100	0	100	0	100	0	100
P22	2	95	2	100	1	100	0	100	1	100	0	100	0	100

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