The role of Cdx2 in Barrett’s metaplasia

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The role of \textit{Cdx2} in Barrett’s metaplasia

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Abstract
Barrett's metaplasia describes a condition in which the stratified squamous epithelium of the oesophagus switches to intestinal type columnar epithelium. The molecular mechanisms underlying the switch to intestinal epithelium is poorly understood but the transcription factor CDX2 has been implicated in the pathogenesis of Barrett's metaplasia and is sufficient to provoke an intestinal metaplasia in the stomach of transgenic mice. To address the molecular basis of Barrett's, I developed an innovative explant system for adult mouse oesophageal epithelium in which the full repertoire of stratified squamous cell types is maintained for prolonged culture periods. In adult oesophageal cultures, cells expressing p63, K14, K4 and loricrin were detected. The ability of Cdx2 to induce intestinal genes in this model as well as in a human oesophageal cell line and foetal mouse oesophageal cultures was assessed.

Cdx2 was sufficient to induce intestinal markers in Het-1A cells and foetal oesophageal epithelium but not in adult oesophageal explants. Following infection with Cdx2, Het-1A cells expressed four intestinal genes, Cdx1, Muc2, villin and K20. Embryonic oesophagus responds similarly and Muc2 and villin mRNA and Muc2 protein were detected. In contrast, infection of adult oesophageal explants did not provoke the expression of intestinal genes. These data suggest that additional factor(s) to Cdx2 are required in the conversion of adult oesophagus towards an intestinal phenotype as seen in Barrett's metaplasia.

HNF4α is a candidate factor to cooperate with Cdx2 in intestinal development and therefore Barrett's metaplasia. Herein I demonstrate that HNF4α is sufficient to induce a columnar phenotype and the expression of intestinal genes within adult squamous oesophageal cells. The resultant phenotype is consistent with that seen in Barrett's metaplasia. Furthermore HNF4α and Cdx2 synergise to further enhance intestinalisation. This data suggests a hitherto unknown potential role for HNF4α in Barrett's metaplasia.
List of Abbreviations

APTS  3-aminopropyltriethoxysilane
ATRA  All-trans retinoic acid
BECs  Biliary epithelial cells
BM   Barrett’s metaplasia
BMDSC Bone marrow-derived stem cell
BME  Basal Medium Eagle
BMP4 Bone morphogenetic protein 4
BSA  Bovine serum albumin
CA   Cholic acid
Cdx  Caudal type homeobox transcription factor
CE   Cell envelope
CMV  Cytomegalovirus
COX  Cyclooxygenase
DAB  Diaminobenzidine
DAPI 4’,6-Diamidino-2-phenylindole dihydrochloride
DCA  Deoxycholic acid
DHCA  Dehydrocholic acid
DMEM Dulbecco’s Modified Eagle’s Medium
DMSO Dimethyl sulfoxide
DSHB Developmental Studies Hybridoma Bank
E    Embryonic day
E. coli Escherichia coli
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene glycol tetraacetic acid
FGF  Fibroblast growth factor
FBS  Fetal bovine serum
FITC Fluorescein isothiocyanate
GATA GATA binding protein
GORD Gastro-oesophageal reflux disease
HCl  Hydrochloric acid
HNF  Hepatocyte nuclear factor
H2O2 Hydrogen peroxide
hrGFP Humanized recombinant green fluorescent protein
HRP  Horseradish peroxidase
IL   Interleukin
IM   Intestinal metaplasia
IRES Internal ribosome entry site
iu/ml Infectious units per milliliter
K    Cytokeratin
kb   Kilobase
kDa  KiloDalton
LOH  Loss of heterozygosity
LPS  Lipopolysaccharides
MEM Minimum Essential Medium Eagle
MOI Multiplicity of infection
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino] propane sulfonic acid</td>
</tr>
<tr>
<td>Muc2</td>
<td>Mucin 2</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>OA</td>
<td>Oesophageal adenocarcinoma</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pdx1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton pump inhibitor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted from chromosome 10</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAREs</td>
<td>Retinoic acid response elements</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SI</td>
<td>Sucrase isomaltase</td>
</tr>
<tr>
<td>SOX</td>
<td>Sex determining region Y-box</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>Tff3</td>
<td>Trefoil factor 3</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TH</td>
<td>T cell helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isomer R</td>
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Chapter 1 - Introduction

1.1 Metaplasia and tissue phenotype specification

1.1.1 What is metaplasia?

Metaplasia is a pathological term which describes the phenomenon of a cell or tissue type acquiring a different phenotype during postnatal life (Slack 1985; Tosh and Slack 2002; Slack 2007). It is not an uncommon phenomenon and results in the acquisition of a focus of ectopic tissue. Metaplasia can occur as the result of a change in phenotype of tissue-specific stem cells, differentiated cells or any of the intermediate phenotypes. Stem cells are undifferentiated cells that can both self-renew and generate specialised (functional) cell types (Burke, Thowfeequ et al. 2007). Adult stem cells generally populate one tissue type. For example, an intestinal stem is restricted to progeny that can form the 4 intestinal cell types (absorptive, goblet, paneth and enteroendocrine). There are numerous examples of metaplasia but they are especially common in epithelium, often in the context of chronic inflammation. Clues for the molecular mechanisms that are responsible for metaplasia are evident from normal developmental embryology.

It is common for metaplasia to arise in tissues that are embryonic neighbours, for example intestinal metaplasia arising in the stomach (Slack 2000) and gastric metaplasia developing in the duodenum (Fitzgibbons, Dooley et al. 1988). These tissues are adjacent in the developing endoderm of the embryo and remain so in the adult. Although these tissues are adjacent structures in the adult, the importance of embryonic anatomy is seen in cystitis glandularis. Cystitis glandularis is a pathological term for patches of colonic mucosa that are acquired in the bladder (Bell and Wendel 1968). These regions represent distinctly separate organs in the adult, but in embryo the bladder develops from a neighbouring region of hindgut and explains the propensity for these tissues to interconvert.

Heterotopia describes a congenital anomaly in which one tissue phenotype is found in an ectopic anatomical location, in contrast to the acquired nature of
metaplasia. Heterotopic tissue arises as a result of either mis-specification of phenotype at the embryonic stage or through failure to migrate to the correct anatomical position, and by definition is present from birth. A common example of heterotopia is the oesophageal inlet patch, in which gastric mucosa is found in the oesophagus following a failure of squamous epithelialisation during embryogenesis (Akbayir, Alkim et al. 2004).

Dysplasia describes the transformation of a tissue to an abnormal phenotype that has no counterpart in the normal body and usually marks the beginning of a progression towards neoplasia. Metaplasia is an important pathological condition because it is often an intermediate step in the development of dysplasia and cancer.

1.1.2 Tissue type specification during normal development
In order to determine the molecular mechanisms that are responsible for the phenomenon of metaplasia it is important to understand how tissue types are specified during development. Embryogenesis involves a tightly regulated temporal and spatial hierarchical expression of genes that determine cell types.

I will consider the important regulators of tissue type during embryogenesis and their commitment to normal development and metaplasia. Specifically I will examine genes that are essential for oesophageal and intestinal specification before exploring their roles in a particular type of metaplasia, Barrett’s metaplasia.

1.1.3 Master switch genes
Metaplastic tissue arises because of an altered pattern of expression of genes that determine tissue types during development (Tosh and Slack 2002; Burke, Thowfeequ et al. 2007). Key tissue type regulatory genes, that are mis-expressed in metaplasia, are referred to as homeotic or master switch genes (Tosh and Slack 2002). Organ development during embryogenesis results from a common sheet of cells that differentiate or divide in response to soluble factors or morphogens, such
as fibroblast growth factors (FGF), bone morphogenetic protein (BMP), sonic hedgehog (SHH) and retinoic acid (RA). Transcription factors, encoded for by master switch genes, are activated or repressed accordingly and different tissues specified. Master switch genes can dictate antero-posterior body patterning, as in the case of Hox genes, or specific tissue types, for example MyoD specifying muscle (Davis, Weintraub et al. 1987). Given that metaplastic tissue commonly arises from embryologically neighbouring tissues, it is hypothesised that it is distinguished from the surrounding region by the state of one or a few master switch genes. If this is the case then the ectopic expression (or repression) of a master switch gene in postnatal life could be responsible for the development of metaplasia (Figure 1.1).

1.1.4 Homeobox Genes
Genes that contain an evolutionary conserved 180 base pair DNA motif (the homeobox) are designated Homeobox genes. A set of homeobox genes are responsible for body patterning during embryogenesis and can give rise to homeosis on mutation. The homeobox encodes a protein domain (homeodomain) which binds DNA and acts as a transcription factor. Although all homeotic genes (concerned with developmental transformations of body segments) contain a homeobox motif, not all homeobox containing genes convey a homeotic function. The Homeobox genes were first described in Drosophila melanogaster in 1983, but have since been discovered in a wide variety of animals and fungi (Garber, Kuroiwa et al. 1983; Scott, Weiner et al. 1983).
Figure 1.1 Expression of a master switch gene during postnatal life can result in metaplasia.

During embryogenesis, two tissue types arise from a common cell sheet (green or red) in response to a master switch gene activated through the induction of a morphogen. The region of the cell sheet that expresses the gene during development becomes tissue B. In postnatal life induction of the master switch gene in one or a few cells in tissue A induces metaplasia to tissue B. The converse mechanism, inhibition of a transcription factor, provides an alternative explanation for metaplasia (adapted from (Quinlan, Colleypriest et al. 2007)).
1.1.5 Hox Genes
The Homeobox gene family is a highly conserved set of genes which contain a helix-turn-helix DNA binding motif conveying homeotic functions. A subgroup of Homeobox genes, known as ‘Hox genes’, control antero-posterior patterning of the developing body axis. Hox genes encode homeodomain transcription factors that regulate genes important for body patterning and cell fate determination. Whilst Drosophila contains a single set of hox genes, the mammalian homologues are dispersed into 4 Hox clusters. These 39 Hox genes are organized into 13 paralogous groups on four separate chromosomes (Scott 1992), in humans HOXA, HOXB, HOXC and HOXD on chromosomes 7, 17, 12 and 2 respectively. These genes are expressed in a colinear pattern along the length of the developing embryo and gut (Deschamps, van den Akker et al. 1999; Beck, Tata et al. 2000). That is, genes at one end of the Hox cluster are expressed and pattern the anterior region and genes at the other end pattern the posterior embryo. The Hox code exhibits spatial colinearity, the genes are temporally organised so that genes are activated in sequence from one end to the other. Thus the organisation of the Hox gene cluster tightly regulates the function of the genes involved in anterior-posterior axis of animals.

There are three stages responsible for the appropriate expression of Hox genes in the mouse – initiation, establishment and maintenance (Deschamps, van den Akker et al. 1999). Initiation begins in the posterior part of the primitive streak (a midline ectodermal ridge that forms in the caudal embryo and later gives rise to the mesoderm and definitive endoderm). A hierarchical expression of Hox genes then spreads anteriorly and results in ordered expression of positional information along the antero-posterior axis (Deschamps, van den Akker et al. 1999; Beck, Tata et al. 2000). The progeny of cells originating in a specific region of the streak continue to express ancestral Hox genes and this ensures maintenance of expression. The tightly regulated and ordered expression of genes in various regions of the developing embryo ensure that cells from the endoderm, lateral plate mesoderm and neural crest develop into the foregut, midgut and hindgut. Hox genes regulate
antero-posterior axial specification but there is no convincing evidence that the same is true for the gut.

1.1.6 The Caudal type homeobox transcription factor (CDX) Genes

The ParaHox cluster, consisting of CDX, PDX1 and GSH genes, represents a set of homeobox genes with high sequence identity (within the homebox) to Hox genes. These genes are felt to represent a paralogue or evolutionary sister of the Hox cluster (Brooke, Garcia-Fernandez et al. 1998). The *Caudal type homeobox transcription factor 2 (CDX)*, located on chromosome 13 in humans and chromosome 5 in the mouse, is a member of the ParaHox cluster. CDX2 belongs to a class of genes encoding transcription factors that constitute mammalian homologues of the Drosophila gene *Caudal* (Schulz and Tautz 1995). *Caudal* is a homeobox gene with roles in early patterning of the posterior segment and later development of the hindgut (Lengyel and Iwaki 2002). *Caudal* homologues have been identified in a number of organisms including humans and mice and have essential roles in intestinal development and maintenance of the epithelia (Silberg, Swain et al. 2000). In mice and humans there are 3 caudal homologues CDX1, CDX2 and CDX4 of which only CDX1 and CDX2 have roles in development of the gastrointestinal tract.

In the mouse embryo, expression of Cdx2 is first evident on embryonic day (E) 3.5 and is confined to the trophectoderm and persists in the extraembryonic ectoderm. From E8.5 Cdx2 is expressed in the posterior gut endoderm, neural tube and tail bud (Guo, Suh et al. 2004). By E12.5, Cdx2 is restricted to the endoderm of the gut (Beck, Erler et al. 1995) and its expression increases significantly during the transformation of endoderm into a columnar epithelium (E14-17) (Silberg, Swain et al. 2000).

Cdx2 null mice emphasise the importance of Cdx2 in the initiation of an axial anterior-posterior axis (Beck, Erler et al. 1995). Given the early role of Cdx2 in trophoblast specification it is not surprising that Cdx2 null mutants are embryonic
lethal and fail to implant (Chawengsaksophak, James et al. 1997). However, Cdx2$^{+/}$ heterozygous embryos show an anterior homeosis of skeletal structures, so that the anatomy of a vertebra in the mutant resembles a more anterior vertebra in the wild type (van den Akker, Forlani et al. 2002).

Postnatally, Cdx2 expression is limited to the small intestine and colonic epithelium of the gastrointestinal tract, importantly it is not normally observed in the stomach or oesophagus (James, Erler et al. 1994). The expression of Cdx2 is highest in the caecum and decreases proximally and distally towards the duodenum and rectum respectively (James and Kazenwadel 1991; James, Erler et al. 1994). Cdx2 is expressed in the majority of intestinal epithelial cells along the crypt-villus axis apart from the proximal intestine region where Cdx2 is absent from the villus tip (Silberg, Swain et al. 2000). Cdx2 has been shown to transcriptionally regulate many genes that are found in the intestinal epithelium; alkaline phosphatase (ALP), trefoil factor 3 (TFF3), sucrase isomaltase (SI), Villin (VIL1) and lactase-phlorizin (Troelsen, Mitchelmore et al. 1997; Tung, Markowitz et al. 1997; Braunstein, Qiao et al. 2002; Yamamoto, Bai et al. 2003; Alkhoury, Malo et al. 2005; Shimada, Koike et al. 2007).

The importance of Cdx2 in specifying and maintaining the intestinal phenotype and intestinal metaplasia will be considered later.

1.1.7 p63 – The master switch for stratified squamous epithelium

In order to understand the phenomenon of metaplasia in the oesophagus, it is important to consider genes that are responsible for establishing its phenotype. The oesophagus is lined by a multilayer (stratified) squamous epithelium, similar to the epidermis. The transcription factor p63, a candidate master switch gene for squamous epithelium, is essential for the commitment of cells to a stratified squamous differentiation pathway. It is a member of the p53 family of transcription factors, and exists in 6 isoforms. The human p63 gene, on chromosome 3, is transcribed from two promoter regions generating two classes of protein, the transactivating (TA) and truncated at the N-terminal isoforms (ΔN). Alternative
splicing produces 3 different C-terminus isoforms α, β and γ. The combination of N and C-terminus modifications provide the 6 possible isoforms (TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β and ΔNp63γ).

*p63* has a distinct expression pattern within the oesophageal epithelium that is different in human and murine oesophagus. In human oesophageal epithelium all basal cells, the majority of suprabasal cells and minimal number of differentiated squames are positive for *p63* (Glickman, Yang et al. 2001). Interestingly, the columnar lining of the oesophageal glands also expresses *p63* (Glickman, Yang et al. 2001). However, in the mouse, only the basal cells in contact with the laminar propria express *p63* (Daniely, Liao et al. 2004). *p63* is switched off as basal cells in the oesophagus differentiate. The basal cells of multilayered epithelium, an intermediate between squamous and columnar epithelium, also express *p63*, but BM cells do not (Glickman, Chen et al. 2001; Glickman, Yang et al. 2001).

There are two lines of evidence implicating *p63* with an integral role in the development of squamous epithelium. Firstly *p63* mutations are responsible for a number of congenital syndromes associated with epidermal malformations. Heterozygous mutations in *p63* contribute to the ectodermal dysplasias, a group of diseases associated with skin, hair, teeth, nails and sweat gland malformations (Rinne, Brunner et al. 2007). These syndromes often demonstrate craniofacial, limb, mammary and prostate abnormalities and are characterised by epidermal fragility.

Secondly, the generation of pan*p63* knockout mice in 1999 revealed very similar phenotypes to these human disorders (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999). Knockout mice demonstrated craniofacial abnormalities and lacked epithelial appendages such as mammary glands, hair follicles and teeth. Furthermore these mice had no epidermis nor squamous epithelium and malformed prostate and urothelium.
The majority of evidence concerning the role of p63 in squamous epithelium relates to epidermal development and homeostasis. Two separate p63 null mice have been described, the phenotype of which was very similar, although different conclusions for the role of p63 were initially drawn (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999). The lack of any squamous epithelium was interpreted by one group as a failure of stem cells to commit to a squamous lineage. (Mills, Zheng et al. 1999). McKeon and colleagues identified small patches of skin that expressed differentiated markers, such as loricrin and involucrin, and concluded that the stem cell pool had become depleted in response to the lack of p63 (Yang, Schweitzer et al. 1999). Careful examination of the epidermis and thymus in p63 null mice suggests that although p63 is essential for normal stratified squamous epithelium occasional squamous cells are found. The observation of differentiated thymic cells demonstrates that p63 is not essential for commitment to terminal differentiation of the thymus gland, but null animals develop hypoplasia (Senoo, Pinto et al. 2007). The epidermis of p63 null mutants, delivered by careful caesarean section, demonstrates similar findings of scattered terminal squames (Senoo, Pinto et al. 2007). It has been suggested that p63 is essential to maintain squamous stem cells and in keeping with this, p63 positive epidermal and thymic cells have greater clonogenic and proliferative capacity in vitro, a feature that is dramatically reduced with p63 knock down (Senoo, Pinto et al. 2007). In fact p63 has, in some tissues, been proposed as a marker of stem cells, although it often highlights a subset of cells with high proliferative potential.

Experiments in p63 null mice prove that the squamous phenotype can be partially rescued by ΔNp63 but not TAp63 isoforms driven from the K5 promoter (Koster, Kim et al. 2004). These experiments demonstrated that the ΔNp63 isoforms are the most important in squamous epithelium but that p63 is not necessary for the commitment to a squamous differentiation pathway, given that the K5 promoter must be active.
Further evidence that p63 is the master switch gene for squamous epithelium is highlighted in bronchial tissue. Ectopic expression of p63 alone is sufficient to provoke a squamous metaplasia in the normal columnar lung epithelium in mouse models. Interestingly terminal differentiation of the metaplastic squamous tissue did not occur (Koster, Kim et al. 2004).

The implication that squamous identity can be controlled by one gene has important implications for the oesophagus. In 2004 the development of oesophagus and trachea was studied in p63 null mice indicating an essential role for p63 in both tissues (Daniely, Liao et al. 2004). The oesophagus and trachea have common origins in the foregut endoderm at E9.5 and both tissues initially develop into a p63 positive pseudostratified ciliated epithelium (Wells and Melton 1999). Post-natally the oesophagus is lined by stratified squamous epithelium that maintains p63 expression in the basal cells and the trachea is a p63 negative glandular mucociliary mucosa (Daniely, Liao et al. 2004). p63 null mice developed oesophagus and trachea, but both exhibited a grossly abnormal epithelium; a ciliated simple columnar epithelium that neither stratified in the oesophagus nor pseudostratified in the trachea (Daniely, Liao et al. 2004). Intriguingly, the absence of p63 in this model, provokes a squamous to columnar switch which characterises that seen in Barrett’s metaplasia (BM).

1.2.1 Barrett’s Metaplasia
Barrett’s metaplasia (BM) describes the pathological condition characterised by a phenotypic switch in the distal oesophageal epithelium from the normal stratified squamous mucosa to an intestinal columnar type (Conio, Filiberti et al. 2002) (Figure 1.2). BM is synonymous with Barrett’s oesophagus and is an example of an intestinal metaplasia. All four intestinal cell types (enterocytes, goblet cells, paneth cells and enteroendocrine cells) can be present in BM. Although BM develops in the context of chronic reflux, it is an inherently asymptomatic condition often detected incidentally on endoscopy (Falk 2002).
Whilst the majority of countries require the presence of specialised intestinal metaplasia in the oesophagus to diagnose BM, the British Society of Gastroenterology (BSG) specify that endoscopically visible columnar metaplasia in the oesophagus is sufficient. The BSG argue that ‘intestinal metaplasia can always be identified in endoscopically-visible columnar metaplasia providing a sufficient number of biopsies are taken over an adequate time-scale’ (Playford 2006). The development of BM is important because it is the major risk factor and only known pre-cursor for oesophageal adenocarcinoma (OA). BM conveys an increased risk of developing OA of between 30-125 times that of the general population (Shaheen, Crosby et al. 2000), equating to a 1 in 20 lifetime risk (Wild and Hardie 2003). Males are 2-3 times more likely to be affected with BM (Peters, Hagen et al. 2004).

The prevalence of BM in the population is difficult to ascertain, but some studies suggest that it is rising (van Soest, Dieleman et al. 2005). There are significant discrepancies in the literature estimating incidence and prevalence of BM within the general population, which in part relates to the epidemiology of the study population and the heterogeneity of the definition of BM used. For example studies require varying lengths and types of metaplastic mucosa in the oesophagus to diagnose BM. In Scotland an increase in prevalence from 1 in 1000 endoscopies in the 1980s to 60/1000 in the late 1990s was demonstrated (Prach, MacDonald et al. 1997). An increase in incidence from 14.3 to 23.1 cases per 100 000 people between 1997 and 2002 was observed in a Dutch study (van Soest, Dieleman et al. 2005). Given that during the study time the absolute number of endoscopies decreased, the number of cases of BM per 1000 endoscopies rose from 19 to 40 (van Soest, Dieleman et al. 2005). The study confirmed a three fold increase in incidence of OA from 1.7 per 100 000 in 1997 to 6.0 in 2002 (van Soest, Dieleman et al. 2005). Obviously there is a degree of ascertainment bias because most patients requiring endoscopy are symptomatic and not representative of the whole population. Published estimate of population prevalence in asymptomatic patients vary significantly from 0% to 25% (Csendes, Smok et al. 2000; Gerson, Shetler et
A novel study by Rex and colleagues found long and short segment BM (<3cm) in 0.36% and 5.6% of patients attending for colonoscopy without reflux symptoms respectively (Rex, Cummings et al. 2003). The corresponding figures for the symptomatic patients were 2.6% and 8.3%. A Swedish population study found long segment (>2 cm) and short segment BM (with SIM) in 0.5% and 1.1% of a randomly selected population of 20-80 year old people (Ronkainen, Aro et al. 2005). 8.3% of the study population had columnar lined oesophagus and would meet the criteria for diagnosis of BM in the UK (Ronkainen, Aro et al. 2005). Further limited information is highlighted by two post mortem studies which found BM in 0.4% and 0.9% of autopsies (Cameron, Zinsmeister et al. 1990; Ormsby, Kilgore et al. 2000). Interestingly the number of clinically recognised cases of BM was 10 fold lower than that found in the same population at autopsy (Ormsby, Kilgore et al. 2000).

The magnitude of increased risk of progression to OA from BM varies in the literature where smaller studies often report higher cancer risks (Shaheen, Crosby et al. 2000). The majority of recent studies suggest an annual risk of progression of approximately 0.5% -1% (Drewitz, Sampliner et al. 1997; Eckardt, Kanzler et al. 2001; Jankowski, Provenzale et al. 2002) and UK figures concur with an incidence of 0.9% (Jankowski, Provenzale et al. 2002).

Unfortunately there is at present no widely available long-term curative treatment available for BM and surveillance programs are advised to detect histological evidence of progression towards cancer. The rationale for surveying this high risk population is related to the survival statistics for oesophageal cancer, which closely relates to tumour staging at diagnosis. Early tumours convey a more favourable prognosis. The 5-year survival for surgically resected mucosal/\textit{in situ} carcinoma is 70% compared with less than 20% for invasive disease (Farrow and Vaughan 1996).
Primary adenocarcinoma of the oesophagus arises in metaplastic columnar epithelium in the distal oesophagus (Chandrasoma, Wickramasinghe et al. 2007); or rarely within heterotopic gastric mucosa. Oesophageal columnar epithelium may be of three types: gastric-fundic-type (also termed oxynto-cardiac and is similar to the epithelium of the gastric fundus), cardiac (similar to the columnar epithelial mucous cells of the gastric cardia) or intestinal type (Chandrasoma 2005). The majority of the literature accepts that specialised intestinal metaplasia is the epithelial type that progresses to adenocarcinoma, rather than cardiac or oxynto-cardiac tissue (Wang and Sampliner 2008). However, recent publications demonstrating OA in metaplastic epithelium without specialised cells and DNA abnormalities, such as aneuploidy, in gastric cardia mucosa, have reopened this debate (Liu, Hahn et al. 2009; Takubo, Aida et al. 2009). OA develops from metaplasia through a progression from low to high-grade dysplasia. At diagnosis 15-25% of patients with established BM already have low grade dysplasia and 5% high grade (Sharma, Morales et al. 1997). Studies have shown variable rates of progression from high grade dysplasia to adenocarcinoma with estimates between 15 and 60 % over 5-8 years (Schnell, Sontag et al. 2001). It is important to remember when making treatment decisions that historically up to 40% of oesophagectomy specimens resected for high grade dysplasia have a foci of incidental adenocarcinoma (Collard 2002), although more recent data suggests that this figure is closer to 20% (Williams, Watson et al. 2007). There are few clinical studies predicting the time frame for progression from high grade dysplasia to adenocarcinoma. Current studies suggest an interval between 6-42 months with multi-focal disease progressing more quickly (Cossentino and Wong 2003). Surveillance strategies employing a higher number of biopsies result in a greater yield of adenocarcinoma (Reid, Blount et al. 2000).

The finding of high grade dysplasia marks the end point for surveillance. Traditionally oesophagectomy has been the only treatment option; consequently screening ceased at a point when the patient would not be fit for, or consider, oesophagectomy. Over the last decade, local ablative therapeutic options have
Figure 1.2 Histology of normal squamous epithelium (A and D), Barrett’s metaplasia (B and E) and oesophageal adenocarcinoma (C and F). A) Normal stratified squamous epithelium. The basal cell layer (which is in contact with the lamina propria) is arrowed. B) Barrett’s metaplasia - the multilayer squamous epithelium has been replaced by a columnar glandular mucosa showing intestinal metaplasia. The lamina propria is arrowed. C) A biopsy of oesophageal adenocarcinoma (marked with arrow). D) Stratification within the squamous epithelium. The basal layer of cuboidal cells (arrowed) progressively flattens and enlarges as differentiation occurs following loss of contact with the basal layer. E) Intestinal metaplasia within a Barrett’s biopsy. Note the organised glandular structure with the presence of goblet cells (arrowed). F) Adenocarcinoma is demonstrated by the presence of disorganised architecture and cytological atypia. Malignant cells have invaded through the epithelial basement membrane to infiltrate the lamina propria. Adapted from (Colleypriest, Palmer et al. 2009).
been used more successfully. Following careful staging and patient selection, laser ablation, photodynamic therapy, endomucosal resection, radiofrequency ablation and cryotherapy are techniques gaining experience (Barr, Stone et al. 2005).

The pathogenesis of BM concerns damage resulting from the reflux of acid and bile which provokes the oesophageal squamous cells to be replaced with columnar epithelium (Vaezi and Richter 1999), although the mechanism is not fully understood. Pharmacological acid suppression, usually with proton pump inhibitors (PPI), currently provides the main stay of medical treatment for BM. Whilst PPIs provide an effective treatment for reflux symptoms, oesophagitis and stricture prevention, a complete reversal of BM is not seen and evidence for preventing the progression to OA is conflicting (Carlson, Lechago et al. 2002; El-Serag, Aguirre et al. 2004; Garcia Rodriguez, Lagergren et al. 2006). Long term PPI use increases serum gastrin levels and this presents a theoretical risk of tumour progression in the context of cancer. Adenocarcinoma cell lines show increased proliferation and decreased apoptosis in response to gastrin (Haigh, Attwood et al. 2003).

Cancer of the oesophagus often remains asymptomatic until late in the disease and consequently carries a poor prognosis, with a 5 year survival of between 5 and 15% (Devesa, Blot et al. 1998; Bollschweiler, Wolfgarten et al. 2001; Newnham, Quinn et al. 2003; McManus, Olaru et al. 2004).

An understanding of the molecular steps that result in BM may offer the potential for targeted interventions at a metaplastic or pre-metaplastic stage.

1.2.2 Oesophageal cancer

In 2002 oesophageal cancer was the sixth commonest cause of cancer death worldwide accounting for 386 000 deaths and 462 000 new diagnoses (Parkin, Bray et al. 2005). Oesophageal cancer is currently responsible for over 7000 deaths per year in the United Kingdom and is consequently the 5th most common cause of death from cancer (2005). There are 2 main types of oesophageal cancer, squamous and adenocarcinoma. Globally, squamous cell carcinoma is the
commonest but in Western areas OA is being diagnosed at a rapidly rising rate (Vizcaino, Moreno et al. 2002). The epidemiology of OA has changed significantly over the last 35 years (Figure 1.3). In the early 1970’s squamous cell carcinoma accounted for the vast majority of oesophageal tumours and only 5-10% were of the adenocarcinoma type. The incidence of squamous carcinoma has remained stable whereas that of adenocarcinoma has increased approximately 4-fold (Blot, Devesa et al. 1991; Devesa, Blot et al. 1998). The rate of increase in incidence of OA over the last 30 years is faster than any other cancer (Pohl and Welch 2005). During a 25 year period from 1979-2004 the incidence in white Americans has increased 5-fold and continues to rise (Brown, Devesa et al. 2008), corresponding to an increase of 8% per year (Devesa, Blot et al. 1998). The reported increase in annual incidence varies from 1% to 17% in the literature (Winters, Spurling et al. 1987; Rex, Cummings et al. 2003). In the highest risk group, white males, the yearly rate of increase is over 10% (Blot, Devesa et al. 1991). The United Kingdom currently has the highest worldwide incidence of oesophageal adenocarcinoma (Bollschweiler, Wolfgarten et al. 2001).

1.2.3 Gastro-oesophageal reflux

Gastro-oesophageal reflux (GORD) is a major risk factor for the development of OA (Conio, Filiberti et al. 2002). GORD conveys an increased risk of between 2-9 times that of a symptom free population. A case controlled study found reflux symptoms to be associated with a six-fold increased risk of OA (Conio, Filiberti et al. 2002). A Swedish study confirmed the link, but found a 10-fold increased magnitude of risk (Johansson, Hakansson et al. 2007). In a Swedish population study the odds ratio for long standing or severe reflux was 43.5 (Lagergren, Bergstrom et al. 1999). The risk is highest with severe symptoms of long term duration (Lieberman, Oehlke et al. 1997; Avidan, Sonnenberg et al. 2001). Acid and bile salts, the main constituents of gastro-oesophageal reflux, have been shown to provoke DNA damage in oesophageal cell lines (Jolly, Wild et al. 2004).
Oesophageal adenocarcinoma has a strong male preponderance with a 7:1 male:female divide, the reason for this striking sex distribution remains unclear. A recent Scottish retrospective analysis of upper gastrointestinal cancer demonstrated that the age related risk for females lags 17 years behind that of males (Derakhshan, Liptrot et al. 2009). Two main theories exist to explain the protective effect of female sex. Firstly, oestrogen may offer a protection against the development of OA. In epidemiological studies, HRT use is associated with a 50% reduction in the risk of gastric adenocarcinoma, but this is not true for OA (Lindblad, Garcia Rodriguez et al. 2006). Oestrogens may offer their protective effects by inhibiting inflammation (reviewed in (Harnish 2006)). The second theory relates to decreased iron stores as a result of menstrual loss. Interestingly in a rat model of BM, OA did not develop unless the animals were given iron supplementation (Goldstein, Yang et al. 1997). Smoking carries a moderate increased risk of OA but alcohol is not reported to (Gammon, Schoenberg et al. 1997; Lagergren, Bergstrom et al. 2000). Helicobacter Pylori (H. Pylori) infection has been shown to have a protective effect and an inverse relationship with the risk of developing OA (Chow, Blaser et al. 1998; Ye, Held et al. 2004).
1.2.4 Oesophageal anatomy and stem cell compartment

The cellular origin of BM and the mechanism that results in a change in phenotypic is unknown. In order to hypothesise about this, it is important to understand the normal oesophageal anatomy.

The oesophagus functions to transport masticated food from the oral cavity, via the pharynx, to the stomach. Mouse and human oesophagus are both lined with stratified squamous epithelium, but vary because the murine epithelium is keratinised. The histological description of oesophageal epithelium is similar to that of epidermis and can be divided into 4 layers. The basal layer of epithelium, the stratum basale, consists of a single layer of proliferating cells. An epibasal differentiating zone, the stratum spinosum, contains transit amplifying and early differentiating cells. The layers of cells that are differentiating, the stratum granulosum, begin to flatten and contain granules. The outermost differentiated flattened squamous keratinised (in the mouse) epithelium is called the stratum corneum (Seery and Watt 2000). These layers can be characterised immunohistochemically depending on the expression of intermediate filament and constituent proteins of the cornified cell envelope (CE) (Figure 1.4).
proliferating basal cells express the intermediate filaments cytokeratin (K)5 and K14. At the onset of differentiation K4 and K13 replace K5 and K14, and the proteins of the CE, such as involucrin, are synthesised. Loricrin and filaggrin are late markers of differentiation and mainly found in terminally differentiated squames (Nemes and Steinert 1999) (Figure 1.4).

At regular intervals along the epithelium of the human oesophagus the lamina propria invaginates into the epithelium and forms papillae. Consequently the epithelium can be divided into papillary and interpapillary regions that possess different characteristics e.g. their proliferation rates and symmetry of cell division. In the papillary region, basal cells are 5 times more often found to be mitotic (Ki67-positive) compared to the interpapillary region (Seery 2002). Cell division in the papillary region results in two symmetrical daughter cells. However, basal cells within the interpapillary region often divide asymmetrically producing progeny in the basal and epibasal layer (Seery and Watt 2000).

The oesophageal epithelium is populated by stem cells possessing the ability to give rise to differentiated squames and to maintain a stem cell pool (Slack 2000). The stem cells give rise to transit amplifying cells that differentiate after a finite number of cell divisions, thus enabling a small number of stem cells to produce a large numbers of differentiated progeny (Potten and Loeffler 1990). Stem cells are inherently slowly proliferating cells, in contrast to their daughter transit amplifying cells. Proliferating cells are more commonly found in the epibasal layer than the basal layer and on this basis are likely to be the transient amplifying population of cells (Seery 2002). The asymmetric divisions found within the interpapillary region yield one progeny in the basal layer and one that enters the epibasal layer (Potten and Loeffler 1990). It is proposed that the cell remaining in the basal layer in contact with the lamina propria is a stem cell. Cells located around the papilla undergo more frequent and symmetrical mitosis which would be consistent with transit amplifying cells that are fuelled by the interpapillary stem cells.
The epibasal layer contains cells that will differentiate into mature squames. The whole epithelium is populated by stem cells, but at present there is no reliable marker of these cells and speculation of their location is based on observed characteristics. Using tritiated thymidine pulse labelling and chasing techniques, it has been demonstrated that all S phase cells are located in the basal layer, and that all cells within this layer could be labelled with 5 days of thymidine treatment (Leblond 1964). Within 48 hours of a single pulse, 50% of labelled cells had moved into the supra basal layers (Marques-Pereira and Leblond 1965). Further work on mice suggests that the oesophageal epithelium is arranged into clonal units on the observational basis of expression of chimeric X-linked genes in clusters (Thomas, Williams et al. 1988; Croagh, Thomas et al. 2008).

Given the parallels between epidermis and oesophagus, putative epidermal stem cell markers have been experimentally assessed within oesophageal epithelium. In tissues consisting of more than one differentiated cell type, putative stem cells have to be able to reconstitute all types and consequently this characteristic can be used as an assessment of stemness. Functional studies on oesophageal stem cells are complicated because there is only one differentiation pathway, and colony forming potential is assessed as a stem-like feature. The membrane bound receptors, the integrins, which facilitate attachment between cells and their surroundings, including extracellular matrix have been identified as epidermal stem cell markers. \( \beta_1 \)-integrin bright cells (high expressing), selected by FACs analysis, represent a subset of human epidermal keratinocytes that possess the highest colony forming potential (Jones and Watt 1993). Interestingly this is not true of oesophageal cells, it is the \( \beta_1 \)-integrin dull cells (low expressing) in human oesophagus, located in the inter-papillary regions, that have the highest colony forming potential (Seery and Watt 2000).

When surface markers are examined in mouse oesophageal epithelial cells a subpopulation of \( \alpha_6 \)-integrin bright / CD71 dim keratinocytes were label retaining and able to regenerate a normal epithelium \textit{in vitro} (Croagh, Phillips et al. 2007).
The neural stem cell marker, neurotrophin receptor p75ntr, has also been demonstrated to highlight human oesophageal cells with a high proliferative capacity in vitro (Okumura, Shimada et al. 2003). Although these markers have all been proposed as stem cell markers, the logical conclusion is that they highlight a heterogeneous population of cells within which the stem cells reside.

Inflammation is a common precipitant of metaplasia and the luminal contents of the oesophagus may provide this stimulus. Normal oesophageal epithelium usually provides an effective barrier to the luminal contents, however in the presence of reflux epithelial permeability increases considerably exposing immature keratinocytes (and the putative oesophageal stem cells) to noxious luminal contents. Tobey et al demonstrated that the normal oesophagus, which is impermeable to dextrans with a molecular weight of 4kDa, when exposed to refluxate, becomes permeable to 20kDa dextrans (Tobey, Hosseini et al. 2004). Bile salts are known to induce oxidative stress, DNA and mitochondrial damage and, in combination with acid, increase COX2, prostaglandin E2, mitogen-activated protein kinase and NFkB - all key inflammatory mediators (Dvorak, Chavarria et al. 2007). Whether acid and bile exposure to the deep squamous cells of the oesophagus in a permissive inflammatory environment, or the direct action of cytokines on key transcription factors and signalling pathways is responsible for BM remains unknown.
Epithelial expression of intermediate filaments and p63 found within native oesophagus.

Figure 1.4 Intermediate filaments expression in the stratified squamous epithelium.

Terminal differentiation of squamous epithelium involves the construction of an insoluble protective cornified envelope from structural proteins. The cornified envelope is a protein lipid layer that replaces the protein membrane. A temporal expression of structural proteins, including cytokeratins, is seen as the cells terminally differentiate. Adapted from (Quinlan, Colleypriest et al. 2007).

1.2.5 Cellular origins of Barrett’s metaplasia

It is not known how the reflux of acid and bile results in a change from a squamous to an intestinal phenotype nor which cells are responsible for instigating this change. A ‘Barrett’s stem cell’ must be present in the metaplastic tissue, given that once BM has developed it usually persists throughout the life of the patient. The location of the putative BM ‘stem cell’ is at present unknown and at least two possibilities exist. The first is that differentiated cells from the squamous epithelium
transdifferentiate to a columnar phenotype. A paradigm for a direct transdifferentiation is seen during the normal development of the mouse oesophagus, when squamous cells arise directly from columnar basal cells independent of both cell division and apoptosis (Yu, Slack et al. 2005). During embryogenesis, a proportion of the primitive columnar cells coexpress markers of squamous (K14) and columnar (K8) tissue. A reverse transformation, of differentiated squamous to columnar cells, could account for the switch in phenotype seen in BM. However, this seems unlikely given that the differentiated oesophageal cell would need to acquire an intestinal stem cell phenotype to produce and maintain all four intestinal cell types and self-renew.

The second possibility is of a change in the commitment of a resident oesophageal stem cell to that of an intestinal stem cell. The differentiation and proliferation of these stem cells into committed progenitor cells would result in intestinal tissue, containing all four cell lineages and an ability to self-renew. There are three possible stem cells residing in the gastro-oesophageal region that could give rise to BM (Figure 1.5). First, the putative squamous oesophageal stem cells located in the interpapillary basal zone of the epithelium (Seery 2002). Second, a stem cell from the duct of the columnar lined submucosal glands (analogous to the bulge region of the hair follicle), has been proposed as the cellular origin of BM (Rochat, Kobayashi et al. 1994; Glickman, Chen et al. 2001). Stem cells located in this region could theoretically repopulate an area of ulcerated mucosa with columnar epithelium. A reversion to squamous fate of these stem cells could account for the squamous islands seen within BM in close proximity to the ducts in association with PPI use (Paulson, Xu et al. 2006; Leedham, Preston et al. 2008). A potential problem with the oesophageal gland stem cell theory is the description of BM-like lesions in rodent oesophagus following oesophagojejunostomy (in which the oesophagus and jejunum are artificially linked to create free reflux). The rodent oesophagus does not contain glands and yet develops an IM that progresses to adenocarcinoma (Miyashita, Ohta et al. 2006).
The third possibility is the migration of a gastric stem cell to the gastro-oesophageal junction, which then repopulates the area with columnar epithelium. This theory is not favoured because the resultant tissue would be of a gastric not intestinal type and thus would require a further phenotypic change to account for BM. Animal studies have demonstrated that intestinal metaplasia of the canine oesophagus will develop in regions of oesophagus not in continuation with the GOJ (Gillen, Keeling et al. 1988).

An alternative stem cell with the potential plasticity to account for BM is a bone-marrow-derived stem cell (BMDSC). BMDSC have been shown to contribute to IM found within the gastric mucosa of a murine model of gastritis (Houghton, Stoicov et al. 2004). Recently, evidence of bone-marrow-derived stem cells contributing to IM in the oesophagus has been presented in a rat model of oesophagitis (Sarosi, Brown et al. 2008). The main criticism of these experiments is the possibility of cell fusion events between the BMDSC and gastric mucosal cells accounting for their presence and is intrinsically unlikely from the standpoint of developmental biology.

The most likely interpretation of these theories is that either the stem cells of the squamous epithelium or oesophageal glands are the most likely source of BM. A single point mutation in p16 has been demonstrated in microdissected squamous duct epithelium in continuation with adjoining BM, suggesting that a mutant population from one region has expanded to occupy the other (Leedham, Preston et al. 2008). There is a possibility that BM may arise from both or either of these stem cell regions and compete for clonal expansion with the other. A mutation in the intraepidermal stem cell compartment could result in BM, thus explaining the rodent model of BM and the presence of squamous islands in association with wild type squamous ducts. Similarly a mutation in the duct may repopulate the epithelium with BM and compete with the progeny of a wild type squamous epithelial stem cell. An analogous situation is demonstrated in the skin when multipotent stem cells of the interfollicular epidermis or hair follicle can regenerate both structures (Watt, Lo Celso et al. 2006).
Figure 1.5. Origin of Barrett’s metaplasia. The origin of the metaplastic tissue seen in BM is currently unknown. The stratified squamous epithelium is shown in blue, inflammatory cells are red, gastric cardia green and stem cells in yellow. At least three sources (yellow arrows) have been proposed for the BM stem cell (1) intrinsic oesophageal stem cells, (2) stem cells present in the submucosal gland (grey) and (3) stem cells located in the gastric cardia. Adapted from (Colleypriest, Palmer et al. 2009).
1.3 Metaplasia, Inflammation and Cancer

Metaplasia and cancer both share inflammation as an aetiology, for example squamous metaplasia that develops within the bronchi of smokers. (Peters, Morice et al. 1993) The role of chronic inflammation in the development of cancer was first suggested by Rudolf Virchow in 1863, following the observation that leukocytes were present in cancerous tissue and is estimated to account for 1 in 4 cancers (Hussain and Harris 2007). Understanding the mechanisms involved in the link between chronic inflammation and carcinogenesis, may offer opportunities for therapeutic intervention. There is epidemiological data linking chronic inflammation with the development of many cancers (Kundu and Surh 2008). Likewise, genetic polymorphisms in genes involved in the inflammatory cascade have been shown to convey a susceptibility to cancer. For example, mutations in genes encoding Toll-like receptors (TLRs), have been linked to prostate cancer and polymorphisms within the interleukin-1 (IL-1) locus have been associated with gastric cancer (El-Omar, Carrington et al. 2000; Sun,Wiklund et al. 2005). Tumour necrosis factor alpha (TNFα), an archetypal inflammatory cytokine, is overexpressed in a variety of premalignant lesions such as gastritis induced by *Helicobacter pylori*, BM and colitis (Kundu and Surh 2008). Similarly, TNFα is present in many cancerous tissues, including oesophageal (Arnott, Scott et al. 2004), and *TNFa* gene mutations confer a poor prognosis in patients with non-Hodgkin’s lymphoma (Cerhan, Liu-Mares et al. 2008). Interruption of TNFα signalling may confer resistance to the development of cancer in certain models (Arnott, Scott et al. 2004).

Given that oesophagitis and GORD seem to be inherently linked to the development of BM and in the progression to OA, anti-inflammatory drugs are a logical choice for chemoprevention. There is good epidemiological evidence that non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin prevent oesophageal cancers of both subtypes, but specifically adenocarcinoma (Sadeghi, Bain et al. 2008). A meta-analysis of 9 studies incorporating 1813 cases demonstrated a 43% risk reduction with any exposure to aspirin or NSAIDS (Corley, Kerlikowske et al.
2003). A multi-centred randomised controlled trial, AsPECT, designed to demonstrate the benefits of PPI and/or aspirin in chemoprevention for BM is currently under way (J de Caestecker and Jankowski 2009).

1.3.1 The role of inflammation in BM and OA

Whilst the major risk factors for developing BM are known, namely the reflux of acid and bile, the sequelae of molecular events invoked by oesophageal inflammation are poorly understood. Recent papers have linked inflammatory mediators and active components within refluxate with key signalling pathways and transcription factors inherent to BM.

The immune response, as defined by cytokine profile and mediated by T-lymphocytes, can be divided into 3 types, T-helper (Th) 1, Th2 and Th17. The contribution of Th17 cells to BM is largely unexplored, but at some point during the development of BM the immune profile switches from a Th1 to Th2 type response (Fitzgerald, Onwuegbusi et al. 2002; Moons, Kusters et al. 2005). It is not known whether this is causative.

Direct evidence for the role of inflammation in the development of IM has been demonstrated using mouse models of *H. pylori* gastritis in immunodeficient mice. *H. pylori* infection fails to cause either inflammation or metaplasia in recombinase-activating gene (Rag) deficient, severe combined immunodeficiency (SCID) and T-cell-deficient mice, suggesting that an intact immune system is required for metaplasia (Roth, Kapadia et al. 1999; Smythies, Waites et al. 2000). Mice deficient in only B-cells, but with reconstituted T-cells, develop severe atrophy and IM in response to *H. pylori*. These results confirm that *H. pylori*-induced gastritis and IM are reliant on Th1 cells in the stomach (Eaton, Mefford et al. 2001). However, in BM a Th2-predominant response is observed in contrast to Th1-type observed in oesophagitis. In a human biopsy study, BM was demonstrated to have a higher number of Th2-type inflammatory cells, plasma cells and mast cells but similar quantities of Th1 effectors (Moons, Kusters et al. 2005). Although BM is
characterised by an increased in Th2 cells, Th1 cells characterise the response in oesophagitis. The observational and epidemiological link between chronic inflammation and BM/OA has been suspected for a long time. Recently the molecular mechanisms that link the inflammatory cascade, metaplasia and cancer are beginning to be dissected.

1.3.2 Oesophagitis
BM is more common in patients with oesophagitis and is usually the result GORD (Winters, Spurling et al. 1987; Vaezi and Richter 1996). The key to understanding the pathogenesis of BM lies in unravelling the complex molecular interactions that arise as a result of acid and bile refluxing into the oesophagus. OA develops as the last step along an oesophagitis–metaplasia–dysplasia pathway, and consequently the development of BM heralds the change from an inflammatory condition – oesophagitis – to a pre-malignant lesion - BM.

BM occurs in 6–12% of patients with symptoms of GORD compared with 0.2–2% of the general population, supporting a primary role for inflammation in the development of metaplasia (Falk 2002). Oesophagitis comprises a cellular inflammatory response provoked by the reflux of acid and bile and is mediated by the release of inflammatory cytokines. Endoscopic studies on patients with persistent reflux, prior to proton pump inhibitors, show 42% to be normal, 45% to have oesophagitis and 12% to have BM (Winters, Spurling et al. 1987). Interestingly, only a proportion of patients with reflux oesophagitis develop BM, suggesting that factors other than reflux are necessary to develop the premalignant condition (Jankowski, Harrison et al. 2000).

1.3.3 Inflammatory Mediators in Oesophagitis
The levels of proinflammatory cytokines and chemokines interleukin (IL) IL-1β, IL-8 and interferon (IFN) IFN-γ are elevated in biopsies of oesophagitis compared to BM. Interestingly, this variation in inflammatory profile occurs irrespective of whether acid reflux is suppressed, suggesting that GORD is not wholly
responsible. In the presence of continued reflux, there is an inherent difference in
the response to acid after BM develops. IL-10 levels are elevated equally in BM
and oesophagitis compared to normal, whereas IL-4 is increased in BM compared
with both oesophagitis and normal squamous tissue (Fitzgerald, Onwuegbusi et al.
2002). Furthermore, the cytokine profile of BM contrasts to control duodenal
samples, proving that the presence of intestinal-type tissue per se is not
responsible for the increased IL-4 level.
The inflammatory cytokine/chemokine/transcriptional profile of BM, oesophagitis,
normal squamous oesophagus and intestine are all different (Figure 1.6). The fact
that the cytokine microenvironment of BM and intestine is different may explain the
variation of malignant potentials seen in these histologically similar tissues. BM has
a life time malignant potential approaching 1 in 20, but small bowel
adenocarcinoma is rare with an annual incidence of 3-6 per 1000000 patients
(Neugut, Jacobson et al. 1998).

The chemokines RANTES/CCL-5 and MCP-1/CCL2, that recruit T-lymphocytes
and monocytes respectively, are elevated in oesophagitis. The levels of IL-8 in
oesophagitis decrease in response to pharmacological acid suppression. In
contrast to research by Fitzgerald et al (Fitzgerald, Onwuegbusi et al. 2002),
differences in IL-8 and IL-1β expression between BM and oesophagitis were not
seen by O'Riordan et al, who found that these cytokines were significantly elevated
in both BM and oesophagitis (O'Riordan, Abdel-latif et al. 2005). An explanation for
this discrepancy relates to the precise position of biopsies within the BM. An
inflammatory gradient exists within BM, with higher expression of IL-8 and IL-1β
observed at the neosquamous junction and within the proximal BM (Fitzgerald,
Abdalla et al. 2002).

Nuclear factor kappa B (NFκB) is a transcription factor with key roles in
inflammation, differentiation and cell growth (Hayden and Ghosh 2004). NFκB has
been implicated in the pathogenesis of a multitude of inflammatory conditions,
including rheumatoid arthritis and inflammatory bowel disease (O'Riordan, Abdel-
Activated NFκB stimulates secretion of IL-8 and IL-1β, which in turn completes an auto-regulatory feedback loop to increase NF-κB. In contrast to IL-8 and IL-1β, NFκB expression increases at each step of the pathway from normal oesophagus through oesophagitis, BM and on to OA, suggesting that additional factors to NFκB act to govern the progression to premalignancy. A more detailed discussion on the role of NFκB in inflammation and cancer is described below.

The overexpression of IL-6 has been linked to the pathogenesis and prognosis of many cancers and is transcriptionally downregulated by the tumour suppressor p53. BM biopsies show higher levels of IL-6 than normal squamous or intestinal samples from the same patient and are consistent with a Th2 response (Dvorakova, Payne et al. 2004). IL-6 also promotes cell survival by activating the anti-apoptotic signal transducers and activators of transcription 3 (STAT3) pathway. In support of an anti-apoptotic role for IL-6, the levels of STAT3 increase along the metaplasia–dysplasia–adenocarcinoma pathway (Dvorak, Chavarria et al. 2007).

In summary, the inflammatory profile of normal squamous epithelium, oesophagitis (Th1 response) and BM (predominantly Th2 response) are different irrespective of continued GORD.

1.3.4 Cyclooxygenase-1 and 2 (COX)
The COX (PTGS) genes encode proteins that catalyse the synthesis of prostaglandin from arachidonic acid. There are 2 isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and COX-2 is induced in response to inflammation (Dannenberg, Altorki et al. 2001). COX-2 expression has been implicated in the development of cancer and is elevated in premalignant lesions, such as BM (Kandil, Tanner et al. 2001). COX-2 is not expressed in normal (non-inflamed) gastrointestinal epithelium but is present in oesophagitis, BM and IM of the stomach, increasing from metaplasia to cancer (Morris, Armstrong et al. 2001; Ferguson, Wild et al. 2008).
Epidemiological data suggests that COX2 may be contributing to the pathogenesis of OA because of the decreased risk observed with the use of selective and non-selective COX inhibitors (Dannenberg, Altorki et al. 2001). The relative risk of developing oesophageal cancer is decreased by 73% with regular exposure to NSAIDs (which act as inhibitors of COX) (Harris, Beebe-Donk et al. 2005). In an in vivo rat model of reflux-induced oesophageal cancer, COX-2 inhibitors decreased the risk of OA and prevented the development of oesophagitis in response to GORD (Buttar, Wang et al. 2002; Oyama, Fujimura et al. 2007). COX-2 is induced in response to the reflux of bile into the oesophagus (Zhang, Altorki et al. 2001), and unconjugated bile salts increase expression of COX-2 in BM and OA derived cell lines (Song, Guha et al. 2007). In the same model of oesophagitis, COX-2 expression is induced, along with the inflammatory cytokines IL-1β and TNF-α (Hamaguchi, Fujiwara et al. 2003). Monitoring of clinical acid reflux magnitude correlates with COX-2 levels (Hamoui, Peters et al. 2004). Recent evidence has linked specific single nucleotide polymorphisms (SNP) within regulatory regions of the COX-2 gene with an increased risk of oesophageal cancer (both types), suggesting a role for COX-2 in the progression to cancer (Zhang, Miao et al. 2005; Moons, Kuipers et al. 2007; Ferguson, Wild et al. 2008). For example the -1195 G to A SNP in the COX-2 promoter increases promoter activity and conveys an increased risk of oesophageal cancer.
**Figure 1.6. Profile of inflammatory mediators through the oesophagitis–Barrett’s metaplasia–oesophageal adenocarcinoma pathway.** (A) Schematic of progression to cancer from normal oesophageal stratified squamous epithelium (SSQ) through oesophagitis (Oes) to Barrett’s metaplasia (BM) and adenocarcinoma (OA).

Proteins that can be used to characterise the cell types are marked. The cytokeratins found within squamous epithelium changes from K5/14 to K4/13 when the basal cells differentiate. The transcription factor p63 is located in the nuclei of the basal layer of squamous cells. The epithelium of columnar intestinal mucosa is typified by K8/18 and K7/20. Inflammatory cells are red. (B) Expression of inflammatory mediators IL-1β, IL-8, IFN-γ, IL-4, IL-10, IL-6, NFκB, TNFα and COX2 at the different stages of the pathway. The level of expression is shown by – (absence) to ++++ (highly expressed). References for data are i) (Fitzgerald, Onwuegbusi et al. 2002), ii) (O’Riordan, Abdel-latif et al. 2005), iii) (Dvorakova, Payne et al. 2004), iv) (Tselepis, Perry et al. 2002) and v) (Marshall, Morrison et al. 1996). Adapted from (Colleypriest, Palmer et al. 2009).

<table>
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<th>Mediator</th>
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1.4 Molecular clues in the pathogenesis of BM

1.4.1 Intercalary regeneration

The first indication that \textit{Cdx2} may be important in the pathogenesis of BM came from Beck in 1999 (Beck, Chawengsaksophak et al. 1999). He demonstrated that mice heterozygous for wild type \textit{Cdx2} gene develop colonic polypoidal lesions consisting of stratified squamous mucosa, similar to human oesophagus and mouse forestomach. These areas of metaplastic squamous mucosa lacked \textit{Cdx2} expression as assessed by immunohistochemistry. It is hypothesised that loss of the wild type allele, through somatic mutation or epigenetic processes, and consequent loss of \textit{Cdx2} expression results in an anterior shift in phenotype to squamous epithelium. Interestingly, the squamous mucosa is flanked in a circular shaped pattern by all of the interposing tissue types found along the antero-posterior axis. Areas of tissues representative of gastric corpus, gastric fundus and small intestine were clearly demonstrated with their appropriate cell types in between the squamous polyp and the native colonic epithelium. The process of ordered filling in is termed intercalary regeneration (Figure 1.7). The phenomenon of intercalary regeneration suggests the intriguing possibility that the development of IM in the oesophagus may require or result in an interposing gastric phenotype.

The importance of \textit{Cdx2} in specifying intestinal-type epithelium has been reinforced by conditional knock out studies. When \textit{Cdx2} is selectively deleted in early endoderm under the control of the FoxA3 promoter, an anterior homeosis of the gut is seen (Gao, White et al. 2009). In these studies the gut is lined by a stratified squamous epithelium, intestinal genes are down-regulated and oesophageal genes up-regulated. When this evidence is considered along with the lack of a gut phenotype for \textit{Cdx1} null mice it further suggests that \textit{Cdx2}, rather than \textit{Cdx1}, is the master switch for intestine.
**Intercalary Regeneration Around Squamous Polyps in Colonic Mucosa of Heterozygous cdx2 mutant mice**

![Diagram showing epithelial distribution]

**Figure 1.7 - Intercalary regeneration.**

Mice heterozygous for wild type *Cdx2* develop multiple stratified squamous polyps in the colon lacking any expression of *Cdx2*. Between the native colonic mucosa and the squamous oesophageal tissue representative of gastric corpus, gastric fundal and intestinal epithelium could be seen. This ordered filling in of the missing tissues has been termed ‘intercalary regeneration’.

SSQ - Stratified squamous polyp in columnar epithelium  
GAC - Gastric antral columnar epithelium with parietal cells  
GCC - Gastric corpus columnar epithelium with mucus secreting cells  
SIE - Small intestinal epithelium with Paneth and Goblet cells  
CCE - Normal columnar Colonic epithelium
1.4.2 Cdx2 and intestinal metaplasia

The importance of Cdx2 in establishing and maintaining the intestinal phenotype suggests a putative role for CDX2 in the acquisition of ectopic intestinal tissue. IM has been demonstrated in a wide variety of tissue types for example oesophagus, stomach (Mizoshita, Inada et al. 2001), gall bladder (Kozuka and Hachisuka 1984), biliary tree (Kozuka, Kurashina et al. 1984), urinary tract (Osawa, Kita et al. 2004), bladder (Steininger, Mueller et al. 2005) and liver (Tatematsu, Kaku et al. 1985). Furthermore, the presence of IM in each of these locations is accompanied by ectopic CDX2 expression. Gastric IM, which is normally associated with chronic inflammatory conditions such as H. Pylori infection, has been shown to aberrantly express CDX2 (Bai, Yamamoto et al. 2002; Almeida, Silva et al. 2003). IM within the gall bladder and bile ducts is associated with chronic choledocholithiasis (the presence of gallstones in the bile duct) and demonstrates CDX2 expression (Ikeda, Sasaki et al. 2007; Sakamoto, Mutoh et al. 2007). Cystitis glandularis describes a columnar metaplasia of the bladder epithelium of 2 subtypes. The first type is a simple columnar lining which replaces the normal transitional epithelium and contrasts to the more complex second type which is a true IM. Only the second subtype exhibits CDX2 expression (Steininger, Mueller et al. 2005; Sung, Lopez-Beltran et al. 2006).

In 2003 Eda et al demonstrated CDX2 expression in BM and these findings have been repeated subsequently by other groups (Eda, Osawa et al. 2003; Phillips, Frierson et al. 2003; Moons, Bax et al. 2004). Interestingly CDX2 mRNA is found in the native squamous epithelium above the Barrett’s segment in one third of patients (Moons, Bax et al. 2004), suggesting that the expression of the transcription factor may precede the switch in phenotype.

A causal role for Cdx2 in gastric IM has been suggested by 2 transgenic mouse studies. In both studies Cdx2 expression directed to the stomach mucosa was sufficient to provoke IM. Silberg et al used the FoxA3 promoter and demonstrated IM histologically with alcian blue staining of goblet cells and intestinal-specific gene
expression (Silberg, Sullivan et al. 2002). Mutoh and colleagues used the H+/K+ ATPase β-subunit gene promoter to drive gastric Cdx2 expression (Mutoh, Hakamata et al. 2002). The resultant gastric intestinal metaplasia contained goblet, enteroendocrine and absorptive enterocytes. Incredibly the neo-mucosa is functional in its absorptive capacity given that it can rescue transgenic mice with extensive intestinal resection (Mutoh, Satoh et al. 2005), suggesting that Cdx2 alone is sufficient to specify fully functional absorptive enterocytes in the stomach. Given that, in theory, the proton pump starts to function post-natally, in this model, Cdx2 should only be expressed in differentiated gastric epithelium. Thus the resultant IM arises from mature post natal gastric cells providing the proton pump promoter is not active prior to birth.

In a rat model, in which free bile reflux is achieved by anastomosing the oesophagus and jejunum, IM, expressing Cdx2, develops in 30% of animals (Kazumori, Ishihara et al. 2006). Furthermore, transfection of a Cdx2 construct into primary cultured rat oesophageal keratinocytes is sufficient to provoke MUC2(a mucin expressed in intestinal cells and a direct target of CDX2) (Kazumori, Ishihara et al. 2006). Further observational evidence of the importance of CDX2 in specifying the intestinal phenotype is seen in gastric metaplasia of the duodenum which lacks CDX2 expression (Faller, Dimmler et al. 2004).

1.4.3 The role of CDX1 in Barrett’s Metaplasia

The CDX1 gene has been implicated in BM. A transgenic mouse model, identical to the Cdx2 experiments, directed ectopic Cdx1 expression in the stomach under control of the H+/K+ ATPase β-subunit gene promoter provoked similar, although not identical, IM (Mutoh, Sakurai et al. 2004). The gastric mucosa of Cdx1 expressing mice contained the full repertoire of all 4 types of intestinal cell; enterocytes, goblet cells, paneth cells and enteroendocrine cells. Cdx2 expression only provoked 3 types of intestinal cell and failed to produce paneth cells. Given that Paneth cells are rarely seen in BM, the Cdx2 transgenic could be considered a
better model. Like Cdx2, Cdx1 is found in biopsies from Barrett’s metaplasia (Wong, Wilding et al. 2005).

Interestingly Cdx1 null mice have no gut phenotype suggesting that Cdx2 is more important in the initiation of the intestinal phenotype (Subramanian, Meyer et al. 1995). There is considerable overlap and redundancy between Cdx1 and Cdx2 during development. The skeletal phenotype of the Cdx1 null mouse is rescued when Cdx2 is knocked in (on the Cdx1 promoter) (Savory, Pilon et al. 2009). A compound Cdx1+/−/Cdx2+/− has been described in terms of axial abnormalities but unfortunately not the intestinal tract (van den Akker, Forlani et al. 2002).

1.4.4 Regulation and provocation of Cdx2 expression

Acid and bile salts, the major constituents of refluxate, influence CDX2 expression in adenocarcinoma cell lines. The colonic cell lines HT29 and Caco-2 both show increased CDX2 expression in response to cholic and dehydrocholic acid (Kazumori, Ishihara et al. 2006). These findings have been confirmed in four oesophageal cell lines (Hu, Williams et al. 2007). In these studies, CDX2 and MUC2 mRNA is induced following bile acid treatment. Cdx2 expression was induced in a primary cell culture of mouse oesophageal cells exposed on the apical aspect to acidified culture media (Marchetti, Caliot et al. 2003). How the extracellular components of bile and acid influence the transcription or activation of Cdx2 is not known, although NFκB activity is essential (Kazumori, Ishihara et al. 2006).

Phosphoinositide 3-kinase (PI3K)-dependent signalling cascades have recognised roles in many aspects of cell growth and proliferation as well as differentiation (Crabbe, Welham et al. 2007). It is interesting, therefore, that the lipid phosphate PTEN (phosphatase and tensin homologue deleted from chromosome 10) stimulates CDX2 protein expression in a colonic cancer-derived cell line (Kim, Domon-Dell et al. 2002). PTEN dephosphorylates PI(3,4,5)P3, the lipid product of PI3K to inhibit downstream signalling and it is interesting that PI3K inhibitors [that mimic the decrease in cellular PI(3,4,5)P3] also stimulate CDX2 activity. The effect
of the PI 3-kinase signalling pathway on CDX2 expression is mediated through members of the NFκB transcription factor family. NFκB has two putative binding sites within the CDX2 promoter region (Kazumori, Ishihara et al. 2006). The upregulation of CDX2 by NFκB activity is suggestive of a mechanistic link between the inflammatory cytokine profile seen in oesophagitis and induction of CDX2. Interestingly, in the same study, the proinflammatory cytokine TNFα decreased CDX2 protein levels. It is proposed that the CDX2 promoter is positively regulated by the NFκB subunit, p50 homodimers, yet negatively regulated by p50–p65 heterodimers. Furthermore, abrogation of PI3K signalling, either by PTEN overexpression or inhibition of PI3K, overcomes p65-mediated inhibition by TNFα. The induction and regulation of CDX2 is positively regulated by inflammatory mediators suggesting a putative mechanistic link between oesophagitis and BM.

A comprehensive study characterising the Cdx2 promoter revealed interactions with multiple transcription factors (Benahmed, Gross et al. 2008). HNF4α, β-catenin, Tcf4 and to a lesser extent GATA6 all independently stimulated promoter activity of a Cdx2-luciferase construct in co-transfection experiments in a colonic cell line. There was synergy between GATA6 and HNF4α and with all four transcription factors together in up-regulating promoter activity. Furthermore the combinations of GATA6 and HNF4 or β-catenin and Tcf4 or all 4 factors provoked the induction of Cdx2 and intestinal genes sucrase isomaltase and LI-cadherin. There is further evidence that the Wnt signalling pathway may be involved in regulation of CDX2 expression, given that transfection of an endometrial carcinoma cell line with an active form of beta-catenin increased CDX2 protein by 2.5-fold (Saegusa, Hashimura et al. 2007). The Wnt5A ligand also induces CDX2 protein in the colorectal adenocarcinoma cell line HT-29, suggesting a role for Wnt signalling pathway in the regulation of CDX2 (Figure 1.8) (Pacheco and Macleod 2008). Recently, CDX2 has been found to up regulate its own promoter activity in a human oesophageal adenocarcinoma cell line (Kazumori, Ishihara et al. 2009). A similar response was demonstrated on the CDX2 promoter following CDX1 transfection. In summary, CDX2 is regulated by transcription factors important in
intestinal development and homeostasis as well as key mediators of the inflammatory cascade.

![Diagram of Cdx2 expression regulation](image)

**Figure 1.8 Potential mechanisms for regulation of Cdx2 expression.**

The blue oval is the nucleus and the site for CDX2 transcription. Acid (H+), bile acids and inflammatory cytokines induce CDX2 expression by an unknown mechanism. Inflammatory intermediates induce CDX2 expression through TLRs and an NFkB-dependent mechanism. PTEN, acting via NFkB, upregulates CDX2 and is antagonised by PI3-K signalling. Beta-catenin signalling and upstream ligands increase CDX2 expression. BMP4 upregulates CDX2 transcription via phosphorylation of SMAD 1, 5 and 8 which complexes with SMAD 4 and translocates to the nucleus. The transcription factors HNF4α, Tcf4 and, to a lesser extent, GATA6, induce CDX2 expression and are antagonized by SOX9, SOX2 and KLF4. Adapted from (Colleypriest, Palmer et al. 2009).
1.4.5 The innate immune system and Cdx2

The regulation of Cdx2 is complicated by studies showing that inflammatory cytokines and various bacterial pathogen associated molecular patterns (PAMPs) can induce Cdx2 and Muc2 in cholangiocytes (Zen, Harada et al. 2002; Ikeda, Sasaki et al. 2007; Ikeda, Sasaki et al. 2008) (Figure 1.8). PAMPs are conserved pathogen associated molecular motifs that are recognised by toll-like receptors (TLRs) and active the innate immune system. Murine biliary epithelial cells up-regulate Muc2 and Muc5AC expression in response to the gram negative bacterial component lipopolysaccharide (LPS) (Zen, Harada et al. 2002). In the same model, TNFα was up-regulated by LPS and could independently provoke Muc2 and Muc5AC transcription. Furthermore, TNFα inhibitors partially inhibited the mucin response related to LPS, suggesting either incomplete inhibition or an alternative mechanism for mucin upregulation. Upregulation of mucin in response to inflammation has been demonstrated in other tissue. Muc2 and Muc5ac expression is increased in response to either LPS or TNFα in bronchial epithelium, nasal and middle ear cells (Levine, Larivee et al. 1995; Li, Dohrman et al. 1997; Lin, Haruta et al. 2000). The effects of LPS on lung epithelium are mediated through NFkB, which, as demonstrated previously is key to Cdx2 regulation and increased in BM (Li, Feng et al. 1998).

Some of the key inflammatory mediators in oesophagitis can be induced by LPS and in turn influence Cdx2. IL-8 expression is increase in response to LPS in lung epithelial cells, via an NFkB dependent mechanism (Li, Johnson et al. 2003). IL-1β is over-expressed in BM and increases both Muc2 and Muc5ac protein levels in bronchial cells (Kim, Kwon et al. 2002). Interestingly, COX-2 induction is seen in response to LPS and is integral to oesophageal inflammation seen in oesophagitis-adenocarcinoma pathway. The fact that putative NFkB binding sites are demonstrated in the COX 2 promoter region suggests some commonality between the inflammatory cascade seen in BM and Cdx2 regulation. Further evidence linking inflammation and Cdx2 arises in the rat biliary system. Treatment of primary rat biliary epithelial cells with various PAMPs, such as LPS, lipoteichoic acid (LTA),
peptidoglycan (PGN) and the synthetic lipopeptide Pam3Cys-sKKKK (Pam3), provokes the induction of Cdx2 and Muc2 (Ikeda, Sasaki et al. 2007). The response was blocked with antibodies to the appropriate toll like receptors (TLR) or by inhibiting NFkB, demonstrating that NFkB acts downstream of TLRs. In biliary epithelial cells, TNF-α, IL-1β, IL-6 and interferon-γ all induce Cdx2 mRNA through an NFkB dependent mechanism (Ikeda, Sasaki et al. 2008). The action of cytokines and PAMPs in these studies suggests a direct link between inflammation and Cdx2/Muc2 expression through NFkB, albeit in biliary cells. The possibility of similar mechanisms provoking the change in oesophageal phenotype seen in BM needs to be further examined. There are considerable differences between biliary and oesophageal epithelium not least the cytokeratin expression profile and morphology of columnar versus squamous mucosa. Oesophageal epithelium expresses the squamous subset of cytokeratins in contrast with that found in columnar tissue.

There is no evidence that bacterial PAMPs play a role in the development of BM and these observations, if applicable in oesophageal cells, are likely to represent surrogate activation of the inflammatory cascade. Although recently the resident bacteria of the distal oesophagus in normal patients and in oesophagitis/BM was shown to be significantly different (Yang, Lu et al. 2009). BM and oesophagitis were more commonly associated with gram negative anaerobes.

1.5 Role of Retinoic acid in Barrett’s Metaplasia

Retinoic acid (RA) is important for epithelial cell differentiation and acts as a morphogen during development (Fuchs and Raghavan 2002; White, Nie et al. 2007). RA is a candidate factor for the induction of BM. Following treatment with all-trans retinoic acid (ATRA), a primary explant culture of human squamous oesophagus undergoes conversion to columnar epithelium, similar to that seen in BM (Chang, Lao-Sirieix et al. 2007). The bile acid lithocholic acid is a competitive agonist with retinoic acid at retinoid X receptors (RXR), suggesting a potential mechanism of action for bile salts in the development of BM (Radomsinska-Pandya and Chen 2002). Similarly, RA biosynthesis is increased in Barrett’s epithelium.
(Chang, Lao-Sirieix et al. 2007) and furthermore RA has been shown to induce expression of the intestinal gene MUC2 in primary cultures of human oesophageal cells (Cooke, Blanco-Fernandez et al. 2008). Although CDX2 transcriptionally regulates MUC2, it has not been directly linked to the effects of RA on oesophageal cells and may suggest an alternative pathway exists for MUC2 regulation.

The necessity for RA in normal columnar differentiation and its ability to inhibit squamous differentiation also suggests a role in BM. Moreover, the RA receptor increases PI3K expression and activation in teratocarcinoma cells, once again highlighting a potential role for PI3K signaling in CDX2 regulation (Bastien, Plassat et al. 2006).

It could be hypothesised that a reversion to a columnar phenotype, similar to that seen with ATRA, could be necessary before intestinal differentiation related to ectopic Cdx2 expression occurs.

1.6 Bone Morphogenetic Proteins (BMPs)

Bone morphogenetic proteins (BMPs) are secreted peptides that were originally identified and named because of their ability to induce bone and cartilage formation in muscle (Reddi 1994). BMPs are members of the transforming growth factor beta (TGF-β) family of growth factors and signal through membrane bound type I and type II serine/threonine receptor subtypes. Following BMP binding, the type II receptor phosphorylates a short series of amino acids (the GS box) in the type I receptor, activating its kinase domain. The activated type I receptor phosphorylates downstream targets, such as the SMAD proteins 1, 5 and 8 which complex with SMAD4, translocate to the nucleus and regulate transcription of target genes (Heldin, Miyazono et al. 1997). The binding specificity of ligand to receptor is dictated primarily by the type I receptor and to a lesser degree by the partner type II receptor (Carcamo, Weis et al. 1994; Massague 1996). Three type I receptors, BMP type IA (Bmpr1a), Bmpr1B and ACVR1 and three type II receptors BmprII, activin type II A (AcrRIIA) and ActRIIB recognise BMPs (Kishigami and Mishina 2005). BMP can, in addition to SMAD signalling, activate alternative pathways such
as PI3 kinase, MAPK and PKC (de Caestecker 2004). BMP signalling is negatively regulated prior to receptor binding by extracellular proteins, such as noggin, which prevent receptor binding (Sasai 2001).

1.6.1 BMP during gastrulation and germ layer patterning

During embryogenesis Bmp4 has early and essential roles, specifically during the development of the primitive streak and early patterning of the mesoderm. Bmp4 is abundantly expressed in the extraembryonic ectoderm and primitive streak at the time of gastrulation (Winnier, Blessing et al. 1995; Lawson, Dunn et al. 1999; Ying and Zhao 2001). The posterior region of the primitive streak in Bmp4 mutant embryos develops abnormally, but can be rescued using wild type/Bmp4 mutant tetraploid chimeric embryos (Fujiwara, Dehart et al. 2002). In the chimeric embryo the mutant Embryonic stem (ES) cells contribute to the embryonic region while the extraembryonic region is wild type, suggesting normal primitive streak formation is dependent on Bmp4 expression in the extraembryonic rather than embryonic region (Nagy, Rossant et al. 1993). Mutations in Bmpr1a, the receptor for Bmp2 and 4, are embryonic lethal at the stage of gastrulation (Mishina, Suzuki et al. 1995). Conditional epiblast knockout of Bmpr1a, using a Mox2-cre Bmpr1a floxed transgenic line, rescued the Bmpr1a mutant phenotype demonstrating that BMP signalling through Bmpr1a in the extraembryonic region is critical for normal gastrulation (Davis, Miura et al. 2004). Bmp4 mutant embryos are embryonic lethal and develop eye, germ cell and posterior body defects along with a lack of allantois (Winnier, Blessing et al. 1995; Furuta and Hogan 1998; Lawson, Dunn et al. 1999).

1.6.2 BMP4 and Embryonic stem cells

ES cells are derived from the inner cell mass and have the ability to form differentiated cells from all three germ layers (Evans and Kaufman 1981; Martin 1981). Mouse ES cells require the cytokine, leukaemia inhibitory factor (LIF), in order to maintain pluripotency (Stewart, Kaspar et al. 1992). LIF enables ES cells to self renew by activating the transcription factor STAT3 (Niwa, Burdon et al. 1998). However, in serum free conditions, ES cells slowly differentiate into sox1
expressing, neural precursor cells and lose pluripotency (Ying and Smith 2003). This process has been named the 'Neural default'. Bmp4 promotes ES cells to differentiate into a mesodermal phenotype (Wiles and Johansson 1999) and in combination with LIF maintains a pluripotent cell type (Ying, Nichols et al. 2003). Bmp4 inhibits the p38 MAP kinase pathways maintaining ES cells in an undifferentiated state (Qi, Li et al. 2004). This p38 dependent mechanism is confirmed by the formation of ES cells from Bmpr1a null embryos, which is not normally possible, when p38 inhibitors are used (Qi, Li et al. 2004).

1.6.3 BMP4 during epidermal development

Clues to the developmental mechanisms involved in oesophageal epithelial organogenesis can be gleaned from studying the formation of stratified squamous epidermis from neuroectodermal precursors. During embryogenesis the epidermis develops from the ectoderm, and dermis from the mesoderm (Aberdam 2008). A gradient of Bmp4 and Bmp4 antagonists (such as Noggin) from ventral to dorsal ectoderm respectively controls differentiation of the ectoderm (Hemmati-Brivanlou and Melton 1997). In Xenopus embryos and mammals, Bmp4 plays a similar role in differentiating neuroectodermal precursor cells into epidermal cells (Wilson and Hemmati-Brivanlou 1995; Hemmati-Brivanlou and Melton 1997). In the presence of Bmp4, ectoderm forms epidermis and in the absence of Bmp4 the default state of the tissue is to become neural (Hemmati-Brivanlou and Melton 1997). Initially the ectoderm, which will develop into the stratified squamous epidermis, consists of a single layer of columnar epithelial cells expressing K8/K18 (Lechler and Fuchs 2005). This situation is mirrored in the oesophageal epithelium where the initial K8/18 positive epithelium differentiates into stratified squamous epithelium expressing K5/K14 (Yu, Slack et al. 2005).

In vitro ES cells can form epidermal keratinocytes when sequentially cultured with Bmp4 and then Bmp4 with serum (Coraux, Hilmi et al. 2003; Aberdam 2004). Bmp4 induces epidermal differentiation by inhibiting neural development through apoptosis of Sox1 positive neural precursors (Gambaro, Aberdam et al. 2006). The
switch to epidermal commitment occurs as a result of ΔNp63 activation (Medawar, Virolle et al. 2008), which, in zebrafish, is a direct target of Bmp4 signalling (Bakkers, Hild et al. 2002). The ΔNp63 isoform of p63, in Bmp4 null zebrafish embryo, is sufficient to specify ectodermal differentiation of neuroectodermal precursors (Bakkers, Hild et al. 2002). In mouse ES cells treated with Bmp4, ΔNp63 gene activation occurs after ectodermal K8/K18 positive cells have formed (Medawar, Virolle et al. 2008). Only a small number of ectodermal cells coexpress ΔNp63 and K8/18, but the epidermal keratinocytes (K5/14 positive), formed from ectoderm, all express ΔNp63 (Medawar, Virolle et al. 2008). Exogenous expression of ΔNp63 in ectodermal cells provokes differentiation into epidermal keratinocytes (Aberdam, Gambaro et al. 2007). Furthermore ΔNp63 is able to partially rescue the epidermis in p63 knockout mice and activate the K14 promoter (Candi, Rufini et al. 2006; Romano, Birkaya et al. 2007). RNA interference knockdown of ΔNp63 in an organotypic model of keratinocyte growth decreases cell proliferation, activates K8/18 genes and abolishes stratification (Truong, Kretz et al. 2006). Interestingly epidermal precursors can be reprogrammed to pluripotency by injecting into a blastocyst or dedifferentiated by Oct-4 (Liang and Bickenbach 2002; Grinnell, Yang et al. 2007).

The embryonic mouse oesophagus is lined by columnar epithelium expressing sonic hedgehog, a gene important in the development of foregut structures (Litingtung, Lei et al. 1998). The developing oesophagus undergoes a squamous transformation in response to the (BMP4) antagonist noggin (Que, Choi et al. 2006). Given the role of BMP4 antagonism in the columnar to squamous transformation seen during development it is possible that this pathway could be involved in the opposite metaplasia found in BM. Human oesophageal cells, when exposed to BMP4, begin to express columnar cytokeratins (7/20) not normally found in squamous epithelium (Milano, van Baal et al. 2007). Furthermore BMP 4 is expressed in the paraoesophageal stroma of BM and oesophagitis patients but not normal squamous epithelium. Again this evidence links the pathogenesis of BM
with oesophageal development. The role of BMP4 in oesophageal development, homeostasis and BM are further discussed in Chapter 4.

1.7 Therapeutic Implications
The evolving understanding of how chronic inflammation provokes metaplasia and cancer offers the potential for therapeutic interventions. The presence of an inflammatory component throughout the oesophagitis-metaplasia-dysplasia-carcinoma sequence suggests interventions may be possible over a considerable time-frame. The controversy over the role of PPIs in the development and progression of BM has been described previously. PPI use remains theoretically attractive because acid is one of the main protagonists of oesophageal inflammation and will pragmatically continue because of the symptomatic benefit to patients. Modifying the inflammatory cascade seen in oesophagitis and BM is an attractive option. Whilst the epidemiological effect of anti-inflammatory drugs, such as NSAIDS, aspirin and COX2 inhibitors, in the prevention of oesophageal cancer are known, conclusive trial evidence is awaited. The AsPECT trial, assessing the chemo preventative role of aspirin and PPI, is underway and should provide interesting data.

Carcinogenesis occurs in the context of inflammation via multiple mechanisms, such as the accumulation of genetic mutations, epigenetic events and alterations in key signalling pathways. In combination, these effects result in a change in cell phenotype associated with increased proliferative potential, decreased apoptosis and ability to metastasise. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated by inflammatory cells, are one potential mechanism that link DNA damage and the consequent activation of oncogenes or deactivation of tumour suppressor genes, leading to a cancerous phenotype. BM is characterised by levels of increased DNA damage compared with normal squamous epithelium (Olliver, Hardie et al. 2003). Antioxidants could offer a potential therapeutic tool to prevent OA as a treatment for BM or even earlier at the oesophagitis stage. There is some evidence for this from rat models of BM and OA, where iron (as a catalyst
for ROS) increases and the antioxidant vitamin E decreases the prevalence of cancer (Chen, Ding et al. 2000; Chen, Mikhail et al. 2000). In a similar rat model, superoxide dismutase prevented both the development of IM and progression to cancer, suggesting potential benefits at the oesophagitis stage (Piazuelo, Cebrian et al. 2005). Curcumin found in turmeric has antioxidant properties and also acts on NFkB and COX2. Trials assessing the use of curcumin as a chemopreventative agent in BM are underway. Given the central role of NFkB in the initiation and propagation of inflammation along with carcinogenesis, it may hold the key to future therapeutic interventions.

There is also a potential for inflammatory profiling to be used as a biomarker for either the development of BM in the context of oesophagitis or the progression towards OA. A further understanding of the molecular events contributing to BM along with inherited genetic predisposition may allow for individualised targeted treatment or risk assessment.

A critical understanding of the role of CDX2 in BM and the molecular mechanisms that regulate or induce the transcription factor may offer opportunities for intervention.

1.8 Summary
BM is the only known precursor for OA, a cancer with a poor prognosis and rapidly rising incidence. The molecular mechanisms that control the development of BM are not fully understood. The CDX2 gene has been implicated in BM and is sufficient to provoke gastric intestinal metaplasia. BM develops in an inflammatory environment and demonstrates a specific cytokine profile. Inflammatory mediators and bile have been shown to induce Cdx2 in a variety of experimental models. Furthermore BMP4 and RA have been shown to induce a BM-like change in phenotype in oesophageal cells. It is not currently known if Cdx2 is sufficient to induce BM or if additional transcription factors and signalling pathways are necessary. There are two lines of evidence to suggest that the ectopic expression of CDX2 is not sufficient for the development of BM. First, human intestinal crypt
cells, unlike rat intestinal crypt cells, do not differentiate to mature intestinal cells in response to CDX2 expression alone, and it is suggested that this relates to the absence of the transcription factors HNF1α and GATA4 (Escaffit, Pare et al. 2006). Secondly a recent abstract publication presented a K14 promoter driven transgenic Cdx2 mouse with no intestinal phenotype in the oesophagus (Jianping Kong* 2009).
1.9 Hypothesis and aims of project

Hypothesis

Ectopic expression of Cdx2 is sufficient to provoke intestinalisation of adult mouse oesophageal squamous cells *in vitro*.

Aims

1. Characterise the squamous oesophageal cell line HET-1A
2. Develop a robust model of adult mouse oesophageal epithelium, that could be applied to human tissue.
3. Determine the effects of exogenous factors linked with the development of Barrett’s metaplasia and induction of Cdx2 on adult mouse oesophageal cells. Specifically ATRA, BMP4, acid, bile salts, IL-1β and TNFα.
4. Establish a protocol for transgene expression (GFP, Cdx2 and HNF4α) in adult mouse squamous oesophagus using adenoviral vectors.
5. Determine the effect of ectopic Cdx2 expression on squamous and intestinal/columnar markers in oesophageal cells.
6. Determine the effect of ectopic HNF4α expression on squamous and intestinal/columnar markers in oesophageal cells.
Chapter 2 Material and Methods

2.1 Cell lines and culture media.

The cell lines Caco-2 (passage 20), HT-29 (passage 8) and HEK-293 (passage 5) were obtained from the European Collection of Cell Cultures (ECACC, Porton Down, U.K). The HET-1A cell line was a generous gift from Dr Jonathan Bury, University of Sheffield Medical School, UK (passage unknown). The culture medium for each cell line was as follows. Caco-2 was maintained in Minimum Essential Medium Eagle (MEM) with Earl’s salts (Sigma) supplemented with 10% gamma irradiated fetal bovine serum (FBS (Invitrogen)), Penicillin (100U/ml (Sigma)), Streptomycin (100µg /ml (Sigma)), 2mM L-glutamine (Sigma) and 1% non-essential amino acids (NEAA (Life Technologies)). Experiments were performed on passage 20 or 21. HT-29 cells were cultured in McCoy’s 5A medium (Sigma) supplemented with 10% gamma irradiated FBS, 2mM L-glutamine, Penicillin (100U/ml) and Streptomycin (100µg /ml). HET-1A cells were maintained in Basal Medium Eagle (BME) with Earle’s salts (Sigma) supplemented with 20% gamma irradiated FBS, Penicillin (100U/ml), Streptomycin (100µg /ml) and 2mM L-glutamine. Experiments on HT-29 cells were performed between passages 8-12. HEK-293 (passage cells were cultured in DMEM (Gibco) containing 2mM L-glutamine and supplemented with 10% heat inactivated FBS, Penicillin (100U/ml) and Streptomycin (100µg /ml). Culture medium was changed every 2 days and cells were passaged at 90% confluence. Adenovirus was amplified in HEK293 passage number 5-12.

For subculture, the media was removed and the cells washed with sterile phosphate buffered saline (PBS (Oxoid)). Adherent cells were incubated with 5ml trypsin-EDTA (Gibco) and 5ml PBS for 5 minutes at 37°C. The HT-29 cell line was incubated with 10ml trypsin-EDTA for 10 minutes. Cells were detached from the culture flask by gently tapping the side and transferred to a 15ml tube. A cell pellet was obtained following centrifuging at room temperature at 1000 rpm using a
benchtop MSE Mistral 1000 centrifuge (ISTCP). The cell pellet was resuspended in 8-12ml of the appropriate culture medium after aspiration of the supernatant to obtain a subculture ratio of 1:8 – 1:12. All cell lines were cultured at 37°C in an atmosphere of 5% CO₂ / 95% air in a humidified incubator.

2.2 Storage of Cell Lines

For long-term storage of cell lines, a cell pellet from a T-75 tissue culture flask (Cellstar) was suspended in 1ml of freezing medium (FBS with 10% v/v Dimethyl sulfoxide (DMSO), VWR International). The suspended cells, in freezing medium, were frozen at -20°C for 1 hour in a cryogenic vial (Nalgene), maintained at -80°C overnight before long-term storage in liquid nitrogen. Cells were resuscitated by thawing at 37°C for 2 minutes and resuspended in 10 ml of appropriate tissue culture medium. The cell suspension was centrifuged at 1000 rpm for 4 minutes, supernatant aspirated and the cell pellet resuspended in 15ml of medium in a T-75 flask.

2.3 Culture of adult mouse oesophagus

An oesophagectomy was performed on an adult CD1 mouse (Charles River Laboratories) that has been sacrificed by cervical dislocation. The thoracic cavity was opened via a left lateral approach and the left hemi-diaphragm divided to expose and mobilise the distal oesophagus. Exposure of the oesophagus along its full length was achieved by removing the heart and left lung. The oesophagus was transected at the proximal and distal end leaving a cuff of oesophageal tissue in situ to ensure that the gastric and bucal mucosa were not attached (Figure 2.1). Whole gastric and intestinal tissue (for immunohistochemistry or RNA extraction) were isolated during this procedure by extending the incision down the lateral abdomen exposing the tissue, division of the mesentery and resection across the lumen. The resected tissue was washed three times with MEM with Hank’s salts (Sigma) and supplemented with 10% gamma irradiated fetal bovine serum (FBS), Penicillin (100U/ml), Streptomycin (100µg /ml), 2mM L-glutamine and gentamicin (20µg/ml).
Using a Leica MZ6 Stereomicroscope, the whole oesophagus was opened longitudinally with a pair of scissors and the epithelium stripped from the submucosal using a pair of forceps following dissection into this plane (Figure 2.2). Following three washes in supplemented MEM, the epithelium was cut into 1mm\(^2\) sections and each oesophagus treated separately (Figure 2.2). 10-15 sections of epithelial tissue were then encouraged into furrows etched on a plastic coverslip (Structure Probe Incorporated) in a 35mm tissue culture dish and covered with 1.5ml of Basal Medium Eagle (BME) with Earle’s salts supplemented with 20% gamma irradiated FBS, Penicillin (100U/ml), Streptomycin (100µg /ml), L-glutamine (2mM L-glutamine) and gentamicin (20 µg /ml). The tissue was cultured for the number of days indicated in the text in a humidified 5% CO\(_2\) / 95% air environment at 37\(^\circ\) C, replacing the supplemented BME every 2 days.

For experiments culturing oesophageal explants under low calcium conditions, M153 (Autogen Bioclear, Wiltshire, UK) media was used and supplemented with L-glutamine (6mM), human epidermal growth factor (5ng/ml), ethanolamine (6.1µg/ml), α-phosphoethanolamine (14.1µg/ml), hydrocortisone (0.5µg/ml) and bovine insulin (5µg/ml) (all Autogen Bioclear).

The majority of the work presented in this thesis concerns adult mouse squamus oesophageal explants and uses two medium. BME is used for normal calcium concentration experiments and MCDB153 for low calcium conditions. An intermediate media, Quantum153, is used for two experiments in Chapter 4, but its manufacture was discontinued and thus not used further.

2.3.1 Quantification of infection efficiency and cell counting

Mouse oesophageal explants cultured in media with a normal calcium concentration (BME) were multilayered and quantification using cell counting was not possible. Experimental results were only commented on if the effect was reproducible and dramatic or if there was induction of an intestinal gene not normally resident in the oesophagus. Oesophageal explants cultured in low
calcium conditions (MCDB153) were monolayer and cell counting was possible. The cells were also reproducibly homogenous and changes within cell phenotype were easier to observe. Under these conditions 100% of cells express p63 and 0% express K8 and consequently any p63 negative or K8 positive cells represented a change in phenotype.

2.3.2 Culture of human adult squamous oesophagus and Barrett’s oesophagus

Human oesophageal biopsies were taken following informed consent from patients with Barrett’s metaplasia on a routine surveillance program. The protocol and collection of all tissue is approved by the Bath Local Research and Ethics Committee (RD: 1110, BA123) see appendix 1. Two additional biopsies were taken from either mid section of the Barrett’s section or 5cm above the Z-line. The tissue was washed three times in MEM with Hank’s salts (Sigma) and supplemented with 10% gamma irradiated fetal bovine serum (FBS), Penicillin (100U/ml), Streptomycin (100µg /ml), 2mM L-glutamine and gentamicin (20µg/ml). The samples were kept in this media and transported on ice within three hours to the laboratory. Using a Leica MZ6 Stereomicroscope, each biopsy sample was dissected into using a pair of scissors and forceps into approximately 50 1mm² pieces. 10-15 sections of epithelial tissue were then encouraged into furrows etched on a plastic coverslip (Structure Probe Incorporated) in a 35mm tissue culture dish and covered with 1.5ml of Basal Medium Eagle (BME) with Earle’s salts supplemented with 20% gamma irradiated FBS, Penicillin (100U/ml), Streptomycin (100µg /ml), L-glutamine (2mM L-glutamine) and gentamicin (20 µg /ml). The tissue was cultured for the number of days indicated in the text in a humidified 5% CO₂ / 95% air environment at 37° C, replacing the supplemented BME every 2 days.
Alternative medium were used in additional experiments using squamous and Barrett’s human tissue. The four medium used were as follows:

1) DMEM supplemented with 10% FBS, Penicillin (100U/ml), Streptomycin (100µg /ml), L-glutamine (2mM), amphotericin B (2.5µg/ml) and gentamicin (20µg/ml - Invitrogen)

2) BME supplemented with 20% FBS, Penicillin (100U/ml), Streptomycin (100µg /ml), L-glutamine (2mM), amphotericin B (2.5µg/ml) and gentamicin (20µg/ml)

3) Keratinocyte serum free media (KSFM – Gibco) supplemented with keratinocyte-SFM supplement pack (human recombinant epidermal growth factor and bovine pituitary extract – 37000-015), Penicillin (100U/ml), Streptomycin (100µg /ml), amphotericin B(2.5µg/ml) and gentamicin (20µg/ml)

4) RPMI medium 1640 (Gibco) supplemented with 10% FBS, Penicillin (100U/ml), Streptomycin (100µg /ml), L-glutamine (2mM), amphotericin B (2.5µg/ml) and gentamicin (20µg/ml)
Figure 2.1 – Isolation of oesophagus.
(A) A longitudinal incision is made to expose the left thoracic cavity and intra-abdominal organs. (B) The liver and left lung were removed to expose the distal oesophagus and stomach. (C) The whole oesophagus is mobilised and transected at the proximal end and removed with stomach and proximal small intestine. (D) An example of the oesophagus, stomach and proximal small intestine following dissection.
Figure 2.1 Isolation of Mouse Oesophagus
**Figure 2.2** – Dissection and culture of oesophageal epithelium.

(A) A freshly isolated oesophagus which is (B) opened longitudinally with a pair of microscissors. (C+D) The epithelium and muscle layers are grasped with forceps and pulled apart. (E) The epithelium is chopped into approximately 1mm x 1mm pieces. (F) Single explants are attached to scratched coverslips with the luminal epithelium uppermost.
Figure 2.2 – Dissection and culture of oesophageal epithelium
2.4 Isolation and culture of embryonic mouse intestine, stomach and oesophagus

Female CD1 outbred mice (Charles River) were mated and stage specific embryos were obtained following observation of the copulatory plug (E 0.5). Pregnant animals were sacrificed by cervical dislocation and uteri were isolated into ice-cold sterile PBS (Oxoid). Embryos were removed from the decidua, decapitated and transferred to MEM (with Earl's salts (Sigma) supplemented with 10% gamma irradiated fetal bovine serum (FBS (Invitrogen)), Penicillin (100U/ml (Sigma)), Streptomycin (100µg /ml (Sigma)) and 2mM L-glutamine (Sigma). Using a tungsten needle and fine forceps, the gut tube, from pharynx to intestine, was identified and isolated. The oesophagus was transected clear of the stomach and trachea and lung buds were removed. The intestine was isolated and any residual dorsal pancreatic bud removed.

The embryonic organs were cultured on subbed coverslips, which were also utilised initially for adult explants. Glass coverslips were rinsed in 95% ethanol / 0.1% acetic acid. After drying, the coverslips were immersed in 2% 3-aminopropyltriethoxysilane (APTS (Sigma)) in acetone for 10 minutes followed by rinsing in acetone and then sterile water. The coverslips were then dried at 37°C and sterilised by baking at 180°C for 3 hours. 40 µl of fibronectin (50 µg/ml (Invitrogen)) was placed on the centre of the subbed coverslip and dried in a class II tissue culture hood. When dry, coverslips were placed into 35mm tissue culture dishes and a sterile cloning ring was placed at the centre of the dried fibronectin. The required tissue was transferred to the centre of the sterile cloning ring and 2ml of supplemented BME was added and incubated at 37°C in 5% CO₂. The cloning ring was removed after 24 hrs and the BME replaced. The culture was incubated for the desired duration replacing the supplemented BME every 2 days.

2.5 Fixation of cultures

For fixation, the coverslips were removed from the tissue culture dish and washed in PBS three times. The fixation conditions depend on the embryonic or adult origin of the tissue and the desired antigen. For immunostaining of cytoskeletal proteins
all tissues were fixed in acetone / methanol (1:1) at -20°C for 5 minutes. For cytosolic and nuclear epitopes, tissue was fixed in 3.8% formaldehyde in MEMFA (0.15M 3-[N-Morpholino] propane sulfonic acid (MOPS), 2mM ethylene glycol tetraacetic acid (EGTA) and 1mM magnesium sulphate) pH 7.4 at room temperature for 1 hour if adult origin or 30 minutes for embryonic tissue or cell line.

2.6 Fluorescent immunohistochemistry of explant cultures and cell lines

Formaldehyde-fixed samples were permeabilised for one hour in 1% Trition X-100 (Sigma) at room temperature. This permeabilisation step was omitted from cultures fixed with acetone:methanol. Tissue to be stained for cytoskeletal proteins were antigen retrieved at 37°C in pH 6 citrate buffer (Lab Vision Corporation) for 1 hour. Following 3 rinses in PBS, non-specific binding sites were blocked using 2% blocking buffer. A 10% stock solution of blocking buffer was made by dissolving Blocking Reagent (Roche) in maleic acid buffer. Primary antibodies (Table 2.1) at the appropriate dilution in 2% blocking buffer (200 µl total) were applied to the coverslip overnight at 4°C. Coverslips were washed three times in PBS for 5 minutes and species specific fluorescent secondary antibodies diluted in 2% blocking buffer were applied for 2 hours (Table 2.2). The sample was washed in PBS three times for 5 minutes and 4´6-diamidino-2-phenylindole (0.1µg/ml DAPI) (diluted in PBS) was applied for 10 minutes to stain the cell nuclei. Samples were mounted on slides using Gel Mount media (Sigma) and viewed on a Leica DMRB fluorescent microscope. Pictures were obtained with a SPOT camera or were collected on a Zeiss LSM 510 confocal microscope.

When dual antibody immunohistochemistry was used, the primary antibodies were applied together and the secondary antibodies sequentially to prevent cross-reactivity.

All antibodies used were optimised using their positive control (table 2.1) for antigen retrieval conditions and dilution. Only antibodies that demonstrated staining within the expected cell compartment in the expected cell type were used for experiments (table 2.1).
### Table 2.1 – Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Species</th>
<th>Positive</th>
</tr>
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<td>1/100</td>
<td>Goat polyclonal</td>
<td>oesophagus</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Sigma (1A4)</td>
<td>1/200</td>
<td>Mouse monoclonal</td>
<td>fibroblasts</td>
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<td>E-cadherin (C36)</td>
<td>Transduction labs</td>
<td>1/100</td>
<td>Mouse monoclonal</td>
<td>Caco-2</td>
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<tr>
<td>Involucrin</td>
<td>Covance</td>
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<td>Rabbit polyclonal</td>
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<tr>
<td>Phospho-histone H3</td>
<td>Cell signal technology</td>
<td>1/100</td>
<td>Rabbit polyclonal</td>
<td>Caco-2</td>
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<td>BioGenex</td>
<td>1/100</td>
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<td>Caco-2</td>
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<td>1/100</td>
<td>Mouse monoclonal</td>
<td>oesophagus</td>
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<td>Cytokeratin 5 (AF138)</td>
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<td>Rabbit polyclonal</td>
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<td>Cytokeratin 14 (LL002)</td>
<td>Neomarkers</td>
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<td>Santa Cruz</td>
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<td>Rabbit polyclonal</td>
<td>intestine</td>
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<tr>
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<td>Santa Cruz</td>
<td>1/50</td>
<td>Mouse monoclonal</td>
<td>oesophagus</td>
</tr>
<tr>
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<td>Santa Cruz</td>
<td>1/100</td>
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<td>liver</td>
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<tr>
<td>Villin (CWWB1)</td>
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<td>Mouse monoclonal</td>
<td>intestine</td>
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### Table 2.2 – Secondary Antibodies

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<td>Anti-rabbit Alexa Fluor 546 IgG</td>
<td>Invitrogen</td>
<td>1/250</td>
<td>Goat</td>
</tr>
<tr>
<td>Anti-rabbit Alexa Fluor 633 IgG</td>
<td>Invitrogen</td>
<td>1/250</td>
<td>Goat</td>
</tr>
<tr>
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<td>Vector</td>
<td>1/100</td>
<td>Goat</td>
</tr>
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<td>Anti-mouse fluorescein isothiocyanate (FITC)</td>
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<td>1/100</td>
<td>Goat</td>
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<tr>
<td>Anti-rabbit texas red</td>
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<td>1/100</td>
<td>Goat</td>
</tr>
<tr>
<td>Anti-rabbit FITC</td>
<td>Vector</td>
<td>1/100</td>
<td>Goat</td>
</tr>
<tr>
<td>Anti-goat texas red</td>
<td>Vector</td>
<td>1/100</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>
2.7 Immunohistochemistry of mouse oesophageal and intestinal sections

Whole oesophagus, stomach and intestine were fixed in 3.8% formaldehyde in MEMFA for 24 hours followed by a post fixation in acetone / methanol (1:1) for 2 hours. The fixed tissue was washed 3 times in PBS for 10 minutes and dehydrated in a tissue processor with serial immersions in 70%, 90%, 95%, 100% ethanol and Histoclear (National Diagnostics) twice. The samples were embedded in paraffin wax and allowed to solidify. Sections (5µm) were then cut using a Leica 2155 microtome.

Sections were dewaxed with Histoclear twice for 7 minutes. Graded ethanol, 100%, 100%, 95%, 90%, 70% and 50%, and water was used for 1 minute each to rehydrate samples. Permeabilisation with 1% Triton X-100 for 1 hour was followed by citrate buffer pH 6 antigen retrieval at 95°C for 30 minutes. Slides were left to cool for 30 minutes and then washed in PBS 3 times for 5 minutes each.

Endogenous peroxidase was quenched using DAKO Envisi on peroxidase block for 10 minutes and then washed twice in PBS. Non-specific binding sites were blocked with 10% normal goat serum and 0.5% bovine serum albumin in PBS. Primary antibodies were diluted appropriately in 1% goat serum and 0.5% bovine serum albumin in PBS and incubated at 4°C overnight. Horse radish peroxidase labelled secondary antibody (DAKO Envision kit) was applied to the specimen following 3 x 10 minute washes and left for 30 minutes. 3, 3 Diaminobenzidine (DAB) was used for immuno-detection on sections. After washing twice in PBS, DAB substrate chromogen (DAKO Envision Kit) was used to develop the peroxidase and the reaction was terminated by immersing in water. Depending on the cellular position of the epitope, sections were counterstained either with Ehrlich’s haematoxylin (nuclear) for 3 minutes or eosin (cytoplasmic) for 2 minutes and slides were rinsed in tap water for 5 minutes. The slides were washed in 1% Hydrochloric acid in 70% ethanol for 30 seconds and staining sharpened in 1% ammonia in 70% ethanol for 1 minute before dehydrating with the opposite series of graded alcohol that was used to hydrate the sections. Following 2 incubations with Histoclear (2 minutes each), the slides were mounted with DePex (BDH Chemicals)
2.8 Adenoviral construct preparation

All adenoviral constructs were prepared using the AdEasy™ Adenoviral Vector System (Stratagene). The plasmids pShuttle-Cdx2-IRES-hrGFP2 and pShuttle-VP16Cdx2-IRES-hrGFP2 were constructed by Dr Wei-Yuan Yu, University of Bath. The recombinant adenovirus Ad-CMV-HNF4α was a gift from Dr Ramiro Jover (Unidad de Hepatología Experimental, Valencia, Spain).

Amplification of adenoviral constructs was performed in HEK-293 cells as follows (figure 2.3). Four T-75 flasks were inoculated with 10μl, 1μl, 0.1μl and 0.01 μl of concentrated adenovirus in supplemented DMEM to prepare a prestock. When 50% of HEK cells demonstrate cytopathic effects (usually between 2-4 days), the cells were detached from the flask by gently tapping the side of the flask and transferred to a 10ml tube. A cell pellet was obtained following centrifuging at 1000 rpm for 4 minutes. The pellet was resuspended in 1ml of DMEM and lysed with 4 freeze/thaw cycles in a dry ice/ethanol bath and 37°C water bath. The appropriate quantity of the prestock that caused 50% cytopathic effect of HEK cells in a T-175 flask (Nunc) is inoculated into each of 10 T-175 flasks. The cells were harvested as before and centrifuged at 2000rpm for 10minutes and the supernatant discarded. All 10 cell pellets are resuspended in 5ml 100mM Tris.HCl pH 8, subjected to 4 freeze/thaw cycles and respun at 2000rpm for 5 minutes. To the supernatant, 0.6 volumes of supersaturated caesium chloride 100mM Tris.HCl pH8 was added, and the contents mixed gently before transfer to 2 centrifuge tubes (Beckman 342412). Following a 4 hours centrifuge at 65000rpm in a Beckman ultracentrifuge (LL-TB003) using a Beckman Vti90 rotor, the virus particle band was removed with a 25g needle. The virus particles were added to 62.5% v/v 100mM Tris.HCl pH 8 / 37.5% caesium chloride supersaturated 100mM Tris.HCl pH8 and respun for 18 hours at 65000rpm. The virus particles were removed in 1ml volumes and dialysed in a Gamma Irradiated Slide-A-Lyzer™ Dialysis Cassette (Pierce Biotechnology) against a buffer containing 10mM Tris.HCl pH 7.5, 1mM MgCl2 and 135mM NaCl for 6 and 12 hours at 4°C. The virus construct was then filtered through a 0.22μm
filter (Millipore, Massachusetts, USA), aliquoted and stored at -80°C. The adenoviral used in this project are recorded in Table 2.3

**Table 2.3 Adenoviral constructs used.**

<table>
<thead>
<tr>
<th>Adenovirus</th>
<th>Titre (IU/ml)</th>
</tr>
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<tbody>
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<td>Ad-RSV-GFP (GFPC)</td>
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<td>Ad-RSV-GFP (GFPD)</td>
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<tr>
<td>Ad-CMV-Cdx2-IRES-hrGFP (Pad1E)</td>
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<tr>
<td>Ad-CMV-Cdx2-IRES-hrGFP (Pad1G)</td>
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<td>Ad-CMV-HNF4α (HNF4A)</td>
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</tr>
<tr>
<td>Ad-Null</td>
<td>5.1 x 10^10</td>
</tr>
</tbody>
</table>

Table 2.3 describes the adenoviral constructs and viral titres used during. The individual constructs are described in detail in the appropriate chapter.

**2.9 Adenoviral titre**

Adenoviral constructs were titred using the Adeno-X™ Rapid Titer Kit (BD Biosciences) according to the manufacturer's instructions. HEK 293 cells in DMEM were seeded into a 12-well tissue culture plate (Nunc) and grown to 90% confluence. To each well, 100μl of 6 serial dilutions, 10^2 to 10^7, of purified virus was added and incubated for 48 hours. The cells were fixed with 1ml per well of methanol at -20°C for 10 minutes after removal of the media. The cells were washed with PBS three times, blocked with 2% blocking buffer for 1 hour at room temperature and incubated with mouse anti-Hexon primary antibody (1/1000 in 2% Blocking Reagent) at 37°C an hour. After 3 washes in PBS, the cells were incubated with rat anti-mouse secondary antibody (HRP conjugate, 1/500 in 2% Blocking Reagent) for 1 hour at 37°C. The peroxidase was developed using DAB
Peroxidase Substrate Kit (Vector) according to the manufacturer’s instructions. Two drops of buffer Ph 7.5, 4 drops of DAB substrate reagent and 2 drops of H₂O₂ were added to 5ml water. To each well, 0.5ml of the DAB solution was added and incubated for 10 minutes at room temperature and then quenched with water. Using an inverted microscope (Leica DMIRB) the mean number of positive cells in a 20x field was calculated from 4 separate fields. The Infectious units (iu) per ml titre was calculated by:

\[
\text{IU/ml} = \frac{\text{(Positive cells per field) x 5730 (number of fields per dish)}}{\text{dilution factor}}
\]

2.10 Adenoviral infection of cell lines and adult explant cultures

HET-1A cells were seeded in 60mm² dishes and maintained in supplemented DMEM until 70% confluent. The number of cells per dish was calculated from the average of 3 fields and the number of virus particles required for multiplicity of infection (MOI) of 10 or 100 was added to 2.5ml of supplemented DMEM. The HEK cells were incubated in this media for 1 day following which the medium was replaced with fresh supplemented DMEM. The cells were incubated for a further 2 days. Total RNA was extracted using TRI reagent (Sigma)

Adult explant oesophageal cultures were incubated with the required concentration of adenoviral vector diluted in 1 ml of supplemented BME. Following incubation at 37 °C in 5% CO₂ for 12 hours, the BME was changed. The cultures were then incubated at 37 °C in 5% CO₂ for the required duration with a change of media every 2 days. Explants were either fixed for immunohistochemistry as described or total RNA was extracted for PCR using TRI reagent.
Figure 2.3 recombinant adenoviral amplification in E1 transformed HEK293 cells
2.11 RT-PCR analysis

Explant cultures were homogenised in 1 ml of TRI reagent using a plastic dounce and adherent cells were lysed using 1ml of TRI reagent per 60mm² dish. Both were stored in DNAase/RNAase free tubes at -80°C prior to processing. Total RNA was extracted from the tissue in TRI reagent as follows. 200μl chloroform per ml of TRI reagent was added to the tube, shaken for 20 seconds and incubated at room temperature for 10 minutes. This was centrifuged at 12 000 rpm for 15 minutes at 4°C and the aqueous phase transferred to a fresh tube, where 500μl of isopropanol was added and mixed by inversion. Following a 10 minute incubation at room temperature, the RNA was pelleted by centrifuging at 12 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet washed in 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water and briefly vortexed. The pellet was collected following centrifuging at 7500 rpm for 5 minutes at room temperature and air-dried. RNA was dissolved in DEPC-treated water and the concentration calculated using a Beckman DU530 UV/Vis Spectrophotometer. The RNA concentration was calculated using the absorbance A₂₆₀ reading multiplied by 40μg/ml.

cDNA was synthesised from mRNA was using SuperScript™ reverse transcriptase (Invitrogen) according to the manufacturers instructions. mRNA was extracted from 1μg of total RNA following incubation at 65°C for 5 minutes with 1 μl oligo dT (500μg/ml) and 1μl 10mM dNTP in a total of 12μl nuclease free water. This was chilled on ice and 4μl of 5x first strand buffer and 2μl of 0.1M 1,4-dimercaptobutane-2,3-diol (DTT) was added and incubated at 42°C for 2 minutes. 1μl (200U) of reverse transcriptase was added, mixed by gentle pipetting and incubated at 42°C for 50 minutes. The reaction was terminated by heating to 70°C for 15 minutes and the cDNA stored at -20°C.

For the PCR reaction 1μl of cDNA (50ng of total RNA) was added, along with 0.5μl of each primer (500nM) to 10μl of Reddymix™ PCR master mix and made up to 20μl with sterile nuclease free water. The reaction was processed in a DNA MJ Research PTC200 thermal cycler under the following conditions: denaturing at 96°C for 5 minutes, followed by the appropriate number of cycles of denaturing at
96°C for 1 minute, annealing for 45 seconds and extension at 72°C for 45 seconds.
This is followed by a final extension step at 72°C for 10 minutes. The primer
sequences and annealing temperatures are shown in Table 2.4. All primers were
designed using DNA and mRNA sequences from the National Centre for
Biotechnology Information (NCBI) website within the following parameters:

1 Length – between 17-24
2 Intron spanning
3 Melting temperature 50-60°C with <5°C between pairs (Calculated with Oligocalc
(simgene.com))
4 GC content 40—60%
5 GC clamp within last 5 bases at 3’ end.
6 Secondary hairpins and dimers were checked for using Oligocalc (simgene.com).
7 Amplicons <600 base pairs

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Table 2.4 – Primers used for reverse transcriptase PCR.
2.12 Reagents used for Retinoic acid, Bile acid, lipopolysaccharide, inflammatory cytokine and BMP4 treatment of oesophageal explants and cell lines.

All-trans retinoic acid (ATRA) (Sigma) was dissolved in DMSO at stock concentrations of 10mM and 100mM and stored in the dark at -20°C. Final concentrations were achieved by dilution in tissue culture medium. Medium was changed every 2 days in the dark with freshly added ATRA.

Lipopolysaccharide (LPS) from Escherichia coli 055:B5 (Sigma) was dissolved in sterile reverse osmosis water at a stock concentration of 10mg/ml. Working concentrations were further diluted in tissue culture media. For LPS treatment media was changed every other day.

Deoxycholic acid (DCA), dehydrocholic acid (DHCA) and cholic acid (CA) were all purchase from sigma. DCA was dissolved in ethanol to a stock concentration of 125mM. DHCA was dissolved in DMSO to a stock concentration of 400mM. CA was dissolved in ethanol to a concentration of 500mM.

Human recombinant BMP4 (Peprotech) was dissolved in 10mM citric acid at pH3 at a concentration of 100µg/ml and further diluted in PBS with 0.2% w/v bovine serum albumin to a10µg/ml stock solution. BMP4 was added to tissue culture media daily.

IL-1β (Peprotech) was diluted in PBS with 0.1% BSA (w/v) to a stock concentration of 5µg/ml and further in tissue culture media. Media was changed daily for IL-1β treatments.

TNFα (Peprotech) was diluted in PBS with 0.1% BSA (w/v) to a stock concentration of 5µg/ml and further in tissue culture media. Media was changed daily for IL-1β treatments.

Fluorescent images

All confocal microscope images were annotated using Zeiss LSM image browser (Carl Zeiss). Fluorescent compound microscope images were annotated using Adobe photoshop 7.0.
Chapter 3: A long-term *in vitro* explant culture model of squamous oesophageal epithelium.

### 3.1 Introduction

#### 3.1.1 Overview

In order to study the molecular mechanisms involved in the conversion of normal stratified squamous epithelium towards an intestinal or columnar mucosa, an *in vitro* model is desirable. An ideal model should (i) recapitulate the full repertoire of cell phenotypes found within the oesophagus (ii) demonstrate the normal three dimensional tissue organisation and (iii) be viable long-term. In order to study the functional effects of overexpression of candidate genes for the induction of BM, it is essential to achieve robust transgene expression within the epithelium. There are three possible *in vitro* techniques available; cell lines, primary cell culture and explant culture. The overall aim of this chapter is to:

i) Characterise the oesophageal and intestinal phenotype of established cell lines.

ii) Develop a representative *in vitro* model of normal stratified squamous oesophagus.

#### 3.1.2 The normal oesophagus.

The human oesophagus and trachea develop from the common foregut endoderm during weeks 3-5 of embryogenesis. A ventral diverticulum in the foregut endoderm elongates and separates to become the trachea. Failure or incomplete separation of the dorsal and ventral foregut results in tracheo-oesophageal fistula formation (Que, Choi et al. 2006). A simple columnar epithelium forms the initial lining of the oesophageal portion of the gut tube at week 8. After week 10, a pseudostratified ciliated columnar epithelium develops (DeNardi and Riddell 1991). Residual islands of ciliated pseudostratified epithelium give rise to the deep oesophageal glands found in the human oesophagus. The stratified squamous epithelium, as seen postnatally, develops during the second half of gestation and has been shown in the mouse to arise directly from columnar cells (Yu, Slack et al. 2005).
The human oesophageal epithelium is a non-keratinising stratified squamous epithelium divided into a basal layer of proliferating cells, an epibasal differentiating zone and a terminally differentiated zone of flattened squamous epithelium at the luminal surface (Seery and Watt 2000). The basal zone consists of small basophilic cells which, upon differentiation progressively flatten and migrate towards the lumen. Immunohistochemical characterisation of intermediate filament type reveals a mixture of phenotypes within the epithelium.

The cells of the interpapillary region have been proposed as the oesophageal stem cells because of the relative paucity of dividing cells within this region (Seery 2002). Further evidence for the stem cells residing in the interpapillary zone is suggested by the asymmetric cell divisions which yield one progeny remaining in the basal layer and one that enters the epibasal layer (Potten and Loeffler 1990). The putative oesophageal stem cells are represented by the daughter cells that remain in contact with the lamina propria. The cells overlying the papilla undergo frequent and symmetrical mitosis suggesting that this region is occupied by transit amplifying cells fed by the interpapillary stem cells. The cells in the epibasal layer proliferate and give rise to the differentiated squames. As previously discussed there are currently no molecular markers for oesophageal stem cells.

p63 knockout mice exhibit a columnar epithelium in the oesophagus lacking stratification (Daniely, Liao et al. 2004). p63 has been suggested as a stem cell marker in human epidermal and corneal cells (Pellegrini, Dellambra et al. 2001). Although the exact role of different p63 isoforms in oesophageal epithelium is not established, it is essential for stratified squamous epithelium and should be expressed in any in vitro oesophageal culture system.

### 3.1.3 Cell phenotypes found in squamous epithelium

The cells that constitute the oesophageal epithelium can be characterised according to the expression of cytokeratins and cornified cell envelope proteins. The cytokeratins are intermediate filaments that provide support and protection for
epithelial cells (Coulombe and Wong 2004). Keratins are divided in two types; acidic keratin (type I intermediate filament) and basic keratin (type II intermediate filament) (Moll, Franke et al. 1982). *In vitro* multiple combinations of acidic and basic keratins copolymerise to form filaments, but *in vivo* expression is limited to specific pairs in an epithelial specific manor (Franke, Schiller et al. 1983; Hatzfeld and Franke 1985). The keratin pair K5/K14 is found in mitotic basal keratinocytes in all squamous epithelium (Nelson and Sun 1983). When basal keratinocytes differentiate, K5/K14 expression is down-regulated and replaced by tissue-specific keratins. In the epidermis and squamous forestomach of the mouse, K1/K10 are expressed upon initiation of the squamous differentiation pathway (Fuchs and Green 1980; Stellmach, Leask et al. 1991). In the cornea, hair follicle outer root sheath and dorsal oral mucosa K5/K14 are replaced by K3/K12 (Moll, Franke et al. 1982; Wu, Zhu et al. 1994), K6/16 (Moll, Franke et al. 1982) and K6/16 respectively (Moll, Franke et al. 1982; Rentrop, Knapp et al. 1986). Only suprabasal differentiating cells within the oesophageal epithelium express K4/K13 (Fuchs and Green 1980; Moll, Franke et al. 1982; van Muijen, Ruiter et al. 1986). Mutations in the squamous keratin genes are responsible for the blistering disorders of the skin epidermolysis bullosa simplex (K5/K14) and epidermolytic hyperkeratosis (K1/K10) (Fuchs and Weber 1994). Both diseases result in blistering because of the accumulation of keratin filaments and cell lysis upon minimal trauma.

One important function for stratified squamous epithelium is of protection and this is, in part, accomplished by the cell envelope (CE) that begins to develop upon differentiation as keratinocyte leave the basal layer. The CE is a specialised 10nm macromolecular structure of insoluble proteins formed on the inside of the plasma membrane (Jarnik, Simon et al. 1998). The CE of keratinised squamous epithelium (in mouse) is described as cornified and contains an extra 5nm extracellular lipid envelope. Many different proteins are involved in the CE assembly, such as filaggrin, involucrin, loricrin, type II keratin intermediate filament proteins, annexin I, cystatin α, elafin, pancornulins and cornifins (Steinert and Marekov 1999). The
onset of differentiation of oesophageal basal cells, in response to an increase in calcium concentration, is marked by the formation of small lipid-containing organelles (Squier and Kremer 2001). During differentiation, there is simultaneous accumulation of lipid and keratin and synthesis of the proteins profilaggrin and involucrin. The lipid-containing lamellar migrate to the apical aspect of the keratinocyte and fuse with the plasma membrane and create an extracellular lipid barrier (Squier and Kremer 2001). Continued differentiation of the cells results in accumulation of aggregated keratin filaments; filaggrin, involucrin and loricrin are deposited on the inner aspect of the plasma membrane. These proteins are cross-linked with $N^\gamma$-(γ-glutamyl)lysine isopeptide and disulphide bonds by transglutaminases, forming an insoluble layer (Nemes and Steinert 1999). Loricrin is the major constituent of the fully formed CE contributing 80% of the protein content (Mehrel, Hohl et al. 1990; Kalinin, Marekov et al. 2001).

The cells of the terminally differentiated mouse oesophagus are keratinised, lack organelles and are anucleate. The human oesophageal epithelium differs from the murine example described because it is not keratinised (Kalinin, Marekov et al. 2001). In this situation the accumulation of lipids and keratins is less marked, but the differentiated cells still flatten and develop a cross-linked protein envelope. The terminally differentiated squamous cells of the human oesophagus exhibit organelles and nuclei.

The hierarchical expression of cytokeratins and components of the cornified cell envelope can be used to characterise the phenotype of oesophageal cells in vivo and in vitro. I will use a selection of markers to assess the phenotype of in vitro oesophageal models (Fig. 1.3 from Introduction).

3.2 The use of a long-term model of squamous adult oesophagus

Whilst the molecular mechanisms involved in the pathogenesis of cancer can be elucidated from the somatic mutations observed in pathological specimens and cancerous cell lines, understanding the initial steps and acquiring functional evidence requires a robust model of normal squamous oesophagus. The
mechanisms involved in the development of oesophageal cancer are being unravelled with tissue samples and cell lines derived from such samples. These strategies look for genetic mutations and abnormalities in signalling pathways already in situ and not at factors responsible for their initial genesis. For example, CDX2 has been observed in biopsy specimens from patients with BM. While these pathological samples implicate CDX2 in the pathogenesis of the disease, the functional role of CDX2 and factors responsible for the initial expression of CDX2 are not understood. Further evidence for the involvement of CDX2 in the development of BM is demonstrated by the appearance of intestinal metaplasia, similar to BM, in the gastric mucosa of transgenic mice with forced gastric expression of Cdx2 (Mutoh, Hakamata et al. 2002; Silber, Sullivan et al. 2002). Although these in vivo studies prove a functional role for the development of gastric IM, neither the effects of Cdx2 on mouse oesophagus nor factors that control the induction of Cdx2 can be concluded. A robust in vitro model of squamous oesophageal epithelium would allow for both the study of initiating factors responsible for Cdx2 induction and the effect on oesophageal cells. Whilst certain cellular characteristics of cancerous cells can be assessed as snapshots in the form of histological specimens or with molecular techniques, an in vitro model allows for dynamic temporal changes to be observed. A robust in vitro model of squamous oesophagus would have many uses outside of studying the pathogenesis of BM and OA. It would provide a versatile system that can be used in many areas of cell biology, particularly to follow normal growth and differentiation characteristics along with elucidating the cellular and molecular basis of oesophageal disease. The mechanisms underlying the control of differentiation from the oesophageal stem cell to the fully mature squame are not fully understood. A model that offers the full spectrum of differentiated cells would allow this to process be examined in detail. Addition of exogenous compounds to the culture media allows for assessment of changes in oesophageal cells and consequently drug toxicity, carcinogen potential, radiation effects and chemotherapy effect can be elucidated. Furthermore, the effects of genetic manipulation such as ectopic gene expression or knockdown experiments using
infection or transfection of transgenes, dominant negative constructs or interfering RNA, could be assessed.

A long term \textit{in vitro} model of squamous oesophagus offers multiple opportunities for studies into carcinogenesis. In the present chapter, I describe a model which recapitulates the full differentiation phenotype of squamous oesophagus \textit{in vivo}. The main utility of this representative \textit{in vitro} model will be to characterise the effects of ectopic Cdx2 expression, specifically the expression of intestinal markers. Currently it is not known to what degree the ectopic expression of Cdx2 can reprogram the phenotype of normal squamous oesophageal cells.

\textbf{3.2.1 \textit{In vitro} models of squamous oesophagus}

Long term culture of oesophageal epithelium has proven difficult and three techniques, immortalised cell lines, primary cell culture from tissue and explant culture, are currently available (Bey, Alexander et al. 1976). Cell lines are established by serial cultivation of cells obtained from tissue specimens, commonly cancer. OE 19 and OE21 (Rockett, Larkin et al. 1997) are examples of oesophageal adenocarcinoma and oesophageal squamous cell carcinoma respectively. Cell lines can be established from normal oesophageal tissue, but often require genetic transformation to establish immortality. For example expression of telomerase reverse transcriptase (Morales, Gandia et al. 2003) or transfection with the SV40 gene can be used to immortalise oesophageal cells (Stoner, Kaighn et al. 1991). Despite their ease of use, cell lines have significant limitations in the extrapolation of experimental results to normal cells. Malignant oesophageal cell lines have a very limited role in investigating early cellular changes and genetic mutations involved in carcinogenesis because of the already abnormal genetic profile and karyotype instability. Immortalised ‘normal’ squamous oesophageal cells behave differently in culture to primary oesophageal epithelial cells, limiting the interpretation of experiments using them, and are known to carry an abnormal karyotype. Immortalised squamous oesophageal cells lack the normal
response to express involucrin, a marker of differentiation, in response to calcium (Inokuchi, Handa et al. 1995).

Primary cell cultures of normal squamous oesophagus have been shown to be viable for at least 20 passages (Oda, Savard et al. 1998; Marchetti, Caliot et al. 2003; Zhang, Gong et al. 2005) although to increase their proliferative capacity some authors have used feeder layers (Compton, Warland et al. 1998). The basal layer of oesophageal cells in vivo is in contact with the lamina propria and influenced by signalling from the connective tissue layer. Primary cell cultures are not in contact with a fibroblast component and consequently signalling between epithelium and connective tissue is absent. Although a fibroblast feeder layer can offer a degree of epithelial-mesenchymal interaction, the cells are not usually genetically identical, may be derived from an anatomically distant site and may even originate from a different species to the original epithelium.

Explant culture offers the advantages of a mixed cell population, thus allowing epithelial mesenchymal interactions, and uses more physiologically relevant cells. Furthermore, explant culture preserves the three dimensional architecture and cellular relationships which are lost during monolayer culture. The main problem associated with explant culture of oesophageal epithelium is selection and overgrowth of fibroblasts within the culture (Freshney 1987). Previous reports of explant culture of the oesophagus have been limited to less than 4 weeks duration (Mothersill, Cusack et al. 1988) or monolayer architecture (Resau, Phelps et al. 1990). The model of oesophagus described in this chapter offers many advantages over existing techniques. The culture, which can be viewed in real time, is viable in culture long term and consists of a full range of epithelial cells identical to the oesophagus.
**Results**

**3.3 Morphology of normal CD1 mouse oesophagus**

Histological sections of adult CD1 mouse oesophagus were stained with haematoxylin and eosin to assess the normal anatomical arrangement of cells. This will permit a comparison with the immunohistochemical sections and therefore aid in interpretation of staining patterns (Fig 3.1).

The adult mouse oesophagus is surrounded on its outermost aspect by a connective tissue adventitia which surrounds the muscularis externa. The muscularis externa consists of 2 distinct muscle layers, an outermost longitudinal layer and inner circular layer (Fig 3.1A). Between the muscularis externa and the mucosa is a layer of loosely packed connective tissue, the submucosa. The mucosa (Fig 3.1B) consists, from the submucosal layer to the lumen, of a muscularis mucosa, the lamina propria and the stratified squamous epithelium. The epithelium (Fig 3.1C) can be divided into a basal cell layer, a differentiating layer and the terminally differentiated stratum corneum.
**Figure 3.1 Histology of adult CD1 mouse oesophagus**

Haematoxylin and eosin stained sections of (A) whole mouse oesophagus, (B) mucosa and (C) epithelium. (A) At the outermost layer of the oesophagus is the connective tissue adventitia which surrounds the two layers of the muscularis externa, longitudinal (outer) and circular (inner). The submucosa is seen as a loosely packed connective tissue layer that surrounds the mucosa. (B) The mucosa consists of an outer thin layer of muscle, the muscularis mucosa, which is in contact with a thin connective tissue lamina propria. The stratified squamous epithelium sits on the lamina propria. (C) The epithelium consists of a regular layer of basal cells, which progressively flatten through the differentiating layer and is covered by the anucleate keratinised stratum corneum.

Scale bar represents 200µm (A), 50µm (B) and 25µm (C)
3.3.1 Specificity of oesophageal antibodies

To determine the spatial expression pattern of oesophageal specific antibodies immunohistochemical analysis was performed on adult mouse oesophageal sections. The squamous markers p63, K14 (two antibodies raised in rabbit or mouse), K4, involucrin and loricrin along with the simple columnar K8 expression was assessed. In addition, the expression of p63 in human oesophageal biopsy sections was assessed.

The expression of p63 within mouse oesophagus is limited to a single basal layer of epithelial cells (Fig 3.2 A and B). K14 is demonstrated only within the epithelium and is confined to the basal layer of cells (Fig 3.3). K4 is only present within the epithelium and has highest expression with the middle differentiating part of the epithelium (Fig 3.4 A-C). Involucrin is found in a similar distribution pattern, but some of the nuclei of the basal cells are positive (Fig 3.4 D-F). Loricrin expression is not seen in the basal cells and is highest within the stratum corneum (Fig 3.4 A-C). The columnar K8 is present within gastric epithelium wholemounts but not oesophageal (Fig 3.5 D-F).

Given that visualisation of primary antibodies for the majority of experiments will be using fluorescent secondary antibodies, the specificity of three antibodies (K14 (mouse antibody), K14 (rabbit antibody) and K4) was retested on oesophageal cryosections (Fig 3.6, 3.7 and 3.8).
Figure 3.2 The squamous transcription factor p63 is expressed in the basal layer of mouse oesophageal sections. p63 immunohistochemistry in mouse oesophageal biopsies (A+B). Within mouse oesophagus, p63 is found in a circular circumferential pattern only within the epithelium (A). A higher power image of the epithelium confirms that p63 is limited to the nuclei of the basal keratinocytes (B).

Scale bar represents 100μm (A) and 50μm (B).
Figure 3.3 Expression pattern for K14 antibodies in mouse oesophagus.
The spatial expression of K14 was assessed using two different antibodies; one raised in mouse (A-C) and the other raised in rabbit (D-F). (A+D) demonstrate a circumferential distribution pattern of K14 expression around the oesophagus for both antibodies. K14 is limited to the oesophageal epithelium (B+E arrowed) and in higher power is shown to mark the basal layer of cells.
Scale bar represents 200µm (A+D), 100µm (B+E) and 50µm (C+F)
Figure 3.4  Expression pattern for the differentiating markers K4 and involucrin within mouse oesophagus.

The spatial expression of K4 (A-C) and involucrin (D-F) in mouse oesophageal epithelium.

(A) Demonstrates a circumferential distribution pattern of K4 expression around the oesophagus. K4 is limited to the oesophageal epithelium (B) and higher power demonstrates expression in the middle layer oesophagus (arrow in C).

(D) Involucrin is expressed symmetrically around the oesophagus in the epithelial compartment (E). The positive cells are mainly within the differentiating portion of the epithelium, but some nuclear staining of the basal cells is seen (F). The arrows mark the positive cells.

Scale bar represents 200μm (A+D), 100μm (B+E) and 50μm (C+F).
Figure 3.5  Expression pattern for loricrin and the simple columnar marker K8.

The specificity of a loricrin antibody with mouse oesophagus (A-C) and a K8 antibody within gastric and oesophageal explant wholemounts (D-F). Loricrin is expression around the oesophagus is a circular pattern (A) which is limited to the epithelium (B). Cells positive for loricrin are restricted to suprabasal cells mainly in the stratum corneum (C). K8 is expressed within the stomach (D+E), but not in oesophagus (F).

Scale bar represents 200μm (A), 100μm (B), 50μm (C, D+F) and 10μm (E).
Figure 3.6  Fluorescent immunohistochemistry for K14 on oesophageal sections.

K14 staining of mouse oesophageal epithelium using a rabbit monoclonal antibody. The structure of the oesophagus is demonstrated by DAPI (A). K14 expression (B) is expressed in a circumferential pattern. (C) Overlay of (A) and (B). High magnification images of the epithelium. (D) shows nuclear DAPI staining and K14 (E) is restricted to the basal cells. (F) overlay of (D) + (E). The K14 positive cells are arrowed.

Scale bar represents 200μm (A-C) and 50μm (D-F)
**Figure 3.7 Fluorescent immunohistochemistry for K14 on oesophageal sections.**

K14 immunostaining of oesophagus using a mouse monoclonal antibody. The structure of the oesophagus is demonstrated by DAPI (A) and K14 expression (B) is in a circumferential pattern. (C) Overlay of (A) and (B). In high power the epithelial shape is demonstrated by DAPI (D) and K14 (E) is restricted to the basal cells. (F) overlay of (D) + (E). The K14 positive cells are arrowed.

Scale bar represents 200μm (A-C) and 50μm (D-F).
**Figure 3.8 Fluorescent immunohistochemistry for K4 on oesophageal sections.**

K4 and nuclear staining of mouse oesophagus (A-C) and epithelium (D-F). The structure of the oesophagus is demonstrated by DAPI (A) and K4 expression (B) is in a circumferential pattern. (C) Overlay of (A) and (B). In high power the epithelial shape is demonstrated by DAPI (D) and K4 (E) is expressed in the suprabasal cells. (F) overlay of (D) + (E). The K4 positive cells are arrowed.

Scale bar represents 200µm (A-C) and 50µm (D-F).
3.3.2 Characterisation of cell lines for oesophageal markers

In order to assess the effects of ectopic Cdx2 expression on oesophageal cell types, it is necessary to characterise any potential culture model according to the expression of oesophageal markers, Cdx2 and intestinal markers. Three cell lines were assessed for the expression of oesophageal and intestinal markers. Firstly Caco-2 cells, derived from a colorectal adenocarcinoma, which differentiate to express intestinal markers spontaneously in culture (Pinto 1983). Secondly an undifferentiated intestinal cell line, HT-29, which arose from a colorectal adenocarcinoma. HT-29 cells can differentiate into varying intestinal phenotypes depending on the culture conditions (Zweibaum, Pinto et al. 1985). Thirdly an immortalised oesophageal cell line, HET-1A, derived from normal human oesophagus (Stoner, Kaighn et al. 1991).

The cell lines were cultured for 2 days after seeding and then fixed and examined for oesophageal and intestinal markers by immunohistochemistry (K4, K8, K14, Cdx2 and p63) and PCR (CDX1, CDX2, chromogranin A, defensin 6, K18, K20, K14, mucin 2, sucrase isomaltase, trefoil factor 3 and villin). We also examined the same markers using PCR seven days post confluence to check for any change in expression pattern.

The columnar cytokeratin K8 is expressed in all 3 cells lines (Fig 3.9), whilst the squamous markers p63 and K14 are not seen in any, on immunofluorescent histochemistry (Fig 3.10 and 3.11). Primary cultures of oesophageal cells were included in the analyses. The intestinal gene CDX2 is only present in Caco-2 cells and localises to the nucleus (Fig 3.12). PCR for intestinal transcripts demonstrates the presence of the following mRNA within the cell lines (Fig 3.13).

1) HT-29 – K18, K20, K14, mucin 2, Trefoil factor 3 (TFF3) and villin
2) Caco-2 - K18, TFF3, villin, CDX1 and CDX2
3) HET-1A - K18, K14 and TFF3
Figure 3.9 - K8 expression in HT-29, Caco-2, HET-1A and primary squamous oesophageal cells.

Immunofluorescent images of K8 expression in the cell lines Caco-2, HT-29 and HET-1A. DAPI nuclear staining of (A) HT-29, (B) Caco-2, (C) HET-1A and (D) primary squamous oesophageal cells. K8 expression is present in (E) HT-29, (F) Caco-2, (G) HET-1A, but not (H) primary squamous oesophageal cells.

Scale bars represent 50μm.
**Figure 3.10- K14 expression in cell lines**

Immunofluorescent images of K14 expression in the cell lines Caco-2, HT-29 and HET-1A. DAPI nuclear staining of (A) HT-29, (B) Caco-2, (C) HET-1A and (D) primary squamous oesophageal cells. K14 expression is absent in (E) HT-29, (F) Caco-2, (G) HET-1A, but present in (H) primary squamous oesophageal cells. Scale bars represent 50μm.
Figure 3.11- p63 expression in cell lines

Immunofluorescent images of p63 expression in the cell lines Caco-2, HT-29 and HET-1A. DAPI nuclear staining of (A) HT-29, (B) Caco-2, (C) HET-1A and (D) primary squamous oesophageal cells. p63 expression is absent in (E) HT-29, (F) Caco-2, (G) HET-1A, but present in (H) primary squamous oesophageal cells.

Scale bars represent 50µm.
**Figure 3.12 CDX2 expression in cell lines**

Immunofluorescent images of CDX2 expression in the cell lines Caco-2, HT-29 and HET-1A. DAPI nuclear staining of (A) HT-29, (B) Caco-2, (C) HET-1A and (D) primary squamous oesophageal cells. CDX2 is expressed in (F), but not (E) HT-29, (G) HET-1A nor (H) primary squamous oesophageal cells.

Scale bars represent 50μm.
Figure 3.13  Expression of oesophageal and intestinal genes in oesophageal and intestinal cell lines.

RT-PCR results for cell lines grown to confluence and for seven days post confluence. Beta actin, CDX1, CDX2, chromogranin A, defensin 6, K18, K20, K14, mucin 2, sucrase isomaltase, trefoil factor 3 (TFF3) and villin mRNA was assessed in Caco-2, HT-29 and HET-1A cell lines.
3.4 Etched plastic cover slips maximise explant attachment

In order to culture mouse oesophageal explants for extended periods of time, it was essential that the tissue attached to a culture material. Different methods to enhance attachment were tested to optimise the culture conditions. Attachment and growth of oesophageal explants were tested under 4 different conditions. APTS-subbed glass coverslips were coated with either the extracellular matrix glycoprotein fibronectin, or the connective tissue component type I collagen and used with glass cloning rings for the first 24 hours. To coat coverslips 40µl of 50µg/ml fibronectin (Invitrogen) or 40µl of 50µg/ml collagen type I (Sigma) was allowed to dry in the centre of the glass coverslip. Plastic coverslips were either used untreated with a glass cloning ring or etched with a grid shaped pattern. Explants attached to the etched coverslips in 82% of cases and this technique was used for the culture model from this point on (Table 3.1). Less than 15% of explants attached to fibronectin coated, collagen coated or plastic coverslips.

<table>
<thead>
<tr>
<th>Attachment conditions</th>
<th>Fibronectin</th>
<th>Collagen</th>
<th>Plastic alone</th>
<th>Etched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number attached at 24 hours</td>
<td>3/20 (15%)</td>
<td>2/20 (10%)</td>
<td>2/50 (4%)</td>
<td>41/50 (82%)</td>
</tr>
</tbody>
</table>

3.4.1 Growth of explant culture.

Adult mouse oesophageal explants attached to the plastic coverslip within 48 hours in greater than 80% of cases. Attached explants develop a surrounding skirt of cells within 3 days. Cells are first visible around the culture on day two post dissection and plating (Fig 3.14). The first cells to become visible were irregular neuronal-like in morphology and had little contact with other cells. On day 3 of culture, regular shaped cells in close proximity to each other began to be observed. A region in closest proximity to the original explant exhibited overlapping cells and cells seen in a higher plane of focus, suggesting a multilayered structure. The size of the outgrowth continued to expand for 2-3 weeks and remained visible for 6 months.
3.4.2 Characterisation of cell phenotypes within the culture model.
The cells surrounding the central core of the explant have a connective tissue morphology. To characterise them further we immunostained the oesophageal explants for alpha smooth muscle actin (SMA), an antibody that recognises both fibroblast or myofibroblast cell types (Fig 3.15). These cells existed both as individual cells (i.e. without any neighbouring cell contact), and in continuous layers. Some of the fibroblasts are mitotically active since they stain for the mitotic marker, phosphohistone H3 (PH3) (Fig 3.15).
Epithelial cells were demonstrated within the culture by the presence of cells expressing E-cadherin and cytokeratins (Fig 3.16). The epithelium appears as a continuous area, with no isolated cells, which was in communication with the original explant. Epithelial cells were always observed intermingled with fibroblasts (Fig 3.16).
The majority of outgrowths, 90%, exhibited both epithelial and connective tissue components. A minority contained only SMA positive cells, but no outgrowth consisted purely of epithelium. The presence and distribution of epithelium was confirmed with an alternative polyclonal antibody to multiple basic cytokeratin filaments, the intermediate filaments unique to epithelial cells, found in squamous epithelium (Fig 3.16).

3.4.3 Cultured oesophageal epithelium expresses markers of differentiation
In order to demonstrate that the explant model is a faithful representation of the native oesophagus, markers for different stages of squamous differentiation were assessed after seven days of culture. Cells analogous to the basal cells of the oesophagus (expressing K5 and K14), were present within the epithelial component of the outgrowth (Fig 3.17). These cells were first visible at day 2 of culture and increased in number as the outgrowth increased in size and were found as sheets of cells (Fig 3.17). Individually, they were regular in outline and often rectangular in shape (Fig 3.18). The expression of K14 was present from the explant to the circumferential edge of the epithelium (Fig 3.18). Some cultures did not contain any regions of epithelial growth and were consequently negative for
K14. However, all K14-positive cultures contained fibroblasts (Fig 3.19). Markers of early differentiation, K4 and involucrin were found within the epithelial component of explant cultures (Fig 3.20). Loricrin (the major component of the cornified cell envelope) is present only in terminally differentiated squamous cells. Cells expressing loricrin were found in the epithelial compartment of the culture outgrowth (Fig 3.20). Cells expressing K14 extended further from the explant that K4-positive cells (Fig 3.21). K4 expressing cells were not found at the edge of the epithelial component of the culture. Some cells co-expressed K4/K14 whilst other cells only expressed one or other cytokeratin (Fig 3.21). The distribution of involucrin-positive cells was different from that of K4. Involutcrin was found throughout and at the edge of the epithelium (Fig 3.22). Involutcrin / K14 co-expression is demonstrated along with individual cells that only express one or other. Numerous p63-positive cells were found within the culture particularly in the original tissue explant (Fig 3.23). P63 positivity was lost in the cells that were furthest from the explant. P63 was present in two discrete populations of cells. The first set of cells were found within the original explant of tissue and did not co-express K14 (Fig 3.24). The second set formed a ring surrounding the explant and co-expressed p63 / K14. The majority of K14-positive cells were negative for p63.

3.4.4 Three dimensional structure of oesophageal explant culture.
To determine the three dimensional structure of the explant model, Z-stack videos were taken using a laser scanning confocal microscope. In order to describe the three dimensional orientation of the model the tissue culture plastic side will be referred to as bottom/underneath and the culture media aspect the top/above. Within the explant of oesophageal tissue a population of cells expressing p63 but not K14 were located at the bottom, underneath a layer of K14 positive cells (Fig 3.24). The basal epithelium (K14) of the outgrowth surrounding the explant was on top of a carpet of fibroblasts (SMA) (Fig 3.25). The differentiating cells (K4) are located on top of the basal layer (K14) (Fig 3.26). A diagrammatic summary of the three dimensional orientation of a typical explant culture in Fig (3.27 A and B).
Figure 3.14 Characteristics of oesophageal explants in culture.

(A-D) Phase contrast images of oesophageal explants. (A) day 0, (B) day 2, (C) day 4 and (D) day 7. An outgrowth of cells from the explant is first seen on day 2 and increases in size daily. E-F) two types of cells were seen within the outgrowth. E) Rectangular shaped cells form a cobblestone appearance in continuation with the explant. F) Neuronal shaped cells are found surrounding the original tissue. Scale bars represent 250μm (A, B, C and D) and 125μm (E and F).
Figure 3.15 Connective tissue components are present in the explant culture model

(A+B) SMA-positive fibroblasts (green) form an epithelial outgrowth around the original explant (arrowed). SMA-positive cells are shown in low and high magnification after seven days in culture. (C) Fibroblasts (green) in mitosis are demonstrated with an antibody to phosphorylated histone H3 (red). (D-F) A typical time course of SMA expression (green) within the outgrowth at day 3 (D), day 5 (E) and day 7 (F). Scale bars represent 500μm (A, D-F) and 50 μm (B+C).
**Figure 3.16 The spatial arrangement of epithelium and connective tissue within explant cultures.**

The epithelial and fibroblast component of explant cultures was examined after seven days in culture. (A) E-cadherin-positive cells (green) form an epithelial outgrowth around the original explant (arrowed) after seven days in culture. (B+C) Epithelial cells (green) are always found in continuation with the original explant, but do not account for all cell types. Non-epithelial cell nuclei (blue DAPI) are arrowed (Fi) and represent fibroblasts. (D) E-cadherin (green) and pancytokeratin (red) antibodies stain the same cells. The edge of the epithelium is arrowed. (E+F) Epithelial cells marked with a pancytokeratin antibody (green) and SMA expressing fibroblasts (red) exist in close proximity within the outgrowth. Scale bars represent 500µm (A+B) and 50 µm (C-F).
Figure 3.17 Time-course for K14 and K5 expression in oesophageal explants.

To identify cells representative of in vivo basal oesophageal cell types, explant cultures were examined for the expression of K5 and K14 after 3, 5 and 7 days in culture. (A-D) K5-expressing cells (green) on day 3 (A), Day 5 (B), Day 7 (C) and at 50x original magnification (D). (E-G) K14-positive cells (green) on day 3 (E), Day 5 (F), Day 7 (G) and at 50x original magnification (H). Scale bars represent 500 μm (A-C and E-G) and 100 μm (D+H).
**Figure 3.18 Morphology of K14-positive cells in explant cultures.**

(A) K14-positive cells (green) surrounding an explant. The nuclei (blue) of fibroblasts are arrowed. (B-D) Cells expressing K14 (green) are cuboidal in shape and always found in continuation with other basal cells. (E) An apparent binucleate cell is arrowed. These are very rare within K14 (green) positive cells and probably represent the shadow from a K14-negative cell overlaying the basal cell. (F) Basal cells (red=K14) extend to the edge of the epithelial component (arrow) (E-cadherin (green)) of the outgrowth. Scale bar represents 100μm (A, B, E and F), 20μm(C) and 10μm (D).
Figure 3.19 Spatial arrangement of fibroblasts and basal epithelial cells

(A) An explant culture with no epithelial component, only SMA-positive (red) fibroblasts are observed. K14 basal cells (green) are absent. (B) Fibroblasts (red) in isolation from other cells (arrowed). Basal cells (green) are always in continuation with other basal cells. (C-E) Fibroblasts (red) are found at the edge (C+D) and within (C+E) the basal epithelium. Scale bar represents 300μm in (A+B), 200μm (C) and 50μm (D+E).
Figure 3.20 Cell representative of differentiating and mature squamous cells are found with explants

(A-F) Expression of the differentiation markers K4 (A+D), involucrin (B+E) and loricrin (C+F) in oesophageal explants cultured for 7 days. (A+D) Cells expressing K4 (green) are shown at both low (A) and high (D) magnification. (B+E) Cells expressing involucrin (green) at low (B) and high (D) magnification. (C+F) Cells expressing loricrin (green) at low (C) and high magnification (F). In (F), nuclear DNA (DAPI) is shown in blue to demonstrate the presence of cells not expressing loricrin. Scale bar represents 100μm in (A,B,C and F) and 20μm in (D+E).
**Figure 3.21 Spatial arrangement of differentiating cells within explant culture.**

After 7 days of culture, explants expressing K4, representative of differentiating cells found in the suprabasal layer of the oesophagus, were found. (A) K14-positive cells (red) found within the outgrowth surrounding the explant. (B) K4 (green) cells within the same outgrowth. (C) Overlay image of (A) and (B) demonstrating that K4 differentiating cells (green) are located more centrally than the K14-positive cells (red). The K14-positive cells extend to the edge of the epithelium. (D-F) Higher magnification images of K4- and K14-expressing cells (green and red respectively). (D) K14-positive cells (red). (E) K4-positive cells (green). (F) Overlay of (D) and (E) demonstrates a cell that co-expresses K14 and K4 (marked with a star) and another cell only positive for K4 (arrowed).

Scale bar represents 200μm (A-C) and 20μm (D-F).
Figure 3.22 Spatial arrangement of involucrin and K4 expression within the epithelium

(A-D) Involucrin (green) and K14 expression (red) within the same 7 day culture. (A) Expression of the differentiating marker Involucrin (green) within seven day old outgrowth of cells. (B) K14 basal cells (red) within the same outgrowth as (A). (C) Overlay image of (A) and (B) to demonstrate the progression of differentiation. Basal cells (red arrow) prior to differentiation lack Involucrin expression. As cells start to differentiate cells co-express K14 and involucrin (green arrow). Some cells further mature and only express involucrin (cell to the right of the star). (D) DAPI nuclear staining has been overlaid onto (C) to highlight non-epithelial fibroblastic cells (double headed arrow).
Figure 3.23 Explant cultures express p63

(A) p63-positive cells (green) are present shown in 7 day old cultures. Note the high number of positive cells within the explant (arrowed) decreasing towards the periphery. (B) Higher magnification image of cells within the explant of tissue. (C) Individual cells within the outgrowth demonstrate nucleolar sparing.

Scale bar represents 200µm (A), 50µm (B) and 20µm (C).
Figure 3.24 Distribution of p63 and K14 is different within oesophageal cultures

(A) K14 (red) and p63 (green) are co-expressed in basal cells within the outgrowth. (B) K14-positive, p63-negative cells are found within the outgrowth and are more frequently found towards the edge of the epithelium. (C-E) p63 positive, K14 negative cells are found within the explant and represent smaller cells compared to the basal population (K14). (C) Occasional p63-positive K14-negative cells are found within the outgrowth. (D) Within the original explant of tissue a high proportion of cells are only positive for p63. The edge of the explant is arrowed. (E) Immediately adjacent to the explant (arrow) cells only expressing p63 are commonly seen. (F) The p63 positive, K14 negative cells within the explant are covered by a layer of K14 positive cells. Within any explant that develops an epithelium, p63 positive / K14 negative cells are found and are located deeper within the culture and than the covering layer of basal cells.

Scale bar represents 50μm (A, C, D and E) and 20μm (B).
Figure 3.25 Three dimensional orientation of oesophageal cultures. Fibroblasts are found under the basal layer. (A-D) Immunostaining for SMA (green) and K14 (red) are shown from a typical culture. (A) K14 positive basal cells (red). (B) SMA positive fibroblasts (green) (C) overlay of images (A) and (B) to show both cell types within the outgrowth. (D) Z-stack section demonstrating basal (red) cells on top of a layer of fibroblasts (green). Scale bar represents 20μm (A-C).
Figure 3.26 Three dimensional orientation of the culture. Differentiating cells are on top of basal cells. 

(A-D) Immunostaining of a typical culture for K14 (red) and K4 (green).

(A) Basal K14-positive cells (red) in a seven days old culture. (B) Differentiating K4 positive cells (green) within the same culture. (C) Overlay of (A) and (B) demonstrates both cell types coexisting in culture. (D) Z-stack section shows red cells forming lower layer of culture (red arrow) with a thick layer of cells coexpressing K14 and K4 (yellow arrow) and a superior layer of only K4 expression (green arrow) Scale bar represents 50μm (A-C).
Figure 3.27 Diagram to summarise the orientation of connective tissue, epithelium and squamous differentiation markers in an *in vitro* explant culture model of adult squamous oesophagus.

When viewed from above SMA-positive fibroblasts extend furthest from the original tissue. K14-expressing basal cells extend to the edge of the epithelium. K4-positive cells are found within the epithelium located more centrally that K14. p63 is found within the bud of tissue and into a small proportion of the outgrowth of cells. A sagittal section reveals the three dimensional structure of the oesophageal explants; the fibroblasts (SMA) are found underneath p63 positive cells, which in turn are covered by basal cells (K14) and differentiating (K4) cells.
3.5 Response of oesophageal cells to changes in extracellular calcium.
The oesophageal culture model exhibits a multilayer connective tissue and epithelial structure. We observed cells at all stages of stratified squamous differentiation. The fact that the model provides a faithful representation of the oesophagus creates two specific technical difficulties during experiments. First, the least mature mitotic cells within the culture reside underneath layers of differentiated and differentiating squamous cells the role of which, in vivo, is to provide protection. In order to study effects of either soluble factors or ectopic genes within all cell types, access to the K14 positive cells deep within the model is required. The second problem relates to multitude of cell types that are represented within the model. Studying the response of cells in respect of inducing or inhibiting differentiation is hampered because cells at all stages of differentiation and cell cycle are present. A solution to these problems would be to arrest the squamous differentiation program and select a set of immature cells, as assessed by p63 and K14 expression. This provides an alternative culture model to allow access to a homogeneous monolayer of immature cells. In order to accomplish this, the calcium concentration was decreased to 0.03mM.

3.5.1 The role of calcium in squamous epithelial differentiation
Although the effects of varying calcium concentration on the morphology of oesophageal cells in culture has been known for some time (Babcock, Marino et al. 1983), the majority of the literature relates to epidermal keratinocytes. Within the epidermis there is an increasing intracellular calcium ion (Ca\(^{2+}\)) concentration from the deep to the outermost cells (Menon, Grayson et al. 1985). The Ca\(^{2+}\) gradient is an important stimulus for initiating the expression of differentiated proteins (Fuchs and Green 1980). In vitro, mouse epidermal keratinocytes cultured in low Ca\(^{2+}\) (<0.05mM) have a high proliferative rate and do not express differentiated genes (Hennings, Michael et al. 1980). Increasing the Ca\(^{2+}\) concentration above 0.1mM induces the sequential expression of proteins found in differentiating and differentiated cells (Yuspa, Kilkenny et al. 1989). A similar response to Ca\(^{2+}\) concentration has been demonstrated in human and rat oesophageal keratinocytes.
Primary cultures of human oesophageal cells grow as monolayers of small cuboidal cells forming poor cell-to-cell contacts under low Ca\(^{2+}\) conditions. Addition of 1mM CaCl\(_2\) provokes stratification, cell to cell contact and expression of the differentiation marker (K13) (Sato and Hitomi 2002). Under low Ca\(^{2+}\) conditions (0.1mM), rat oesophageal cells, are smaller and have higher colony forming efficiency than cells grown in 0.3mM Ca\(^{2+}\) (Babcock, Marino et al. 1983). The addition of 0.3mM Ca\(^{2+}\) causes cells to enlarge and flatten suggesting that calcium is a critical factor in the differentiation of squamous epithelium.

In order to test the response of the oesophageal explant model to calcium concentration, an alternative to BME medium (calcium concentration of 1.8mM) was required. Although it is possible to remove Ca\(^{2+}\) from BME using dialysis a defined supplemented medium, MCBD 153, was used for these experiments.

### 3.5.2 Effect of calcium concentration on differentiation of explant culture

The cells within the outgrowth surrounding explants in MCDB153 media (0.03mM Ca\(^{2+}\)) are smaller and grow in monolayers (Fig 3.28). p63 and K14 co-expression is found within the majority these cells (Fig 3.29). There is very little cell-to-cell contact, no stratification and no expression of K4 seen in these cells (Fig 3.29 and 3.30). Increasing the Ca\(^{2+}\) concentration to 1mM in MCDB 153 medium, provokes the expression of K4, induces stratification and increases the size of oesophageal cells (Fig 3.31).

### 3.5.3 Mitotic cells in BME and MCDB 153 media

Phosphorylation of specific serine residues on histone H3 are only seen during the mitosis stage of the cell cycle and phospho-specific antibodies can be used to highlight these cells (Prigent and Dimitrov 2003). In explants cultured in BME, the only mitotic cells within the outgrowth were fibroblast. None of the K14-positive cells expressed phospho-histone H3 (PHH3) (Fig 3.32). PHH3 positive cells were found within the original explant of tissue. In contrast, cells cultured in MCDB 153, exhibited mitotic cells within the explant as well as the outgrowth (Fig 3.32).
3.5.4 Adult mouse oesophageal epithelium does not contain p63 positive cells that are K14 negative.
The explant model of adult squamous mouse oesophagus contains cells that are p63 expressing but K14 negative. To look for a comparable in-vivo oesophageal cell phenotype, mouse oesophageal sections were dual immunostained with p63 and k14. Within mouse oesophagus, all cells that express p63 are K14 positive (figure 3.33)
Figure 3.28 Phase contrast images of oesophageal explants cultured in BME and MCDB 153.
Phase contrast images of explant cultured in MCDB 153 media (A-C) or BME (D-F). Cells within the outgrowth of MCDB 153 cultures have a circular shape (A) which is different to BME cells (D) (the explant is shown with an arrow). Oesophageal cells cultured in MCDB 153 grow in monolayers (B-C) compared with flattened stratified cells cultured in BME (E-F). There is some evidence of cells detaching in MCDB 153 media (arrow in C). There are no stratified flattened cells in contrast to BME media (arrow in F)
Figure 3.29 Expression of p63 and K14 in oesophageal explants grown in BME and MCDB 153 media.

Confocal analysis of p63 (green) and K14 (red) expression in oesophageal explants cultured in MCDB 153 (A-C) and BME (D-F). The majority of cells found within the outgrowth of cells in MCDB media co-express p63 and K14 (A) in contrast to the BME media in which p63 expression is distributed centrally (D). A cell at the edge of the outgrowth positive for p63 is shown with an arrow in (A and B). The cells under low calcium conditions fail to stratify and show little cell to cell contact (arrow in C) in contrast to the cells in BME (E). In BME the cells overlap and interlock with other epithelial cells (F). Scale bar represents 200μm (A+D), 100μm (B), 50μm (E+F) and 20μm (C).
Figure 3.30 Oesophageal explants cultured in MCDB 153 fail to express K4.

Dual immunostaining for K14 (red) and K4 (green) in oesophageal explants cultured in MCDB 153 (A-C) and BME (D-F). (A-C) The outgrowth of cells under low calcium conditions does not express K4 (green) in any cells. (A) Shows a culture after 5 days and (B, C) are increased magnification to demonstrate individual cells. (D) is a representative image of an explant grown in BME for 5 days in which the distribution of K4 expressing cells (green) can be seen. In contrast to K14 (red), K4 (green) is located more centrally. Higher magnification images (E-F) demonstrates individual cells expressing K14 and K4.

Scale bar represents 100µm (A, B, D and E) and 50µm (C+F).
Figure 3.31 An increase in calcium concentration induces K4 expression.

Dual immunohistochemistry analysis for K14 (red) and K4 expressing cells (green) in 5 day old cultures that have been maintained in control media (A-C) (MCDB 153 0.03mM Ca^{2+}) or increased calcium media (D-F) (MCDB 153 supplemented with 1mM calcium (total calcium concentration is 1.03mM). (A-C) cells following 3 days of treatment with control media express K14 (B) but not K4 (A). (C) overlay image of (A+B).

Cells cultured with the addition of 1mM calcium ions for 3 days begin to express K4 (D) and continue to express K14 (E). Stratification can be seen (arrow) in the overlay image (F).

Scale bar represents 100μm.
Figure 3.32 Mitotic cells in explants cultured in BME and MCDB 153 media.
Dual immunohistochemistry for K14 (green) and the mitotic marker PHH3 (red) in an explant culture in BME (A-C) and MCDB 153 (D-F), Mitotic cells are found outside of the explant in BME cultures, but do not express K14. None of the K14 positive cells in the outgrowth express PHH3 (B). Multiple PHH3 positive cells are located in the explant (C). In MCDB 153 the outgrowth contains mitotic cells (D+E) as demonstrated by PHH3 expression (red), which is localised to the nuclei (F).
Scale bar represents 50μm.
Figure 3.33 In-vivo basal oesophageal cells express K14 and p63.
Dual immunohistochemistry for K14 (red) and p63 (green) in oesophageal sections. (A) example of a whole oesophagus demonstrating the basal distribution of K14 and p63. (B) A high power section shows that p63 positive cells (green) always express K14 (red), in contrast to explant culture. Pictures are representative images from 9 oesophageal sections.
Scale bars equal 100μm in (A) and 20μm in (B).
3.6 Discussion

In this chapter, I have demonstrated a novel *in vitro* model of mouse oesophageal culture that is viable long-term and replicates the full repertoire of cell phenotypes found within the oesophagus. I initially compared the expression of oesophageal markers in the explant culture model with the expression of squamous epithelial markers in the immortalised squamous oesophageal cell line, HET-1A. HET-1A cells do not express detectable K14, K4 nor p63 but instead expressed K8 detectable with fluorescent immunohistochemistry (table 3.2). Thus, for the purposes of future experiments, HET-1A cells aren’t representative of oesophagus. There are 4 possible explanations as to why HET-1A cells do not express squamous markers. Firstly the cell line were established in 1991 (Stoner, Kaighn et al. 1991) and will have been passaged a number of times potentially inducing or selecting for a specific cell type. Secondly the immortalisation process involved stable transfection of the SV40 gene provokes genetic changes which could result in a change in cell phenotype and increased karyotypic instability. The third possibility is that the cell line originally arose from the columnar lined oesophageal glands found within the oesophagus and thus express columnar cytokeratins. The fourth possibility is that the cell line was not HET-1A cells but a contaminant.

The oesophageal epithelium demonstrates a hierarchical arrangement of cells from the progenitor cells adjacent to the lamina propria through the differentiating cells of the suprabasal region to the luminal squames, which are terminally differentiated. The explant culture technique demonstrates for the first time an *in vitro* model that fully recapitulates the *in vivo* epithelial spatial structure. It is viable long-term and has remained in culture for 6 months. Furthermore, cells representative of each layer of oesophageal cells, expressing p63, K14, K4 and loricrin, are present. The model is the first to identify and demonstrate the location of loricrin (Hohl, Lichti et al. 1991) and p63-expressing cells (Pellegrini, Dellambra et al. 2001); only previously described in the culture of cells from other organs such as epidermal keratinocytes.
Table 3.2 Expression of oesophageal and intestinal markers in cell lines and adult mouse oesophageal explants. Blank cells were not tested.

<table>
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<tr>
<th>Marker</th>
<th>Caco2 mRNA</th>
<th>Caco2 Protein</th>
<th>HT-29 mRNA</th>
<th>HT-29 Protein</th>
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<th>HET-1A Protein</th>
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Given the full spectrum of epithelial and fibroblast cell types found, the model offers a number of unique opportunities to study the normal development of these cells as well as pathologies. Manipulation of the media allows for the study of the effects of soluble compounds on the culture offering options for utilisation of the culture system in drug toxicity and radioactivity studies.
The explant technique offers considerable advantages over other *in vitro* systems, because it uses normal cells and maintains three dimensional tissue architecture. Cancer cell lines have inherited significant genetic mutations in genes important in the pathogenesis of cancer, for example p53 (Hollstein, Metcalf et al. 1990), and are consequently of limited value in investigating the early steps involved in carcinogenesis. Primary cell cultures lack the presence of a genetically identical connective tissue compartment which is important in epithelial-connective tissue interactions. These signalling interactions are known to be essential in development and influence differentiation of intestinal epithelial cells (Dahan, Roda et al. 2008). For example, retinoic acid can provoke a columnar epithelial phenotype from the stromal cell compartment in human oesophageal biopsies, a transformation that mirrors the changes observed in Barrett’s metaplasia (Chang, Lao-Sirieix et al. 2007). The biggest advantage of the explant system is that it is simple to set up, reproducible and robust.

The finding of outgrowths that consist solely of fibroblasts but not epithelial cells suggests that the connective tissue layer is essential for epithelial growth. The necessity for fibroblast feeder layers in oesophageal primary cell culture (Compton, Warland et al. 1998) and collagen embedded fibroblasts in attempts to create a neo-oesophagus, confirms their importance (Hayashi, Ando et al. 2004). The organisation of the outgrowth surrounding the explant demonstrates a hierarchical structure representative of oesophageal mucosa. The most peripheral cells are fibroblasts which surround the K14 positive basal cells, which encase the K4 differentiating cells, suggesting that K4 expressing cells require a close proximity to the basal cells and do not exist independently. The finding of cells that co-express K4 and K14 would confirm this observation, suggesting that the basal cells differentiated to produce K4 expression.

The distribution of p63 expression is interesting and warrants further comment. p63 is normally expressed maximally in the basal cells of the oesophagus (Glickman, Yang et al. 2001) and has been suggested as a stem cell marker in the limbal cells
of the cornea (Pellegrini, Dellambra et al. 2001). The finding of K14⁺ p63⁻ cells at
the edge of the outgrowth and K14⁺ p63⁺ cells closer to the explant is similar to the
pattern seen in the oesophagus. K14⁺ p63⁺ cells are consistent with the role of p63
in the squamous differentiation pathway. The finding of K14⁻ p63⁺ cells within and
immediately adjacent to the bud is interesting and raises the possibility of a
prebasal phenotype. The explant contains numerous K14⁻ p63⁺ cells and yet this
phenotype is not observed in vivo (Figure 3.33). Interestingly this is the phenotype
of the primitive embryonic oesophageal epithelium prior to the acquisition of
squamous cells (Daniely, Liao et al. 2004). There are 3 possible explanations for
these cells.

1) An artefact of culture. The cells may express an alternative cytokeratin
that is not found in the oesophagus.
2) An artefact of culture. The cells may express an alternative cytokeratin
that is only found in the oesophagus in pathological states e.g. K6.
3).The cells could represent a phenotype that has been selected for in
culture which is normally present in a low number of normal oesophageal
cells.
The K14⁺ p63⁺ phenotype could represent a progenitor population of cells given
that all the mitotic epithelial cells are located in the same region of the explant.

The role of calcium in the initiation of squamous epithelial cells is known. Culture of
explants in MCDB 153 media with a calcium concentration of 0.03mM provides a
method of selecting a homogenous monolayer population of immature
keratinocytes. The observation of K4 expressing cells after increasing the calcium
concentration of the media proves that the basal keratinocytes behave normally in
this aspect of their biology and suggests that the K14 positive basal cells give rise
to the K4 subset. These findings would be expected based on the epidermal
literature and confirm the results achieved in rat and human oesophageal cells.
The explant model, as a robust representative model of oesophagus, will be used
to study factors involved in the induction of Cdx2 and the effect of Cdx2 expression
in oesophageal cells with a view to understanding the pathogenesis of BM.
Chapter 4: Screening of exogenous factors for re-specification of oesophageal cells to columnar intestinal-type cells.

4.1 Introduction

4.1.1 Overview
A variety of factors have been implicated in the BM phenotype change from a stratified squamous to a columnar intestinal-type epithelium. The ParaHox gene, CDX2 is a candidate for the induction of an intestinal phenotype in oesophageal epithelium. However, it is not currently known if CDX2 induction is sufficient to provoke induction of columnar epithelium in an analogous manner to BM. In order to understand the role of CDX2 in BM, it would be desirable to induce its expression within oesophageal cells in vitro and observe the phenotypic changes. There is now increasing evidence that the induction of CDX2 may be related directly to mediators of inflammation.

In this chapter, I used the explant model of oesophagus (described in chapter 3) to determine changes in the cellular phenotype in the presence of 6 factors that have been linked to either the development BM or the induction of CDX2: retinoic acid (RA), lipopolysaccharide (LPS), bile salts, bone morphogenetic factor 4 (BMP4), tumour necrosis factor alpha (TNFα) and interleukin one beta (IL-1β). Specifically I looked for a loss of the oesophageal phenotype and induction of Cdx2. The ultimate goal was to identify a compound that could be used to induce Cdx2 and determine whether this was sufficient to induce an intestinal phenotype in a physiological model of oesophageal cells. If it is possible to provoke the expression of Cdx2, and induce an intestinal phenotype, in an in vitro explant of oesophagus, this would provide (i) evidence that Cdx2 is the master gene in the development of BM and (ii) a useful model for the pathogenesis of BM.

4.1.2 Evidence for the role of CDX genes in the pathogenesis of BM
CDX1 and CDX2 play a major role in intestinal development and have been linked with a causative role in BM. Recently, Rustgi and colleagues tested the utility of
Cdx1 in combination with c-myc to induce an intestinal phenotype in immortalized human oesophageal keratinocytes (Stairs, Nakagawa et al. 2008). The authors showed that c-myc and Cdx1 cooperate to induce mucin production and changes in keratin expression reminiscent of intestine. Further evidence for a role of Cdx1 is elicited from experiments using the oesophageal adenocarcinoma cell line, KYSE30 as well as tissue from BM patients (Wong, Wilding et al. 2005). Cdx1 is expressed in BM (Wong, Wilding et al. 2005) and addition of the hypomethylating agent 5-azacytidine to KYSE30 cells induces Cdx1 expression. CDX1 expression is further enhanced by treatment with 5-azacytidine and TNFα (Wong, Wilding et al. 2005).

There is circumstantial evidence that CDX2 is involved in BM. CDX2 expression has been demonstrated in IM including: oesophagus (Eda, Osawa et al. 2003), stomach (Bai, Yamamoto et al. 2002), gallbladder (Kozuka and Hachisuka 1984), biliary tree (Kozuka, Kurashina et al. 1984), urinary tract (Osawa, Kita et al. 2004), bladder (Steininger, Mueller et al. 2005), liver (Tatematsu, Kaku et al. 1985) and pancreas (Albores-Saavedra, Simpson et al. 2007). Intriguingly, chronic inflammation has been causally linked to IM in all of these locations, hinting at a common mechanism of action. The finding of CDX2 in normal squamous epithelium proximal to BM in one third of patients (Moons, Bax et al. 2004), suggests that its expression precedes the switch in phenotype.

Cdx2 is sufficient to provoke neo-intestine in the stomach of transgenic mice (Silberg, Sullivan et al. 2002) (Mutoh, Hakamata et al. 2002) that is functional in its absorptive capacity as it can rescue these transgenic mice from extensive intestinal resection (Mutoh, Satoh et al. 2005). These observations suggest that Cdx2 alone is sufficient to specify fully functional absorptive enterocytes. Although in transgenic mice Cdx2 expression alone provokes IM of the stomach, a few studies suggest that this may not be the case in the oesophagus. Overexpression of the cell-cycle regulator cyclin D1 and exposure to demethylating agents are required in addition to ectopic CDX2 expression in order to provoke the expression
of intestinal genes in immortalised oesophageal cells (Kong, Nakagawa et al. 2008). Likewise a recent transgenic mouse in which oesophageal Cdx2 is driven from the K14, squamous epithelial specific promoter, lacked any expression of intestinal genes (Jianping Kong* 2009). Altogether, these data suggest that CDX2 may have a key early role in the development of IM. Therefore, understanding how CDX2 expression is controlled would offer insights into the molecular mechanisms of the disease.

4.1.3 Role of Retinoic acid in Barrett’s Metaplasia

Vitamin A (retinol) and its active metabolites (retinoids) exert pleiotropic roles during embryogenesis, tissue homeostasis, cell proliferation, differentiation and apoptosis (Mark, Ghyselinck et al. 2009) [and references therein]. Retinoids are potent morphogens during development with roles in organogenesis and anterior-posterior specification. Vitamin A is oxidised by alcohol and retinol dehydrogenases to retinaldehyde which is further oxidised to the metabolically active RA by three types of retinaldehyde dehydrogenases (Duester 2008). RA exists in 3 isoforms, all trans-retinoic acid (ATRA), 9-cis-retinoic acid and 13-cis-retinoic acid of which ATRA is the most important during embryogenesis. RA is a ligand for two families of nuclear receptors, the RA receptors (RARα, RARα and RARγ) and the retinoic X receptors (RXRα, RXRα and RXRγ) (Chawla, Repa et al. 2001). RA complexes with heterodimeric receptors and initiates transcription of target genes by binding to cis-regulatory RA response elements (RAREs).

Vitamin A deficiency during embryogenesis is associated with a wide spectrum of congenital malformations and embryonic defects including ocular, central nervous system, pancreas, lung, muscle and limb defects (fully reviewed in (Duester 2008)). The morphogenetic effects that are most relevant to BM relate to defects in endoderm-derived tissue and anterior-posterior patterning. RA is required for the normal development of pancreas from endoderm in zebrafish, frog and mice (Stafford and Prince 2002; Chen, Pan et al. 2004; Martin, Gallego-Llamas et al. 2005). In the mouse embryo, RA is first detectable, using RAREs-lacZ reporter
transgenics, at E7.5 (Rossant, Zirmibl et al. 1991). Mice lacking the enzyme responsible for the conversion of retinal to RA in the pancreas, retinaldehyde dehydrogenase 2 (Raldh2), demonstrate agenesis of the dorsal pancreas. Furthermore the pancreatic transcription factor, Pdx1, is not expressed in Rald2 knockout mice (Martin, Gallego-Llamas et al. 2005) suggesting that Pdx1 may be downstream of RA signalling.

In chick embryos, RA has been shown to play an essential role in developing the anterior-posterior (A-P) axis of the gut endoderm. Development of anterior endodermal organs, such as the thyroid, is inhibited in the presence of RA. RA signalling is necessary for the expression of CdxA, the chick homologue of Cdx1, and development of the pancreas (Bayha, Jorgensen et al. 2009). The disturbance of A-P specification is further demonstrated by the finding of an increased anterior foregut and decreased posterior foregut in retinoid-deficient quail embryos (Quinlan, Gale et al. 2002). These findings were confirmed in zebrafish and, conversely, excess RA results in a posterior shift, so that pancreas and liver are seen in the anterior foregut (Stafford and Prince 2002). There is evidence that the A-P patterning effects of RA arise through interaction with Hox genes. RA induces the expression of Hox genes in embryocarcinoma cells in a temporal and hierarchical fashion similar to that seen during embryogenesis (Simeone, Acampora et al. 1990). Furthermore, exogenous RA provokes anteriorisation of Hox gene expression if administered to E7.5 mice (Kessel and Gruss 1991).

Murine Cdx1, is a direct target of RA and disruption of the Cdx1 RARE results in an anterior homeotic shift similar to that seen in the Cdx1−/− mouse (Houle, Sylvestre et al. 2003). Similarly RA signalling, via Hoxa5, is essential for the normal branching of the lung formed from posterior endoderm (Duester 2008).

During postnatal life, vitamin A deficiency can result in a plethora of manifestations, of which squamous metaplasia is the most interesting in the context of BM. Vitamin A deficiency provokes squamous metaplasia in many epithelium including salivary glands, tongue and pharynx, the respiratory tract (trachea and bronchi), the
cornea, conjunctivas, the renal pelvis, ureters and bladder (Leitner 1951). Even the secretory glands of the epidermis can be involved (Leitner 1951). RA is important for correct epidermal cell differentiation (Fuchs and Raghavan 2002) and has been proposed as a candidate for a factor contributing to the induction of BM (Chang, Lao-Sirieix et al. 2007).

There is considerable in vitro data to suggest that RA has a role in columnar metaplasia. The RA antagonist, citral, provokes a squamous metaplasia in cultures of chick trachea (Aydelotte 1963), replicating the phenotype of vitamin A deficient animals (Aydelotte 1963). In contrast, addition of RA to embryonic chick oesophageal cultures results in failure to form a stratified squamous epithelium, a pseudostratified columnar ciliated mucosa and instead, there is an increase in mucous secreting cells (Aydelotte 1963). Similar findings were demonstrated in two other squamous epithelial cell types, the epidermis and cornea (Aydelotte 1963). Cultures of embryonic rat oesophageal epithelium respond to RA by inhibiting keratinisation and developing a mucous secreting columnar epithelium (Lasnitzki 1963).

RA exerts similar effects in human oesophageal cells. Following treatment with all-trans retinoic acid (ATRA), a primary explant culture of human squamous oesophagus undergoes conversion to columnar epithelium, similar to that seen in BM (Chang, Lao-Sirieix et al. 2007). The columnar epithelium expresses K18 and K7, not normally seen in oesophagus, but present in BM. The bile acid lithocholic acid is a competitive agonist with retinoic acid at retinoid X receptors (RXR), suggesting a potential inductive mechanism for bile salts (Radominska-Pandya and Chen 2002). Likewise, retinoic acid biosynthesis is increased in Barrett’s epithelium (Chang, Lao-Sirieix et al. 2007). It is not known how RA produces a columnar metaplasia and its interaction with the master switch gene for squamous epithelium, p63, in oesophageal cells has not been studied. RA inhibits p63 expression in immortalised nasopharyngeal epithelial cells (Yip and Tsao 2008), but not in primary keratinocytes (Bamberger, Pollet et al. 2002). Furthermore, RA
has been shown to induce expression of the intestinal gene MUC2 in primary cultures of human oesophageal cells (Cooke, Blanco-Fernandez et al. 2008). Although MUC2 is a direct target of CDX2, it has not been directly linked to the effects of RA on oesophageal cells and this may suggest that an alternative pathway exists for MUC2 regulation. In lung epithelium, where CDX2 is not normally expressed, RA is essential for the regulation of MUC2 (and MUC5AC). Normal human tracheobronchial cells cultured in the absence of RA undergo squamous differentiation (Kim, Hong et al. 2007). MUC2 and MUC5AC expression can be re-established with the addition of RA to the culture. The necessity for RA in normal columnar differentiation and its ability to inhibit squamous differentiation suggests a potential role in BM.

4.1.4 BMP4 and oesophageal development
The trachea and oesophagus develop from a common foregut endodermal tube at 4-6 weeks gestation in the human and around embryonic day E9.5 in the mouse (Cardoso and Lu 2006). The ventral portion of the anterior foregut endoderm forms the trachea whilst the dorsal part develops into the oesophagus. Defects in the development of the tracheobronchial septum (which separates both lumen), results in congenital malformations such as tracheoesophageal fistula (TOF), oesophageal atresia or tracheal atresia.

The Bmp signalling pathway is implicated in the development of these congenital malformations (Que, Choi et al. 2006; Li, Litingtung et al. 2007). Phosphorylated Smad 1/5/8 (P-Smad), indicative of active Bmp signalling, is seen in ventral foregut endoderm and mesoderm at the stage of oesophageal tracheal separation (Li, Gordon et al. 2008). Bmp4 expression is closely associated with the ventral foregut mesenchyme surrounding the primitive trachea (Weaver, Yingling et al. 1999). Bmp4 knockout is embryonic lethal, but conditional deletion of Bmp4 in the ventral foregut endoderm and mesenchyme using a Foxg1 cre transgenic mouse, causes failure of oesophageal and tracheal separation (Li, Gordon et al. 2008). The mice developed a single endodermal tube which expressed the oesophageal marker
pax9, but neither of the tracheal markers Nkk2.1 nor collagen type Ila were expressed. Bmp4 is antagonised by the secreted protein Noggin, encoded by Nog. The development of TOF has been linked in humans with deletions of the chromosomal region containing the Nog gene (Marsh, Wellesley et al. 2000). Furthermore, Nog null mice develop TOF and oesophageal atresia in which endoderm and mesenchyme of the fistula and distal oesophagus maintains a tracheal phenotype (Que, Choi et al. 2006). Noggin is normally expressed in the dorsal foregut region that develops into the oesophagus. Taken together, the data suggest that Bmp4 is important for tracheal and lung morphogenesis and Noggin antagonises Bmp4 to specify the oesophageal region.

4.1.5 BMP signalling in the Intestine

The intestinal epithelium consists of 4 cell type (goblet cells, paneth cells, enterocytes and enteroendocrine cells). These cells differentiate from a common intestinal stem cell. The stem cells are located near the base of the crypt and, through a hierarchical set of decisions, differentiate in the upper third of the crypt (Bjerknes and Cheng 2005). The microenvironment surrounding the crypt-villus dictates a precise and specific regulation of genes that control differentiation of the stem cells. Many signalling pathways are involved in this process including Bmps, platelet derived growth factor and Hedgehog (Karlsson, Lindahl et al. 2000; Haramis, Begthel et al. 2004; He, Zhang et al. 2004; Madison, Braunstein et al. 2005). Bmp signalling plays an important role in intestinal development by defining the crypt-villus axis and in the visceral mesoderm, Bmp directs the mesenchymal condensations that form just before villus outgrowth (Batts, Polk et al. 2006).

The main Bmps found in the intestine are Bmp2 and 4 (Roberts 2000). Bmp4 is expressed in the intravillus and intracrypt mesenchymal cells. Bmpr1a is located in a gradient along the crypt-villus axis with highest expression towards the tip and also in the stem cells, but not in the intervening proliferating zone (He, Zhang et al. 2004). Noggin is found in the submucosal region adjacent to the bottom of the crypt, but not along the villus.
Conditional knockout of Bmpr1a in the intestine (epithelium and mesenchyme) interferes with the homeostasis of intestinal epithelial renewal provoking expansion of the stem cell and progenitor cell populations (He, Zhang et al. 2004). Bmp4, though activation of PTEN, controls stem cell homeostasis by decreasing stem cell mitosis. The loss of Bmp4 signalling decreases PTEN signalling and results in nuclear β-catenin and stem cell division. Transient Noggin expression abrogates Bmp4 signalling and allows stem cell division. The resultant conditional Bmpr1a knock out mice resembled the phenotype seen in human juvenile polyposis syndrome, which is linked to mutations in Bmp1a and Smad4 (Howe, Roth et al. 1998; Howe, Bair et al. 2001).

Epithelial specific knockout of Bmpr1a demonstrates a different phenotype to epithelial and mesenchymal knockout mice. These mice developed hyper proliferation within the crypt and elongated villi, but did not develop polyps nor new crypts (Auclair, Benoit et al. 2007). Interestingly, the secretory cell lineages failed to mature properly; the epithelium was deficient in enteroendocrine cells and contained immature and malformed Paneth and goblet cells.

4.1.6 BMP4 and Barrett’s Metaplasia

In BM samples, Bmp4 was found to be increased 19-fold compared with normal squamous tissue, in a serial analysis of gene expression (SAGE) (van Baal, Milano et al. 2005). Bmp4 protein was found to be highly expressed in BM and oesophagitis, but absent in normal squamous mucosa of biopsy specimens (Milano, van Baal et al. 2007). Furthermore, BM and oesophagitis sections demonstrated activation of inhibitor of differentiation 2 (ID2), a direct target of Bmp4 and P-Smad 1/5/8. Treatment of a primary culture of human oesophageal squamous cells with Bmp4 provokes the expression of the intestinal cytokeratins K7/K20 and decreased squamous markers K10/K13 (Milano, van Baal et al. 2007). These results seem to contradict the effects of Bmp4 during epidermal development; Bmp4 directs the neuroectodermal precursor to an epidermal fate in
contrast to oesophageal cells which move away from squamous differentiation. This is in keeping with the varying roles inducing factors have at different stages of development. The epidermal lineage requires ΔNp63 to differentiate from ectodermal to squamous epidermis and it is possible that treatment of oesophageal keratinocytes, having lost p63 expression, with Bmp4, reprograms them. In fact, when epidermal keratinocytes are cultured with Bmp4, ES cell-related genes are activated and the cells can be reprogrammed to a neural cell lineage (Grinnell, Yang et al. 2007). Epidermal stem and transit amplifying cells, treated with Bmp4, begin to express Oct-4, Sox-2, Nanog and rex-1 (Grinnell, Yang et al. 2007).

Bmp4 has been linked to Cdx genes in studies on haematopoiesis; Bmp4, via the Wnt signalling pathway, up regulates Cdx1 and Cdx4 expression to direct mesoderm towards a blood cell phenotype (Lengerke, Schmitt et al. 2008). A link between Cdx2 and Bmp4 was recently demonstrated in gastric intestinal metaplasia and gastric carcinoma cell lines (Barros, Pereira et al. 2008). H. pylori gastritis results in increased Bmp2 and 4 signalling in the stomach (Bleuming, Kodach et al. 2006). The expression patterns of Bmp2/4, P-Smad 1/5/8 Smad4 and Cdx2 correlated with areas of gastric intestinal mucosa compared with normal mucosa (Barros, Pereira et al. 2008). Exogenous Bmp4 up-regulates Cdx2 expression in a gastric cancer cell line, AGS, possibly via direct regulation of the Cdx2 promoter by Smad4.

4.1.7 A link between Cdx2 and the innate immune response
Recent publications provide evidence for a link between the innate immune system and Cdx2 regulation. In cholangiocytes, PAMPS (pathogen associated molecular patterns), such as LPS, induce Cdx2 and Muc2 (Zen, Harada et al. 2002; Ikeda, Sasaki et al. 2007; Ikeda, Sasaki et al. 2008). PAMPs are conserved pathogen-associated molecular motifs that are recognised by pattern recognition receptors (PRRs) such as TLRs (toll-like receptors) and activate the innate immune system. Murine biliary epithelial cells (BECs) upregulate muc2 and muc5AC expression in response to the Gram-negative bacterial component lipopolysaccharide (LPS)
(Zen, Harada et al. 2002). In BECs, TNFα is elevated in response to LPS and provoke Muc2 and Muc5AC transcription. Muc2 and Muc5AC expression is increased in response to either LPS or TNFα in bronchial epithelium, nasal and middle ear cells (Levine, Larivee et al. 1995; Li, Dohrmann et al. 1997; Lin, Haruta et al. 2000). The effects of LPS on lung epithelium are mediated through NFkB, which, as discussed previously is key to Cdx2 regulation and is increased in BM (Li, Feng et al. 1998).

4.1.8 How do acid and bile influence Cdx genes?

BM is more common in the context of GORD and in animal models results from the reflux of jejunal contents, such as acid and bile, into the oesophagus (Bremner, Lynch et al. 1970; Kazumori, Ishihara et al. 2006; Kazumori, Ishihara et al. 2009). Squamous oesophageal mucosa provides a protective barrier between the luminal contents and the basal oesophageal cells. However, in the context of reflux, this barrier increases in permeability allowing bile salts and other toxins access to the stem cell niche (Tobey, Hosseini et al. 2004). Acid and bile have both been demonstrated, in vitro, to induce expression of Cdx genes. Long term exposure of mouse oesophageal cells to acid induces Cdx2, interestingly this was shown to occur in differentiated cells (Marchetti, Caliot et al. 2003). A number of different bile acids at neutral and acidic pH have been shown to induce Cdx2 expression. Kazumori and colleagues treated Caco-2, HT-29 and primary rat oesophageal cells with 11 types of bile acid and found that cholic and dehydrocholic acid increased CDX2 promoter activity (Kazumori, Ishihara et al. 2006). Mutation analysis of the CDX2 promoter suggested that NFκB binding sites were responsible for the effects of these bile acids. The study further demonstrated that transfection of a CDX2 expression vector into rat oesophageal cells induced MUC2 (Kazumori, Ishihara et al. 2006). The same group demonstrated similar effects on the CDX1 promoter in OE33, HET-1A cell lines and primary rat oesophageal cells, when stimulated with a mixture of cholic, glycocholic and taurocholic acid (Kazumori, Ishihara et al. 2009). Induction of CDX2 occurs, through NFκB binding sites in the CDX2 promoter, in
OE-19 cells treated with deoxycholate (Debruyne, Witek et al. 2006). Deoxycholate induced nuclear translocation and CDX2 promoter binding of the NFκBp50 subunit.

The bile acids ursodeoxycholic acid and deoxycholic acid up-regulate CDX2 in a BM adenocarcinoma cell line, OE33 (Burnat, Rau et al. 2007). Deoxycholic acid and chenodeoxycholic acid, but not glycocholic acid induce CDX2 mRNA in HET-1A, SEG-1 and two squamous cell carcinoma cell lines (HKESC-1 and 2) (Hu, Williams et al. 2007). In a continuation of this study, deoxycholic acid at neutral pH induced both CDX2 and MUC2 mRNA after 1 hour of treatment (Hu, Jones et al. 2007). Along with activation of inflammatory mediators, such as NFκB, there is evidence that acid and bile demethylate the CDX2 promoter in HET-1A and SEG-1 cell lines and result in CDX2 induction (Liu, Zhang et al. 2007). An additional mechanism for CDX2 upregulation, activation of the epidermal growth factor receptor (EGFR), in the context of bile acid, has been postulated. Deoxycholic acid, at acidic and neutral pH, activate the EGFR in SEG-1 cells. CDX2 induction in response to deoxycholic acid can be inhibited using antibodies to the EGFR (Avissar, Toia et al. 2009).

Bile acids inhibit nucleostemin, a nucleolar p53 binding protein which regulates the cell cycle in stem cells and cancer cells, in the HT-29 cell line (Sun, Wang et al. 2009). Furthermore, knock down of nucleostemin induces CDX2 expression. Recently, bile salt exposure has been linked to BMP4 induction. Human oesophageal epithelial cells exposed to acid or a mixture of bile salts start to express BMP4 and villin. Interestingly, BMP4 induced CDX2 within these same cells (Zhou, Sun et al. 2009).

**Methods**

**4.2 Retinoic acid treatment of oesophagus.**

To assess the effects of retinoic acid on oesophageal explants, experiments were performed in both BME and MCDB 153 media.
In BME, oesophageal explants were allowed to attach to the plastic substratum for 24hrs and were then treated with vehicle only (DMSO) control or with 10μM or 100μM ATRA. The media was changed daily and cultures were kept in the dark to prevent light mediated degradation of ATRA. After a further 9 days, explants were either fixed for immunostaining or processed for RT-PCR.

To study the effect of ATRA on a homogeneous population of undifferentiated oesophageal cells, explants were cultured in MCDB153 media. The addition of ATRA to the media from day 1 resulted in detachment of the explants, consequently ATRA was added from day 5 of culture for 5 days. Explants cultured in MCDB 153 were more sensitive to ATRA and cells died or detached, hence lower concentrations of 0.1μM or 1μM ATRA were used.

In addition to the BME and MCDB 153 media, an additional medium, Quantum 153, was used during these experiments. Quantum 153 has a calcium concentration of 0.06μM and the addition of EGF to an undefined media manufactured for epidermal keratinocytes.

4.2.1 Retinoic acid induces Cdx2 mRNA and a columnar phenotype along with loss of the squamous phenotype in oesophageal explants.

Oesophageal explants cultured for 9 days with ATRA were smaller and demonstrated a branching pattern to the edge of the epithelium compared to control cultures (Fig 4.1). On occasions islands of epithelium were found that were not in continuation with the original explant (results not shown). Treatment with 10μM or 100μM ATRA (but not DMSO or untreated samples) induced Cdx2 mRNA expression (Fig 4.2). However, the expression of Cdx2 protein is not detectable with immunofluorescence. The edge of the epithelium surrounding an outgrowth normally expresses the columnar marker K8 in a sub-population of cells. K8 expression is increased with 10μM and 100μM ATRA compared with control (Fig 4.3).

More cells within the outgrowth express p63 when explants are cultured in Quantum 153 (Fig 4.4) and so this media was used to assess the effect of ATRA on the expression of the squamous markers K14 and p63. The number of cells...
within the outgrowth of explants that express p63 is reduced in a dose dependent manner with ATRA treatment (Fig 4.5). Under control culture conditions, p63 expression extended into the outgrowth, but following treatment with 10μM ATRA, very few cells were positive for p63 (Fig 4.5). In the cultures treated with 100μM ATRA no cells expressed p63.

Similar results were achieved when explants were cultured in MCDB 153 media (Fig 4.6). In contrast to media with higher calcium concentrations (BME and Quantum 153), all oesophageal cells cultured in MCDB 153 express nuclear p63. Treatment with 0.1μM ATRA resulted in elongation of some of the cells (Fig 4.6), an increase in K8 expression compared to control (Fig 4.7), but no decrease in the number of cells expressing p63 (Fig 4.6). ATRA (1μM) resulted in a decrease in cell density, loss of p63 expression (Fig 4.6), and an increase in K8 positive cells compared to control (Fig 4.7).

Table 4.1 Effect of ATRA on number of K8 positive and p63 negative oesophageal cells in MCDB153 media.

<table>
<thead>
<tr>
<th>ATRA concentration</th>
<th>Number of K8 positive cells</th>
<th>Number of p63 negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DMSO</td>
<td>0.33 +/- 0.58</td>
<td>0.00 +/- 0.00</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>7.67 +/- 1.53 (p=0.046) *</td>
<td>0.33 +/- 0.58 (p=0.31)</td>
</tr>
<tr>
<td>1 μM</td>
<td>7.00 +/- 1.73 (p=0.043) *</td>
<td>2.67 +/- 0.58 (p=0.034) *</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0. ATRA treatments were compared with control.
Figure 4.1 Effects of ATRA on Oesophageal Explants.

Oesophageal explants were cultured in BME in the presence of DMSO (0.1%) and in the absence and presence of ATRA (10μM or 100μM). Phase contrast images are shown. Oesophageal explants were allowed to attach for 24hrs and then treated with either vehicle alone (DMSO) control (A+D) or ATRA at a concentration of either 10μM (B+E) or 100μM (C+F) for a further 9 days. (A-C arrow marks explant) The size of typical explant cultures treated with ATRA is smaller than control cultures (A). (D) Explants treated with DMSO demonstrated a defined convex edge (arrow). In contrast, ATRA-treated cultures developed a branching pattern to the edge of the epithelium (arrow in E). On occasions, islands of epithelium not in continuation with the explant are seen in ATRA explants (arrow in F). Scale bars represent 200μm (A-C) and 100μm (D-F).
**Figure 4.2 ATRA induces Cdx2 mRNA but not protein.**

Immunofluorescence images and RT-PCR for Cdx2 in oesophageal explants cultured in BME in the absence or presence of ATRA. Oesophageal explants were allowed to attach for one day and then treated with either vehicle only control (DMSO) (A+D) or ATRA at a concentration of 10μM (B+E) or 100μM (C+F) for a further 9 days. (A-C) After 10 days in culture following DMSO (A), ATRA 10μM (B) or ATRA 100μM (C) there is no expression of Cdx2 detectable by immunohistochemistry. (D-F) Nuclear staining with DAPI to show distribution of cells within DMSO (D), ATRA 10μM (E) or ATRA 100μM (F) treatments. (G) Oesophageal cells expressing Cdx2 following adenoviral infection as a positive control. (H) Following 35 cycles of RT-PCR Cdx2 mRNA is detectable in oesophageal explants cultured with ATRA but not control cultures.

Scale bars represent 100μM (A-G).
**Figure 4.3 ATRA induces the columnar marker K8 in oesophageal cells.**

Immunofluorescent images of K8 expression in oesophageal explants cultured in BME with ATRA. Oesophageal explants were allowed to attach for one day and then treated with either vehicle only control (DMSO) (A+D) or ATRA at a concentration of 10μM (B+E) or 100μM (C+F) for a further 9 days. (A) As with explant cultures grown in BME, only the cells at the periphery of an explant culture treated with DMSO express K8. The arrow marks the original explant in (A-C). (B-C) There is a marked increase in K8-positive cells within explants treated with ATRA. K8 expression was observed closer to the explant. (D-E) Higher magnification images of (A-C) demonstrated the increase in number and intensity of staining.

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.4 Oesophageal cells grown in Quantum 153 medium demonstrate increased p63 expression.

Immunohistochemistry images for K14 (red) and p63 (green) in oesophageal explants grown in BME (A-C) or Quantum 153 (D-F) media. (A-C) The majority of p63 positive cells are within or immediately adjacent to the original explant in BME media. (A) Overlay image of (B) K14 and (C) p63 expression after one week of culture in BME. (D-F) p63 positive cells are more abundant and extended further into the outgrowth after culture for 7 days in Quantum 153 media. (D) Overlay image of (E) K14 and (F) p63 expression after one week of culture in Quantum 153. The arrows mark the original explant. Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.5 Oesophageal explants cultured in ATRA show decreased p63 expression.

Oesophageal explants were allowed to attach for 24 hrs and then treated with either vehicle only (DMSO) (A+D) or ATRA at a concentration of 10μM (B+E) or 100μM (C+F) for 9 days. K14 (red) / p63 (green) expression was assessed with immunofluorescence. (A+D) p63 positive cells (green) were seen within the explant and outgrowth. The arrow marks the original explant in (A-C). (B+E) There is a marked decrease in p63 positive cells in the outgrowths of cells treated with 10μM ATRA. Positive cells were present within the explant. (C+F) There were very few p63 positive cells following treatment with 100μM ATRA.

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.6 ATRA decreases p63 expression in explants cultured in MCDB 153.

After 5 days of culture in MCBD 153, explants were treated with DMSO (A+D), 0.1μM ATRA (B+E) or 1μM ATRA (C+F) for a further 5 days. (A-C) K14 (red) and p63 (green) expression in treated cultures reveals an increase in intercellular spacing (B) and reduced cell numbers (C). All cells expressed p63 in the DMSO treated controls. The explant is marked with an arrow (A-C). (D-F) Higher magnification shows individual cell morphology and demonstrates the regular cuboidal shape in control cultures (A), the presence of long thin cells in 0.1μM ATRA treatment (arrow in E) and disorganised cells showing a lack of p63 expression (arrows) in (F). The results are representative images from 3 separate experiments. Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.7 ATRA induces K8 expression in explants cultured in MCDB 153.

After 5 days of culture in MCBD 153, explants were treated with DMSO (A+D), 0.1μM (B+E) or 1μM (C+F) ATRA for a further 5 days. (A-C) K8 (green) expression in explants treated with DMSO (A), 0.1μM ATRA (B) or 1μM ATRA (C) is increased following 0.1μM ATRA treatment (B). (D-F) Higher magnification images show individual cell morphology. (D) DMSO control cultures express a faint perinuclear rim of K8, in contrast to the cytoskeletal cytoplasmic expression in ATRA treatments (E+F). The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
4.3 Treatment of oesophageal explants with lipopolysaccharide (LPS).

The effect of *Escherichia coli* 0157 LPS was assessed on oesophageal explants cultured in three different media: BME, Quantum 153 and MCDB 153. Following attachment, LPS (10μg/ml or 100μg/ml) was added to BME or Quantum 153 every other day for 9 days and then explants were processed for immunohistochemistry or RT-PCR. Explants cultured in MCDB 153 were grown for 5 days and then LPS (1μg/ml or 10μg/ml) was added for an additional 5 days.

4.3.1 LPS induces a columnar phenotype and Cdx2 in oesophageal explants cultured in BME.

Oesophageal explants cultured in BME with a concentration of 10μg/ml or 100μg/ml LPS demonstrated a less well defined (irregular) edge to the epithelial outgrowth and an incomplete epithelial monolayer (Fig 4.8). There is induction of Cdx2 mRNA with both LPS concentrations but no protein was detectable by immunostaining (Fig 4.9).

The area of K8 expression that is normally restricted to the edge of the epithelium is expanded with LPS in comparison to vehicle only controls (Fig 4.10). Treatment of oesophageal explants cultured in Quantum 153 medium with 10μg/ml LPS decreased the expression of p63 within the outgrowth (Fig 4.11). Following LPS treatment, p63 expression is more limited to the explant and is found in only a few cells within the outgrowth. Explants cultured in Quantum153 exposed to 100μg/ml LPS demonstrated no expression of p63 in the outgrowth and very few positive cells within the explant (Fig 4.11). The morphology of the epithelium, which is more ‘branched’, and the gaps within the epithelium, is clearly demonstrated with K14 immunohistochemistry (Fig 4.11).

Explants cultured in MCDB 153 medium for 5 days and with LPS (10μg/ml) for a further 5 days, demonstrated an increase in K8 expression (Fig 4.12) (Table 4.2). A decrease in cell density and loss of p63 in some cells was visible with the highest LPS concentration (10μg/ml) (Fig 4.13) (Table 4.2).
Table 4.2 Effect of LPS on number of K8 positive and p63 negative oesophageal cells in MCDB153 media.

<table>
<thead>
<tr>
<th>LPS concentration</th>
<th>Number of K8 positive cells</th>
<th>Number of p63 negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 +/- 0.00</td>
<td>0.00 +/- 0.00</td>
</tr>
<tr>
<td>1µg/ml</td>
<td>0.33 +/- 0.58 (p=0.32)</td>
<td>0.00 +/- 0.00 (p=1.00)</td>
</tr>
<tr>
<td>10µg/ml</td>
<td>3.00 +/- 1.00 (p=0.037)*</td>
<td>2.67 +/- 0.58 (p=0.034)*</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0. LPS treatments were compared with control.
**Figure 4.8 The effect of lipopolysaccharide (LPS) on oesophageal explant culture.**

Phase contrast images of oesophageal explants cultured in BME with LPS. Oesophageal explants were allowed to attach for 24hr and then treated with either vehicle only control (water) (A+D) or LPS at a concentration of 10μg/ml (B+E) or 100μg/ml (C+F) for 9 further days. (A-C arrow marks explant) The edge of the epithelium in treated cultures (B+C) is irregular and the outgrowth lacked epithelium in places. (D) Explants treated with LPS demonstrated a defined convex edge. In contrast, LPS-treated cultures develop a branching pattern to the edge of the epithelium (arrow in E). Gaps within the epithelium are seen in LPS- treated culture (arrow in F).

Scale bars represent 200μm (A-C) and 100μm (D-F).
**Figure 4.9 Lipopolysaccharide (LPS) induces Cdx2 mRNA but not protein.**

Immunofluorescent images and RT-PCR of oesophageal explants cultured in BME and treated with LPS are shown. Oesophageal explants were allowed to attach for 24hr and then treated with either vehicle only control (water) (A+D) or LPS at a concentration of 10μg/ml (B+E) or 100μg/ml (C+F) for 9 further days. (A-C) After 10 days in culture following control (A), LPS 10μg/ml (B) or LPS 100μg/ml (C) there is no expression of Cdx2 detectable by immunohistochemistry. (D-F) Nuclear staining with DAPI shows the distribution of cells within control (D), LPS 10g/ml (E) or LPS 100μg/ml (F) treatments. (G) Oesophageal cells expressing Cdx2 following adenoviral infection as a positive control. (H) Following 35 cycles of RT-PCR, Cdx2 mRNA is detectable in oesophageal explants treated with 10μg/ml and 100μg/ml but not control. Scale bars represent 100μm (A-G).
**Figure 4.10** Lipopolysaccharide induces the columnar marker K8 in oesophageal cells.

Oesophageal explants in BME were allowed to attach for 24hr and then treated with either vehicle only control (water) (A+D) or LPS at a concentration of 10μg/ml (B+E) or 100μg/ml (C+F) for 9 further days. (A) As with explant cultures grown in BME, only the cells at the periphery of the control explant expressed K8. The arrow marks the original explant in (A-C). (B-C) There is a marked increase in K8 positive cells within explants treated with LPS. K8 expression was seen closer to the explant. (D-E) Higher power images of (A-C) demonstrated the increase in number and intensity of staining for K8 following LPS 10μg/ml and 100μg/ml treatment compared with control. The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.11 Oesophageal explants cultured with lipopolysaccharide demonstrate less p63 positive cells.

Oesophageal explants cultured in Quantum153 were allowed to attach for one day and then treated with either vehicle only control (water) (A+D) or LPS at a concentration of 10μg/ml (B+E) or 100μg/ml (C+F) for 9 further days and K14 (red) / p63 (green) expression was assessed. (A+D) p63 positive cells (green) are seen both within the explant and outgrowth. The arrow marks the original explant in (A-C). (B+E) There is a marked decrease in p63 positive cells in the outgrowths of cells treated with 10μg/ml LPS, only a small rim surrounding the explant is visible. Positive cells were present within the explant. (C+F) p63 positive cells remain visible within the explant but not within the outgrowth of cultures treated with 100μg/ml LPS. The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
**Figure 4.12 Lipopolysaccharide (LPS) induces K8 expression in explants cultured in MCDB 153.**

After 5 days of culture in MCBD 153, explants were treated with control (A+D), 1μg/ml LPS (B+E) or 10μg/ml (C+F) LPS for a further 5 days. (A-C) K8 (green) expression in explants treated with control (A), 1μg/ml LPS (B) or 10μg/ml LPS (C). More positive K8 positive cells are seen in the 10μg/ml LPS treatment. (D-F) Higher magnification shows individual cell morphology of K8 expression in LPS treatments. The results are representative images from 3 separate experiments. Scale bars represent 200μm (A-C) and 100μm (D-F)
Figure 4.13 Lipopolysaccharide (LPS) reduced cell number and p63 expression in explants cultured in MCDB 153.

After 5 days of culture in MCBD 153, explants were treated with control (A+D), 1μg/ml LPS (B+E) or 10μg/ml LPS(C+F) for a further 5 days. (A-C) K14 (red) and p63 (green) expression in treated cultures reveals an increase in intercellular spacing (B) and reduced numbers of cells (C). All cells in the DMSO control (A+D) and LPS 1μg/ml (B+E) expressed p63, whereas p63 negative cells were seen in the LPS 10μg/ml treatment group. (D-F) Higher magnification shows individual cell morphology and demonstrates the regular cuboidal shape in all treatments and the expression of p63 (green) in all cells (D+E). Cells that have lost p63 expression are found following 10μg/ml LPS (arrows in F). The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
4.3.2 Effects of LPS and ATRA on CDX2 expression in HET-1A cells

In order to assess the effects of LPS and ATRA on the expression of CDX2 in HET-1A cells, three different concentrations of either compound were tested at two different time points. HET-1A cells were allowed to grow to 50% confluence before treatments so that dividing cells were exposed to the appropriate stimulus. LPS (1μg/ml, 10μg/ml or 100μg/ml) and ATRA (1μM, 10μM or 100μM) were added at the concentrations shown and mRNA extracted following 5 or 10 days of treatment. CDX2 expression was examined by RT-PCR. LPS or ATRA treatment did not induce CDX2 expression in HET-1A cells (Fig 4.14).

Figure 4.14

![Image](image.png)

**Figure 4.14** Neither Lipopolysaccharide (LPS) nor all trans-retinoic acid (ATRA) induces CDX2 mRNA in HET-1A.

mRNA expression of CDX2 was assessed with 35 cycles of RT-PCR. HET-1A cells were treated at 70% confluence with LPS (1μg/ml, 10μg/ml or 100μg/ml) for either 5 or 10 days. Following treatment, CDX2 mRNA was not detected. HET-1A cells were treated at 70% confluence with ATRA (1μM, 10μM or 100μM) for either 5 or 10 days. Following treatment CDX2 mRNA was not detected. Caco-2 mRNA was used for a positive control.
4.4 Treatment of HT-29 and HET-1A cell lines with Bile acid.

Bile salts are candidates for the induction of CDX2 in BM and would be useful agents to provoke an intestinal phenotypic change in oesophageal cells. The effect of the unconjugated bile salt deoxycholic acid (DCA) at neutral pH on two cell lines, HT-29 and HET-1A was determined. Cells at 70% confluence were treated for 24 hours with 3 concentrations of DCA, 111μM, 333μM or 1mM, or vehicle only control, for 24 hours. CDX2 and MUC2 expression were then examined using RT-PCR. These concentrations were chosen because they have been used to successfully induce CDX2 in HET-1A cell lines previously (Hu, Williams et al. 2007). HET-1A cells were more sensitive to DCA and cells did not survive the highest concentration (1mM) treatment for 24 hours. CDX2 and MUC2 were induced following 24 hours of 111μM DCA in HET-1A cells (Fig 4.15). HT-29 cells treated with 333μM or 1mM DCA expressed CDX2 and MUC2, which were not seen with 111μM DCA or ethanol only control (Fig 4.15 A).

4.4.1 Bile and acid do not induce Cdx2 or Muc2 in oesophageal explants.

The effect of DCA on oesophageal explants was assessed at the 3 concentrations that induced CDX2 and MUC2 in HT-29 and HET-1A cells (111μM, 333μM and 1mM). Treatment with 1mM for either 24 hours or three 10 minute pulses, resulted in the loss of outgrowths surrounding oesophageal explants and so the maximum tolerated concentration, 333μM, was used for further experiments. Oesophageal explants were grown for 7 days and then treated with various combinations of 333μM DCA and acidified media:

i) ethanol control
ii) three x 10 minute pulses of pH 4 media in 24 hours
iii) three x 10 minute pulses of 333μM DCA at pH 7.4 in 24 hours
iv) three x 10 minute pulses of 333μM DCA at pH4 in 24 hours
v) 333 μM DCA at pH7.4 for 24 hours

Neither Cdx2 nor Muc2 mRNA was detectable with RT-PCR following any treatment (Fig 4.15 B). An alternative bile acid, dehydrocholic acid (DHCA), was also used to attempt to provoke Cdx2 in mouse oesophageal explants, because it
has previously been demonstrated to do so in HT-29, Caco-2 and primary rat oesophageal keratinocytes (Kazumori, Ishihara et al. 2006). One week old oesophageal explants were cultured in the presence of 100μM, 400μM DHCA or vehicle only control for 24 or 48 hours. RT-PCR demonstrated no induction of Cdx2 or Muc2 under any of these conditions (Fig 4.15 C).

Having shown that various bile acid treatments did not induce Cdx2 mRNA during 48 hours, longer duration treatment was utilised. Oesophageal cultures were grown for 5 days and treated with deoxycholic acid 500μM, 400μM dehydrocholic acid or 500μM cholic acid at pH7.4 for a further 5 days. Cdx2 protein was not detectable using immunofluorescence (Fig 4.15 D)
Figure 4.15 The effect of bile acids on \textit{CDX2} and \textit{MUC2} in HET-1A and HT-29 cells.

All RT-PCR were performed for 30 cycles. (A) CDX2 and MUC2 mRNA is induced by deoxycholic acid at pH 7.4 in HT-29 cells and HET-1A cells following 24 hours of treatment with concentrations of 333μM or 1mM and 111μM respectively. Human small intestine mRNA was used as a positive control (B) Oesophageal explants were cultured for 7 days in BME media. These cultures were then treated as follows: (i) with three 10 minute pulses in 24 hours of media at pH4; (ii) with three 10 minute pulses in 24 hours of 333μM DCA at pH 7.4 in media; (iii) with three 10 minute pulses in 24 hours of 333μM DCA at pH4 in media or (iv) with 333μM DCA pH7.4 for 24 hours in media

Neither Cdx2 nor Muc2 mRNA were detected at 24 hours with RT-PCR.

(C) Oesophageal explants were cultured for 7 days in BME and then treated with DHCA at either 100μM or 400μM for 24 and 48 hours. Cdx2 or Muc2 mRNA was not induced.

(D-G) Oesophageal cultures were grown for 5 days and treated with deoxycholic acid 500μM (D), 400μM dehydrocholic acid (E) or 500μM cholic acid without the induction of Cdx2 protein. (G) Embryonic mouse intestine cultured for 5 days was used as a positive control.
4.5 BMP4 does not induce a columnar phenotype nor suppress K14 or p63 expression in oesophageal cells.

The effect of BMP4 on oesophageal cells was assessed using oesophageal explants cultured in MCDB 153 media. Quantum 153 media, which increases the proportion of p63-positive cells in culture and had been used in previous experiments, was discontinued prior to the BMP4 experiments. Oesophageal explants cultured in MCDB 153 were more sensitive to LPS and ATRA compared to culture in BME and this observation combined with the more homogeneous expression of K14 and p63 determined the use of low calcium containing media for BMP4 treatment. Oesophageal explants were cultured for 5 days and then with vehicle control, BMP4 (10ng/ml or 100ng/ml) was added to the media for a further 5 days. K14, p63 and K8 expression was determined by immunofluorescence detection. Although the density of cells cultured in BMP4 was decreased in comparison to controls there were no effects of K14, p63 (Fig 4.16) or K8 expression (Fig 4.17)

4.6 TNFα provokes a columnar phenotype and loss of p63.

Oesophageal explants cultured in MCDB 153 for 5 days were treated with control or TNFα (1ng/ml or 10ng/ml) for a further 5 days. 1ng/ml TNFα reduced the density of cells within the outgrowth, but did not affect p63, K14 or K8 expression (Fig 4.18). The higher concentration of 10ng/ml TNFα resulted in a loss of expression of p63 and an induction of K8 in cells within the outgrowth not seen in control cultures (Fig 4.18 and Fig 4.19)

4.7 IL-1β does not induce K8 nor decrease p63/K14 expression in oesophageal explants.

Five day old oesophageal explants in MCDB were cultured for a further 5 days with either vehicle, 1ng/ml IL-1β or 10ng/ml IL-1β in the media. IL-1β did not reduce p63 expression (Fig 4.20) nor induce significant K8 expression.
4.8 IL-1β, TNFα or BMP4 do not induce CDX2 or MUC2 in oesophageal cells

To determine the effect of IL-1β, TNFα or BMP4 on Cdx2 and Muc2 expression in oesophageal cells, treatment of 5 day old explant cultures were performed in BME and MCDB 153 media. Following 5 days of growth in either media, 100ng/ml BMP4, 10ng/ml TNFα or 10ng/ml IL-1β were added to the explants daily for 6 days. These concentrations were chosen because they had an effect on the morphology of cells in MCDB 153 and had demonstrated effects in other culture models. Cdx2 and Muc2 expression were tested with RT-PCR. Neither Cdx2 nor Muc2 induction was observed in oesophageal cells cultured in either media with the addition of BMP4, TNFα or IL-1β (Fig 4.22).

Table 4.3 Effect of BMP4, TNFα or IL-1β on number of K8 positive and p63 negative oesophageal cells in MCDB153 media.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of K8 positive cells</th>
<th>Number of p63 negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP 4 control</td>
<td>0.33 +/- 0.58</td>
<td>0.00 +/- 0.00</td>
</tr>
<tr>
<td>BMP4 10ng/ml</td>
<td>0.00 +/- 0.00 (p=0.317)</td>
<td>0.00 +/- 0.00 (p=1)</td>
</tr>
<tr>
<td>BMP4 100ng/ml</td>
<td>0.67 +/- 0.58 (p=0.456)</td>
<td>0.00 +/- 0.00 (p=1)</td>
</tr>
<tr>
<td>TNFα control</td>
<td>0.00 +/- 0.00</td>
<td>0.00 +/- 0.00 (p=1)</td>
</tr>
<tr>
<td>TNFα 1ng/ml</td>
<td>0.67 +/- 0.58 (p=.114)</td>
<td>1.33 +/- 1.15 (p=.114)</td>
</tr>
<tr>
<td>TNFα 10ng/ml</td>
<td>6.00 +/- 1.00 (p=0.037)</td>
<td>5.33 +/- 1.15 (p = 0.34)</td>
</tr>
<tr>
<td>IL-1β control</td>
<td>0.00 +/- 0.00</td>
<td>0.00 +/- 0.00</td>
</tr>
<tr>
<td>IL-1β 1ng/ml</td>
<td>0.33 +/- 0.58 (p=0.317)</td>
<td>0.00 +/- 0.00 (p=1)</td>
</tr>
<tr>
<td>IL-1β 10ng/ml</td>
<td>0.00 +/- 0.00 (p=1)</td>
<td>0.33 +/- 0.58 (p=0.317)</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0. All treatments were compared with control.
Figure 4.16 Treatment of oesophageal explants cultured in MCDB 153 with bone morphogenetic protein 4 (BMP4) does not effect p63 expression.

After 5 days of culture in MCDB 153, explants were treated with control (A+D), 10ng/ml BMP4 (B+E) or 100ng/ml BMP4 (C+F) for a further 5 days. (A-C) K14 (red) and p63 (green) expression in treated cultures reveals a decrease in density of cells (B+C) compared to control (A). (D-F) Higher magnification shows individual cell morphology and demonstrates the regular cuboidal shape in all treatments and the expression of p63 (green) in all K14 positive cells. The results are representative images from 3 separate experiments. This experiment was performed in parallel with that from figure 4.7 which acts as a positive control.

Scale bars represent 200μm (A-C) and 100μm (D-F).
**Figure 4.17 Bone morphogenetic protein 4 does not induce K8 in oesophageal explants grown in MCBD 153**

Immunostaining for K8 (green) at low (A-C) and high (D-F) magnification following treatment with BMP4. After 5 days of culture in MCBD 153 explants were treated with control (A+D), 10ng/ml BMP4 (B+E) or 100ng/ml BMP4 (C+F) for a further 5 days. There is no increase in K8 expression following BMP4 exposure. The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
**Figure 4.18 Oesophageal explants treated with tumour necrosis factor alpha (TNF-α) lose p63 expression.**

After 5 days of culture in MCBD 153, explants were treated with control (A+D), 1ng/ml TNFα (B+E) or 10ng/ml TNFα (C+F) for a further 5 days. (A-C) The number of K14 (red) positive cells decreases with TNFα treatment (B+C) compared to control (A). (D-F) Higher magnification shows individual cell morphology and demonstrates a decrease in cell number following 1ng/ml TNFα treatment (E) and a loss of p63 expression in cells after 10ng/ml TNFα (arrow in F) compared to control (D) The results are representative images from 3 separate experiments..

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.19 Oesophageal explants treated with tumour necrosis factor alpha (TNF-α) express K8.

After 5 days of culture in MCBD 153, explants were treated with control (A+D), 1ng/ml TNFα (B+E) or 10ng/ml TNFα (C+F) for a further 5 days. (A-C) The number of K8 (green) positive cells increases with 10ng/ml (C), but not 1ng/ml TNFα treatment (B) compared to control (A). (D-F) Higher magnification shows individual cell morphology of K8 positive cells in TNFα treatment (F) The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.20 Oesophageal explants treated with interleukin 1β (IL-1β) continue to express p63

After 5 days of culture in MCBD 153 explants, were treated with control (A+D), IL-1β 1ng/ml (B+E) or IL-1β 10ng/ml (C+F) for a further 5 days. (A-C) The density of cells, as assessed by immunostaining for K14 (red) and p63 (green), is reduced following treatment with IL-1β 10ng/ml (C) (D-F). Higher magnification images show that p63 expression is maintained in all cells in each treatment group. The results are representative images from 3 separate experiments. Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.21 Treatment of oesophageal explants with interleukin 1β (IL-1β) does not induce K8

After 5 days of culture in MCBD 153, explants were treated with control (A+D), IL-1β 1ng/ml (B+E) or IL-1β 10 ng/ml (C+F) for a further 5 days. Expression of K8 (green) was assessed with immunohistochemistry following treatment with: control (A+D), IL-1β 1ng/ml (B+E) or IL-1β 10 ng/ml (C+F). There was no increase in K8 expression following treatment. The results are representative images from 3 separate experiments. This experiment was performed in parallel with that from figure 4.7 which acts as a positive control.

Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 4.22 Treatment of oesophageal explants with BMP4, TNFα or IL-1β does not induce either Cdx2 or Muc2.

RT-PCR results for Cdx2 and Muc2 following treatment of a 5 day old cultures with BMP4 100ng/ml, TNFα 10ng/ml or IL-1β 10ng/ml for 5 days. These treatments were performed on oesophageal explants cultured in BME and MCDB. RT-PCR was performed at 30 cycles. Neither Cdx2 nor Muc2 was induced. mRNA isolated from mouse intestine was used as a positive control.
Discussion

In this chapter I have assessed the effects of ARTA, LPS, bile acids, BMP4, TNFα and IL-1β on oesophageal cells. If a candidate factor that induced Cdx2 protein could be identified then the molecular mechanism and downstream effects, specifically on intestinal genes, could be assessed. The results from these experiments are summarised in table 4.4.

As previously discussed, ATRA is known to induce a glandular phenotype in various models of squamous epithelium and it is not surprising to observe the increased expression of K8 within oesophageal explants following treatment with this compound. The precise sequence of events that result in BM are not known and, according to BSG guidelines, the presence of columnar K8 expression in the oesophagus is the same phenotype as Barrett's metaplasia. Retinoic acid (RA) is known to influence the keratin expression in many cell types. RA inhibits differentiation, stratification and the synthesis of the cornified cell envelope in cultured keratinocytes (Yuspa and Harris 1974), furthermore lack of RA promotes increased differentiation and stratification (Eckert 1989). Expression of columnar keratins present in the LP-9 ovarian tumour cell lines, K7, K8, K18 and K19, is dependent of RA in the media (Kim, Stellmach et al. 1987; Glass and Fuchs 1988). In the absence of RA, keratin expression decreases and vimentin expression increases suggesting an epithelial-mesenchymal transition. RA is known to enhance the intestinal phenotype in CaCO2 cells, as assessed by intestinal alkaline phosphatase expression (Baltes, Nau et al. 2004). ATRA induces a columnar phenotype, K8 expression, in squamous oesophageal biopsies (Chang, Lao-Sirieix et al. 2007). Interestingly, ATRA induces MUC2 in primary human oesophageal keratinocytes through an unknown mechanism (Cooke, Blanco-Fernandez et al. 2008). It could be conceived that the first step in the development of BM is the induction of a columnar phenotype, which could be mediated by RA.
Table 4.4 Effects of ATRA, LPS, BMP4, TNFα, IL-1β, acid and bile treatment of explant cultures, HET-1A and HT-29 cells. Blank cells were not tested.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cdx2 mRNA</th>
<th>p63 expression</th>
<th>K8 expression</th>
<th>Muc2 mRNA</th>
<th>Cdx2 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA explants BME medium</td>
<td>Induction</td>
<td>Decreased</td>
<td>Increased</td>
<td>No induction</td>
<td></td>
</tr>
<tr>
<td>ATRA explants MCDB medium</td>
<td>Decreased</td>
<td>Increased</td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATRA HET-1A cells</td>
<td>No induction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS explants BME medium</td>
<td>Induction</td>
<td>Decreased</td>
<td>Increased</td>
<td>No induction</td>
<td></td>
</tr>
<tr>
<td>LPS explants MCDB medium</td>
<td>Decreased</td>
<td>Increased</td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS HET-1A cells</td>
<td>No induction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP 4 explants MCDB medium</td>
<td>No induction</td>
<td>No effect</td>
<td>No effect</td>
<td>No induction</td>
<td></td>
</tr>
<tr>
<td>TNFα explants MCDB medium</td>
<td>No induction</td>
<td>Decreased</td>
<td>Increased</td>
<td>No induction</td>
<td></td>
</tr>
<tr>
<td>IL-1β explants MCDB medium</td>
<td>No induction</td>
<td>No effect</td>
<td>No effect</td>
<td>No induction</td>
<td></td>
</tr>
<tr>
<td>DCA HT-29 24 hours</td>
<td>Induction</td>
<td></td>
<td>Induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCA HET-1A 24 hours</td>
<td>Induction</td>
<td></td>
<td>Induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid pulse explant BME</td>
<td>No induction</td>
<td></td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCA pulse explant BME</td>
<td>No induction</td>
<td></td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid and DCA explant</td>
<td>No induction</td>
<td></td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCA explant BME 24 hours</td>
<td>No induction</td>
<td></td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCA explant BME 24 hours</td>
<td>No induction</td>
<td></td>
<td>No induction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The loss of p63 seen with ATRA treatment may account for the induction of a columnar phenotype and is known to exert a direct effect on squamous keratins. K5, K14, K1 and K10 are all repressed by ATRA (Stellmach, Leask et al. 1991) and RAREs are found in the promoter of K5, K10 and K14 (Tomic-Canic, Sunjevaric et al. 1992). The interaction between p63 and RA in postnatal cells has not been explored in detail. RA prevents the down-regulation of the ΔNp63α isoform of p63 in in vitro cultured epidermal keratinocytes thereby preventing terminal differentiation (Bamberger, Pollet et al. 2002). Although RA prevents the down-regulation of ΔNp63α, the RARs found in skin (RARα and RARγ) are not essential to epidermal keratinocyte growth (Goyette, Feng Chen et al. 2000). Goyette and colleagues established epidermal cell lines from RARα, RARγ and RARαγ deficient mice and demonstrated that the growth suppression effect of RA on keratinocytes was mediated via RARγ (Goyette, Feng Chen et al. 2000). The down-regulation of p63 expression by RA has only been shown in immortalised nasopharyngeal squamous carcinoma cells (Yip and Tsao 2008).

Studies on human embryonic stem cells (hESCs) reveal that RA has a dose dependent and temporal effect on differentiation. Treatment of hESCs with RA causes induction of K18 and p63 followed by induction of K14 in prolonged culture (Metallo, Ji et al. 2008). These effects were dependent on the appropriate dose and timing of RA treatment otherwise a neural fate was achieved. The induction of a columnar phenotype and suppression of squamous markers seen in oesophageal explants is consistent with a role of RA in epithelial differentiating and would be concordant with a function in the pathogenesis of BM.

ATRA induced Cdx2 mRNA in oesophageal explants but translation into protein was not seen. There are various explanations for this finding. It is possible that Cdx2 protein was only expressed at a level that was not detectable by immunohistochemistry. The nature of RT-PCR means that it is not known if Cdx2 mRNA was expressed at a low level in the majority of cells or at high levels in very few cells. If Cdx2 protein is not expressed in any cells within the explant culture
then post transcriptional mechanisms, such as micro RNA (miRNA) may be preventing translation.

There is considerable overlap between the effects of RA and Hox / Parahox genes during development suggesting a potential mechanism for the altered phenotype seen in explants. Anterior-posterior patterning of the vertebrae during early embryogenesis is dependent on temporal and spatial expression of the Hox genes. RARγ null mice demonstrate posterior vertebral homeotic transformations which results in altered expression of some Hox genes. Furthermore certain Hox mutants demonstrate the same phenotype as the RARγ knockout mice (Houle, Prinos et al. 2000). Although some Hox genes contain RAREs, Cdx1 acts as an intermediate in RA regulating other Hox code specification (Houle, Prinos et al. 2000). RARγ null mice exhibit posterior homeotic transformations and, intriguingly, squamous metaplasia in the normally glandular prostate and seminal vesicle epithelium (Lohnes, Kastner et al. 1993). RA signalling is known to be essential for Cdx1 expression during development and increases Cdx1 levels during embryogenesis as direct target effect (Houle, Prinos et al. 2000; Houle, Sylvestre et al. 2003). Furthermore RA causes induction of CDX1 in the embryonal cancer cell line, F9, via RXR-RARγ heterodimers (Chiba, Clifford et al. 1997). The skeletal defects seen in RAR null mice and Hox mutants are reminiscent of Cdx1−/− and Cdx2 +/- mice (Subramanian, Meyer et al. 1995; Chawengsaksophak, James et al. 1997; van den Akker, Forlani et al. 2002). Despite the overlap between the function of Cdx1 and Cdx2 during development and the interplay between their promoters there is currently no evidence that retinoic acid regulates Cdx2 expression.

The effects of ATRA on oesophageal explants may result from non-specific mechanisms. RA is toxic in excess and it is possible that toxicity could result in loss of p63 expression as an early apoptotic mechanism.

Explaining the variation in response to ATRA shown by oesophageal explants and HET-1A cells may relate to their different cell phenotypes. As I have shown, the
HET-1A cells are a monolayer columnar culture that does not express K14 nor p63. Interestingly HET-1A cells began to express CDX2 in the presence of demethylating agents but not in response to ATRA. The phenotype of HET-1A cells resembles gastric more than oesophageal type and the difference in response to ATRA may reflect this variation. It is not known which pathway is responsible for CDX2 induction by ATRA nor whether RAR/RXR is responsible for this. The mechanism of action could be further examined using either specific RAR or RXR agonists and antagonists in combination with ATRA or oesophageal explants from RAR/RXR null mice to determine specific receptor responsible for RA signalling.

LPS is a key inflammatory protagonist that induces NFκB activity via TLR4 and has a potential role for the induction of Cdx2 in models of BM. The inflammatory response is inherently linked with the development of epithelial metaplasia (Colleypriest, Palmer et al. 2009). The response of oesophageal cells to LPS is similar to that seen with ATRA. There is induction of K8 and Cdx2 (mRNA) and loss of p63 expression. A link between LPS and Cdx2 has only been demonstrated once previously. Cdx2 is induced in rat biliary epithelial cells in response to LPS via a mechanism that is dependent on TLR4 and NFκB activity (Ikeda, Sasaki et al. 2007). LPS has been demonstrated in many cell types to induce mucin expression. For example MUC2 and MUC5AC are induced and up-regulated respectively in respiratory epithelial cell cultures (Dohrman, Miyata et al. 1998; Voynow, Gendler et al. 2006). Interestingly the induction of MUC2 and up-regulation of MUC5AC provoked by LPS treatment in human pulmonary mucoepidermoid carcinoma cell lines is abrogated by a RARα inhibitor, suggesting that RA signalling is involved and potentially explaining the common effects on oesophageal cells (Koo, Kim et al. 2002). Both Muc2 and Muc5AC are upregulated in biliary epithelial cells in response to LPS (Zen, Harada et al. 2002; Ikeda, Sasaki et al. 2007). The effect of LPS on oesophageal cells has not previously been studied. The possibility exists that the effects of LPS on oesophageal represent a general toxic effect rather than a specific inflammatory one. The mechanism of action for this could be further
examined using TLR4 antibodies and NFκB inhibitors to dissect the exact inflammatory pathway responsible.

The loss of p63 expression in response to LPS in oesophageal cells is of interest in BM. Within the epithelial explant culture p63 loss correlated with an increase in K8 expression and could be hypothesised as a first step in BM. The effects of inflammation on p63 have only been studied briefly, but some publications hint at a role in metaplasia. p63 expression along with other markers of squamous differentiation, such as involucrin and K14, are induced in bronchial epithelial cells in response to TGF-β (Murata, Ota et al. 2007). The interaction of LPS and p63 has not been previously examined, but the proinflammatory cytokine TNFα is known to induce degradation of the ΔNp63α isoform of p63 in immortalised epidermal keratinocytes and in a cervical cancer cell line (Lee, Lee et al. 2007). The induction of K8 that is seen with loss of p63 expression following ATRA, LPS and TNFα treatment is consistent with p63 knock down experiments in keratinocytes. Truong and colleagues demonstrated an increase in K8 and K18 expression in epithelium reconstituted from keratinocytes treated with p63 siRNA (Truong, Kretz et al. 2006).

The lack of effect of IL-1β on K8, K14, p63 or Cdx2 on oesophageal explants is unexpected. BECs treated with IL-1β, TNFα or LPS demonstrate an induction of Cdx2 (Ikeda, Sasaki et al. 2008). In these experiments TNFα is sufficient to provoke induction of Muc2 suggesting that it has a more integral role. The lack of any effect of IL-1β could arise because of a number of different reasons. Firstly the IL-1β may have been used at a lower equivalent dose, either reflecting a decreased half-life or lower absolute dose compared with LPS. The fact that the IL-1β treated cells were at a decreased density relative to control suggests that the absolute dose was adequate, although LPS is likely to provoke a more robust and prolonged inflammatory reaction. There is evidence from mononuclear cells that the half-life of IL-1β mRNA is increased in the presence of LPS (Schindler, Clark et al. 1990). The lack of induction of Cdx2 mRNA following treatment with TNFα and
IL-1β may reflect a similar dose effect, given that BECs cells respond in such a way (Ikeda, Sasaki et al. 2007).

Although LPS are specific PAMPs the effect demonstrated on oesophageal cells may not be representative of the situation in BM. The LPS may be a surrogate activator of the inflammatory cascade (through NFκB) which in vivo is related to GORD, rather than a specific bacterial effect.

BMP4 has been demonstrated to provoke the expression of intestinal keratins K7 and K20 in cultures of primary oesophageal cells (Milano, van Baal et al. 2007). In mouse oesophageal cultures hrBMP4 did not induce K8 expression nor inhibit squamous markers. This may reflect a difference between mouse and human oesophageal cells or could relate to the use of hrBMP4 on mouse cells, although human and murine sequences exhibit >97% homology (Barros, Pereira et al. 2008). The expression of K7 and K20 was not assessed in oesophageal explants treated with BMP4 although Cdx2 was not found. BMP4 causes induction of CDX2 in human gastric carcinoma cell lines but fails to do so in oesophageal explants (Barros, Pereira et al. 2008), which may represent a difference either between oesophageal and gastric cells or between normal and carcinoma. The presence of BMP receptors in oesophageal cells was not assessed and may be responsible for the lack of response. It is possible that oesophageal explants require longer treatment to induce Cdx2. Further experimentation using murine BMP4, longer treatments and combinations of factors would be interesting along with determining the expression of K7 and K20. BMP4 has been shown to induce pluripotent markers in keratinocytes and assessment of Oct4 in treated oesophageal explants may be interesting.

Neither acid nor bile provoked the induction of Cdx2 in oesophageal explants and needs to be discussed in reference to the literature. Acid is known to induce Cdx2 in murine cells but the experimental model was considerably different to the explant model described. First primary cultures were used on supports so that independent apical and basal access could be achieved in contrast to the explant...
cultures. Second acidification of the media was continued for 18 days post confluence, which was not possible in explants.

The majority of literature corresponding to bile acid treatment and the induction of CDX2 pertains to cell lines, which as evidence from this chapter, respond quite differently to the more physiological explant culture system. Furthermore, the majority of the cell lines used were either derived from cancer, commonly colorectal, and/or do not reflect a squamous phenotype.

DHCA has been demonstrated to induce Cdx2 in a primary rat oesophageal cell culture, but this was not seen in mouse explants. This difference could be related to species differences or to the structure of the explant culture. The differentiated squamous layers may have protected the deeper immature cells from the effect of bile acids and would not be the case in primary cells (monolayer). The oesophageal explants cultured in low calcium conditions should allow access to the immature cells, did not survive bile acid treatment as all cells detached.

The current chapter demonstrates that some of the candidate factors for the induction of BM in vivo have significant effects on the squamous oesophageal epithelium phenotype. ATRA, LPS and TNFα down regulate p63 and induce K8, a phenotype that is seen in BM. A combination of factors may have further columnar/intestinal effects on the phenotype and should be investigated further. Unfortunately none of these factors was sufficient in isolation to induce Cdx2 protein, although mRNA expression was seen with ATRA and LPS. In future chapters alternative strategies will be employed to provoke ectopic Cdx2 expression in squamous oesophageal epithelium.
Chapter 5: Ectopic expression of Cdx2 in an in vitro model of adult squamous oesophagus.

5.1 Overview
The aim of the research described in this chapter was to determine whether ectopic Cdx2 expression was sufficient to provoke an intestinal phenotype in the oesophagus. I utilised the model described in chapter 3 in order to test this hypothesis. Although some of the candidate factors used in Chapter 4 induced a change in phenotype, none of these factors tested resulted in expression of Cdx2 protein. Two different oesophageal explant culture models described in chapter 3, utilising BME and MCDB 153 culture media, allow for the assessment of the effect of Cdx2 on normal differentiating squamous oesophageal epithelium and on an immature subset of p63 / K14 positive cells. I also describe a protocol to established ectopic Cdx2 expression using first generation recombinant adenoviral vectors and characterise any resultant change in mRNA or protein expression.

5.1.1 Evidence implicating CDX2 in Barrett’s metaplasia
Cdx2 is essential for the normal development of the gastrointestinal tract and has been implicated in the metaplastic lesion BM (James and Kazenwadel 1991). Loss of Cdx2 in the mouse intestine and colon (both conditional deletion and in Cdx2+/-mice) results in squamous metaplasia (Chawengsaksophak, James et al. 1997; Gao, White et al. 2009). A causative role for Cdx2 in gastric IM is also demonstrated by two transgenic mice studies (Mutoh, Hakamata et al. 2002; Silberg, Sullivan et al. 2002). Both confirmed that Cdx2 is sufficient to establish IM in the stomach. The observation of ectopic Cdx2 in BM an IM within the stomach (Mizoshita, Inada et al. 2001), oesophagus (Eda, Osawa et al. 2003), biliary tree (Kozuka and Hachisuka 1984), pancreatic ducts and urinary bladder (Steininger, Mueller et al. 2005) provides circumstantial evidence. Acid, bile and inflammatory intermediates induce Cdx2 expression in cell lines and primary oesophageal cell cultures linking the main protagonists of BM with the condition (Marchetti, Caliot et

The demonstration of ectopic Cdx2 in BM and, interestingly, Cdx2 mRNA in the native squamous epithelium above the Barrett’s segment in one third of patients, suggests that the transcription factor expression may precede the switch in phenotype and further implicates Cdx2 as an integral and early mechanism (Eda, Osawa et al. 2003; Phillips, Frierson et al. 2003; Moons, Bax et al. 2004).

Immortalised ‘normal’ and cancer derived oesophageal cell lines are of limited use in dissecting the role of Cdx2 on oesophageal intestinalisation. Cell lines derived from squamous cancers have already progressed beyond the initial metaplastic phenotype and have acquired considerable genetic abnormalities. Primary cells and explant culture provide a more physiological model to study the transformation from normal to metaplastic tissue. For example, the immortalised squamous oesophageal cell line HET-1A expresses K8, rather than squamous cytokeratins, and fail to respond to increased calcium concentration as expected with involucrin expression (Inokuchi, Handa et al. 1995). Furthermore, Cdx2 expression can be induced in the HET-1A cell line by promoter demethylation, suggesting that the phenotype is closer to the intestine cell than a normal oesophageal cell (Liu, Zhang et al. 2007).

For the reasons described above the oesophageal explant model described in Chapter 3 was used to ectopically express Cdx2. In this Chapter 5 I will characterise the phenotype of the explant model of adult squamous oesophagus following ectopic Cdx2 expression.

5.2 Recombinant adenovirus.

There are 51 human adenovirus (Ad) serotypes which are numerically classified on their ability to be neutralised by specific animal antisera. Further subdivision into subgroups A-F is based on the capacity to agglutinate rat, human and monkey erythrocytes and tumour forming ability in rodents (Russell 2009). The human Ad5
virus, a member of species C, has been widely used for the transfer of genes in recombinant form (Russell 2009). The Ad5 genome consists of a linear double stranded 36Kb DNA molecule, which is divided into two regions, early and late, related to the onset of viral DNA synthesis within host cells (Kremer and Perricaudet 1995; He, Zhou et al. 1998). There are 6 early regions, E1A, E1B, E2A, E2B, E3 and E4, and one late region. The switch from early to late gene expression occurs around 7 hours after infection (Kremer and Perricaudet 1995). The E1 genes are involved in adenoviral replication and are the only genes transcribable without adenovirus coded transcription factors and are consequently essential for successful replication. Ad5 binds to the coxsackie adenovirus receptor (CAR) which is an integral part of the tight junction and an immunoglobulin (Philipson and Pettersson 2004). Internalisation of the adenovirus is facilitated by α integrins (Wickham, Mathias et al. 1993). First generation recombinant adenovirus has been rendered replication deficient by deletion of the E1 region allowing for the insertion of a transgene cassette up to 8Kb in size. The E3 region, involved in evasion of host immunity, is also deleted in first generation recombinant adenovirus (Bett, Haddara et al. 1994). Recombinant adenoviral vectors are amplified in cell lines stably expressing the E1 genes such as HEK293 or human embryonic retinoblast 911 cell line (Graham, Smiley et al. 1977; Fallaux, Kranenburg et al. 1996). Recombinant Ad5 confers many advantages in the expression of transgenes in mammalian cells:

i) Infection of a wide variety of mammalian cells.

ii) Infection of replicating and non-replicating cells.

iii) Transfer of genetic material up to 8Kb

iv) Does not integrate into the host genome remaining epichoromosomal

5.2.1 Recombinant adenovirus used.

Three replication deficient first generation recombinant Ad5 vectors have been used in this chapter.
Ad-RSV-GFP (a gift from Harry Heimberg, Diabetes Research Centre, University of Brussels) – the Rous sarcoma virus (RSV) promoter drives expression of GFP. This construct was used to optimize infection parameters and as a control for adenoviral infection.

Ad-CMV-CDX2-IRES-hrGFP. The constitutively active cytomegalovirus (CMV) promoter drives the expression of the murine coding region of Cdx2 (the Cdx2 plasmid was a gift from Dr Debra Silberg, University of Pennsylvania). The internal ribosomal entry site (IRES) allows for the initiation of transcription of a humanised recombinant GFP sequence in the middle of an mRNA molecule (Pelletier and Sonenberg 1988). In turn, this allows for visualisation of the GFP as a surrogate for Cdx2 expression. Ad-CMV-CDX2-IRES-hrGFP was constructed by Wei-Yuan Yu, University of Bath.

Ad-CMV-VP16Cdx2-IRES-GFP. The CMV promoter drives the expression of a Herpes Simplex viron protein 16 (VP16) murine Cdx2 fusion sequence. The VP16 codes for a transactivation domain that is important for immediate early virus gene transcription and consequently boosts Cdx2 expression levels (Triezenberg, Kingsbury et al. 1988). The IRES initiates GFP transcription. Ad-CMV-VP16Cdx2-IRES-GFP was constructed by Wei-Yuan Yu, University of Bath.

I will demonstrate that ectopic gene expression within the epithelium of oesophageal explants is possible using adenoviral infection. I will also describe the results obtained following characterisation of the phenotype for any expression of intestinal genes. All of the experiments described have been repeated at least three times.
5.3 Ectopic GFP expression within oesophageal explants following Ad-RSV-GFP infection

The Ad-RSV-GFP virus was used to test the ability of adenoviral vectors to express transgenes within the explant cultures and to optimise the infection protocol. Oesophageal explants were cultured in BME for 7 days and then incubated with a range of Ad-RSV-GFP virus particles for either 2 hours or 12 hours. To determine the most effective number of particles required to provoke ectopic gene expression oesophageal cultures were exposed to 2.4x10^4, 2.4x10^5 and 2.4x10^6 particles per explant for either 2 or 12 hours. GFP expression was observed after 3 days only in the 2.4x10^6 particles per explant dish and was stronger in the 12 hour infection (Fig 5.1). In order to ensure that GFP expression was present in the epithelial component of the explants, cultures were fixed and immunostained with a pan-cytokeratin antibody. This is a polyclonal antibody raised to multiple epitopes found in squamous epithelium. GFP expression is provoked in the epithelium and fibroblasts found surrounding oesophageal explants (Fig 5.2 and 5.3). The connective tissue expression will serve as an internal control if there is induction of intestinal genes.
Figure 5.1. Expression of GFP in oesophageal explant culture following infection with Ad-RSV-GFP virus.

(A) Explant cultures were cultured for 7 days and then infected with $2.4 \times 10^6$ Ad-RSV-GFP particles per explant for 12 hours. Following infection, the explants were cultured for a further 5 days. A phase contract image is shown, the original bud of tissue is arrowed and the epithelium (E) and mesenchyme (M) are marked with lettered arrows. (B) GFP fluorescence within the same explant culture. (C) Overlay image of (A) and (B) demonstrating the expression of GFP in both epithelium and connective tissue, but with higher levels in the former.

Scale bar represents 200μm.
Figure 5.2. Expression of GFP within the epithelium following infection with Ad-RSV-GFP virus.

(A) The epithelium (red) of a whole explant culture was grown for 7 days, infected with Ad-RSV-GFP and cultured for a further 5 days. Epithelium was detected using a pancytokeratin (red) antibody. (B) GFP (green) expression is demonstrated within the same culture. (C) Overlay of (A) and (B) to demonstrate cells co-expressing GFP and pancytokeratin.

Scale bars represent 200μm.
Figure 5.3. Expression of GFP within the epithelium and connective tissue following infection with Ad-RSV-GFP. (A) An explant culture was grown for 7 days, infected with Ad-RSV-GFP and cultured for a further 5 days. Epithelium is shown by expression of pancytokeratin (red). Epithelial cells (E) and connective tissue (M) cells are arrowed. (B) GFP (green) expression is demonstrated within the same culture. (C) Overlay of (A) and (B) to demonstrate GFP expression within epithelial cells and connective tissue in the outgrowth of cells surrounding an explant. Scale bars represent 100μm.
5.4 Expression of Cdx2 within oesophageal explant cultures using adenoviral vectors.

Two adenoviral vectors were utilised in this study in order to provoke Cdx2 expression, Ad-CMV-Cdx2-hrGFP and Ad-CMV-VP16Cdx2-hrGFP. A similar infection protocol to that used for Ad-RSV-GFP was followed for both Cdx2 constructs, but the efficiency proved poor. Three days after infection, less than 10% of cells expressed Cdx2 (by immunohistochemistry). Under these conditions Cdx2 expression was localised to the periphery on the epithelium. In order to improve the infection efficiency, a 20-fold increase in virus particles was used, so that each explant was exposed to $5 \times 10^7$ particles for 12 hours. After 3 days 62% oesophageal cells infected with Ad-CMV-Cdx2-hrGFP and 73% of Ad-CMV-VP16Cdx2-hrGFP expressed nuclear cdx2 (table 5.1).

Oesophageal explants were cultured in BME for 7 days and then incubated with $5 \times 10^7$ adenoviral particles per explant of Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP for 12 hours. Explants were cultured for varying lengths of time after Cdx2 adenoviral infection, fixed and then examined for Cdx2 expression. Both adenoviral constructs resulted in robust Cdx2 expression within a high proportion of epithelium (Fig 5.4, 5.5, 5.6 and 5.7) and K14 positive basal cells (Fig 5.8 and 5.9) after 3 or 5 days of culture. Expression of Cdx2 remains, but at a reduced level, in basal cells after 10 days.

Table 5.1 number of cells expressing cdx2 protein, assessed by immunohistochemistry, three days following infection with Ad-CMV-Cdx2-hrGFP and Ad-CMV-VP16Cdx2-hrGFP.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ad-CMV-Cdx2-hrGFP</th>
<th>Ad-CMV-VP16Cdx2-hrGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115/164 (70.1%)</td>
<td>20/26 (76.9%)</td>
</tr>
<tr>
<td>2</td>
<td>219/388 (56.4%)</td>
<td>41/59 (69.5%)</td>
</tr>
<tr>
<td>3</td>
<td>25/42 (59.5%)</td>
<td>232/320 (72.5%)</td>
</tr>
<tr>
<td>Mean +/- SD</td>
<td>62% +/- 7.2</td>
<td>73% +/- 3.7%</td>
</tr>
</tbody>
</table>
5.5 Expression of Cdx2 in HET-1A cells induces intestinal gene expression

We have demonstrated that adenoviral vectors can be used to express Cdx2 within oesophageal cells. In order to demonstrate that the resultant protein was functional, we decided to utilise the HET-1A cell line. Since HET-1A cells do not normally express CDX2, we determined the effect of overexpression of the transcription factor on intestinal gene expression. Stably transfected HET-1A cells have been shown to up-regulate intestinal genes, so we wished to assess for the effects of transient Cdx2 expression (Liu, Zhang et al. 2007). We therefore incubated HET-1A cells with either Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP at three different MOIs for 12 hours and observed GFP as a surrogate marker for Cdx2 expression (Fig 5.10). Cdx2 expressed from either adenoviral construct (Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP) was sufficient to induce four intestinal genes: Cdx1, K20, mucin 2 and villin at 3 days following infection in HET-1A cells (Fig 5.11). None of the intestinal markers were observed with control adenoviral infection alone.

5.5.1 Cdx2 expression does not induce intestinal genes in an explant model of squamous oesophagus

Explants were grown for 5 days, incubated with media containing virus for 12 hours and intestinal markers were assessed by immunohistochemistry and RT-PCR after 3, 5, 7 and 10 days of culture. Incubation with Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP results in expression of Cdx2 within the majority of epithelial and basal squamous cells within the oesophagus (Fig 5.4-5.9). Cdx2 protein was detectable two days after infection and persisted for at least 10 days. Cdx2 protein was expressed within the nucleus of epithelial and K14-positive cells and also Cdx2 mRNA was present. Ectopic expression of Cdx2 does not result in the induction of any intestinal genes; mucin 2, sucrase isomaltase, villin, lactase, trefoil factor 3, alkaline phosphatise, cryptdin 1 and chromogranin A as assessed by RT-PCR (Fig 5.12). K20 was expressed at a transcript level prior to Cdx2 infection.
Figure 5.4. Expression of Cdx2 within the epithelium of oesophageal explant culture following infection with Ad-CMV-Cdx2-hrGFP.

(A-C) An oesophageal explant was cultured for 5 days, infected with Ad-CMV-Cdx2-hrGFP for 12 hours and cultured for a further 3 days. Explants were immunostained for (A) Cdx2 (green) and (B) pancytokeratin (red). (C) is an overlay of (A) and (B). The Cdx2 (green) localises to the nuclei of multiple epithelial cells (red). The results are representative images from 3 separate experiments.

Scale bars represent 200μm.
Figure 5.5. Nuclear expression of Cdx2 within epithelium of oesophageal explant culture following infection with Ad-CMV-Cdx2-hrGFP.

(A-C) An oesophageal explant cultured for 5 days and then infected with Ad-CMV-Cdx2-hrGFP and cultured for a further 5 days. Explants were immunostained for (A) Cdx2 (green) and (B) pancytokeratin (red). (C) is an overlay of (A) and (B). The Cdx2 (green) localises to the nuclei of squamous epithelial cells (red). The results are representative images from 3 separate experiments.

Scale bars represent 50μm.
Figure 5.6. Expression of Cdx2 within epithelium of oesophageal explant culture following infection with Ad-CMV-VP16-Cdx2-hrGFP.

(A-C) An oesophageal explant was cultured for 5 days and then infected with Ad-CMV-VP16-Cdx2-hrGFP for 12 hours and cultured for a further 3 days. Explants were immunostained for (A) Cdx2 (green) and (B) pancytokeratin (red). (C) is an overlay of (A) and (B). Cdx2 (green) expression can be seen within the nuclei of multiple epithelial cells (red). The results are representative images from 3 separate experiments. Scale bars represent 200μm.
Figure 5.7. Expression of Cdx2 within the epithelium of oesophageal explant culture following infection with Ad-CMV-VP16-Cdx2-hrGFP.

(A-C) An oesophageal explant was cultured for 5 days and then infected with Ad-CMV-VP16-Cdx2-hrGFP for 12 hours and then cultured for a further 5 days. Explants were immunostained for (A) Cdx2 (green) and (B) pancytokeratin (red). (C) is an overlay of (A) and (B). Cdx2 (green) expression is seen within the nuclei of the majority of squamous epithelial cells (red). The results are representative images from 3 separate experiments. Scale bars represent 100μm.
Figure 5.8. Nuclear expression of Cdx2 within the basal epithelial cells of oesophageal explant culture following infection with Ad-CMV-VP16-Cdx2-hrGFP.

(A-C) An oesophageal explant was cultured for 5 days and then infected with Ad-CMV-VP16-Cdx2-hrGFP and then cultured for a further 5 days. Explants were immunostained for Cdx2 (green) and K14 (red). (A+B) multiple basal cells within the oesophageal explant express Cdx2. (C) Cdx2 (green) protein is localised to the nuclei of K14 positive cells (red). The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A), 50μm (B) and 20μm (C).
Figure 5.9. Expression of Cdx2 within the basal epithelial cells of oesophageal explant culture following infection with Ad-CMV-Cdx2-hrGFP.

(A-C) An oesophageal explant has been cultured for 5 days and then infected with Ad-CMV-Cdx2-hrGFP for 12 hours and cultured for a further 5 days. Explants have been immunostained for Cdx2 (green) and K14 (red). (A+B) multiple basal cells within oesophageal explant express Cdx2. (C) Cdx2 (green) protein is localised to the nuclei of K14 positive cells (red). The results are representative images from 3 separate experiments. Scale bars represent 200μm (A), 50μm (B) and 20μm (C).
5.5 Expression of Cdx2 in HET-1A cells induces intestinal gene expression

We have demonstrated that adenoviral vectors can be used to express Cdx2 within oesophageal cells. In order to demonstrate that the resultant protein was functional, we decided to utilise the HET-1A cell line. Since HET-1A cells do not normally express CDX2, we determined the effect of overexpression of the transcription factor on intestinal gene expression. Stably transfected HET-1A cells have been shown to up-regulate intestinal genes, so we wished to assess for the effects of transient Cdx2 expression (Liu, Zhang et al. 2007). We therefore incubated HET-1A cells with either Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP at three different MOIs for 12 hours and observed GFP as a surrogate marker for Cdx2 expression (Fig 5.10). Cdx2 expressed from either adenoviral construct (Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP) was sufficient to induce four intestinal genes: Cdx1, K20, mucin 2 and villin at 3 days following infection in HET-1A cells (Fig 5.11). None of the intestinal markers were observed with control adenoviral infection alone.

5.5.1 Cdx2 expression does not induce intestinal genes in an explant model of squamous oesophagus

Explants were grown for 5 days, incubated with media containing virus for 12 hours and intestinal markers were assessed by immunohistochemistry and RT-PCR after 3, 5, 7 and 10 days of culture. Incubation with Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP results in expression of Cdx2 within the majority of epithelial and basal squamous cells within the oesophagus (Fig 5.4-5.9). Cdx2 protein was detectable two days after infection and persisted for at least 10 days. Cdx2 protein was expressed within the nucleus of epithelial and K14-positive cells and also Cdx2 mRNA was present. Ectopic expression of Cdx2 does not result in the induction of any intestinal genes; mucin 2, sucrase isomaltase, villin, lactase, trefoil factor 3, alkaline phosphatase, cryptdin 1 and chromogranin A as assessed by RT-PCR (Fig 5.12). K20 was expressed at a transcript level prior to Cdx2 infection.
Figure 5.10. Expression of Cdx2 within HET-1A cells following infection with Ad-CMV-VP16-Cdx2-hrGFP.

(A-C) HET-1A cells were cultured to 50% confluence and incubated for 12 hours with Ad-CMV-VP16-Cdx2-hrGFP virus and grown for a further 3 days. (A) phase contrast image of HET-1A cells. (B) GFP, as a surrogate marker for Cdx2, expression can be seen in the majority of cells 3 days after infection. (C) overlay image of (A) and (B) demonstrates that the GFP corresponds to HET-1A cells.

Scale bar represents 100μm.
Figure 5.11 Induction of the intestinal genes *CDX1*, *Cdx2*, *MUC2*, *K20* and villin in HET-1A cells following ectopic Cdx2 expression.

(A+B) RT-PCR was performed for human intestinal genes in HET-1A cells following ectopic Cdx2 expression with (A) AD-CMV-Cdx2-hrGFP or (B) AD-CMV-VP16-Cdx2-hrGFP. HET-1A cells were grown to 50% confluence and then incubated with AD-CMV-Cdx2-hrGFP or AD-CMV-VP16-Cdx2-hrGFP for 12 hours. After a further 3 days, mRNA was extracted and RT-PCR (30 cycles) was performed for intestinal genes. Three different MOIs are demonstrated for AD-CMV-Cdx2-hrGFP (A) and AD-RSV-GFP is used as a control for both experiments. Induction of *CDX1*, *Cdx2*, *MUC2*, *K20* and villin is seen following infection with both adenoviral constructs, but not control infections.
Figure 5.12 RT-PCR for intestinal genes after ectopic Cdx2 expression in oesophageal explants.

(A+B) RT-PCR (35 cycles) was performed for intestinal genes in oesophageal explants following ectopic Cdx2 expression for (A) 3 days and (B) 5 days. (A) Oesophageal explants were cultured in BME for 5 days and then incubated with Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16-hrGFP for 12 hours. RT-PCR was performed for beta actin, Cdx2, Muc2, sucrase isomaltase (SI), villin, K20, lactase-phlorizin, trefoil factor 3, cryptdin 1, intestinal alkaline phosphatase (ALP) and chromogranin A after 3 (A) and (B) 5 days. Cdx2 mRNA is present using both adenoviral constructs, but there is no induction of intestinal genes.
5.5.2 Ectopic Cdx2 in low calcium cultures

Within oesophageal explants cultured in BME, the majority of the cells infected were in the epithelial outgrowth. In Chapter 3, I identified a subset of p63 expressing cells located mainly within the original explant of tissue. These cells are likely to represent the least mature and most plastic cells within the model and conversely because of their location, under layers of squamous cells, the hardest to infect. In order to determine if these p63 positive cells respond differently to the remainder of oesophageal cells, the low calcium containing, MCDB 153, media protocol was established (See Chapter 3). Oesophageal explants cultured with low calcium (0.03mM) demonstrate a different phenotype to those in normal calcium (1.8mM). The cells cultured in low calcium grow as monolayers, fail to form cell-cell contacts and do not stratify. Expression of p63 is maintained in most of these cells cultured in low calcium and is found co-expressed with K14. There is no stratification of cells and K4 expression is not observed. We initially used the same titre of virus used for BME culture infections, but this resulted in a large amount of cell death and so we reduced the titre 100-fold to achieve similar infection efficiency (5x10^5 IU/explant) (Table 5.2) (Fig 5.13). At this titre, the viability of the cultures was maintained and Cdx2 expression was observed in the majority of K14-positive cells (Fig 5.14). Following three days of Cdx2 expression, p63 is lost from some of the cells expressing Cdx2, but not from cells infected with control adenovirus (Fig 5.15, 5.16 and table 5.3). This is true using either of the Cdx2 constructs. After 3 days of Cdx2 expression, but not in control cultures, a few cells within the outgrowth begin to express the columnar marker, K8 (Fig 5.17, 5.18 and table 5.3). Ectopic Cdx2 infection provoked a low level of Muc2 and villin mRNA in oesophageal cells cultured in MCDB 153. Faint bands were visible in the RT-PCR after 35 cycles, but no protein was evident on immunostaining (Fig 5.19).

Table 5.2 Number of oesophageal cells positive for cdx2 following infection with Ad-CMV-VP16-Cdx2-hrGFP for 3 days.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive cells</td>
<td>90/96 (93.7%)</td>
<td>33/35 (94.3%)</td>
<td>82/86 (95.3%)</td>
<td>94.4% +/- 0.8%</td>
</tr>
</tbody>
</table>
Table 5.3 Number of K8 positive and p63 negative oesophageal cells following three days of infection with Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16-Cdx2-hrGFP in MCDB medium. Results shown from three separate experiments.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number of K8 positive cells per high power field</th>
<th>Number of p63 negative cells per high power field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-RSV-eGFP</td>
<td>0.00 +/- 0</td>
<td>0.33 +/- 0.58</td>
</tr>
<tr>
<td>Ad-CMV-Cdx2-hrGFP</td>
<td>1.33 +/- 0.58 (p=0.034)*</td>
<td>4.33 +/- 1.53 (p=0.046)*</td>
</tr>
<tr>
<td>Ad-CMV-VP16-Cdx2-hrGFP</td>
<td>1.00 +/- 0.00 (p=0.025)*</td>
<td>3.67 +/- 1.53 (p=0.046)*</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0. All treatments were compared with control.
5.13 Expression of ectopic Cdx2 in oesophageal explants in BME and MCDB 153 media.

(A-D) A one week old culture in BME was incubated with Ad-CMV-VP16-Cdx2-hrGFP for 12 hours and grown for a further 3 days. (A) A phase contrast image of oesophageal culture in BME 3 days after adenoviral infection is shown. The explant (Ex) and outgrowth (O) are marked. (B) GFP after 3 days of infection is shown. (C-D) Higher magnification images of (A+B) demonstrate individual cells 3 days post infection with Ad-CMV-VP16-Cdx2-hrGFP in (C) phase contrast and (D) GFP. The nucleus of an individual cell, expressing GFP, is shown with an arrow.

(E-H) A one week old culture in MCDB 153 was incubated with Ad-CMV-VP16-Cdx2-hrGFP at a titre that is 100 fold less than (A-D). (E-F) Three days after infection the explant (Ex) and (O) are shown in phase contrast (E) and GFP (F). (G+H) Higher magnification images of (E and F) demonstrating a high proportion of GFP positive cells. An arrow marks the nucleus of a positive cell.
5.14 Ectopic Cdx2 expression in basal cells of oesophageal explant in MCDB 153 media.

(A-C) A 5 day old oesophageal explant cultured in MCDB 153 infected for 12 hours with Ad-CMV-VP16-Cdx2-hrGFP and grown for a further 3 days. Ectopic Cdx2 expression (GFP from virus) is demonstrated in the majority of basal K14 positive (red) cells (A). The higher magnification images (B+C) demonstrate nuclear Cdx2 expression (Green) (arrow in C) in basal cells (red). The results are representative images from 3 separate experiments.

Scale bars represent 200μm (A), 100μm (B) and 50μm (C).
Figure 5.15 Loss of p63 expression following ectopic Cdx2 expression.

Five day old oesophageal explants cultured in MCDB 153 were infected with either Ad-CMV-Cdx2-hrGFP (A-C) or Ad-RSV-GFP (D-F) for 12 hours and fixed after a further 3 days. (A-C) A group of cells infected with Ad-CMV-Cdx2-hrGFP. Ectopic Cdx2 expression is demonstrated by the GFP in (A). (B) Three cells are highlighted with arrows that have lost p63 expression (red). (C) The overlay image demonstrates the position of the cells that have lost p63. Cells within the control infection (D-F) are all positive for p63. Two cells are marked with an arrow to demonstrate that the cells expressing GFP (D) to the highest extent are positive for p63 (red) (E). Scale bars represent 200μm (A-C) and 100μm (D-F).
Figure 5.16. Loss of p63 expression following ectopic Cdx2 expression.

Five day old oesophageal explants cultured in MCDB 153 were infected with either Ad-CMV-VP16-Cdx2-hrGFP (A-C) or Ad-RSV-GFP (D-F) for 12 hours and fixed after a further 3 days. (A-C) A group of cells infected with Ad-CMV-VP16-Cdx2-hrGFP. Ectopic Cdx2 expression is demonstrated by the GFP in (A). (B) Three cells are highlighted with arrows that have lost p63 expression (red). (C) The overlay image demonstrates the position of the cells that have lost p63. Cells within the control infection (D-F) are all positive for p63. Three cells are marked with an arrow to demonstrate that the cells expressing GFP (D) to the highest extent are positive for p63 (red) (E).

Scale bars represent 200μm (A-C) and 50μm (D-F).
Figure 5.17. Ectopic Cdx2 expression provokes K8 positive cells.

Five day old oesophageal explants cultured in MCDB 153 were infected with either Ad-CMV-Cdx2-hrGFP (A-C) or Ad-RSV-GFP (D-F) for 12 hours and fixed after a further 3 days. (A-C) A group of cells infected with Ad-CMV-Cdx2-hrGFP. Ectopic Cdx2 expression is demonstrated by the GFP in (A). (B) A cell is marked with an arrow that is positive for K8 (red) (C) The overlay image demonstrates the K8 (red) positive cell corresponds to a GFP (green) expressing cell.

Cells within the control infection (D-F) were negative for K8. Two cells are marked with an arrow to demonstrate that the cells expressing GFP (D) are negative for K8 (red) (E).

Scale bars represent 100μm (A-F).
Figure 5.18. Ectopic Cdx2 expression provokes K8 positive cells in oesophageal explant.

Five day old oesophageal explants cultured in MCDB 153 were infected with either Ad-CMV-Vp16-Cdx2-hrGFP (A-C) or Ad-RSV-GFP (D-F) for 12 hours and fixed after a further 3 days. (A-C) A group of cells infected with Ad-CMV-VP16-Cdx2-hrGFP. Ectopic Cdx2 expression is demonstrated by the GFP in (A). (B) three cells are marked with arrows that are positive for K8 (red) (C) The overlay image demonstrates the K8 (red) positive cells corresponds to a GFP (green) expressing cell.

Cells within the control infection (D-F) are negative for K8. Two cells are marked with an arrow to demonstrate that the cells expressing GFP (D) are negative for K8 (red) (E).

Scale bars represent 200μm (A-F).
Figure 5.19. RT-PCR for intestinal markers following ectopic Cdx2 infection in MCDB 153 media.

Oesophageal explants were grown in MCDB153 media for 5 days and then incubated for 12 hours with either Ad-CMV-Cdx2-hrGFP or Ad-CMV-VP16Cdx2-hrGFP. After a further 3 days in culture, mRNA was extracted and 35 cycles of RT-PCR performed for intestinal genes. (A) Following 35 cycles of PCR, there is a faint band for villin and Muc2 in explants infected with either Cdx2 adenoviral construct. This band is not observed with control Ad-RSV-GFP infection. (B) There is no induction of any other intestinal genes following 4 days of infection.
5.5.3 Cdx2 causes induction of mucin 2 and villin in an *in vitro* model of embryonic oesophagus

We previously established an embryonic mouse oesophageal explant culture system to investigate the normal development of the oesophagus (Yu, Slack et al. 2005). To test whether ectopic Cdx2 expression could induce intestinalisation in an embryonic setting, we infected embryonic oesophagus and followed the expression of oesophageal and intestinal gene expression patterns. Embryonic oesophagi were dissected and briefly treated with dispase, to increase adenoviral infections. Each oesophagus was incubated with $5.4 \times 10^8$ Ad-CMV-Cdx2-hrGFP virus particles for 2 hours and then cultured on fibronectin coated coverslips. Embryonic oesophagus can be cultured *in vitro* for 7 days following ectopic expression of Cdx2 or GFP. Cdx2 was expressed in oesophageal cells following Ad-CMV-Cdx2-hrGFP incubation, but not in control infected cultures. The intestinal mucin, muc2, is provoked by Cdx2 expression and demonstrated with immunohistochemistry after 7 days (Fig 5.20). Villin, Muc2 and Muc5AC mRNA are induced in embryonic oesophagus after Cdx2 expression (Fig 5.21).

The same protocol was used for E14.5 stomach and RT-PCR after 7 days revealed the presence of Muc2 mRNA (Fig 5.21) and this has been confirmed with immunostaining by a previous PhD student in the lab (Quinlan 2008).
Figure 5.21. Ectopic Cdx2 expression provokes the expression of Muc2 and villin in embryonic oesophageal and gastric cultures.

E15.5 mouse embryonic oesophagus and E14.5 mouse embryonic stomach were incubated for 2 hours with either Ad-CMV-Cdx2-hrGFP or control Ad-RSV-GFP and then cultured on fibronectin coated coverslips for a further 7 days when mRNA was extracted. RT-PCR performed for 30 cycles for intestinal genes. Following ectopic Cdx2 expression in the oesophagus there is induction of Muc2, Muc5AC and villin. Ectopic gastric Cdx2 provoked the expression of Muc2 and Cdx1.
Figure 5.20. Ectopic Cdx2 expression provokes the expression of Muc2 in embryonic oesophageal cultures.

E15.5 mouse embryonic oesophagus was incubated for 2 hours with Ad-CMV-Cdx2-hrGFP or control Ad-RSV-GFP and then cultured on fibronectin coated coverslips for a further 7 days and examined for GFP (green) and mucin 2 (red) expression using fluorescent antibodies. (A) Ad-RSV-GFP infected virus demonstrate GFP (green) expression but not mucin 2 (red). (B+C) Oesophagus infected with Ad-CMV-Cdx2-hrGFP show induction of mucin 2 (red) and GFP (green). Pictures are representative of results from two separate experiments.

Scale bars represent 20μm (A+C) and 10μm (B).
5.6 Discussion

In this chapter I tested the utility of *Cdx2* to induce the conversion of oesophageal cells to intestinal cells (Table 5.4). Three model systems were utilised; a human oesophageal cell line (HET-1A), an embryonic oesophageal culture model and an organotypic model of normal adult squamous oesophagus to address the role of *Cdx2* in BM. Whilst *Cdx2* is able to induce a degree of intestinalisation in the first two models, our results show that *Cdx2* alone is not sufficient to provoke the expression of intestinal genes in adult oesophageal cells.

HET-1A cells and embryonic oesophagus are not ideal models for adult oesophagus and so we developed an explant culture model that recapitulates the full repertoire of cell types found in the oesophagus. HET-1A cells do not express the squamous epithelial markers p63, K4 nor K14, but maintain a columnar phenotype. The explant culture model is viable long term and also maintains the normal three-dimensional organisation of the oesophagus. The presence of a myofibroblastic connective tissue layer that is correctly orientated, deep to the basal cells, allows for appropriate epithelial-mesenchymal interactions which are important during normal development and may be involved in BM (Plateroti, Freund et al. 1997; Chang, Lao-Sirieix et al. 2007). Above the connective tissue layer, the K14 positive basal cells differentiate and start to express the differentiation markers, involucrin and K4, prior to the formation of the cornified cell envelope in fully mature squames (Seery and Watt 2000). Loricrin is the major component of the cornified cell envelope found in terminally differentiated squamous cells and has been demonstrated in epidermal keratinocyte cultures but has not been demonstrated previously in any *in vitro* oesophageal model (Hohl, Lichti et al. 1991). Loricrin is located in the epithelial component of the outgrowth demonstrating that all stages of oesophageal squamous cell differentiation are represented. Consequently, this model of squamous oesophagus allows for the assessment of the effects of ectopic genes (including *Cdx2*) on squamous differentiation in the context of intestinal metaplasia.
Table 5.4. Summary table of the effects of ectopic Cdx2 expression within all models of oesophagus. Blank cells were not tested.

<table>
<thead>
<tr>
<th>Marker</th>
<th>HET-1a</th>
<th>Adult mouse explants(BME)</th>
<th>Adult mouse explants(MCDB)</th>
<th>Embryonic oesophagus</th>
<th>Embryonic stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdx1 mRNA</td>
<td>Induced</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Induced</td>
</tr>
<tr>
<td>Cdx2 mRNA</td>
<td>Induced</td>
<td>Induced</td>
<td>Induced</td>
<td>Induced</td>
<td>Induced</td>
</tr>
<tr>
<td>Muc2 mRNA</td>
<td>Induced</td>
<td>Nil</td>
<td>low level Induced</td>
<td>Induced</td>
<td>induced</td>
</tr>
<tr>
<td>K20 mRNA</td>
<td>Induced</td>
<td>present</td>
<td>present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>SI mRNA</td>
<td>nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Villin mRNA</td>
<td>Induced</td>
<td>Nil</td>
<td>low level Induced</td>
<td>Induced</td>
<td>Present</td>
</tr>
<tr>
<td>Lactase mRNA</td>
<td>nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>TFF3 mRNA</td>
<td>present</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
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<td>nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>ALP mRNA</td>
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<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Chr A mRNA</td>
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<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Muc5AC mRNA</td>
<td></td>
<td></td>
<td>Induced</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>K8 protein</td>
<td>present</td>
<td>Nil</td>
<td>Induced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p63 protein</td>
<td>nil</td>
<td>reduced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muc2 protein</td>
<td></td>
<td></td>
<td>Induced</td>
<td>Induced</td>
<td>Induced</td>
</tr>
</tbody>
</table>

Cdx2 has been implicated in the development of BM and IM of other organs and act as the master switch gene for intestine development (Quinlan, Colleypriest et al. 2007). Selective deletion of Cdx2 from the gut endoderm within the developing mouse embryo, using Foxa3Cre mice, results in a highly disorganised epithelium that fails to terminally differentiate and develops a squamous phenotype (Gao, White et al. 2009). Cdx2 deficient posterior intestinal epithelium demonstrated a stratified squamous histology with the expression of the oesophageal genes p63, K5, K14 and K13. Furthermore, transcription factors important in intestinal differentiation, such as Cdx1, HNF4α, HNF1α and Math1 were decreased and foregut transcription factors, Sox2 and Pax9, enriched (Gao, White et al. 2009).
The absence of expression of intestinal markers, following Cdx2 expression in adult oesophageal cells is surprising. Two separate transgenic mouse studies have demonstrated that ectopic expression of Cdx2 in the murine stomach is sufficient to provoke intestinalisation, whereas the data presented here suggest that alternative or additional factors are necessary in the oesophagus (Mutoh, Hakamata et al. 2002; Silberg, Sullivan et al. 2002). There are considerable differences between the gastric epithelium utilised in these studies and the present explant model which may explain the differences observed. Firstly the stomach shares many similarities with the intestinal mucosa, both are simple columnar glandular epithelia and both contain more than one cell lineage. Likewise, the intestine and stomach are derived from regions that are neighbouring in the embryo and as indicated by Cdx2+/− mice may be more likely to interconvert.

The oesophageal squamous epithelium contains only one type of cell which differentiates along one lineage and expresses transcription factors that are absent from the hind stomach, such as p63. In one of the transgenic studies, Cdx2 was conditionally expressed under the control of the FoxA3 promoter, which is active during embryogenesis, presumably when the epithelium is more plastic and may explain the induction of IM (Silberg, Sullivan et al. 2002; Yu, Slack et al. 2005). There are two considerable differences between the embryonic and adult oesophagus that could account for the variation in response to Cdx2 observed in this chapter. Firstly, the embryonic oesophagus, at the time of dissection, is lined by a columnar epithelium similar to the gastric mucosa, in contrast to the adult explant stratified squamous phenotype. This similarity to a gastric phenotype may account for the ability of Cdx2 to convert embryonic oesophagus to an intestinal phenotype akin to the gastric Cdx2 expressing transgenic mice. The columnar oesophagus develops into a squamous epithelium in response to p63 expression, but also can be transformed into intestine with ectopic Cdx2 expression. These findings would be in keeping with the only oesophageal Cdx2 transgenic, which does not express any intestinal markers (Jianping Kong* 2009). In this situation Cdx2 is driven from the K14 promoter and will consequently only be expressed in
squamous epithelium. It would seem that the tissue fate decision of embryonic oesophagus is made at the point when \( p63 \) is expressed invoking a squamous phenotype. Up until this point \( Cdx2 \) can induce intestinal genes, but afterwards the effect is minimal. Secondly, the columnar oesophageal epithelium found during embryogenesis is likely to represent a more plastic tissue than adult oesophagus and could account for Muc2 and villin induction in following \( Cdx2 \) induction.

Stable transfection of \( CDX2 \) in HET-1A cells induces intestinal genes such as \( Muc2, sucrase isomaltase, villin \) and \( K20 \) (Liu, Zhang et al. 2007). The present study also demonstrates Muc2, K20, villin and \( Cdx1 \) mRNA expression in HET-1A cells infected with \( Cdx2 \) and suggests that our Cdx2 adenoviral construct is correctly translated and is functional. The absence of sucrase isomaltase transcription in adenovirus infected HET-1A, compared with stably transfected cells, could be due to either to differences in the duration of \( Cdx2 \) expression or species variation in transgene. Adenoviral Cdx2 expression is transient and persists for at most 10 days. We also used mouse cDNA to create the adenovirus whereas the human cDNA was used in the stable transfection HET-1A studies.

The difference in intestinal gene induction in the HET-1A but not in oesophageal explants needs some explanation. Although the HET-1A cell line was derived from normal oesophageal epithelium it does not recapitulate the normal oesophageal characteristics. For example it fails to express involucrin as a marker for differentiation in response to calcium and expresses cytokeratin 8, a simple columnar marker (Inokuchi, Handa et al. 1995). We have shown that HET-1A cells do not express p63 or K14 protein (unlike oesophageal explants). HET-1A cells also express Cdx2 in response to demethylation distinct from oesophageal cells (Liu, Zhang et al. 2007). Consequently there are considerable differences between studies on the HET-1A cell line and the explant model presented here. In fact the HET-1A cell line phenotype would be more consistent with gastric epithelium supporting the theory that \( Cdx2 \) is sufficient in this type of cell (gastric, embryonic oesophagus and HET-1A) and not squamous epithelium.
Ectopic Cdx2 induces Muc2 expression in rat primary oesophageal cells (Marchetti, Caliot et al. 2003; Kazumori, Ishihara et al. 2006). We have not observed the same affect of Cdx2 expression on adult mouse oesophageal cultures, suggesting a stability of the oesophageal phenotype.

The K14 positive cells, that mark the basal layer of the oesophageal culture, demonstrate a high degree of Cdx2 expression following adenoviral infection as assessed by immunohistochemistry. From this observation it can be concluded that ectopic expression of Cdx2 within the basal cells of the oesophagus, does not provoke the transcription of intestinal genes. The transcription factor p63 can also be used as a marker for basal cells and interestingly varied from K14 expression (Daniely, Liao et al. 2004). The edge of the outgrowth that surrounded the explant contained predominantly K14⁺ p63⁻ cells, which changed to a K14⁺ p63⁺ pattern closer to the centre of the outgrowth. Interestingly, immediately adjacent to and within the original explant are a subset of cells that were K14⁻ p63⁺, a phenotype that is absent in the native oesophagus (data not shown). These cells are deep to the basal layer, contain all the mitotic epithelial cells and speculatively could represent a ‘prebasal phenotype’ within which the stem cells reside. Given the thickness of the original tissue and the orientation of the p63⁺ cells underneath the basal cells, a consistent level of Cdx2 expression in these cells was not achieved and could account for the lack of effect of Cdx2. In order to address this problem, the explants were cultured in a medium with a low calcium concentration. Calcium is an essential determinant of epidermal keratinocyte proliferation and differentiation (Hennings, Michael et al. 1980; Hennings and Holbrook 1983). Mouse epidermal keratinocytes cultured in media containing less than 0.1mM calcium do not stratify, proliferate rapidly and contain wide intercellular distances (Hennings, Michael et al. 1980; Hennings and Holbrook 1983). Calcium at concentrations higher than 0.1mM provoke a decrease in proliferation and an increase in stratification, terminal differentiation and cell-cell contacts (Hennings, Michael et al. 1980; Hennings, Steinert et al. 1981). Oesophageal cells grown
under low calcium conditions behave in a similar way with increased proliferation, lack of stratification and differentiation. Increasing the calcium concentration provokes the formation of cell-cell contacts and differentiation markers, such as K4. p63 and K14 expression are maintained in 99% of cells surrounding the explants and enables targeting of Cdx2 expression to p63^+ cells. Cdx2 expression within p63^+ cells reduced p63 expression, suggesting an inhibitory mechanism between Cdx2 and p63. Cdx2 and p63 are not co-expressed in any normal cell type and loss of Cdx2 expression during intestinal development and postnatal life results in induction of p63 and the squamous differentiation pathway, consistent with mutual inhibition (Chawengsaksophak, James et al. 1997; Gao, White et al. 2009).

Likewise, Cdx2 induced a columnar marker K8. Two intestinal genes, Muc2 and villin, are induced at low levels by Cdx2 expression in p63^+ cells, but the mRNA is not translated into protein. The difference in response of cells under low calcium conditions, with regards the induction of Muc2 and villin, could be explained by their immaturity or the un-physiological culture method. The immature cells are more likely to interconvert. Likewise the low calcium culture technique results in oesophageal cells without cell-cell contact and could result in altered behaviour due to lack of signalling. It is possible that occasional immature cells under these conditions were able to overcome the inhibition of Cdx2 targets and express these genes. Muc2 and villin protein was not detectable in these cells and the low level of mRNA induced would not be considered as evidence for intestinalisation, in contrast to the HET-1A and embryonic models.

Although evidence from transgenic studies suggests that Cdx2 expression alone provokes IM of the stomach, this is not true in oesophageal cells. These findings are in keeping with two other publications. Immortalised oesophageal cells require overexpression of the cell-cycle regulator cyclin D1 along with demethylating agents before ectopic CDX2 expression provokes the expression of intestinal genes (Kong, Nakagawa et al. 2008). These results suggest that CDX2 provokes cell cycle arrest and that CDX2 target genes are methylated in oesophageal cells. Likewise, a recent transgenic study presented in abstract form, in which Cdx2 is
driven from the K14 promoter, demonstrated a lack of intestinal gene expression in the oesophagus (Jianping Kong* 2009). In this situation Cdx2 will be expressed as soon as the embryonic oesophagus is squamous and within the basal cells post-natally. A similar inability of Cdx2 to induce intestinal genes is seen in human intestinal crypt cells. Unlike rat intestinal crypt cells, human crypt cells do not differentiate to mature intestinal cells in response to Cdx2 expression alone, and it is suggested that this relates to the absence of the transcription factors HNF1α and GATA4 from human intestinal crypt cells (Escaffit, Pare et al. 2006).

The study presented here is the first to compare and contrast the effects of Cdx2 expression in a variety of models of oesophageal epithelium. Cdx2 is not sufficient to provoke intestinalisation in an organotypic model of *in vitro* oesophagus. These findings contrast with the induction of *Cdx1*, *Muc2*, *villin* and *K20* seen in HET-1A cells and *muc2* and *villin* in embryonic oesophageal cultures. It appears that, in contrast to gastric epithelium, factors other than, or in addition to Cdx2 are required to induce an intestinal phenotype in postnatal oesophageal cells. It is not known whether the inability of Cdx2 to reprogram adult squamous cells relates to the inherent ability of Cdx2 to induce an intestinal phenotype or in the accessibility of the target genes.
Chapter 6: Explant culture of human oesophageal biopsies

6.1 Introduction

In Chapter 3, I described a robust *in vitro* explant model of oesophagus that maintains the correct three dimensional organisation and demonstrates the presence of all the expected cell phenotypes. There are significant differences between the human and murine oesophagus and in vitro models for both would be desirable. The first difference is the presence of deep oesophageal glands in the submucosa of human oesophagus (Long and Orlando 1999). Second, human oesophageal lamina propria invaginates into the epithelium forming papillae that are not seen in mouse. Thirdly the human oesophagus is non-keratinising and consists of an increased number of layers of squamous cells. Finally the expression pattern of the transcription factor p63 is limited to a single basal cell layer in mouse but extends into the suprabasal oesophageal cells in humans. In this Chapter I will test the explant culture technique on samples of squamous and Barrett’s epithelium obtained from patients at endoscopy.

6.2 *In vitro* models of human oesophageal mucosa.

In order to determine the molecular sequence of events that convert normal squamous oesophagus to BM, it would be desirable to culture both types of epithelium. Normal human oesophageal explants have previously been cultured for 2-4 weeks, but are commonly overgrown with fibroblasts during this time (Mothersill 1995). The culture model described by these authors was not characterised according to squamous epithelial markers. Other investigators using oesophageal explants obtained at endoscopy have limited their use to days (Chang, Lao-Sirieix et al. 2007). Originally fibroblast culture layers were used to prolong the proliferative potential of primary oesophageal cells obtained from surgical resection, but were still only viable for 4 passages (Compton, Warland et al. 1998). These primary cells expressed K5/K14 and involucrin. Primary cell culture from oesophageal biopsies and BM can be routinely cultured for 2-3 weeks and express the appropriate
cytokeratins K10/13 and K7/20 respectively (Milano, van Baal et al. 2007). Oda described a method for primary culture from surgical specimens which allow for 11 passages of cells with the expression of K13 (Oda, Savard et al. 1998). A long-term robust model of human squamous epithelium would therefore be beneficial.

The first description of in vitro culture of BM was published in 1992 (Garewal, Leibovitz et al. 1992). Garewal described a non-enzymatic technique to establish a primary cell culture from endoscopic biopsies and demonstrated pan cytokeratins, PAS and Alcian blue staining of the cells. In 1994, Washington cultured BM explants and primary Barrett’s cells from resection specimens (Washington, Gottfried et al. 1994). Both explants and primary cultures were maintained in a modified MCDB 153 medium supplemented with fetal calf serum, hydrocortisone, epidermal growth factor, cholera toxin, adenine and bovine pituitary extract. Cells were cytokeratin positive and stained with alcian blue and were passaged 8 times (Washington, Gottfried et al. 1994). Genetic analysis of in vitro BM cells established using a similar technique revealed that long-term culture was statistically more likely from patients with high grade dysplasia, 17p LOH and p53 inactivation (Palanca-Wessels, Barrett et al. 1998). Further refinement of the media (3:1 DMEM:Ham’s F12) is suggested to limit fibroblast overgrowth and cells grown under these conditions exhibit alcian blue and Periodic Acid-Schiff positivity (Khan, Pillay et al. 1997). Only 2 reports have established culture of BM cells, for more than 4 days, from biopsy samples (Khan, Pillay et al. 1997; Milano, van Baal et al. 2007).

6.3 Human squamous oesophagus can be grown using explant culture

Two separate attempts to culture human squamous epithelium were performed (table 6.1). On the first attempt, 45 plastic coverslips were set with approximately 10 explants of tissue on each in supplemented BME. Of these 450 separate explants only 4 attached and established a visible outgrowth of cells (Fig 6.1). Twelve culture dishes became infected despite supplementation of penicillin and
streptomycin. On the second attempt, 4 different culture media were utilised and amphotericin B and gentamicin was used in addition to the normal supplements. Eight plastic coverslips were set with approximately 10 explants per dish in each of the media conditions described above. The total number of explants that grew were: DMEM 2, BME 1, KSFM 0 and RPMI 3 (total 80 per medium). These were cultured for 18 days on etched plastic coverslips and then immunostained for K14. All explants that attached and developed an outgrowth contained K14 positive cells (Fig 6.2). In an attempt to increase the number of explants that grow successfully an alternative explant culture method was attempted. Explants were sandwiched between two coverslips and immersed in the appropriate medium. This method is successful using mouse oesophageal explants. Two explants were sandwiched between a pair of coverslips and cultured in the medium described. For each medium 80% of the explants developed an outgrowth surrounding the explant (Fig 6.3). These cells were K14 positive irrespective of the medium used (Fig 6.4).

**Table 6.1 Patient demographics for Barrett’s and squamous culture.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Barrett’s</th>
<th>Squamous</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>64</td>
<td>1 biopsy</td>
<td>2 biopsies</td>
<td>1 biopsy</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>52</td>
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</tr>
<tr>
<td>3</td>
<td>M</td>
<td>77</td>
<td>2 biopsies</td>
<td>2 biopsies</td>
<td></td>
</tr>
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<td>4</td>
<td>M</td>
<td>72</td>
<td>2 biopsies</td>
<td>2 biopsies</td>
<td></td>
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<td>M</td>
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<td>M</td>
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<td>11</td>
<td>M</td>
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<td>M</td>
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<td>14</td>
<td>M</td>
<td>77</td>
<td>2 biopsies</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.4 Culture of Barrett’s metaplasia biopsy samples.
When cultured on plastic coverslips with BME, four of the 150 explants grew and 5 dishes became infected (Fig 6.5). In order to improve efficiency, amphotericin B and gentamicin was added to the 4 media described above. From 80 explants in each different media a total 3 grew, all from the RPMI cultures. The original cultures grown in BME and the RPMI samples were examined and determined to express the columnar marker K8.
The sandwich biopsy sample technique resulted in 0 explant growths for BM samples.
Figure 6.1. Human squamous oesophageal explant culture.

Phase contrast pictures of human oesophageal explants culture in BME after 5 days (A+B) and 8 days (C). The bright white lines seen in A and B are scratches in the coverslip to help attachment of the oesophageal explants. Scale bars represent 250μm (A) and 125μm (B+C).
Figure 6.2. Cultured human squamous oesophageal cells express K14.
After 8 days of culture in BME (A+B) or DMEM (C+D), oesophageal cells were fixed and stained for K14 expression. (A) Phase contrast image of cells cultured in BME on day 8. (B) K14 positive cells were seen within the outgrowth of cells around the explant. (C) Phase contrast images of cells cultured in DMEM on day 8. (D) K14 positive cells were found within the outgrowth of cells.
Scale bars represent 125μm (A+C) and 100μm (B+D).
Figure 6.3. Morphology of human oesophageal cells cultured in 4 different types of media sandwiched between coverslips. Phase contrast images of cells cultured in (A) DMEM, (B) BME, (C) RPMI and (D) KSFM between 2 coverslips. The cells cultured within RPMI (C) exist as a continuous sheet of cells. In contrast, the cells maintained in the other 3 media were found in isolation.

Scale bars represent 200μm.
Figure 6.4. Human oesophageal cells cultured between coverslips express K14

Immunostaining for K14 expression in human oesophageal cells cultured between 2 coverslips in (A) DMEM, (B) BME, (C) RPMI and (D) KSFM for 7 days. The morphology of cells within each media is grossly similar. Scale bars represent 100μm.
Figure 6.5 Explant culture of Barrett’s metaplasia samples cultured in BME. (A-C) demonstrate phase contrast images of BM biopsies after 8 days of growth. Scale bars represent 250μm (A) and 125μm (B+C).
Figure 6.6 Barrett’s metaplasia explants cultured in BME and RPMI express K8.

BM explants were cultured in either BME (A-B) or RPMI (C) for 8 days and then immunostained for K8 (green). All of the cultures contained cells that were positive for K8. Scale bars represent 250μm.
6.5 Discussion

Using the technique applied to adult mouse oesophageal epithelium, it was possible to grow human squamous and BM biopsy explants obtained at endoscopy. The number of explants that attached and grew were far fewer in the case of human samples (>80% mouse and <5% human). Infection of the culture was more common with human explants but can be controlled with the addition of gentamicin and amphotericin B. Even with the additional of antimicrobials, the level of success was lower in human biopsies. RPMI proved to be the most effective media but the number of viable explants was still <5%. The coverslip sandwich technique increased the number of viable explants when used for squamous epithelium. This method has significant difficulties for processing of the culture for microscopy. When handling the coverslip sandwich it is not uncommon for them to shear and separate detaching the explant. Coverslip sandwiches require additional time to permeabilise and stain using immunohistochemistry and deliver variable results. All of the explants that grew, from BM and squamous tissue, expressed appropriate cytokeratins. Squamous cultures contained K14-positive and BM explants K8-positive cells. It is possible that human explants were not as successful as mouse explants because of the starting material. Murine explants were obtained from full thickness pieces of oesophagus, whereas human material was from a superficial biopsy. Oesophageal biopsies taken at endoscopy commonly do not include the lamina propria. Consequently the poor results may explain a lack or relative lack of oesophageal stem cells and may apply to BM biopsies. A method to test for this would be to use resection specimens to obtain full thickness material. Another important determinant of success is the tissue culture media, an alternative may offer a potential improvement in success. MCDB153 has been used successfully supplemented with fetal calf serum, hydrocortisone, epidermal growth factor, cholera toxin, adenine and bovine pituitary extract to culture BM cells (Washington, Gottfried et al. 1994). Given that a proportion of squamous and BM explants can be cultured it suggests that with refinement of the technique, starting material and culture media, both types of tissue could be available for experimentation.
Chapter 7 – The role of Hepatocyte Nuclear Factor 4α (HNF4α) in Barrett’s Metaplasia

7.1 Introduction

In the previous chapters, I have shown that ectopic expression of Cdx2 decreases markers of squamous differentiation but is not sufficient to induce an intestinal phenotype in an explant culture of normal oesophageal cells. In contrast, Cdx2 induces an intestinal phenotype in HET-1A cells and embryonic oesophagus. These findings would suggest that either additional or alternative factors to Cdx2 are necessary to induce an intestinal phenotype in normal adult oesophageal cells.

There is a weight of evidence to suggest that Cdx2 is involved in the development of IM, but these results suggest that additional factors are required to complete this transformation in the normal oesophagus. These potential factors can be divided into either transcription factors or soluble factors that are required to create a permissive environment in oesophageal cells. Here, I concentrate on the requirement for additional /alternative transcription factors in order to provoke an intestinal phenotype in oesophageal cells.

Two fundamental theories could be proposed to explain the lack of effect of Cdx2 and the requirement of an additional transcription factor. Firstly, Cdx2 may only be sufficient to induce the intestinalisation of columnar epithelium and this would explain the positive results seen in gastric epithelium, embryonic oesophagus and HET-1A cells. Furthermore the developmental relationship of the oesophagus and intestine raises the possibility that the gastric phenotype may be a necessary intermediate in the process. The finding of intercalary regeneration in Cdx2 +/- mice would support this theory (Beck, Chawengsaksophak et al. 1999). If a columnar phenotype is necessary for Cdx2 to induce an IM then transcription factors responsible for the specification of stomach, or alternative anterior foregut derivates, may need to be activated prior to expression of Cdx2.

Secondly, alternative transcription factors may need to be sequentially expressed after Cdx2 initiation in order to induce maturation of intestinal cells. The differentiation of an intestinal stem cell requires the hierarchical set of cell fate
decisions that are made in response to soluble factors and result in the activation of transcription factors. It is possible that Cdx2 may be sufficient to create immature intestinal cells, but require the subsequent activation of transcription factors necessary for differentiated intestinal cells.

Any candidate transcription factor for the development of IM should be present in BM but not normal oesophagus. There is an extensive literature concerning the differential expression of genes in oesophagus and BM.

### 7.1.1 Genes upregulated in Barrett’s metaplasia

There are currently 14 studies examining differential gene expression between BM and normal oesophagus resulting in a wealth of data (Barrett, Yeung et al. 2002; Selaru, Zou et al. 2002; Xu, Selaru et al. 2002; Pohler, Craig et al. 2004; Fox, Sapinoso et al. 2005; Gomes, Esteves et al. 2005; Helm, Enkemann et al. 2005; Kimchi, Posner et al. 2005; van Baal, Milano et al. 2005; Hao, Triadafilopoulos et al. 2006; Ostrowski, Rubel et al. 2006; van Baal, Diks et al. 2006; Wang, Zhan et al. 2006; Greenawalt, Duong et al. 2007; Ostrowski, Mikula et al. 2007). A recent publication revisited 6 of these studies and applied gene set enrichment analysis techniques to identify statistical differences in pre-defined sets of genes (Wang, Qin et al. 2009). As expected, a multitude of genes expressed in the normal intestine were significantly elevated compared to normal oesophagus, such as K8, K20, TFF3, lysozyme, and villin. Key genes involved with the BMP/TGFβ pathway were also upregulated in BM samples suggesting activation of this pathway. Six transcription factors were identified as potentially important in the development of BM, CDX1, CDX2, HNF1α, HNF4α, SOX9, TTF1 and GATA6 (Wang, Qin et al. 2009). I will consider the functional role of each transcription factor in turn in terms of homeotic function in order to select candidate genes. Of the transcription factors that exhibit differential distribution between BM and normal oesophagus, CDX1, CDX2, HNF1α, HNF4α and GATA6 are all important in the differentiation of the intestinal mucosa (Gao, Sedgwick et al. 1998; Boudreau, Zhu et al. 2001; Almeida, Silva et al. 2003; Lussier, Babeu et al. 2008).
The evidence concerning *CDX1* in BM has been previously discussed. Given that *Cdx1* null mice have no alteration to the normal intestinal phenotype and the redundancy between *Cdx1* and *Cdx2* in development, it would seem likely that *Cdx1* would be unlikely to induce an intestinal phenotype in addition to *Cdx2*. 

*Sox9* is expressed in the intestine and colon within the crypt compartment and also in Paneth cells (Bastide, Darido et al. 2007). *Sox9* represses *Cdx2* expression and is thus an unlikely protagonist in the development of BM (Bastide, Darido et al. 2007). Furthermore *Sox9* deletion in the intestinal mucosa only affects Paneth cells (Mori-Akiyama, van den Born et al. 2007). *TTF1* is involved in the separation of the primitive trachea and oesophagus, as well as the development of the thyroid and consequently would not be a logical choice for IM induction (Stainier 2002). 

*GATA6* has an essential role in the differentiation of the primitive endoderm, establishment of bronchial epithelium and regulation of lung specific genes (Morrisey, Tang et al. 1998; Shaw-White, Bruno et al. 1999). *GATA6* activates the promoter of intestinal genes such as lactase-phlorizin (Fitzgerald, Bazar et al. 1998) as well as tissue specific genes in many tissues including heart (troponin C (Morrisey, Ip et al. 1996)), lung (surfactant A (Bruno, Korfhagen et al. 2000)) and blood (von Willibrand factor (Peng and Jahroudi 2003)). *GATA6* is transcriptionally upstream of *HNF4α* and *TTF1* (Morrisey, Tang et al. 1998; Shaw-White, Bruno et al. 1999).

*HNF4α* has an essential early role in specification of primitive endoderm, gastrulation and the expression of many hepatic genes (Chen, Manova et al. 1994; Duncan, Manova et al. 1994). *HNF4α* expression is limited to the liver, stomach, intestine, colon, pancreas and kidney (Sladek, Zhong et al. 1990; Miquerol, Lopez et al. 1994; Briancon, Bailly et al. 2004). *HNF1α* is known to regulate genes in the liver and intestine and is a direct transcriptional target of *HNF4α* (Kuo, Conley et al. 1992; Serfas and Tyner 1993). The spatial distribution of *HNF4α*, its role in intestinal epithelial differentiation and its transcriptional control of *HNF1α* suggest that it may be involved in the development of IM and is discussed in more detail below.
7.1.2 Hepatocyte Nuclear Factor 4 alpha

*HNF4α* is an orphan member of the nuclear hormone receptor family of transcription factors originally identified in hepatoma cells bound to the transthyretin promoter (Sladek, Zhong et al. 1990). Its transcriptional activity is modulated by the binding of long chain fatty acids, although these are not essential for transcription (Hertz, Magenheim et al. 1998). During development, *HNF4α* has a critical role in the developing visceral endoderm and *HNF4α* null mice are embryonic lethal (Chen, Manova et al. 1994). At E8.5, *HNF4α* expression is restricted to the foregut endoderm in the region of the liver bud and is later found in the pancreas and kidney by E10 (Duncan, Manova et al. 1994; Taraviras, Monaghan et al. 1994). Between E10 and E13.5 *HNF4α* continues to be expressed in the liver, stomach, intestine, colon, pancreas and renal tract (Duncan, Manova et al. 1994). *HNF4α* is not required for hepatocyte specification but is essential for a number of genes present in mature hepatocytes such as apolipoproteins (*Apo*), aldolase B, L-type fatty acid-binding protein (LFABP) and transferrin (TFN) (Li, Ning et al. 2000). The liver and intestinal epithelium has similarities given their neighbouring embryonic identity and the common enzyme expression patterns, for example many apolipoproteins and LFABP are expressed in both tissues, suggesting a common requirement for *HNF4α*. Furthermore, *HNF4α* is essential for normal colonic development and selective deletion results in poor crypt/villus architecture, immature goblet cells and a decrease in multiple enterocyte enzymes (Garrison, Battle et al. 2006). At birth *HNF4α* expression is limited to the liver, stomach, intestine, colon, pancreas and kidney (Sladek, Zhong et al. 1990; Miquerol, Lopez et al. 1994; Briancon, Bailly et al. 2004).

7.1.3 *HNF4α* and the intestine

*HNF4α* is expressed in the stomach, intestine and colon and has been shown to regulate many intestinal specific genes. Apolipoprotein IV (*Apo A-IV*), intestinal alkaline phosphatase (ALP) and sucrase isomaltase (SI) are all associated with differentiated enterocytes and are upregulated by *HNF4α* (Archer, Sauvaget et al. 2005; Olsen, Bressendorff et al. 2005; Lussier, Babeu et al. 2008). Computational
analysis of transcriptome data from crypt, villus and fetal intestine revealed that genes associated with differentiated enterocytes had an overrepresentation of HNF4α binding sites within their promoters (Stegmann, Hansen et al. 2006). Furthermore the transcriptional target of HNF4α, HNF1α, has been shown to cooperate with Cdx2 and GATA4 in the regulation of enterocyte-specific genes. HNF1α positively regulates, in concert with Cdx2, SI (Boudreau, Rings et al. 2002), lactase-phlorizin hydrolase (Mitchelmore, Troelsen et al. 2000), calbindin 3 (Wang, Klopot et al. 2004), liver fatty acid binding protein gene (Staloch, Divine et al. 2005) and claudin 2 (Sakaguchi, Gu et al. 2002).

In a co-culture system of human intestinal mesenchyme and a rat intestinal stem cell line (IEC-6), stable transfection of Cdx2 provoked differentiation of cells to an enterocyte lineage (Lussier, Babeu et al. 2008). The onset of differentiation coincided with and was dependent on the induction of HNF4α and HNF1α. HNF4α expression, in stable Cdx2 transfected IEC-6 cells, provoked the expressed of HNF1α, ALP, Apo A-IV, intestinal FABP and guanylate cyclase 2 without the requirement for intestinal mesenchyme co-culture. When IEC-6 cell were co-cultured with intestinal mesenchyme the cells demonstrated a more developed polarization following HNF4α expression, confirming a sequential role for Cdx2 then HNF4α in enterocyte differentiation.

The utility of HNF4α to induce an intestinal phenotype has recently been assessed in mouse embryonic fibroblasts (NIH-3T3) (Babeu, Darsigny et al. 2009). Apo A-IV, Tff3 and villin were induced by stable HNF4α transfection, and the addition of Cdx2 and GATA4 further provoked intestinal FABP, but not Muc2 nor SI (Babeu, Darsigny et al. 2009).

Taken together, the evidence would suggest that HNF4α is required for the normal differentiation of intestinal epithelium and is necessary following Cdx2 activation and this has obvious implications for BM. The finding of HNF4α protein in BM tissue has only been reported once (Piessen, Jonckheere et al. 2007) and the
effect of ectopic expression of HNF4α on primary oesophageal cells or oesophageal explants is unknown. In this chapter I determine the effects of ectopic HNF4α expression alone and in combination with Cdx2 to induce an intestinal phenotype.

7.2 Ectopic HNF4α expression can be established using adenoviral vectors
The HNF4α expression vector, Ad-CMV-HNF4α (a gift from Dr Ramiro Jover, Unit of Experimental Hepatology, Valencia, Spain), was expanded, purified and titered (5.1 x 10^{10}). The optimum number of virus particles to infect explants cultured in MCDB 153 is 5.1 x 10^5 per explant and in BME is 1 x 10^8 per explant. The Hnf4α antibody was optimized using mouse liver sections and villin antibody using mouse intestinal sections (Fig 7.1) and Cdx2 and HNF4α expression was examined in human sections of mouse intestine, human oesophagus and Barrett’s metaplasia. The human samples used in these experiments were archived samples obtained from Dr L Biddlestone at the Royal United Hospital, Bath.

Oesophageal explants do not express endogenous HNF4α (Fig 7.3). Infection of 5 day old oesophageal explant with Ad-CMV- HNF4α (5x10^5 IU/explant) induces the expression of HNF4α protein, detectable with immunohistochemistry on day 3 post infection, in 95.8% of oesophageal cells (Fig 7.3 and Table 7.1). Three days following HNF4α expression in oesophageal explant cells maintained in MCDB153, begin to group closer together and form patches that are elevated off the plastic coverslip (Fig 7.4). These areas are seen in a higher plane of focus on the microscope. Without HNF4α infection, oesophageal cells remain in monolayer without cell to cell contact. Expression of nuclear HNF4α within the epithelial cells cultured in MCDB 153 is confirmed with counterstaining for K14 protein (K14) (Fig 7.5).

Table 7.1 – Number of oesophageal cells expressing HNF4 α three days after incubation with Ad-CMV- HNF4α (5x10^5 IU/explant).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean+/− SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF4α positive cells</td>
<td>84/92(91.3%)</td>
<td>219/222(98.6%)</td>
<td>297/303(97.4%)</td>
<td>95.8%/3.9%</td>
</tr>
</tbody>
</table>
Figure 7.1 Positive controls for villin and HNF4α antibodies.

Villin expression is not seen in mouse oesophageal sections (A), but is present within the brush border region of mouse intestinal sections (B). HNF4α is expressed in a nuclear pattern within mouse sections of liver (C). Scale bars represent 50µm.
Figure 7.2 HNF4α is expressed in Barrett’s metaplasia

Immunohistochemistry of Cdx2 and HNF4α expression in sections of mouse intestine, human squamous oesophagus and human Barrett’s metaplasia. Nuclear expression of Cdx2 is seen in sections of normal intestine (A) and BM (C) but not normal squamous oesophagus (B). The pattern of expression of HNF4α is similar. HNF4α is expressed in the small intestine (D) and BM (F), but not oesophageal epithelium (E).

Scale bars represent 200μm (A), 100μm (B) and 50μm (C).
Figure 7.3 Ad-CMV-HNF4α induces HNF4α expression in oesophageal cells cultured in MCDB 153.
A mouse oesophageal explant has been cultured in MCDB153 for 5 days and then infected with Ad-null (A+C) or Ad-CMV-HNF4α (B+D) for 3 further days and immunostained for HNF4α (red). (A) An Ad-null infected control culture does not express HNF4α (red). (B) HNF4α expression can be seen in the majority of Ad-CMV-HNF4α infected cells. (C+D) higher magnification images demonstrate the nuclear expression pattern of HNF4α (D).
**Figure 7.4 HNF4α provokes clumping of oesophageal cells.**

Oesophageal explants are grown in MCDB 153 for 7 days and infected with Ad-CMV-\textit{HNF4α} for 3 further days and HNF4α (red) expression is examined (A-C). (A) Areas of cells that are in a higher plane of focus are seen (arrow). (B+C) These areas are shown in higher magnification where the brightest cells are elevated from the plastic coverslip (B+C).
Figure 7.5 HNF4α expression in K14-positive cells.
Oesophageal explants are grown in MCDB 153 for 7 days, infected with Ad-CMV-HNF4α for 3 further days and HNF4α (red) and K14 (green) expression is examined (A-C). (A) The majority of cells positive for K14 (green) also express HNF4α (red). (B) Higher magnification image demonstrates that all cells co-express K14 (green) and HNF4α (red). (C) HNF4α (red) can clearly be seen to localize to the nucleus. The results are representative images from 3 separate experiments.
7.3 *HNF4α* provokes the loss of p63 and gain of K8 in oesophageal cells in MCDB 153

Oesophageal explants cultured in MCDB153 were grown for a week and then infected for 12 hours with Ad-CMV-*HNF4α* 5.1 x 10^5 IU per explant. Three days after infection with Ad-CMV-*HNF4α*, multiple cells within the culture have lost expression of the squamous marker p63 and express HNF4α (Fig 7.6, 7.7 and table 7.2). p63 expression is lost in 67.5% of HNF4 expressing cells but persists in 99% of cells in control cultures infected with ad-null (table 7.3). The cells that lose p63 expression following HNF4α infection maintain K14 expression (Fig 7.5). HNF4α expression, achieved under the same experimental conditions, also induces the columnar K8 marker after 3 days (Fig 7.8, 7.9 and table 7.2). All of the cells that are K8 positive co-express HNF4α. Uninfected control cultures demonstrate occasional cells that express K8 (Fig 7.8).

The change in morphology of the *HNF4α* infected oesophageal cells, to form clumps of elevated cells which are closer together (Fig 7.4), suggests that cell to cell contact may be increased. To asses the formation of cell to cell contact the epithelial junction protein, E-cadherin, expression was examined in HNF4α positive oesophageal cells. Cultures were infected as previously described and following 3 days of *HNF4α* expression oesophageal cells are positive for E-cadherin in a membranous pattern, suggesting cell junctions have formed (Fig 7.10 and 7.11). Uninfected control cultures do not express E-cadherin in MCDB 153 media.

Table 7.2 Effect of LPS on number of K8 positive and p63 negative oesophageal cells in MCDB153 media.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Number of K8 positive cells</th>
<th>Number of p63 negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-CMV-null</td>
<td>0.33 +/- 0.58</td>
<td>2.00 +/- 1.00</td>
</tr>
<tr>
<td>Ad-CMV-<em>HNF4α</em></td>
<td>32 +/- 13.1 (p=0.046)*</td>
<td>23.67 +/- 1.52 (p=0.05)*</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 random high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0.
Table 7.3 Number of cells that lose p63 expression following ectopic HNF4α expression. Results from three random fields in three separate experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total p63(^{-}) Cells</th>
<th>Number of HNF4 positive cells losing p63 expression</th>
<th>Number of HNF4 positive cells with p63 expression</th>
<th>Total p63(^{+})</th>
<th>Total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>Ad-HNF4</td>
<td>25</td>
<td>25 / 31 (80.6%)</td>
<td>6</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Ad-HNF4</td>
<td>24</td>
<td>24 / 41 (58.5%)</td>
<td>17</td>
<td>30</td>
<td>54</td>
</tr>
<tr>
<td>Ad-HNF4</td>
<td>87</td>
<td>87 / 137 (63.5%)</td>
<td>50</td>
<td>146</td>
<td>233</td>
</tr>
<tr>
<td>Mean +/- SD</td>
<td>67.5 +/- (11.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 7.6 Fewer oesophageal cells express p63 following HNF4α infection**

Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-null (A) or Ad-CMV-HNF4α (B) for 3 further days. HNF4α (red) and p63 (green) expression is assessed with immunohistochemistry. (A) All oesophageal cells remain positive for p63 (green) following Ad-null infection. (B) A proportion of cells do not express p63 (green) following HNF4α (red) infection (shown with arrow). Cells that co-express p63 and K14 appear orange and are in the minority. The results are representative images from 3 separate experiments.
Figure 7.7 High magnification images demonstrating loss of p63 following HNF4α infection.
Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-CMV- HNF4α (A-C) or Ad-null (D-F) for 3 further days. HNF4α (red) and p63 (green) expression is assessed with immunohistochemistry. (A-C) Representative image from Ad-CMV-HNF4α infected culture in which the colour channels are shown individually to highlight the decrease of p63 expression: (A) DAPI (B) p63 (C) HNF4α.

(D-F) Representative images from Ad-null infected culture in which the colour channels are shown individually to highlight that all cells are p63 positive: (D) DAPI (E) p63 (F) HNF4α. The results are representative images from 3 separate experiments.
Figure 7.8 Induction of K8 following HNF4α expression

Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-null (A) or Ad-CMV-HNF4α (B) for 3 further days and HNF4α (red) and K8 (green) expression is examined with immunohistochemistry. (A) Following Ad-null infection there are no HNF4α (red) positive cells and minimal K8 (green) expression. (B) Ad-CMV-HNF4α induces expression of HNF4α (red) in the majority of cells and provokes K8 (green) expression. The results are representative images from 3 separate experiments.
Figure 7.9 High magnification images demonstrating induction of K8 following HNF4α infection.

Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-CMV-HNF4α (A-C) or Ad-null (D-F) for 3 further days. HNF4α (red) and K8 (green) expression as assessed with immunohistochemistry. (A-C) Representative images from Ad-CMV-HNF4α infected culture in which the colour channels are shown individually to highlight the induction of K8 positive cells: (A) DAPI (B) K8 (C) HNF4α. (D-F) Representative image from Ad-null infected culture in which the colour channels are shown individually demonstrating the lack of K8 positive cells: (D) DAPI (E) K8 (F) HNF4α.
Figure 7.10 *HNF4α* causes the induction of E-cadherin in oesophageal cells cultured in MCDB153.

Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-null (A) or Ad-CMV-*HNF4α* (B) or for 3 further days. HNF4α (red) and E-cadherin (green) are examined with immunohistochemistry. (A) Following Ad-null infection none of the cells express HNF4α (red) nor E-cadherin (green). (B) HNF4α provokes the induction of E-cadherin (green). All cells that express E-cadherin (green) co-express HNF4α (red). The results are representative images from 3 separate experiments.
Figure 7.11 High magnification images of E-cadherin induction following HNF4α infection.

Oesophageal explants are grown in MCDB 153 for 7 days and infected with either Ad-CMV-HNF4α (A-C) or Ad-null (D-F) for 3 further days. HNF4α (red) and E-cadherin (green) expression is assessed with immunohistochemistry. (A-C) Representative image following HNF4α infections demonstrates the induction of E-Cadherin (green). The image has been separated into individual colour channels: (A) DAPI (B) E-cadherin (green) (C) E-cadherin (green) and HNF4α (red). All E-cadherin positive cells express HNF4α. (D-F) Representative images following Ad-null infection demonstrate the absence of E-Cadherin (green). The images have been separated into individual colour channels: (D) DAPI (E) E-cadherin (green) (F) E-cadherin (green) and HNF4α (red).
7.4 HNF4α is sufficient to provoke the induction of intestinal markers in Oesophageal cells

Following 3 days of HNF4α expression, oesophageal explants cultured in MCDB 153 express Tff3 and villin mRNA. If 1 week old oesophageal explants cultured in MCDB153 are incubated with Ad-CMV-Cdx2-hrGFP (5x10⁶ IU/explant) and Ad-CMV-HNF4α (5.1x10⁶ IU/explant) for 12 hours then ALP and villin mRNA are induced (Fig 7.12). Oesophageal explants cultured in BME for 1 week and incubated with Ad-CMV- HNF4α (5x10⁷ IU/explant) also express Tff3, Muc5ac and villin mRNA after 3 days (Fig 7.13).

HNF4α expression, under the same experimental conditions, is sufficient to provoke the expression of villin protein in oesophageal cells cultured in MCDB 153 (Fig 7.13, 7.14 and table 7.4) and BME (Fig 7.15) media. Villin expression is found around the periphery of cells in MCBD153 cultures and is seen as a villus-like border (Fig 7.14). When neighboring cells express villin, their borders seem to be in contact with each other suggesting the formation of cell junctions. Villin expression is only seen in HNF4α positive cells in MCDB 153 and BME cultures. Oesophageal explants cultured in BME and infected with HNF4α demonstrated expression of HNF4α protein in cells located at the periphery of cultures (Fig 7.15) and probably represents an inadequate titre to infect all cells. Villin expression in these cultures is seen in the peripheral cells that co-express HNF4α. If Ad-CMV-Cdx2-hrGFP (5x10⁷ IU/explant) adenovirus is used in addition to Ad-CMV-HNF4α after 3 days oesophageal cells express villin and Alp mRNA (Fig 7.16). Expression of Tff3, Alp and Muc5AC protein was not assessed.
Table 7.4 Number of oesophageal cells expressing villin, assessed by immunohistochemistry, following Ad-CMV-\textit{HNF4a} infection for 3 days.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total number cells</th>
<th>\textit{HNF4a} positive cells</th>
<th>Villin positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>51</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control 2</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control 3</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control mean</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ad-CMV-\textit{HNF4a}</td>
<td>13</td>
<td>13 (100%)</td>
<td>7 (53.8%)</td>
</tr>
<tr>
<td>Ad-CMV-\textit{HNF4a}</td>
<td>48</td>
<td>41 (85.4%)</td>
<td>8 (19.5%)</td>
</tr>
<tr>
<td>Ad-CMV-\textit{HNF4a}</td>
<td>41</td>
<td>38 (92.7%)</td>
<td>17 (44.7%)</td>
</tr>
<tr>
<td>HNF4a mean</td>
<td>34 +/- 18.5</td>
<td>92.7% +/- 7.3%</td>
<td>39.3% +/- 17.8% (p=0.037)*</td>
</tr>
</tbody>
</table>

Data is expressed as mean +/- standard deviation and represents the number of cells for 3 random high power fields for each treatment. Comparisons were analysed using the Mann-Whitney test and calculated with SPSS statistics 17.0.
Figure 7.12 *HNF4α* induces villin and Tff3 in oesophageal cells.

RT-PCR was run at 30 cycles, for intestinal genes.

(A) Oesophageal explants were grown for 7 days in MCDB 153 and infected with either Ad-CMV-GFP, Ad-CMV-*HNF4α*, Ad-CMV-Cdx2-hrGFP or both Ad-CMV-*HNF4α* and Ad-CMV-Cdx2-hrGFP. Expression of intestinal genes was examined using RT-PCR with the following positive results:

1) Ad-CMV-*HNF4α* – *HNF4α*, Villin and Tff3
2) Ad-CMV-*HNF4α* and Ad-CMV-Cdx2-hrGFP – *HNF4α*, Cdx2, villin and ALP

(B) Oesophageal explants were grown for 7 days in BME and infected with either Ad-CMV-GFP, Ad-CMV-*HNF4α* or Ad-CMV-Cdx2-hrGFP. Expression of intestinal genes was examined using RT-PCR with the following positive results:

1) Ad-CMV- *HNF4α* – *HNF4α*, villin and Tff3

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Figure 7.13 \textit{HNF4\textalpha\ provokes villin expression in oesophageal cells cultured in MCDB153}

Oesophageal explants were grown for 7 days in MCDB 153 and infected with Ad-null (A) or Ad-CMV-\textit{HNF4\textalpha\} (B) for a further 3 days. Expression of villin (green) and HNF4\textalpha (red) was assessed using immunohistochemistry. (A) There is no expression of HNF4\textalpha (red) nor villin (green) following Ad-null infection. (B) Ad-CMV-\textit{HNF4\textalpha\} provokes expression of HNF4\textalpha (red) and villin (green) in oesophageal cells. Villin-positive cells always express HNF4\textalpha. The results are representative images from 3 separate experiments.
Figure 7.14 Induction of villin by HNF4α

Oesophageal explants were grown for 7 days in MCDB 153 and infected with either Ad-null (A) or Ad-CMV-HNF4α (B-F) for a further 3 days. (A) Ad-null infected cells do not express villin (green) nor HNF4α (red). (B) An individual cell expressing villin (green) following HNF4α infection. (C) Multiple cells expressing villin following HNF4α infection. The membranous villin (green) appears to be in contact with other cells. (D+E) Villin (green) and HNF4α (red) expression in HNF4α infected cells demonstrate the villus-like border to these cells. (F). Villin expression is limited to HNF4α-positive cells.
Figure 7.15 *HNF4α* provokes villin expression in oesophageal cells cultured in BME.

Oesophageal explants cultured in BME for 7 days and infected with Ad-null (A-C) or Ad-CMV-\textit{HNF4α} (D-F) for a further 3 days. Expression of villin (green) and HNF4α (red) was examined. (A-C) There is no expression of HNF4α (red) nor villin (green) in Ad-null infected cultures. (D-F) HNF4– positive cells are found around the periphery of the culture (D) and villin (green) positive cells are found in this area also (E-F).
Figure 7.16 *HNF4α* and *Cdx2* co-operate to induce ALP expression.

RT-PCR was run at 30 cycles to determine the expression of intestinal genes. Oesophageal explants were grown for 7 days in BME and infected with either Ad-CMV-GFP, Ad-CMV-*HNF4α*, Ad-CMV-*Cdx2*-hrGFP or both Ad-CMV-*HNF4α* and Ad-CMV-*Cdx2*-hrGFP. Expression of intestinal genes was examined using RT-PCR with the following positive results:

1) Ad-CMV-*HNF4α* – *HNF4α*, Villin and Tff3
2) Ad-CMV-*HNF4α* and Ad-CMV-*Cdx2*-hrGFP – *HNF4α*, *Cdx2*, villin and *Alp*
7.5 Discussion
In this chapter I have assessed the utility of HNF4α to induce the conversion of oesophageal cells to intestinal cells. HNF4α is important in early development, in intestinal maturation and has been demonstrated in BM samples, but the effects of ectopic HNF4α expression on oesophageal cells have not previously been tested. Using the adenovirus, Ad-CMV-HNF4α, it is possible to ectopically express HNF4α within oesophageal cells using the explant system described in Chapter 3.

In low calcium conditions (MCDB153), oesophageal explants do not express E-cadherin nor K8, but are positive for the squamous markers p63 and K14. HNF4α provokes the loss of p63 and induces E-cadherin and the columnar marker K8 under these conditions (table 7.1).

The induction of E-cadherin and K8 would be in keeping with the role HNF4α in epithelialisation and tight junction formation. HNF4α has previously been shown to provoke epithelialisation of a dedifferentiated hepatoma cell line (H5) (Spath and Weiss 1998). HNF4α is essential, in co-operation with dexamethasone, for differentiation and expression of K18 and E-cadherin along with the induction of liver genes in H5 cells. Prior to HNF4α expression, H5 cells display a mesenchymal phenotype and are vimentin positive but E-cadherin and cytokeratin negative. Interestingly, K8 induction was not observed in H5 cells but is evident in oesophageal explants (Spath and Weiss 1998). During liver development, HNF4α is essential for epithelialisation and HNF4α null embryonic liver cells lack E-cadherin expression, along with other adherens junction molecules and exhibit large intracellular gaps (Parviz, Matullo et al. 2003; Battle, Konopka et al. 2006).

The role of HNF4α in cell junction formation is further examined in F9 embryonal carcinoma cells (F9). HNF4α induces multiple adherens junction proteins, the formation of functional tight junctions and redistribution of E-cadherin to the membrane in F9 cells (Chiba, Gotoh et al. 2003).
<table>
<thead>
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<th></th>
<th>Control</th>
<th>HNF4α</th>
<th>Cdx2</th>
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<tr>
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<tr>
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<td>Villin protein</td>
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Table 7.1 – summary of effects of ectopic Cdx2 and HNF4α expression on adult mouse oesophageal squamous cells.

In the intestine, E-cadherin is expressed to a higher extent in differentiated enterocytes found in the villus region compared to the crypt (Escaffit, Perreault et al. 2005). Furthermore, E-cadherin expression has been linked to enterocyte function and differentiation. The Apo A-IV gene is only expressed within differentiated enterocytes and is regulated by HNF4α both in vivo and in vitro (Caco-2 cells) (Archer, Sauvaget et al. 2005). When Caco-2 cells are deprived of calcium, cell to cell junctions breakdown and E-cadherin is redistributed away from the membrane (Peignon, Thenet et al. 2006). Under these conditions the expression of Apo A-IV is perturbed and similar results are achieved using an E-cadherin antibody, suggesting that E-cadherin is essential for Apo A-IV expression. Intriguingly the distribution of E-cadherin resulted in a decrease in HNF4α
expression, but not villin, and suggests that E-cadherin may modulate HNF4α expression.

The loss of p63 in oesophageal cells infected with HNF4α would be consistent with the normal tissue distribution of these transcription factors. HNF4α and p63 are not co-expressed in any tissue in vivo and their interaction has not previously been investigated. The mutual inhibition of p63 and HNF4α would be in keeping with their roles during embryogenesis. Both genes are expressed from E8.5 (HNF4α) and E9.5 (p63) in the anterior foregut endoderm during the specification of oesophagus. HNF4α is essential for the normal development of the liver, intestine and colon and continues to be expressed in the stomach. Whereas p63 determines the oesophageal (and tracheal) epithelium. The varying patterns of HNF4α and p63 expression may result from a mutual regulatory mechanism, but their relationship has not been reported. It is interesting to observe that K14 expression persists within HNF4α positive cells. This observation may be explained by the half life of the K14 protein which persists after p63 has been inhibited and K8 induced. Our data suggests that further studies are required to address the relationship between HNF4α and p63.

Ectopic expression of HNF4α in primary oesophageal cells has not previously been reported, but the evidence from this Chapter suggests that it is sufficient to induce a columnar phenotype. Furthermore the current study demonstrates for the first time the ability of HNF4α to induce intestinal genes in oesophageal cells cultured in MCDB153 or BME. HNF4α was sufficient to provoke the induction of villin and Tff3, and in combination with Cdx2, Alp was also induced. These markers were all robustly detected using RT-PCR and villin was confirmed with immunohistochemistry. Most studies have concentrated on the role of HNF4α in the regulation of hepatic genes and have shown that HNF4α binds to 12% of hepatocyte promoters and is an important regulator of hepatic lipid metabolism (Watt, Garrison et al. 2003; Odom, Zizlsperger et al. 2004). There are similarities in
hepatocytes and enterocytes because both are columnar epithelium concerned with metabolism of lipid and carbohydrate.

The ability of \textit{HNF4α} to induce aspects of an intestinal phenotype in non-intestinal/non-hepatic cells has been demonstrated in NIH-3T3 (mouse embryonic fibroblasts) and MIA PaCa-2 (human pancreatic adenocarcinoma) cell lines. Stable, retrovirally induced \textit{HNF4α} expression provoked the induction of \textit{Apo A-IV} and \textit{villin} in both cell lines as well as \textit{Tff3} mRNA in fibroblasts (Babeu, Darsigny et al. 2009). The translation of any of these transcripts into protein was not assessed. The induction of \textit{Tff3} and \textit{villin} mRNA in oesophageal explants is in keeping with these findings, although villin protein is demonstrated in explants. \textit{HNF4α} has also been shown to induce villin protein and microtubule formation in F9 cells (Chiba, Sakai et al. 2006). \textit{HNF4α} is known to bind to the promoter of Intestinal FABP in a non-intestinal cell line, HeLa (Klapper, Bohme et al. 2007). In the same cells \textit{HNF4α}, in co-transfection experiments increases ALP promoter activity (Olsen, Bressendorff et al. 2005).

In oesophageal explants cultured in MCDB 153 and BME \textit{Cdx2} and \textit{HNF4α} cooperate to induce the expression of \textit{Alp}. The co-operation of \textit{Cdx2} and \textit{HNF4α} in enterocyte differentiation has been demonstrated in 2 recent studies. A rat intestinal embryonic cell (IEC-6) line with stable expression of \textit{Cdx2} does not spontaneously differentiate in culture. However with forced \textit{HNF4α} expression, these cells express \textit{Apo A-IV}, \textit{ALP}, intestinal \textit{FABP}, guanylate cyclase 2c and \textit{HNF1α} mRNA (Lussier, Babeu et al. 2008). These findings confirm the requirement for \textit{Cdx2} and \textit{HNF4α} for \textit{Alp} expression. IEC cells, with stable \textit{Cdx2} expression, cultured on intestinal mesenchyme begin to spontaneously differentiate and this was shown to coincide with the onset of \textit{HNF4α} induction. Furthermore, forced \textit{HNF4α} induction in this situation led to an increased polarization, a microvillus brush border formation and apical basal transport (Lussier, Babeu et al. 2008). The combination of \textit{HNF4α}, \textit{Cdx2} and GATA4 expression in a fibroblast cell line resulted in the additional induction of only one
gene, Intestinal FABP, to that which HNF4α alone provoked, Apo A-IV and Tff3. In postnatal life, villin expression is limited to the intestine, proximal tubules of the kidney, biliary ducts, pancreatic duct and epididymis (Maunoury, Robine et al. 1992). The degree of intestinalisation associated with HNF4α and HNF4α in combination with Cdx2 is not known. An indication of this could be inferred by performing a microarray of oesophageal explants infected with either or both of these constructs in comparison to uninfected cells. The exact roles of HNF4α and Cdx2 need to be further explored with infection of oesophageal cells using varying titers of each virus, for various periods of time and in different orders. In the present experiments, both genes were expressed at the same point and it is possible that altering the order of expression will increase co-operation in an intestinal phenotype. Further evidence from in vivo transgenic studies with oesophageal expression of HNF4α and/or Cdx2 would help to untangle the extent to which an IM can be provoked. It is of course possible that other transcription factors may be required for a complete IM in the oesophagus, such as GATA4, GATA6 and Cdx1. All of these genes have been shown to co-operate with Cdx2 or HNF4α in intestinal cells. Likewise HNF1α is involved in the transcription of many intestinal genes and is a target of HNF4α. HNF1α expression in oesophageal cells infected with HNF4α was not assessed.

In summary, HNF4α provokes the expression of E-cadherin, K8, villin and Tff3 in oesophageal cells, and in combination with Cdx2 also induces Alp. The induction of E-cadherin, villin and Tff3 has been demonstrated previously in cell lines, but not primary, nor oesophageal cells. K8 and Alp induction by HNF4α has not been seen before outside of intestinal cell lines. The resultant phenotypic change that accompanies ectopic HNF4α alone or in combination with Cdx2 expression is consistent with that seen in the development of BM:

1) Loss of p63 expression
2) Induction of K8 expression
3) Induction of the brush border protein villin, as a marker of differentiated enterocytes
4) Induction of Alp – found in differentiated enterocytes
5) Induction of Tff3 – found in goblet cells.

These results leave some outstanding questions concerning HNF4 and BM.
1) Does HNF4α actually reprogram squamous oesophageal cells or just activate individual target genes?
2) Does the combination of ectopic Cdx2 and HNF4α enhance the intestinal phenotype more than HNF4α alone?
3) Are additional genes required in addition to Cdx2 and HNF4α?
4) Are the effects of HNF4α specific to oesophageal cells or will the same effects exist on multiple cell types.
Chapter 8  Final discussion and future work

The work described in this thesis described the development and characterisation of an *ex vivo* model of mouse squamous oesophageal epithelium. I used this model to investigate candidate soluble factors and transcription factors in the pathogenesis of BM. The aim of this research was to gain a more detailed understanding of the molecular steps (and their provocateurs) that lead to the loss of a squamous and development of a columnar/intestinal phenotype in the oesophagus. The candidate factors tested were chosen from the literature relating to (i) BM, (ii) control of Cdx2 expression, and (iii) understanding the developmental biology of the gastrointestinal tract. Three specific transcription factors, *p63*, *Cdx2* and *Hnf4α*, have been highlighted for their homeotic roles in oesophagus and intestine.

8.1 The explant model of oesophagus provides a new tool for understanding oesophageal pathobiology. It is viable long-term and fully representative in cell phenotype and three dimensional architecture of the oesophagus *in vivo*. Furthermore, it is the first time that *p63* and loricrin have been identified in an *in vitro* model of squamous oesophagus. The spatial orientation of oesophageal markers within the explant model offers an insight into normal biology. The stem cells of this model presumably reside amongst the p63+ K14- cells within the original explant because mitotic epithelial cells are only ever seen in this region and differentiated cells are found in increasing frequency away from the bud. This observation is in keeping with the theory that *p63* is a gate keeper for oesophageal stem cells. Further work using modern label retaining techniques, such as brainbow, will offer further insights into oesophageal stem cell biology and control of differentiation.

The ability to isolate a homogeneous monolayer of differentiation arrested basal cells, with manipulation of the calcium concentration, offers a complementary explant model. The advantages of oesophagus cultured under these conditions are
increased transgene expression efficiency (using adenovirus) and a more sensitive test for observing phenotypic change. The p63\(^+\) K14\(^+\) positive cells that arise under such conditions are in keeping with the transit amplifying cells of the native oesophagus. These cells reach senescence in culture in less than 4 weeks confirming that p63 expression per se is not sufficient to maintain a stem cell phenotype. This observation suggests that in low calcium concentration conditions the stem cell niche is lost, presumably relating to a lack of cell to cell contact and loss of soluble factors. The nature of the signal that maintains stem-ness in the explant model is unknown, but the observation that cells in isolation senesce raises the possibility that it either arises in neighbouring epithelial cells or within mesenchymal fibroblasts. Further characterisation of the growth kinetics of both explant model systems will offer insights into the oesophageal stem cell. Likewise, further examination of the p63\(^+\) K14\(^-\) cells located in the explant may help determine the significance of these cells in terms of their progenitor status. Given their potential to become intestinal cells, it is also possible that under the appropriate conditions, the cells may generate other endodermal cell types.

8.2 The origins of Barrett’s metaplasia
The deep oesophageal glands have been proposed as the cellular origin of BM. Laser capture microdissection techniques have enabled precise mapping of specific genetic mutation within BM and adjacent epithelium. These techniques have identified BM in continuation with an oesophageal gland both of which contain the same single point mutation in p16 (Leedham, Preston et al. 2008) In contrast neosquamous islands, also arising from oesophageal glands, were found to be wild-type. This evidence has been interpreted to suggest that oesophageal glands can give rise to BM, if mutant, and also squamous islands if wild type. The apparent ability of Cdx2 to intestinalise columnar mucosa and not squamous epithelium would support this theory. There are however problems in attributing all oesophageal IM to deep oesophageal glands. Firstly, the lack of oesophageal glands in rodents requires an additional mechanism to explain the rat model of BM. The molecular steps that result in IM in rodent oesophagus, in the context of reflux,
can presumably occur in human BM. Secondly, the finding of identical point mutations in continuous oesophageal ducts and BM could be explained by a retrograde transformation of phenotype resulting from a mutation in the intraepithelial stem cell. Thirdly, the distal oesophageal duct lining remains squamous even in the context of BM (Coad, Woodman et al. 2005). If the gland was responsible for the replacement of squamous epithelium with columnar cells then it would seem logical to occur within the squamous duct as well. Finally, isolated islands of BM within squamous epithelium are not reported, but would be expected if gland stem cells are responsible.

The work presented in this thesis would suggest that a two-hit hypothesis is required to convert a resident oesophageal stem cell to an intestinal phenotype. First, the acquisition of a columnar phenotype either in response to inflammation, ATRA, loss of p63 or the induction of HNF4α. Secondly intestinalisation in response to induction of Cdx2. The first step in which a columnar phenotype is achieved may be represented by the appearance of columnar mucous secreting cardia-type mucosa within the oesophagus. Cardia mucosa or oxynto-cardiac mucosa is a common finding in the context of reflux disease and its length relates to the severity of GORD (Oberg, Peters et al. 1997; Chandrasoma, Lokuhetty et al. 2000). Although cardia mucosa is often considered to be a normal finding in the distal oesophagus, it is absent in a significant proportion of the population and is proposed to always represent a pathological finding (Chandrasoma 2005). In post-mortem examination and endoscopies on normal and symptomatic patients cardiac mucosa is absent in 56%, 65% and 38% respectively (Jain R 1998; Chandrasoma, Der et al. 2000; Marsman, van Sandick et al. 2004). Cardiac mucosa, which arises in the context of chronic reflux, and is of greater length with increasing GORD, has the potential to progress into IM in response to Cdx2 and Hnf4α. If cardiac mucosa is the origin of BM, where does this epithelium arise? The migration theory of a gastric stem cell repopulating the oesophageal epithelium cannot be dismissed. If BM is a two-step process a columnar epithelium arising from the proximal gastric region this would explain the observation that BM is always continuous with the
GOJ and the resultant tissue is likely to intestinalise in response to Cdx2. Experiments in canine oesophagus in which columnar tissue could be provoked under reflux conditions even in the presence of an intact ring of squamous epithelium at the GOJ would suggest that the cell of origin of BM is resident in the oesophageal mucosa (Gillen, Keeling et al. 1988). Once an area of columnar of intestinal mucosa has arisen it would compete with adjacent mucosa for clonal expansion. Laser capture studies have demonstrated a degree of genetic heterogeneity in neighbouring BM crypts in point mutations in p16 and p53.

8.3 The role of Cdx2 in Barrett’s metaplasia
Cdx2 has been firmly implicated in BM and is sufficient to provoke gastric IM. A recent publication, using a rodent model of oesophageal IM, has demonstrated that Cdx2 is expressed in squamous epithelium near reflux ulcers, multilayered epithelium and IM suggesting an early role in disease pathogenesis (Ingravallo, Dall’olmo et al. 2009). The work described in Chapter 5 determined that Cdx2 is not sufficient, in an oesophageal explant model, to induce an intestinal phenotype in oesophageal cells. To add scientific weight to and further understand this negative finding, Cdx2 was also expressed in HET-1A cells and embryonic oesophageal cultures. The induction of Cdx1, K20, Muc2 and villin, and Muc2 and villin in HET-1A and embryonic oesophagus is in keeping with the literature as previously described. Furthermore, these results confirm that the both Cdx2 adenoviral constructs produced functional Cdx2 protein excluding translational problems as a reason for its ineffectiveness. The lack of effect of ectopic Cdx2 protein, even on direct transcriptional targets, demonstrates that additional factors are necessary to induce intestinal genes. Within oesophageal cells in culture, Cdx2 targets must be inactive, which could be related to gene promoter methylation or histone modifications. Further work using methylation-specific PCR and CHiP analysis would help to explain the lack of effect of Cdx2 on target genes.
Cdx2 expression produced very mild effects on oesophageal cells cultured in MCDB 153, such as loss of p63, occasional gain of K8 and also low level induction of Muc2 and villin mRNA.

When all of the experiments expressing ectopic Cdx2 are considered together, there is an obvious difference between the cell types that do and do not intestinalise in response to Cdx2; their columnar phenotype. HET-1A cells, although originally oesophageal in nature, express K8, embryonic oesophagus is columnar lined and gastric mucosa is a glandular columnar type. The conversion of stratified squamous oesophageal epithelium may require an intermediate columnar step in order for Cdx2 to induce IM. The acquisition of a non-intestinal columnar phenotype could be compared to the cardia-type mucosa that has been proposed as an intermediate in BM. In Chapter 4 a variety of substances, ATRA, LPS and TNFα induced a columnar phenotype in oesophageal cells. Whilst retinoic acid is a potent morphogen and controls epithelial differentiation, LPS and TNFα are inherently linked to the inflammatory pathway via NFκB. A two hit hypothesis for the conversion of oesophagus to intestine would suggest that a columnar phenotype develops first, potentially as a result of inflammation and/or ATRA effect prior to the induction of Cdx2 and intestinal genes. The temporal co-operation between factors that induce a columnar phenotype and ectopic Cdx2 expression would be important to investigate further using explant culture.

The results relating to ectopic Cdx2 expression confirm the findings of one other group, published in abstract, demonstrating no intestinalisation of ectopic Cdx2 expression in the oesophagus driven from the K14 promoter.

8.4 HNF4α and Barrett’s metaplasia

In chapter 7, I demonstrated that ectopic HNF4α expression is sufficient to intestinalise oesophageal cells. HNF4α has not previously been expressed in oesophageal cells and has only been identified in BM once, but there are several lines of evidence suggesting it is involved. Embryologically the liver, stomach, oesophagus and proximal duodenum all develop from the foregut endoderm and
the liver and intestine have overlapping roles in adult life, e.g. lipid metabolism. The temporal and spatial expression pattern of HNF4α during development would be in keeping with a role as a master switch gene for the gastrointestinal tract posterior to the oesophagus (i.e. the oesophagus does not express HNF4α). HNF4α is known to induce a columnar epithelial phenotype, induction of K8 and E-cadherin, in dedifferentiated hepatoma cells. Likewise, HNF4α provokes the intestinal genes, Apo A-IV, villin and Tff3, in fibroblasts. Our results show for the first time that HNF4α induces a columnar and intestinal phenotype in oesophageal cells (K8, villin and Tff3). Villin is a component of the brush border and found on enterocytes and goblet cells and Tff3 suggests the induction of a goblet cell lineage. The molecular mechanisms or reflux components responsible for HNF4α expression in the context of BM are unknown and warrants further work. It is not known how often BM is associated with HNF4α expression or if HNF4α is found in oesophagitis. Likewise, the extent of the induction of intestinal genes following HNF4α expression could be further characterised using micro-array technology. The effect of oesophageal expression of HNF4α in vivo could be studied using a transgenic approach. HNF4α further co-operates with Cdx2 for the induction of the enterocyte specific intestinal Alp.

It is important to determine the sequential role of Cdx2 and HNF4α in the induction of IM. It is possible to argue this either way. HNF4α may induce a columnar/gastric phenotype within which Cdx2 functions to intestinalise. Alternatively, Cdx2 may specify progenitor intestinal cells which require HNF4α to allow maturation of intestinal cells in keeping with its expression pattern and promoter targets. It is conceivable that HNF4α has a dual function in the initial genesis of BM prior to Cdx2 induction and then again later to mature intestinal phenotype. Developmentally, both genes are expressed at a similar time point, but with different distribution. HNF4α is expressed in the stomach, liver, intestine and colon whereas Cdx2 is restricted to the intestine and colon. The oesophagus does not normally express either HNF4α or Cdx2 in contrast to the stomach which only lacks Cdx2. This master switch differential may explain the utility of only Cdx2 to induce
gastric or columnar cells to intestine, but not oesophageal cells. If this is the case, the order of transcription factors may not be important. The stem cells of BM are unknown but a two hit hypothesis requires that the expression of ectopic transcription factor is maintained. If this memory resides in an oesophageal stem cell the inhibition of p63 by HNF4α and Cdx2 suggests that p63 would be absent in these cells. How the newly acquired phenotype competes and expands within the squamous epithelium is unknown. The sequence of HNF4α and Cdx2 expression at various intervals and the effect on intestinal genes within explants could explain their relative roles.

The control of tissue identity during embryogenesis implicates certain transcription factors with a role in BM. Conditional loss of Cdx2 results in an anterior homeosis of the GI tract epithelium. Squamous oesophageal transcription factors, such as p63 and Sox2 are expressed within the intestinal domain. Furthermore, the integral intestinal transcription factors Cdx1, HNF1α and HNF4α are downregulated. The combination of Cdx2, Cdx1, HNF1α and HNF4α transcriptionally control a large proportion of the intestinal transcriptome. Given that Cdx2 and HNF4α are transcriptionally upstream of Cdx1 and HNF1α respectively it is not surprising that this combination of transcription factors reprogrammes oesophageal cells. It would seem logical that the squamous transcription factors p63 and Sox2 need to be inhibited in order for Cdx2 and HNF4α to exert an effect. The role of Sox2 in BM is largely unexplored.

In summary, Cdx2 is not sufficient to provoke an IM in oesophageal cells. HNF4α induces markers of a columnar intestinal phenotype and further co-operates with Cdx2 in order to induce Alp. The relationship between Cdx2 and HNF4α in inducing an intestinal phenotype needs further investigation. Transgenic studies provoking oesophageal expression of HNF4α and Cdx2 would complement the in vitro findings presented in Chapter 7.
References

"ISD online."
"Northern Ireland Cancer Registry."


van den Akker, E., S. Forlani, et al. (2002). "Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation." Development 129(9): 2181-93.


