The role of HNF4α in Barrett’s metaplasia development

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A thesis submitted for the degree of Doctor of Philosophy

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Abstract

Barrett’s metaplasia (BM) is the condition whereby the stratified squamous epithelium at the lower end of oesophagus is replaced by intestinal-like columnar epithelium. BM is associated with chronic cell injury caused by acid and bile salts refluxing from the stomach (so-called Gastro-oesophageal reflux disease (GORD)). BM is important clinically as it is the only known precursor to oesophageal adenocarcinoma. Metaplasia is associated with the aberrant expression of key (master switch) transcription factor(s). It is therefore possible that intestinal transcription factors such as those involved in regulation of intestinal gene expression may be involved in the development of BM. In addition, the development of BM may be linked to exogenous factors such as acid, bile salts and inflammatory mediators, which leads to an inflammatory response and the premalignant condition. Therefore, the aims of the current project are three-fold to investigate (i) the role of HNF4α in the development of BM, (ii) the role of HNF1α, CDX2, FOXA2 and PDX1 in the development of BM and (iii) the effects of exogenous factors involved in the inflammatory response and the Wnt and Notch pathways on HNF4α and CDX2 expression in the development of BM. The data showed that ectopic HNF4α, CDX2 or HNF1α expression induces intestinal gene expression in the human normal oesophageal epithelial HET1A cells, but FOXA2 or PDX1 ectopic expression did not provoke intestinal gene expression. Furthermore, ectopic HNF4α expression in mouse oesophageal epithelial explants induced the squamous-to-columnar cell type conversion as well as VILLIN protein expression. These results suggest that ectopic expression of HNF4α, CDX2 and HNF1α might be involved in the initiation of BM. Finally, exogenous factors involved in the inflammatory and the Wnt and Notch pathways treatment failed to induce intestinal gene expression in the HET1A cells, but further studies such as extending the treatment time and the use of the 3D organotypic models or animal models could be tested.
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<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>5’Aza</td>
<td>5’-Azacytidine</td>
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<tr>
<td>AcH3</td>
<td>Histone 3 acetylation</td>
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<td>Akp3</td>
<td>alkaline phosphatase 3</td>
</tr>
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<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<td>APO</td>
<td>Apolipoprotein</td>
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<td>BM</td>
<td>Barrett’s metaplasia</td>
</tr>
<tr>
<td>BME</td>
<td>Basal Medium Eagle</td>
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<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Cyclin dependent kinase 1</td>
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<td>CDX</td>
<td>Caudal-type homeobox</td>
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<td>CEBP/β</td>
<td>CCAAT enhancer binding protein β</td>
</tr>
<tr>
<td>ChIP-chip</td>
<td>Chromatin Immunoprecipitation-chip</td>
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<tr>
<td>CIAP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CKI</td>
<td>Casein kinase I</td>
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<td>COX2</td>
<td>Cyclooxygenase 2</td>
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<td>CsCl</td>
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<td>CYP3A4</td>
<td>Cytochrome P450 3A4</td>
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<td>4',6-diamidino-2-phenylindole</td>
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<td>N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-buty1 ester</td>
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<td>Dibenzazepine</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s medium</td>
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<td>DNMTs</td>
<td>DNA methyltransferases</td>
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<td>DppIV</td>
<td>Dipeptidyl peptidase IV</td>
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<td>Dithiothreitol</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>EMR</td>
<td>Endoscopic mucosal resection</td>
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<td>Fabp1</td>
<td>Fatty acid binding protein 1</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>iFabp</td>
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<td>MAPK</td>
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<td>NICD</td>
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<td>Trefoil factor 3</td>
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<td>Transforming growth factor β</td>
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<td>Toll-like receptor 4</td>
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<td>Melting temperature</td>
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Chapter 1
Introduction
1.1 Anatomy and development of the mouse oesophagus

1.1.1 Oesophagus anatomy

The oesophagus is a tube-like organ which connects the pharynx and stomach. Food from the oral cavity is delivered through the pharynx and the oesophagus to the stomach for digestion. The gastrointestinal contents can also be pushed out from stomach and intestine under vomiting and reflux conditions (Kuo & Urma, 2006). The adult mouse oesophagus is a multi-layered structure, composed of stratified squamous epithelium surrounded by connective tissue layers: lamina propria, muscularis mucosa, submucosa, muscularis externa and adventitia, from the innermost to the outermost layer (Gartner & Hiatt, 2000).

There are differences between human oesophageal epithelium and mouse oesophageal epithelium. For example, there are more cell layers in human oesophageal epithelium than in the mouse oesophageal epithelium. In addition, unlike mouse, human oesophageal epithelium lack a cornified layer (Seery & Watt, 2000). Furthermore, human (but not mouse) oesophageal epithelium displays a papillae structure (the folding by submucosal projections) and also contains mucosal and submucosal glands, (Croagh et al., 2008; Squier & Kremer, 2001; Seery & Watt, 2000).

1.1.2. Oesophageal epithelium development in mouse

In the mouse, the oesophagus is derived from the foregut endoderm at around embryonic day 9.5 (E9.5) (Wells & Melton, 1999). At this stage, the oesophageal epithelium is a single layer of columnar epithelium expressing the columnar cell marker, cytokeratin 18 (K18). The epithelial cells gradually differentiate and then become stratified (multiple layers) squamous epithelium. At about E15.5, the oesophageal epithelium is lined by the stratified squamous epithelial cells which express markers such as K5 and K14 at the basal region, and K1 and K10 at the suprabasal epithelium. This replacement of columnar cells by squamous cells is considered as a direct cell type conversion (transdetermination). At birth, a cornified layer develops in mice at the outermost layer of epithelium and the epithelium consists of cells with the expression of the different intermediate filaments (cytoskeletal components) depending on the differentiated stage (Fig 1.1) (Moll et al., 1982; Yu et al., 2005).
Figure 1.1. Intermediate filament and p63 expression in the mouse oesophageal epithelium

The Figure illustrates the different intermediate filament expressed in the mouse stratified squamous oesophageal epithelium. Oesophageal epithelial cells differentiate from the cells at the basal region and migrate toward the surface of the epithelium. Cells at basal region express the squamous transcription factor p63. During the terminal differentiation, cells express the different types of intermediate filaments. (Figure is modified from Colleypriest, 2010).
1.2. Anatomy and development of the mouse small intestine

Following gastrulation in the mouse, the pseudostratified endoderm forms and then develops into the intestinal epithelium at about E14.5. Cytodifferentiation starts between E14.5 and E17.5. During cytodifferentiation, the pseudostratified endoderm develops into a single layer of columnar cells. In addition, the villi are also formed at this stage (Fig. 1.2). The region between villus (intervillus region) then invaginates and becomes a crypt-like structure. At the adult stage, the small intestine epithelium is shown in villus-crypt architecture with single layer of columnar cells (Sancho et al., 2004; de Santa Barbara et al., 2003; Totafurno et al., 1987).

The main function of the small intestine is to aid in the digestion and absorption of food. The small intestinal epithelium can be divided into two regions: the villi (finger-like protrusions) and crypts (found at the base of the villi). Intestinal stem cells are thought to be located in the crypt region and can differentiate into 4 main types of intestinal epithelial cells including enterocytes, enteroendocrine cells, Goblet cells and Paneth cells. Enterocytes comprise about 80% of intestinal epithelial cells and contribute to nutrient absorption. Enteroendocrine cells secrete hormones e.g. serotonin, substance P to regulate intestinal functions such as intestinal movement, homeostasis and secretion. Goblet cells produce mucus to prevent microbial invasion and to protect the intestinal epithelium from the proteolytic activity of digestive enzymes. Paneth cells secrete antimicrobial protein and peptides including α-defensin, lysozyme and cryptidins to against microbial invasion. Therefore Paneth cells play a critical role in the gut innate immune response (Schonhoff et al., 2004; Stappenbeck, 2009).

In addition to the 4 main cell types, M cells and Tuft cells are also found in the intestinal epithelium. M cells are found on the luminal surface of the mucosal lymphoid follicles. M cells sample antigen from the gut lumen and transport antigen to the immune cells (Jang et al., 2004). Tuft cells account for only 0.4% of intestinal epithelial cells and Tuft cell function remains to be discovered although they are believed to secrete prostaglandins (Gerbe et al., 2012).
In the adult, the small intestinal epithelial cells renew every 5 to 7 days. Stem cells located at the crypts are considered as the origin of epithelial cells of small intestine (Marshman et al., 2002). As the intestinal epithelial cells differentiate, cells including enterocytes, enteroendocrine cells and goblet cells migrate from the crypt to villus region while Paneth cells inhabit the crypt region (Gartner & Hiatt, 2000). The Notch and Wnt pathways have been suggested to determine intestinal cell fate. Notch signalling contributes to the differentiation towards the absorptive lineage (enterocytes), while Wnt signalling leads to the secretory cell types (Paneth cells, enteroendocrine cells and goblet cells) (van der Flier & Clevers, 2009).

1.3. Metaplasia and Transdifferentiation
Metaplasia is defined as “the transformation of one tissue type into another” whereas transdifferentiation is more restricted to the phenomenon of “the transformation of one differentiated cell type into another”. The transformation process in metaplasia might include the conversion of tissue-specific stem cells, progenitor cells or differentiated cell lineages, while transdifferentiation is a subtype of metaplasia which refers to the direct conversion between differentiated cell types (Slack, 2007; Eberhard & Tosh, 2008).

One of the current models explaining the underlying molecular mechanisms of metaplasia suggests that the activation (or inactivation) of certain key transcription factors is critical to the switch in phenotype. At the embryonic stage, the expression of the key transcription factor(s) affect the transcription of downstream genes, so gives the tissue identity with its unique gene expression profile (Fig. 1.3). However, in the adult tissue, the expression profile of the key (master switch gene) transcription factors might be altered due to a genetic mutation or changes in environmental factors (which then alters gene expression). Thus, either the induction of the new transcription factor or the loss of the original transcription factor might change gene expression pattern and induce a new cell type. For example, CEBP/β (CCAAT enhancer binding protein β) is a transcription factor expressed in liver, but is not expressed in the pancreas. When CEBP/β is introduced into the pancreatic exocrine cell line AR42J-B13, this leads to a pancreatic-to-hepatic cell type switch (Shen et al., 2000).
**Figure 1.2. The development of intestinal epithelium**

The embryonic small intestinal epithelium initially forms stratified epithelial epithelium which then develops into single layer epithelium with finger-like villi structure at late fetus stage. Subsequently, villi elongate and crypts are formed by invagination and the villus-crypt units are finished as adult small intestine feature. (Figure is modified from de Santa Barbara et al. 2003)
Figure 1.3. Concept of metaplasia

(a). The expression and of transcription factor X determines the tissue type between neighbouring regions during embryogenesis. (b). In postnatal life, loss of transcription factor X leads to metaplasia (Figure modified from Slack, 2007).
Metaplasia in postnatal life is usually associated with pathological conditions. For example, Barrett’s metaplasia is the conversion of the stratified squamous cells at the lower end of the oesophagus to columnar intestinal cells and is linked to gastro-oesophageal reflux disease. Barrett’s metaplasia has been recognised as the only known precursor of oesophageal adenocarcinoma (Badreddine & Wang, 2010) (see section 1.4 for further details). Another example in which metaplasia appears is Crohn’s disease. Crohn’s disease is a chronic inflammatory bowel disease and gastric metaplasia can be found in the sites of ulceration associated with the disease (Kushima et al., 1997; Kaneko et al., 2008). Given that metaplasia may be related to stimuli from the cellular environment, studying the mechanisms underlying the initiation and progression to metaplasia could benefit disease diagnosis and aid in treatment. For example, identifying markers which are involved in the formation of metaplasia could be applied to disease diagnosis (Shen et al., 2004). Understanding the factor(s) that initiate metaplasia might also help to manage disease progression by avoiding risk factors. In addition, understanding the mechanisms underlying the development of metaplasia is also crucial for developing cell therapy treatments in regenerative medicine. As the shortage of organs and tissues for transplantation has been an issue, converting one cell type to another cell type to produce the desired cells could be a strategy for tissue regeneration and cellular therapy (Shen et al., 2004).

1.4. Barrett’s metaplasia (BM)

1.4.1. What is Barrett’s metaplasia?
Barrett’s metaplasia (BM) is the pathological condition in which the normal stratified squamous epithelium is replaced by columnar type epithelium at the distal end of the adult oesophagus. The columnar cells display characteristics of small intestinal epithelium (Shaheen & Richter, 2009). BM has clinical importance as it is the only known precursor to oesophageal adenocarcinoma (Thrift et al., 2012). The disease progression is from normal oesophageal, oesophagitis, BM, dysplasia to oesophageal adenocarcinoma (Jankowski et al., 2000; Conio et al., 2002). The prevalence of BM is approximately 1.6%. Around 0.33% of BM patients go on to develop oesophageal adenocarcinoma annually (Desai et al., 2012). Indeed patients with BM have 30–125 times higher risk of developing oesophageal adenocarcinoma than the normal population (Paulson & Reid, 2004; Pohl & Welch, 2005; Hahn et al., 2010). As the
incidence of oesophageal adenocarcinoma has been increasing over the last 30 years (Pohl & Welch, 2005), and BM is the only known precursor, it is therefore important to understand the molecular mechanisms underlying the development of BM.

1.4.2 Current treatments for BM

Because the risk of oesophageal adenocarcinoma in non-dysplastic BM is relatively low (~0.33% per year), repeat endoscopic surveillance is suggested for the patient with non-dysplastic BM (Desai et al., 2012; Fitzgerald et al., 2014). For the dysplastic BM, various treatments are available. The treatments aim to prevent the progression to cancer. Some treatments also aim to remove the BM tissue (Spechler, 2014; Fitzgerald et al., 2014). Current treatments for dysplastic BM include medical treatment, anti-reflux surgery, endoscopic treatment and surgical resection methods.

In conventional medical treatment, proton pump inhibitors (PPIs) are currently the most effective available medicines for inhibiting gastric acid secretion through inhibiting proton pump (H⁺/K⁺-ATPase) function (Sachs et al., 2006). The inhibition of gastric acid secretion may help to prevent the acid-induced chronic inflammation at the lower end of the oesophagus as well as the secretion of cancer-promoting cytokines (such as interleukin 6 (IL-6) and IL-8) by the oesophageal epithelial cells (Rafiee et al., 2009; Spechler, 2014). Acid suppression may also prevent acid-induced genetic mutation and abnormal cell proliferation (Clemons et al., 2007; Spechler, 2014). Therefore, PPI treatment might prevent the neoplastic progression in BM.

Anti-reflux surgery aims to improve the closing function of the lower oesophageal sphincter function in order to prevent the reflux of the gastric acid to the lower oesophagus. The anti-reflux operation is called Fundoplication in which the gastric fundus is wrapped around the lower end of the oesophagus and sewn in place (Jamieson et al., 1994). Surgical treatment might be more effective than the PPI treatment in preventing carcinogenesis in BM (DeMeester & DeMeester, 2000). PPIs only prevent gastric acid secretion, but surgical treatment could prevent the gastric juice reflex including both gastric acid and bile salts, to the lower end of the oesophagus. Bile salts could also potentially promote carcinogenesis in the BM (Jenkins et al., 2007; Spechler, 2014). However, the surgical risk, and the cost and
effects of anti-reflux surgery need be taken into consideration for BM treatment (Spechler, 2014). Therefore, it is proposed that surgical treatment was suggested for patients who have poor response to acid suppression treatment (Fitzgerald et al., 2014).

BM tissue might be eliminated by endoscopic ablative techniques such as radiofrequency ablation (RFA), endoscopic mucosal resection (EMR), photodynamic therapy (PDT) and cryotherapy. RFA is performed through an endoscope-mounted or a balloon-mounted catheter which was attached to the end of the endoscope. When the electrodes (covered in the catheter) targets BM tissue, the balloon inflates and the electrodes generate radiofrequency energy to ablate the BM tissue (Konda et al., 2014). PDT is used for treatment of dysplastic BM. During the PDT treatment, a photosensitizer (a light sensitive drug) is given to the patient and the photosensitizer is selectively absorbed by the malignant cells. The laser light is then introduced to the BM region which will induce the photosensitizer to produce an oxidative reaction in turn destroying the BM dysplastic tissue (Sánchez et al., 2010). The principle of EMR is based on using the endoscopic resection technique to perform a cut or snare resection procedure to remove BM mucosa (Seewald et al., 2007). In cryotherapy treatment, liquid nitrogen is sprayed onto BM region. Therefore, BM tissue is frozen and destroyed (Gosain et al., 2013). Overall, endoscopic treatments are recently used primarily for dysplastic BM treatment and the endoscopic treatments seem to be cost-effective. However, long-term effects still need to be studied to evaluate the benefits of using endoscopic treatment for preventing oesophageal adenocarcinoma (Spechler et al., 2011; Fitzgerald et al., 2014). Finally, surgical resection, which is known as oesophagectomy, may be recommend for the young patients with extensive and multifocal high-grade dysplasia (Shaheen & Richter, 2009).

1.4.3 The cellular origins of BM

There are four potential mechanisms to explain the appearance of columnar type cells in the oesophagus (Fig. 1.4). The first is that the BM might arise as the transformation from an oesophageal stem cell to an intestinal stem cell with subsequent differentiation to intestinal cell types (goblet cells, enterocytes, Paneth cells and enteroendocrine cells) (Barbera & Fitzgerald, 2010). The second is that the BM arises
from the stem cells in the submucosal glands (Leedham et al., 2008; Nicholson et al., 2012). The third may involve the reverse of the cell type conversion observed in embryonic development (i.e. conversion from a stratified squamous to columnar-type epithelium) (Yu et al., 2005; Barbera & Fitzgerald, 2010). The fourth is that the BM cells originate from the gastro-oesophageal junction or gastric cardia (Wang et al., 2011; Quante et al., 2012).

In terms of the first possibility, the columnar-like intestinal cells in BM might differentiate from the oesophageal epithelial stem cells. However, the location and identity of the oesophageal epithelial stem cells is still controversial. A study showed that CD34+ cells at the basal layer have stem cell capacity for self-renewal and differentiation (Kalabis et al., 2008). A further study identified α6 integrin^bright CD71^dim basal cells as stem cells and these cells are able to reconstitute oesophageal epithelium in vitro (Croagh et al., 2007). Interestingly, a recent study demonstrated that the oesophageal epithelium is maintained and repaired by proliferating progenitor cells in the basal layer of the stratified squamous epithelium rather than the quiescent and self-renewing stem cells (Doupé et al., 2012). Further studies still need to address the identity of the epithelial stem cells.

In the second explanation, the theory proposed is that BM cells arise from stem cells in the glandular neck region of sub-mucosal glands (Barbera & Fitzgerald, 2010). Evidence from serial sections of BM tissue revealed that in some BM samples, oesophageal gland ducts open onto the overlying Barrett's epithelium suggesting the relationship between gland ducts and BM (Glickman et al., 2001b). In addition, characterization of pig oesophageal sub-mucosal gland cultures showed a similar K7 and K20 expression profile to that in BM (Abdulnour-Nakhoul et al., 2007). Finally, clonal analysis of BM sections demonstrated that BM cells display genetic heterogeneity suggesting that BM cells originate from different clones (Leedham et al., 2008; Nicholson et al., 2012). It was also observed that BM cells have the same mutation as the adjacent gland ducts suggesting gland ducts are the source of BM.

The third explanation for the appearance of BM is transdifferentiation. The oesophageal epithelium during embryonic development initially forms a simple
columnar epithelial layer which expresses the columnar epithelial marker K8. From around E11.5, the epithelial layer progressively turned into stratified squamous epithelium. Cells at basal region begin to express the squamous epithelial marker K14. K8/K14 co-staining and in vitro lineage tracing experiments demonstrated that the columnar-to-squamous cell type change is a direct cell type conversion referred to as transdetermination because it occurs in the embryonic stage of development (Yu et al., 2005).

In the fourth theory, the BM cells may originate from cells migrating from the gastro-oesophageal junction or gastric cardia (Wang et al., 2011; Quante et al., 2012). Recently two reports using mouse models support the idea that BM cells may arise from the gastro-oesophageal junction or the gastric cardia. In the first report, it was observed that p63-null mouse embryos develop a BM-like phenotype (Wang et al., 2011). Tracking the cell source revealed that these BM-like cells may be the residual embryonic cells. These embryonic residual cells are usually located at squamous-columnar junction in postnatal life. When squamous epithelium is damaged, these residual embryonic cells proliferate and migrate into the oesophagus to replace the original squamous epithelium. However, there is a lack of robust lineage tracing experiments in this study. In the second report, IL-1β was overexpressed in the mouse oesophageal epithelium, and this leads to BM and oesophageal adenocarcinoma (Quante et al., 2012). Lineage tracing experiments revealed that BM cells might arise from gastric cardiac leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5)-expressing stem cells at the squamous-columnar junction. Indeed, LGR5 is a marker for stomach and intestinal stem cells and LGR5 expression is found in the BM tissue (Barker et al., 2007, 2010; Becker et al., 2010). These LGR5-positive gastric cardiac stem cells presumably migrate into the oesophagus due to an inflammatory signal (Quante et al., 2012).
Figure 1.4. Potential cellular origins of BM

BM cells may originate from (a) oesophageal epithelial stem cells, (b) stem cells in the submucosal gland, (c) cells transdetermined from oesophageal epithelial cells or (d) stem cells located at gastric or oesophageal-gastric junction. (Figure is amended from Colleypriest et al. 2009 and Barbera & Fitzgerald 2010.)
1.4.4 Molecular mechanisms underlying the development of BM

The main risk factor for the development of Barrett’s is GORD (gastro-oesophageal reflux disease). Around 10% of GORD patients develop Barrett’s metaplasia (Westhoff et al., 2005). The key components of the gastric refluxate are bile acids. Bile acids are derived from cholesterol in the liver, and are stored and concentrated in the gallbladder. After food intake, the duodenum secretes cholecystokinin which stimulates the gallbladder to release bile. Bile is then delivered into the duodenum to emulsify fats for absorption. In the intestinal lumen, more than 95% of bile acids will be reabsorbed from the intestine back to the liver through the portal vein (the so-called enterohepatic circulation) (Dawson et al., 2009; Li & Chiang, 2013).

The bile acid family can be divided into primary and secondary bile acids according to where they are produced. Primary bile acids including cholic acid and chenodeoxycholic acid are produced in the liver. Secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid are modified from primary acids by bacteria in the intestine (Lefebvre et al., 2009).

Bile acid exposure in the oesophagus is associated with the induction of intestinal transcription factor gene expression. Treatment of rat oesophageal keratinocytes with cholic acid or DCA have been shown to induce the expression of intestinal transcription factor CDX2 (see 1.5.1 section for further details of CDX2)(Kazumori et al., 2006). In addition, it has been shown that DCA is able to activate NF-κB signalling pathway in OE33 cells (Jenkins et al., 2004, 2008). Because the CDX2 gene is a target of NF-κB (Kim et al., 2002; Kazumori et al., 2006), it is therefore possible that CDX2 induction by DCA is mediated through an NF-κB-dependent mechanism. Furthermore, treatment of OE33 cells, Het-1A (human normal oesophageal cells) cells and rat oesophageal keratinocytes with a mixture of cholic acid, glycocholic acid and taurocholic acid induces the expression of the intestinal transcription factors CDX1 and gut-enriched Krüppel-like factor (KLF4) (Kazumori et al., 2009, 2011). Taken together these studies suggest that bile acids are highly associated with the pathogenesis of BM.
1.5 Candidate transcription factors in BM development

The exposure of gastric refluxate might result in the conversion of oesophageal squamous cells to intestinal-like columnar cells. The theory about how metaplasia arises suggests that metaplasia formation is linked to the mis-expression of few key transcription factors (Slack, 2007). It is therefore possible that ectopic expression of intestinal transcription factors is associated with the development of BM.

1.5.1 Role of CDX2 in the intestine and BM

The homeodomain transcription factor caudal-type homeobox (CDX) family proteins CDX1 and CDX2 are specifically expressed in the differentiated intestinal epithelium. In the mouse, Cdx2 protein is first expressed at E3.5 in the trophectoderm and Cdx1 is detected at E7.5 along the embryonic axis. By E8.5, Cdx2 is found in the hindgut endoderm, while Cdx1 begins to be expressed in the hindgut endoderm from E12.5 (Beck et al., 1995; Subramanian et al., 1995; Silberg et al., 2000). Cdx1 and Cdx2 participate in axial patterning and Cdx2 also regulates anterior-posterior patterning of the intestine epithelium (Guo et al., 2004; Kaestner, 2010). In addition, Cdx2 plays a central role in initiating intestinal epithelial differentiation. Forced expression of Cdx2 in the undifferentiated rat intestinal epithelial cell line, IEC-6, initiates differentiation (Suh & Traber, 1996). It was also shown that Cdx2 regulates intestinal gene expression such as sucrase-isomaltase (Si) and lactase (Suh & Traber, 1996; Lorentz et al., 1997).

CDX2 is absent in the normal oesophageal epithelium, but CDX2 is expressed in BM. Therefore CDX2 has been considered to be a marker of BM (Eda et al., 2003; Moons et al., 2004; Burnat et al., 2007). Research utilizing transgenic mice models showed that Cdx2 homozygous knockout mice died between E3.5-E5.5 due to implantation failure (Chawensaksophak et al., 1997; Strumpf et al., 2005). Cdx2 heterozygous knockout mice are viable, but intercalary regeneration (or order filling-in of the tissue) in the form of stratified squamous epithelium was discovered in the proximal colon (Tamai et al., 1999; Beck et al., 1999). In addition, studies from conditional Cdx2 knockout mice (Cdx2\textsuperscript{loxP/loxP}; Foxa3-Cre) in which Cdx2 expression is ablated around E9.5, lead to the absence of colon and the loss of intestinal characteristics. The posterior intestinal cells displayed characteristics of oesophageal epithelium such as...
expression of p63 and K13 (Gao et al., 2009). More importantly, in Foxa3-Cdx2 transgenic mice, ectopic expression of Cdx2 in gastric mucosa promotes the intestinalization of gastric epithelium, suggesting the critical role of Cdx2 in the development of intestinal metaplasia (Silberg et al., 2002).

*In vitro* studies have demonstrated that ectopically expressing Cdx2 in primary rat oesophageal epithelial cells induces mucin 2 (*Muc2*) expression (an intestinal goblet cell marker) suggesting that the transcription factor may play a role in the development of BM (Kazumori et al., 2006). However, *K14-Cdx2* transgenic mice in which the *K14* promoter drives *Cdx2* expression in the basal layer of oesophageal epithelium, did not induce the expression of BM associated genes, but treating *K14-Cdx2* mice with 5′-Azacytidine (5′Aza), which is a DNA methyltransferase inhibitor initiated the expression of BM associated markers including K18 and Cdx1. This suggests chromatin modification might also be required in the development of BM (Kong et al., 2011b). Furthermore, it was suggested that the exposure of bile and acid is associated with the induction of Cdx2 expression in oesophageal epithelium (Marchetti et al., 2003; Debruyne et al., 2006). For example, it was observed Cdx2 protein production was induced by the bile salt or acid treatment in the primary rat or mouse oesophageal epithelial cell cultures (Marchetti et al., 2003; Kazumori et al., 2009).

In addition to CDX2, CDX1 may also be involved in the development of BM (Wong et al., 2005). *Cdx1* null mice are viable and show the anterior homeotic transformations of the vertebrae (Subramanian et al., 1995). Like CDX2, CDX1 is found in BM (Eda et al., 2003; Wong et al., 2005; Stairs et al., 2008). Transgenic mice (*H+/K+-ATPase-Cdx1*) in which Cdx1 is expressed in the gastric mucosa develops gastric intestinal metaplasia (Mutoh et al., 2004). In addition, Cdx1 protein can be induced by a cocktail of bile acids (cholic acid, glycocholic acid and taurocholic acid) in primary cultured rat oesophageal epithelial cells. A positive inter-regulation was observed between CDX1 and CDX2 expression in the OE33 cells. The transfection of CDX1 expression vector increased CDX2 promoter activity. Similarly, CDX1 promoter activity was increased following the transfection of CDX2 expression vector. This suggests that CDX1 and CDX2 together contribute to the initiation of BM
(Kazumori et al., 2009). Although CDX transcription factors may be key regulators in the initiation of intestinal metaplasia, it is not clear whether the expression of CDX transcription factors alone or in combination with other intestinal transcription factors initiate BM.

1.5.2 Role of HNF4α in the intestine and BM

Hepatocyte nuclear factor 4α (HNF4α) is an orphan nuclear receptor that functions as a transcription factor. HNF4α is mainly expressed in the liver, pancreas, intestine and kidney. HNF4α is essential for embryonic development. In mouse, as early as E4.5, Hnf4α is specifically expressed in visceral endoderm and regulates protein secretion for gastrulation. Hnf4α knockout mice die before E10.5 due to the disruption of gastrulation (Chen et al., 1994; Duncan et al., 1997).

HNF4α is expressed in the differentiated intestinal epithelium and regulates differentiation, cell proliferation and homeostasis. Lack of Hnf4α in conditional Hnf4α knockout mice (Villin-Cre-ERT; Hnf4αloxP/loxP) showed abnormal homeostasis, cell structure and barrier function in intestinal epithelium. Hnf4α deletion alters intestinal cytodifferentiation resulting in increased numbers of Goblet cells and the inhibition of enterocyte and enteroendocrine maturation (Babeu et al., 2009; Cattin et al., 2009). Observations from in vitro models also showed that Hnf4α is involved in the differentiation of intestinal epithelium. For example, forced Hnf4α expression in IEC-6 cells (an undifferentiated intestinal epithelial cell line) was able to induce cell differentiation in a co-culture model (Lussier et al., 2008).

Interestingly, overexpression of HNF4α in non-intestinal cells (including NIH-3T3 cells (mouse embryonic fibroblast) and Mia PaCa-2 (Human pancreatic carcinoma)) promotes the transcription of intestinal genes such as apolipoprotein A-IV (APOA-IV), Trefoil factor 3 (TFF3) and VILIN. In addition, the formation of apical microvilli and dense ultrastructures reminiscent of intestinal epithelial cell characteristics was also observed in NIH-3T3 cells in which HNF4α was overexpressed (Babeu et al., 2009). More importantly, HNF4α is not expressed in oesophageal epithelium, but it is present in the glands and epithelium of oesophageal tissue in BM (Piessen et al., 2007; Colleypriest, 2010). Indeed, forced expression of Hnf4α in primary mouse
oesophageal epithelial cells provoked intestinal Villin gene and protein expression (Colleypriest and Tosh personal communication). Therefore, HNF4α is a potential key transcription factor for intestinal epithelium identity and may play a critical role in BM development.

1.5.3 Role of HNF1α in the intestine and BM
Hepatocyte nuclear factor 1 α (HNF1α) is a homeodomain-containing transcription factor and binds to DNA as homodimers. HNF1α is expressed in the liver, pancreas, intestine, stomach, and kidney (Baumhueter et al., 1990; Pontoglio et al., 1996). In mouse intestinal epithelium, HNF1α protein is mainly detected in the villus region and HNF1α plays a role in regulating intestinal gene expression. Studies demonstrated that HNF1α regulated intestinal Si gene transcription by binding to promoter region in the Caco2 cell line (human colorectal adenocarcinoma cells) (Boudreau et al., 2002). Further studies also suggested that HNF1α worked in combination with CDX2 to promote intestinal differentiation in HIEC cells (human intestinal epithelial crypt cells)(Benoit et al., 2010).

HNF1α and HNF1β may have complementary roles in regulating intestinal epithelium. *Hnf1α* and *Hnf1β* double knockout (*Hnf1α<sup>−/−</sup>, *Hnf1β<sup>fl<sub>ox</sub>/fl<sub>ox</sub>; Villin-CreER*) mice displayed an alteration in the marker expression of both the enterocyte and secretory lineage cells, the increase of goblet cell population and the absence of mature Paneth cells, suggesting the HNF1 protein family plays an important role in regulating intestinal cell differentiation and cell identity (D’Angelo et al., 2010). *Hnf1α* knockout mice also suggest that Hnf1α is required for the normal adult intestinal architecture. Hnf1α controls cell growth and cell fate decisions in the adult intestinal epithelium (Lussier et al., 2010). In addition, HNF1α becomes expressed in BM tissue (Piessen et al., 2007). Therefore, HNF1α may be involved in the development of BM.

1.5.4 Regulatory network of CDX2, HNF1α and HNF4α in the intestine
Intestinal cell proliferation and differentiation are controlled by a transcription factor regulatory network (Olsen et al., 2012). The aberrant expression of one intestinal transcription factor could affect the expression of the other intestinal transcription factors. The regulatory network among CDX2, HNF1α and HNF4α is the most
studied transcription factor network in the intestinal epithelium. Details of CDX2, HNF1α and HNF4α interactions are shown in Table 1.1 and the model of their regulatory network is illustrated in Figure 1.5.
Table 1.1. Transcriptional regulatory network in the intestinal epithelium

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Target gene</th>
<th>Regulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDX2</td>
<td>CDX2</td>
<td>Autoregulation, Cdx2 activates its own enhancer and promoter</td>
<td>(Xu et al., 1999; Boyd et al., 2010)</td>
</tr>
<tr>
<td>HNF1α</td>
<td>CDX2</td>
<td>Activates CDX2 binds to HNF1α promoter</td>
<td>(Gao et al., 2009)</td>
</tr>
<tr>
<td>HNF4α</td>
<td>CDX2</td>
<td>Activates HNF4α expression through HNF4α enhancer and promoter</td>
<td>(Boyd et al., 2010; Gao et al., 2009)</td>
</tr>
<tr>
<td>HNF1α</td>
<td>CDX2</td>
<td>Activates CDX2 gene expression through CDX2 enhancer</td>
<td>(D’Angelo et al., 2010)</td>
</tr>
<tr>
<td>HNF1α*</td>
<td>HNF1α</td>
<td>Autoregulation, inhibits HNF1α expression through its own promoter</td>
<td>(Kritis et al., 1993)</td>
</tr>
<tr>
<td>HNF4α*</td>
<td>HNF4α</td>
<td>Activates HNF4α gene expression through HNF4α promoter</td>
<td>(Odom et al., 2004)</td>
</tr>
<tr>
<td>HNF4α</td>
<td>CDX2</td>
<td>Activates CDX2 gene expression through CDX2 promoter</td>
<td>(Boyd et al., 2009)</td>
</tr>
<tr>
<td>HNF1α</td>
<td>HNF1α</td>
<td>Activates HNF1α gene expression through HNF1α promoter</td>
<td>(Lussier et al., 2008; Boyd et al., 2009)</td>
</tr>
<tr>
<td>HNF4α*</td>
<td>HNF4α</td>
<td>Autoregulation, inhibits HNF4α expression through its own promoter</td>
<td>(Magenheim et al., 2005)</td>
</tr>
</tbody>
</table>

Table 1.1. Transcriptional regulatory network in the intestinal epithelium

Table showing the regulation of the transcription factors CDX2, HNF1α and HNF4α in the differentiated intestinal epithelium. * Note that the regulation of HNF4α expression by HNF1α and the autoregulation of HNF1α or HNF4α in the intestine has not been reported, so data presented here was from the research in the liver.
Figure 1.5. Model of transcriptional regulatory network in the intestinal epithelium

Figure is the summary of Table 1.1 and illustrates the regulatory network of CDX2, HNF1α and HNF4α in the differentiated intestinal epithelium. CDX2, HNF1α and HNF4α activate each other’s expression. CDX2 positively autoregulates its own expression, but HNF4α and HNF1α might negatively autoregulate their own expression. The regulation presented in solid line is based on studies in the intestine and the regulation shown in the dotted line is based on studies in the liver.
1.5.5 Role of FOXA2 in the intestine and BM

Forkhead box A2 (FOXA2) (also known as hepatocyte nuclear 3β (HNF3β)) belongs to the forkhead DNA-binding protein family. FOXA2 protein functions as a transcription factor binding to DNA through a winged helix motif (Besnard et al., 2004). In the developing mouse intestine, Foxa2 can be detected at E10.5 stage and in the adult, Foxa2 is mainly expressed in the crypt region (van der Sluis et al., 2008; Suzuki et al., 2009). FOXA2 regulates intestinal gene expression. For example, Foxa2 controls Muc2 expression via binding to the Muc2 promoter in CMT-93 cells (mouse rectal carcinoma cells) (van der Sluis et al., 2008). Loss of Foxa2 in mice (Foxa2-/-) is embryonic lethal and this is primarily due to the failure of normal node and notochord formation (Ang & Rossantt, 1994). Studies of Foxa1 and Foxa2 deletion in the intestine have been reported (Ye & Kaestner, 2009). Animal models of intestinal Foxa1 and Foxa2 deletion was created by using Foxa1loxP/loxP, Foxa2loxP/loxP, Villin-Cre mice. Intestinal Foxa1 and Foxa2 null mice demonstrated the decrease of goblet and enteroendocrine cell population. It was found that the ablation of Foxa1 and Foxa2 decreased the expression of enteroendocrine cell transcription factors including paired box transcription factors 6 (Pax6) and Islet-1 (Isl-1). The reduction of Pax6 and Isl-1 resulted in the down-regulation of their downstream targets, preproglucagon gene (glucagon-like peptide 1/2, Glp 1/2) and Somatostatin respectively (Leonard et al., 1992; Hill et al., 1999; Ye & Kaestner, 2009). In addition, Foxa1 and Foxa2 ablation also decreased Muc2 at the transcription level. These results suggest that Foxa1 and Foxa2 regulate intestinal enteroendocrine cell and goblet cell differentiation (Ye & Kaestner, 2009).

1.5.6 Other transcription factor involved in BM

Pancreatic and duodenal homeobox 1 (PDX1) transcription factor may be involved in the development of BM. PDX1 expression is found in the anterior part of the intestine, pancreas and stomach (Miller et al., 1994; Larsson et al., 1996). It was shown that Pdx1 promoted the differentiation toward the enteroendocrine cell lineage in the undifferentiated rat intestinal IEC-6 cells (Yamada et al., 2001). In addition, Pdx1 conditional deletion in mouse intestine (Pdx1loxfloxtloxflox;Villin-Cre) led to the reduction in gene expression of enterocytes (such as alkaline phosphatase 3) and enteroendocrine cells (such as gastric inhibitory polypeptide and somatostatin) (Chen et al., 2009;
Chen & Sibley, 2012). Pdx1 also regulates enterocyte gene expression including Si, fatty acid binding protein 1 and Lactase (Wang et al., 2004; West & Oates, 2005; Chen et al., 2012b).

Intestinal transcription factors GATA-binding protein 4 (GATA-4), GATA-6, KLF4 and SRY-box containing gene 9 (SOX9) have been reported to have a potential role in the development of BM (Chen et al., 2008; Haveri et al., 2008; Wang et al., 2010; Kazumori et al., 2011). For example, it was proposed that bile acid triggers oesophageal epithelial cells to produce Sonic hedgehog (SHH) protein. SHH stimulates mesenchymal cells to produce BMP4 which then induces SOX9 expression in the oesophageal epithelium. Ectopic SOX9 expression drives columnar differentiation of oesophageal squamous epithelium and also induces the expression of glycoprotein A33. A33 glycoprotein is exclusively expressed in the intestine and A33 is also a BM marker (Wong et al., 2006; Clemons et al., 2012; Wang et al., 2010).

Homeobox (HOX) genes may play a role in BM. The HOX gene family is known to play a central role in patterning anterior-posterior axis during the embryonic development (McGinnis & Krumlauf, 1992). It was shown that the HOX gene subfamily HOXB5, 6, and 7 (which are highly expressed in the colon) are expressed in BM but not the normal stratified squamous epithelium. Indeed, forcing HOXB5, 6, and 7 expression in the normal human oesophagus-derived NES cells induces BM phenotype with VILLIN, K20 and MUC2 expression all detected (di Pietro et al., 2012).

1.5.7 Squamous epithelial transcription factor, p63
Activation of intestinal transcription factors in oesophageal epithelium might cause intestinal metaplasia. In contrast, down-regulation of the key transcription factor in squamous epithelial differentiation is also observed. p63, a homologue of the tumour suppressor p53, is required for squamous epithelial development and is essential for squamous cell differentiation as mice lacking p63 fail to develop stratified squamous epithelium (Mills et al., 1999; Yang et al., 1999). In the oesophagus, p63 is expressed in the basal layer of epithelium (Glickman et al., 2001a; Daniely et al., 2004).
However, p63 expression is absent in BM (Glickman et al., 2001a; Wang et al., 2011). There is a potential link between bile acid and p63 expression. It was shown that bile acid exposure is able to down-regulate the level of p63 in primary oesophageal cell culture (Roman et al., 2007).

1.6 Inflammation and BM

Inflammation may be defined as the response of the immune system to tissue injury or infection. The purpose of an inflammatory response is to protect the tissue from infection and restore homeostasis (Medzhitov, 2010; Ben-Neriah & Karin, 2011). However, there is increasing evidence showing that chronic inflammation can promote cancer formation. It has been estimated that about a quarter of cancer cases is associated with inflammation suggesting a potential link between the two (Eiró & Vizoso, 2012).

Two pathways connecting inflammation and cancer have been defined: the intrinsic pathway and the extrinsic pathway. In the extrinsic pathway, inflammation and infection of extrinsic stimuli may promote cancer development. However, the intrinsic pathway is linked to genetic alterations which cause neoplasia leading to an inflammatory microenvironment. The activation of these two pathways result in activation of transcription factors such as nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) which then increase the production of inflammatory mediators including cytokines, chemokines (such as IL-1β and tumour necrosis factor α (TNF-α)) and cyclooxygenase 2 (COX2). The inflammatory mediators further recruit inflammatory cells and also activate transcription factors including NF-κB and STAT3 in the inflammatory cells to produce more inflammatory mediators. Therefore, a cancer-related inflammatory environment is generated which results in cell transformation and tumour-promoting effects (Mantovani et al., 2008; Gibson et al., 2013).

In BM development and carcinogenesis, the extrinsic pathway of inflammation is activated by the chronic bile acid irritation caused by GORD. Bile acid exposure also induces the intrinsic pathway. This is due to bile acid exposure in oesophageal cells inducing reactive oxygen species (ROS) and nitric oxide (NO) production which in
turn leads to DNA damage (Dvorak et al., 2007; Jenkins et al., 2007, 2008; Jolly et al., 2009; McAdam et al., 2012). A number of studies have shown that exposure to bile acid can activate the pro-carcinogenic NF-κB pathway in both the oesophageal epithelial cell lines and the human oesophageal biopsy cultures (Jenkins et al., 2004, 2008). The STAT3 pathway (in response to IL-6) is activated in BM (Dvorakova et al., 2004). In addition, the production of cytokines and chemokines such as IL-1β, IL-6, IL-17, TNFα and interferon-γ (IFN-γ), and COX2 are increased in BM (Dvorakova et al., 2004; Bannister et al., 2012; Taddei et al., 2013). Taken together, the activation of both extrinsic and intrinsic pathway generate an inflammatory microenvironment and lead to BM (Gibson et al., 2013).

It is possible that the NF-κB pathway might initiate BM development through activating CDX2 expression. NF-κB acts a transcription factor. In the cytoplasm, NF-κB binds to the inhibitory protein nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα), so NF-κB is maintained in the inactive state. Once IκBα is phosphorylated by IκB kinase (IKK) following an extracellular stimulation, IκBα will be degraded. NF-κB will then translocate into the nucleus and enhance gene transcription. NF-κB targets include inflammation-related genes (Bollrath & Greten, 2009). Interestingly, it was found that there are two putative NF-κB binding sites in the CDX2 promoter region. In addition, bile acid treatment was able to induce CDX2 promoter activity and initiate CDX2 protein expression in rat oesophageal epithelial cells (Kazumori et al., 2006). However, using telomerase-immortalized NES cells as a model, bile acids (a mixture of glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid, and taurodeoxycholic acid) only activated CDX2 mRNA and protein expression in NES cells which were derived from GORD patients with BM, but not in the NES cells which were originated from GORD patients without BM. It was also found that the CDX2 promoter activity is increased through NF-κB signalling activation (Huo et al., 2010). Whether the different results observed are due to the difference in of cell types used and/or bile acid utilized remains to be investigated.
1.7 Models for BM studies

Current models used for studying BM or BM-related carcinogenesis research include tissue culture, organotypic culture and *in vivo* studies on animals (Pavlov & Maley, 2010). Cell culture models can be subdivided into cells originating from oesophageal squamous epithelium, BM tissue or oesophageal adenocarcinoma. In cell culture models, HET-1A, NES and EPC2-hTERT cell lines have been used for the research of BM development. HET-1A cell line is SV40 T antigen immortalized human oesophageal epithelial cells (Stoner et al., 1991). NES and EPC2-hTERT cell lines are both human telomerase reverse transcriptase transformed oesophageal epithelial cells but were developed by different groups (Morales et al., 2003; Harada et al., 2003). BAR-T, CP-A, CP-B, CP-C and CP-D cell lines are derived from human BM tissue and are all immortalized by hTERT transfection. BAR-T and CP-A are non-dysplastic BM cell lines, and CP-B, CP-C and CP-D are high-grade dysplastic BM cell lines (Palanca-Wessels et al., 2003; Jaiswal et al., 2007). Cell lines derived from human oesophageal adenocarcinoma includes OE33 and FLO cell lines (Rockett et al., 1997; Boonstra et al., 2010).

Two-dimensional tissue culture of primary human normal oesophageal squamous cell has been tested. However, the cell replicative life span is limited and the telomere length was progressively shortened (Harada et al., 2003). Three-dimensional organotypic culture models have been developed. Three-dimensional organotypic models have the advantage of prolonged viability in primary cells and more importantly, it mimics the *in vivo* microenvironment which is important for BM initiation and malignant progression (Okawa et al., 2007; Souza et al., 2011). In organotypic culture system, oesophageal epithelial cells grow on the top of a fibroblast layer. EPC2-hTERT cells, mouse and human primary oesophageal epithelial cells have been shown to differentiate and recapitulate the stratified squamous epithelium structure in the organotypic culture system (Harada et al., 2003).

Rat surgical models are the most commonly used BM animal models (Pavlov & Maley, 2010). Rat surgical models include oesophagogastrroduodenal anastomosis (OGDA), oesophagogastrjejunostomy (OGJ), oesophagoduodenal anastomosis (ODA), oesophagojejunostomy (OJ) and total gastrostomy (Kresty et al., 2011). The surgery induces stomach acid and/or bile salts reflex to the lower end of the
oesophagus in order to induce oesophagitis, BM, dysplasia and adenocarcinoma (Su et al., 2004; Cheng et al., 2005; Lu et al., 2009; Aiyer et al., 2011). Nevertheless, it is also worth noting that, unlike rodent, human oesophageal epithelium lacks cornified layers (Seery & Watt, 2000), but contains papillae structures, mucosal and submucosal glands. In addition, in humans, the squamous-columnar junction of gut is between oesophagus and stomach (Seery & Watt, 2000; Croagh et al., 2008), but the squamous-columnar junction in rodent is shifted posteriorly to the middle of the stomach (Wang et al., 2011). Therefore, using rat surgical models for BM research may not fully represent the BM pathogenesis in human.

1.8 Aims
The current hypothesis is that the intestinal transcription factors HNF4α, CDX2, HNF4α, HNF1α, PDX1 and FOXA2 are linked to intestinal metaplasia. Exogenous factors including bile acids and inflammatory mediators may be a key stimulus for BM development. The aims of the project were to

(1) Test the role of ectopic HNF4α expression in the development of BM.
(2) Test the role of ectopic CDX2, HNF4α, HNF1α, PDX1 and FOXA2 in the development of BM.
(3) Screen exogenous factors which may be involved in the pathogenesis of BM.
Chapter 2
Materials and Methods
2.1 Isolation and culture of mouse embryonic oesophagus

Pregnant female CD1 mice were scarified by cervical dislocation and the uteri were transferred to ice-cold PBS (Fig. 2.1). Embryos at E11.5 stage were then separated from the uteri and placed in ice-cold dissection medium Minimum Essential Medium (MEM medium (Sigma) with 10% fetal bovine serum (FBS)(GIBCO), 2 mM L-glutamine (Sigma), penicillin/Streptomycin (50U, Sigma), Gentamycin (20mg/ml ,GIBCO) and Fungizone (1U/ml, GIBCO)). The oesophagus was then isolated using forceps and tungsten needles under a Leica MZ6 stereomicroscope (Fig 3.1). For plating, 35mm diameter round coverslips were placed in 4-well plate and 35μl of fibronectin (50 µg/ml, Invitrogen) was added to the middle of coverslip. After the fibronectin was dry, a plastic cloning ring was placed onto the fibronectin-coated region. 0.5 ml complete Basal Medium Eagle (BME) medium (BME medium (Sigma) with penicillin/streptomycin, 2 mM L-glutamine and 10% FBS) was added to the well and the oesophagus was then transferred into the centre area inside the plastic ring. Embryonic organs were cultured in in a humidified incubator at 37°C supplied with 5% CO₂/95% air. The plastic cloning rings were removed the next day and the BME medium was replaced with fresh BME medium. Medium was then changed every 2 days thereafter.

2.2 Adult mice oesophageal epithelium culture

Adult CD1 mice were sacrificed by cervical dislocation. The oesophagus was isolated and transferred to ice-cold dissection medium (Fig. 2.2). The oesophagus was opened longitudinally using scissors under a Leica MZ6 stereomicroscope. The epithelium was then stripped from the submucosal tissue and then cut into 1-2 mm² pieces. Approximately 15 pieces of oesophageal epithelium were placed on the furrows of a scratched plastic coverslip in a 35mm dish with 2ml of complete BME medium or Epilife medium (Invitrogen). The epithelial tissue was cultured in a 37°C incubator supplied with 5% CO₂/95% air. The medium was first changed after 2-days of incubation and then every 2 days thereafter.
Figure 2.1. Isolation of mouse embryonic oesophagus

(A) CD1 mouse embryonic day 12.5 (E12.5) embryo in the uterus. (B) E12.5 mouse embryo was removed from the uterus and the organ tree was isolated. Figures show (C) front view and (D) back view. (E) After liver and heart were removed, oesophagus was shown. (F) Oesophagus was isolated.
Figure 2.2. Dissection of adult mouse oesophageal epithelium

(A) Adult CD1 mouse was opened and oesophagus was identified. (B) Closer view of oesophagus. (C) Oesophagus was removed and was placed in the dissection medium. (D) Oesophagus was longitudinally opened. Oesophageal epithelium and the muscular layer were seen. (E) Oesophageal epithelium was isolated and (F) was cut into small pieces (approximately 1-2 mm$^2$) for culturing in a scratched plastic slide in a culture dish.
2.3 Culture of cell lines
HET-1A cells were obtained from American Type Culture Collection (ATCC, Middlesex, UK). OE33, KYSE30 cells, HT29, HEK-293 and Caco2 cells were obtained from European Collection of Cell Cultures (ECACC, Porton Down, U.K). HET-1A cells were maintained in complete BME medium (Sigma), OE33 and KYSE30 cells were maintained in complete RPMI-1640 medium (Sigma), HT29 and HEK-293 were maintained in complete Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen), and Caco2 cells were maintained in MEM medium (Sigma). All cell lines were cultured at 37°C under an atmosphere of 95% air, 5% CO₂. BME, RPMI and DMEM complete medium all contains 10% (v/v) fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (Sigma), penicillin/Streptomycin (50U, Sigma).
Culture medium was replaced every 2 days, and cells were subcultured (1:10-20) every 5 to 7 days. Subculturing of cell lines included the following steps. After washing the cells with PBS, cells were incubated with 5ml Trypsin/Ethylendiaminetetraacetic acid (Trypsin/EDTA) (Gibco) at 37°C for 5 minutes. 5 ml of the appropriate complete medium was added to stop the reaction. The side of the flask was tapped gently then the cells were transferred to a 15 ml tube. After centrifugation at 1000 rpm for 3 minutes, the supernatant was discarded, and 1ml of complete medium was added to re-suspend cells. Finally, 100 µl of cell suspension was transferred into a T75 flask supplied with 10 ml completed medium.

2.4 Storage of cell lines
To store cell lines, cell pellets were resuspended in freezing medium containing 90% FBS and 10% v/v Dimethyl sulfoxide (DMSO) (Sigma). The cell suspension in a cryogenic vial was then transferred to a Mr. Frosty™ freezing container and was frozen at -80°C overnight. Cells were then transferred into liquid nitrogen for long-term storage.

To resuscitate cells, cells from the liquid nitrogen were thawed in a 37°C water bath and then were slowly added into 10ml culture medium in a 15 ml tube. The cell suspension was then centrifuged at 1000rpm for 3 min. The supernatant was discarded and the cell pellet was resuspended in 10 ml culture medium before transfer to a T75 culture flask.
2.5 Adenovirus preparation

To amplify adenovirus for large scale preparation (as a prestock), 1, 2, or 8 μl of adenovirus stock was added to 70% confluent HEK-293 cells cultured in a T75 flask in complete DMEM medium. After 2 days of infection, about 50% cells showed morphological changes with the cells displaying more rounded characteristics (cytopathic effect), cells were then removed by tapping the edge of the flask and then transferred into a 15ml tube. Cells were harvested by centrifuging at 1000rpm for 4 minutes and the supernatant was then discarded. Cells were suspended in 1 ml of complete DMEM medium. Four freeze/thaw cycles were used to lyse the cells and release virus particles. This was performed on a dry ice/ethanol bath before switching to a water bath at 37°C. To test the amount of the virus needed for the large scale preparation, 1, 10 or 100 μl of adenovirus prestock was added to 70% confluent HEK-293 cells cultured in a T75 flasks in complete DMEM medium. The cytopathic effects were observed 2 days after infection. To prepare the virus on a large scale, 10 x T175 flasks of HEK-293 cells were infected with prestock virus for 2 days. The amount of virus added was referred to the prestock testing step which caused cytopathic effects in about 50% cells. Cells were then centrifuged at 2000rpm for 10 minutes and the medium was removed. 5 ml of 100mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8 was used to resuspend cell pellets and 4 cycles of freeze/thaw were performed to lyse cells. Cells were spun at 2000rpm for 5 minutes and the virus in the supernatant was transferred to a 50ml tube. 0.6 volume of caesium chloride (CsCl) supersaturated 100mM Tris-HCl, pH 8 of supernatant was added and transferred to 2 centrifuge tubes (Beckman 342412). After centrifuging at 65000rpm for 4 hour (Beckman ultracentrifuge LL-TB003, Vti90 rotor) a 25G needle was used to remove the virus particles band. Virus particles was added to [one volume of 100mM Tris-HCl: 0.6 volume CsCl saturated supersaturated 100mM Tris-HCl, pH 8] and centrifuged at 65000rpm for 18 hours. Virus particles were transferred to a Gamma irradiated slide-A-Lyzer™ dialysis cassette (Pierce Biotechnology) and then dialysed overnight in [10mM Tris-HCl, pH 7.5, 1mK MgCl2, 135mN NaCl]. The virus particles were filtered through a 0.22 μm filter (Millipore), aliquoted and stored at -80°C.
2.6 Adenoviral infection of oesophageal epithelial explants and HET-1A

For adenoviral infection of adult mice oesophageal epithelium, explants were cultured for 7 days in a 35mm dish. Complete medium (2ml) which contained 5.1 x 10^5 of Ad-CMV-HNF4α or the equivalent titre of Ad-Null viral particles (Table 2.1) was added to explants cultures. For HET-1A cells, 10^5 cells were seeded in a 6 cm plate 24 hrs prior to infection. Adenoviral particles (Table 2.1) were diluted in culture maintenance medium supplied with dextran (5µg/ml) (Sigma) and were then added to the cultures. For both explants and HET-1A cells, after 24 hours infection, culture medium was replaced by complete medium. Medium was then changed every two days.

Table 2.1. Adenoviral constructs

<table>
<thead>
<tr>
<th>Name (Bench)</th>
<th>Titre (IU/ml)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-CMV-VP16-Cdx2-ires-hrGFP (CDX2-H)</td>
<td>6.0 x 10^{10}</td>
<td>Wei-Yuan Yu, University of Bath, UK</td>
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<tr>
<td>(CDX2-I)</td>
<td>1.8 x 10^{11}</td>
<td></td>
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<tr>
<td>Ad-CMV-HNF1α</td>
<td>7.3 x 10^{9}</td>
<td>Marco Pontoglio, Paris Descartes University, France</td>
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<tr>
<td>Ad-CMV-HNF4α (HNF4α-A) (HNF4α-B)</td>
<td>5.1 x 10^{10}</td>
<td>Ramiro Jover, Unidad de Hepatologia Experimental, Valencia, Spain</td>
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<td></td>
<td>2.0 x 10^{11}</td>
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<tr>
<td>Ad-CMV-PDX1-VP16-eGFP</td>
<td>2.3 x 10^{10}</td>
<td>Harry Heimberg, Vrije Universiteit Brussel, Belgium</td>
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<td>Ad-RSV-GFP</td>
<td>9.9 x 10^{10}</td>
<td>Emma Regardsoe, University of Oxford, UK</td>
</tr>
<tr>
<td>Ad-Null</td>
<td>6.6 x 10^{10}</td>
<td>Harry Heimberg, Vrije Universiteit Brussel, Belgium</td>
</tr>
</tbody>
</table>

IU/ml: Infectious units per milliliter

2.7 Creation of Lentiviral plasmid

Lenti-HNF4α-50FCD2 (Lenti-mutant HNF4α)

To create a cloning site, N’ and C’ truncated HNF4α insert DNA was amplified from pcDNA3-HNF4α-50FCD2 construct by PCR (provided by Dr Chia-Ning Shen) and the (BamHI)- HNF4α-50FCD2-(BamHI) insert DNA was created. The primers designed are shown in Table 2.2. Insert DNA was then ligated into the pL-S-PURO vector, which was obtained by removing FOXA2 from pL-S-FOXA2-PURO plasmid using BamHI, and pL-S-HNF4α-50FCD2 was generated.
**Lenti-HNF4α-2A-CDX2-2A-HNF1α (Lenti-HCH)**

FMDV (Foot and Mouth Disease Virus) 2A sequence from Ad-Pdx1-2A-Ngn3-2A-MafA polycistronic construct (generously provided by Dr James Dutton, University of Minnesota) was amplified by PCR to create EcoRI and EcoRV restriction enzyme sites. The primer sequence is shown in Table 2.2. The 2A sequence was ligated into the pcDNA3 plasmid at EcoRI and EcoRV sites to create pcDNA3-2A plasmid. (EcoRV)-CDX2-(XbaI) cDNA fragment was amplified from HT29 cells by PCR with the translational termination codon replaced by XbaI site. By using EcoRV/XbaI site, CDX2 was cloned into pcDNA3-2A as pcDNA3-2A-CDX2 plasmid. Similarly, (EcoRV)- HNF1α-(XbaI) was created and was cloned into pcDNA3-2A as pcDNA3-2A-HNF1α, but the translational termination codon of HNF1α was still maintained. HNF4α cDNA was amplified from HT29 cells as (KpnI-BamHI)-HNF4α-(EcoRI) fragment and the translational termination codon was replaced by EcoRI site. The fragment then was ligated into pcDNA3-2A-CDX2 by using KpnI/EcoRI sites as pcDNA3-HNF4α-2A-CDX2. PCR was then performed in order to create (XbaI)-2A-HNF1α- (BamHI-XbaI) fragment from pcDNA3-2A-HNF1α. XbaI site was used to clone (XbaI)-2A-HNF1α- BamHI-(XbaI) into pcDNA3-HNF4α-2A-CDX2 as pcDNA3-HNF4α-2A-CDX2-2A-HNF1α. Finally, by using the BamHI site, HNF4α-2A-CDX2-2A-HNF1α was cloned into lentiviral vector which was obtained by removing FOXA2 from lenti-FOXA2 plasmid. Therefore, pL-S-HNF4α-2A-CDX2-2A-HNF1α was created.
Table 2.2. Sequence of primers used for cloning

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF4A BamHI 50FCD2 F</td>
<td>gCGGATCCatgcgtgtgccccatctgt</td>
</tr>
<tr>
<td>HNF4A BamHI 50FCD2 R</td>
<td>cCGGATCCcaggttaatgtctctcag</td>
</tr>
<tr>
<td>EcoRV HNF1A_F</td>
<td>CAGATATCatggttctaaactgacccgc</td>
</tr>
<tr>
<td>XbaI HNF1A_R</td>
<td>aaTCTAGAttactggaggaagagccca</td>
</tr>
<tr>
<td>EcoRV CDX2_F</td>
<td>CAGATATCatgtacgtgacactcctct</td>
</tr>
<tr>
<td>XbaI CDX2_R</td>
<td>ttTCTAGActgggtgacggtggggtta</td>
</tr>
<tr>
<td>XbaI 2A_F</td>
<td>agTCTAGAAAATTGTCGCTCCTGTCAA</td>
</tr>
<tr>
<td>XbaI BamHI HNF1A_R</td>
<td>aaTCTAGAGGATCCttactggaggaagagccca</td>
</tr>
<tr>
<td>KpnI BamHI HNF4A_F</td>
<td>TTGGTACCGGATCCatgcgtctccaaacccctgtTga</td>
</tr>
<tr>
<td>EcoRI HNF4A_R</td>
<td>TTGAATTCgataacctctgctggtgta</td>
</tr>
</tbody>
</table>

2.8 Cloning methods

To clone and insert the transgene DNA into the plasmid, insert DNA was amplified by PCR using Phusion high-fidelity DNA polymerase (Thermo). Insert and vector DNA were then digested with the appropriate restriction enzymes. Calf intestinal alkaline phosphatase (CIAP) (Promega) digestion step was performed for vector DNA if only one type of restriction enzyme was used for digestion. Insert and vector DNA was then run on an agarose gel and the percentage of the gel (0.6~2% w/v) varied depending on the DNA size. The required DNA fragment was visualised and cut out on a UV transilluminator (UVP). DNA was purified by using SV Gel and PCR clean-up kit (Promega). In the ligation step, T4 DNA ligase (Promega) was used, and the vector and insert DNA molar ratio was 1:3. In the transformation step, 10 µl of ligation mixture was incubated with DH5α competent cells on ice for 30 minutes and was then heated at 42°C for 90 seconds. 200 µl of LB medium was added and the competent cell culture was shaken at 225 rpm for 1 hour at 37°C. Competent cells were spread on an ampicillin (100µg/ml) LB agar plate and the plate was incubated at 37°C for 16 hours. Single colonies were picked and further cultured in 5 ml of LB with ampicillin (100µg/ml) at 37°C for 16 hours. 1.5 ml of bacteria in LB medium was transferred in to a 1.5ml tube. The bacteria lysate was collected by centrifuging at 12000rpm for 1 minute and then the supernatant was discarded. Plasmid was purified from bacteria lysate by using SV Miniprep kit (Promega). Plasmid was assessed by restriction enzyme digestion or PCR before sending for sequencing (Source bioscience). Large amounts of plasmid for lentiviral particles.
production was prepared from 100ml bacteria culture by using Endo Free Plasmid Midi kit (Qiagen).

### 2.9 Lentivirus preparation

The lentiviral expression plasmid pL-S-HNF4α-I-EGFP and pL-S-FOXA2-I-PURO was a gift from Dr Michael Bock (Hannover Medical School, Germany) and the plasmid constructs were previously reported (Iacob et al., 2011). Plasmid pL-S-mutant HNF4α (50FCD2), pL-S-HNF4α-2A-CDX2-2A-HNF1α construction was created as described in session 2.7. To prepare lentiviral particles, $3.75 \times 10^6$ HEK293T cells were seeded in a T75 flask one day before transfection. Four hours prior to transfection, the medium was replaced with fresh 10 ml of HEK 293T maintenance medium. To prepare plasmid and transfection reagent mixture, the viral vector (12.84µg), pREV (7.71µg), pGAG/Pol/PRE (7.71µg) and pVSV-G (3 µg) were mixed and ddH$_2$O was added to a total volume of 62.75 µl. 90 µl of the transfection reagent TransIT-LT1 (MIR 2300, Geneflow UK) and 750 µl of plain RPMI/25mM HEPES media were mixed and then incubated at RT for 5 minutes. Plasmid mixture was then slowly added into the mixture of the RPMI/25mM HEPES medium and the transfection reagent. The transfection mixture was incubated at room temperature for 45 minutes before adding to HEK293T cells. After 48 hours incubation, the medium which contained viral particles was collected and was centrifuged at 1000 rpm for 3 minutes. The supernatant containing the viral particles was collected and aliquoted into 1ml per tube. Lentivirus stock was stored at -80°C.

### 2.10 Lentiviral infection and stable clone selection

The day before lentiviral infection, $6.9 \times 10^4$ Het1a cells were seeded in a 35mm dish. On the day of infection, the lentivirus stock was thawed on ice for 2 hour. The lentivirus was diluted in culture maintenance medium supplied with Dextran (5µg/ml) and then added to the culture medium. Twenty-four hours after virus infection, the medium was changed and thereafter the medium was changed every 2 days. To select single colonies, cells were split and seeded into a 96 well format at a density of 1 cell per well. Single cells were subsequently selected and expanded. Transgene expression was confirmed by immunostaining and RT-PCR.
2.11 Analysis of mRNA expression (RT-PCR)

RNA purification

Cells cultured on a 6cm-dishes were washed with PBS twice and 1ml of TRI reagent (Sigma) was added to harvest cells. Cells from explants were further homogenized by using a sterile homogenizer in TRI reagent in RNase-free sterile 1.5 ml tubes and incubated for 5 minutes at RT. 200 μl of chloroform (Sigma) was then added to separate the RNA aqueous phase. The tubes were vortexed for 15 seconds and then incubated at RT for 15 minutes. The RNA aqueous layer was separated by centrifuging for 15 minutes at 12000 x g at 4 °C, and then the top RNA aqueous layer was transferred into a new 1.5 ml tube without taking any of the white DNA phase at the interface. 500 μl of isopropanol was added to precipitate the RNA and the solution was shaken briefly. Samples were incubated at RT for 10 minutes before collecting the RNA by centrifuging for 10 minutes at 12000 x g at 4 °C. After centrifugation, the supernatant was carefully removed so as not to disrupt the pellet and discarded. The RNA pellet was then washed with 1ml 75% ethanol, and samples were centrifuged for 5 minutes at 7600 x g at 4 °C. The ethanol was discarded and the remaining ethanol was removed by air drying at 37 °C on a heated plate until the RNA pellet appeared glassy. Finally, the RNA was dissolved in 20μl diethylpyrocarbonate (DEPC)-treated ddH₂O and then incubated for 10 minutes at 60°C. RNA concentration was measured by NanoPhotometer™ (Implen) and the RNA was stored at -80°C.

DNase treatment

DNase treatment was used to remove contaminating DNA by using an Ambion Kit. The process included preparing a mixture of 3 μg RNA with 1μl DNase, 3μl 10x Buffer, and RNase-free water to a final volume of 30 μl and the mixture was incubated at 37 °C for 30 minutes. 0.77 μl of 200 mM EDTA was then added to the sample and incubated at 75 °C for 10 minutes. After DNase treatment, genomic DNA contamination can be checked by amplifying β-actin gene by PCR (35 cycles) in a PCR machine (Techne). The DNase-treated RNA was stored at -80°C.
Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To generate the cDNA from RNA, SuperScript™ II Reverse Transcriptase kit (Invitrogen) was used. 9μl RNA from DNase-treated RNA sample, 1μl 10mM dNTP (Invitrogen), 1μl oligo (dT) primer (0.5μg/ul) (Invitrogen) and 1μl RNase-free H₂O to the final volume of 12 μl were mixed and then incubated at 65°C for 5 minutes. The mixture was then placed on ice for 1 minute, and 8μl of prepared PCR mixture including 4μl 5x First-Strand buffer, 2μl 0.1M DTT, 1μl RNase-free water and 1 μl SuperScript™ II Reverse Transcriptase was added. Samples were then incubated at 42°C for 52 minutes and followed by 70°C for 15 minutes. The cDNA was stored at -20°C.

Polymerase Chain Reaction (PCR)

To check for DNA contamination after DNase treatment or to analyse cDNA after RT-PCR, genomic DNA or cDNA was amplified by PCR. For PCR, the forward and reverse primers (Table 3.3), and H₂O were mixed in a 10μl volume, prior to addition of 10μl 2X PCR Master Mix (Thermo). The reaction was carried out in PCR machine using the following program: 94 °C for 5 minutes, amplify the products (24 to 35 cycles) of 94 °C for 1 minute, annealing temperature for 1 minute, and 72 °C for 30 seconds, and followed by the final extension at 72 °C for 10 minutes. PCR products then run on 1.5% agarose gel with 100 base pair (bp) DNA ladder (Thermo) and images were obtained by using an Alpha Imager™ 3400 system.
<table>
<thead>
<tr>
<th>Primer name*</th>
<th>Sequence (5’→3’) Forward</th>
<th>Sequence (5’→3’) Reverse</th>
<th>Tm (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M)β-ACTIN</td>
<td>AAGAGCCTATGAGCCTGCCTGA</td>
<td>TACGGATGTCAACGTCACAC</td>
<td>54</td>
<td>160</td>
</tr>
<tr>
<td>(M)ALPI</td>
<td>TGGATGCTGCAAGAAGCTGC</td>
<td>AGAGATAGGCGGTGGCTGTCG</td>
<td>56</td>
<td>243</td>
</tr>
<tr>
<td>(M)CDX2</td>
<td>CCATCACCGCGCATCACACGCG</td>
<td>AGTGAACCTCTTCTCCAGGCTCCAGC</td>
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<td>272</td>
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<tr>
<td>(M)FOXA2</td>
<td>CAAGTGTGAGAAGCAACTGG</td>
<td>GATAAGAAGGGGGTGTGTTGA</td>
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<td>373</td>
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<tr>
<td>(M)HNF4A</td>
<td>ACAGGAGAGGGTCAGAAGCA</td>
<td>GATGTGTCACAAACACAGG</td>
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<td>180</td>
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<tr>
<td>(M)HNF4A-2**</td>
<td>GAAAGTGCTTCGGGCTGCG</td>
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<td>(M)MUC2</td>
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<td>(M)PDX1</td>
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<td>CCACCCAGTATTACAAGCTCTC</td>
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<td>325</td>
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<tr>
<td>(M)TFF3</td>
<td>AGATTACCTGGCCTGTCCTCC</td>
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<td>341</td>
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<tr>
<td>(M)SI</td>
<td>GGCAGATACGGCTGTGCG</td>
<td>CGCAGCTCTTGGAAAGGCC</td>
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<td>271</td>
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<td>(M)VILLIN1</td>
<td>TATGATATACCTACTGGATGGGC</td>
<td>GCTTGAATGGCAAGCCTAGCG</td>
<td>54</td>
<td>586</td>
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<tr>
<td>(H)β-ACTIN</td>
<td>ATGGATGATGATATCGCGCGG</td>
<td>ATCTGGTGTACCTTCTCGCG</td>
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<td>365</td>
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<tr>
<td>(H)CDX2</td>
<td>AGAGCAAAAGGAGAGAAATCAAC</td>
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<td>281</td>
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<tr>
<td>(H)CDX2-2***</td>
<td>AGAGCAAAAGGAGAGAAATCAAC</td>
<td>GTGAGCGTGGGTTTAGCA</td>
<td>58</td>
<td>224</td>
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<tr>
<td>(H)FOXA2</td>
<td>GCGACCCCAAGACCTACAG</td>
<td>GGTTCCTGGCCGTGAGAAGGG</td>
<td>58</td>
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<tr>
<td>(H)K14</td>
<td>CTGGAAGCAGAATATCCGCTGC</td>
<td>TCTCGTCTTGGCCGCTGTCCG</td>
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<tr>
<td>(H)K18</td>
<td>AGAACAGAGAAGGAGACATGC</td>
<td>GGCAATTGCTCACATTTTGGC</td>
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<tr>
<td>(H)K20</td>
<td>AACCTAATGACGCTGTTATGC</td>
<td>ATCAATTTGCGAGCACACC</td>
<td>58</td>
<td>213</td>
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<tr>
<td>(H)MUC2</td>
<td>AACAACCTCTGCTAAGGTCATC</td>
<td>CAAATGTGGCAGCAGAAGGCA</td>
<td>58</td>
<td>281</td>
</tr>
<tr>
<td>(H)S1</td>
<td>CCGCCTGGGTCACTTTGGAAGCA</td>
<td>ACCACCCGACATGGGGAGC</td>
<td>58</td>
<td>341</td>
</tr>
<tr>
<td>(H)LGR5</td>
<td>TGCTGCTGCTGTGAGTCGCG</td>
<td>GCCAGCAGCGACAGACAGCA</td>
<td>58</td>
<td>242</td>
</tr>
<tr>
<td>(H)ISX</td>
<td>GCAAGAAGAGGTTTGTGGTTC</td>
<td>GGGTGTGCGAGCAGAGTGA</td>
<td>58</td>
<td>422</td>
</tr>
<tr>
<td>(H)K20</td>
<td>AACCTAATGACGCTGTTATGC</td>
<td>ATCAATTTGCGAGCACACC</td>
<td>58</td>
<td>213</td>
</tr>
<tr>
<td>(H)VILLIN1</td>
<td>ATGGATGACCTCTGCAAGGCG</td>
<td>TCTCCATGCGGGTGGTCTTCCG</td>
<td>56</td>
<td>308</td>
</tr>
</tbody>
</table>

*Primers are for: (M) mouse genes or (H) human genes. **For checking full length HNF4α in lenti-HNF4α only ***For checking CDX2 in Lenti-HCH only.
2.12 Immunostaining

PFA fixation
For paraformaldehyde (PFA) fixation, cells cultured on glass coverslips in 35mm dishes were washed twice with 2ml PBS and then fixed with 1.2 ml 4% (v/v) PFA (Sigma) for 20 minutes. After fixation, cells were washed three times with PBS and were then topped-up with PBS and left in the fridge until needed.

Acetone:Methanol fixation
In acetone: methanol fixation, cells in 35mm dishes were washed twice with PBS and fixed with 1.2 ml ice cold acetone:methanol (1:1 v/v) for 10 minutes. After fixation, cells were washed three times with PBS and were then topped up with PBS at 4°C until required.

Permeabilization, antigen retrieval and blocking
Permeabilization was only performed on PFA-fixed cells. For samples of cell culture, 0.5 ml of 1% (v/v) Triton-X100 (Sigma) in PBS was added and incubated for 30 minutes. For cells which required antigen retrieval, prior to the blocking step, cells were incubated in 1X citrate buffer (Lab vision) at 37°C for 1 hour. Blocking was carried out by adding 1.2 ml of 2% (v/v) blocking buffer (Roche) for 30 minutes.

Antibody staining
After blocking, the blocking buffer was removed and the cells were then incubated with 200μl of primary antibody diluted in 2% blocking buffer overnight at 4°C. Antibodies are listed in Table 2.4. After the antibody binding step, cultures were washed gently with PBS three times for 15 minutes on a shaker. To avoid fluorescent bleaching of the signals, the following steps were performed in the dark. 200μl of fluorescent secondary antibody (Table 2.5) diluted in 2% blocking buffer was applied for 2 hours, and any unbound secondary antibody was removed by washing with PBS as described above. To counterstain the nuclei, cells were stained with 1ml 4’,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml) solution (Sigma) for 10 minutes and then washed with PBS three times for 15 minutes. The slides were then mounted with mounting gel (Thermo). Immunostaining images were observed by using Leica DMRB microscope or Zeiss LSM510META confocal microscope.
Table 2.4. Primary antibodies used in the immunostaining

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Supplier</th>
<th>Fixation</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Smooth muscle actin (1A4)</td>
<td>Mouse</td>
<td>Sigma</td>
<td>4% PFA or Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>Cdx2 (88)</td>
<td>Mouse</td>
<td>BioGenex</td>
<td>4% PFA</td>
<td>1:100</td>
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<tr>
<td>E-cadherin (C36)</td>
<td>Mouse</td>
<td>Transduction labs</td>
<td>4% PFA or Acetone/Methanol</td>
<td>1:100</td>
</tr>
<tr>
<td>HNF4α (H171)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>4% PFA</td>
<td>1:100</td>
</tr>
<tr>
<td>K14 (LL002)</td>
<td>Mouse</td>
<td>Neomarkers</td>
<td>Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>K14</td>
<td>Rabbit</td>
<td>Thermo</td>
<td>Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>K8 (Troma 1)</td>
<td>Rat</td>
<td>Developmental studies hybridoma bank</td>
<td>Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>K4</td>
<td>Mouse</td>
<td>Sigma</td>
<td>Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>K20</td>
<td>Mouse</td>
<td>Dako</td>
<td>Acetone/Methanol</td>
<td>1:200</td>
</tr>
<tr>
<td>p63 (4A4)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>4% PFA</td>
<td>1:50</td>
</tr>
<tr>
<td>Sucrose isomaltase</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>4% PFA</td>
<td>1:100</td>
</tr>
<tr>
<td>Villin (CWWB1)</td>
<td>Mouse</td>
<td>Abcam</td>
<td>Acetone/Methanol</td>
<td>1:100</td>
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</table>

Table 2.5. Secondary antibodies used in the immunostaining

<table>
<thead>
<tr>
<th>Name</th>
<th>Host</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rat texas red</td>
<td>Rabbit</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit fluorescein</td>
<td>Horse</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit texas red</td>
<td>Goat</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-mouse fluorescein</td>
<td>Goat</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-mouse texas red</td>
<td>Goat</td>
<td>Vector</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit Alexa Fluor 546 IgG</td>
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<td>1:250</td>
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<tr>
<td>Anti-rabbit Alexa Fluor 633 IgG</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>1:250</td>
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<tr>
<td>Anti-mouse Alexa Fluor 546 IgG</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>1:250</td>
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<tr>
<td>Anti-mouse Alexa Fluor 633 IgG</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>1:250</td>
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</tbody>
</table>
2.13 Quantitative polymerase chain reaction (qPCR)
Quantitative PCR (qPCR), also known as Real-time PCR, was used to quantify and compare gene expression levels. LightCycler FastStart Reaction DNA Master SYBR Green I kit (Roche) was used. To perform Real-time PCR, 8 µl mixture of 0.5 µl of 10mM forward and reverse primers, 0.6 µl of 25mM MgCl₂, 1µl enzyme containing LightCycler FastStart Reaction Mix SYBR Green I and 5.4 µl H₂O was added into LightCycler capillary. 2µl cDNA was then added into the same capillary. Capillaries were centrifuged briefly and then were transferred to LightCycler instrument.
Real-time PCR program includes preincubation at 95°C for 10 minutes, amplification for 40 cycles of 95°C for 10 seconds, primer melting temperature (Tm) for 10 sec and 72°C for (product length/25) seconds. The temperature transition rate is at 20 °C /second unless otherwise indicated. Melting curves analysis starts at 95°C for 0 second, 65 °C for 15 seconds and then increases to 95°C at rate of 0.1 °C /second followed by cooling to 40°C. LightCycler software was used to analyse Real-time PCR results. Primers used for real-time PCR are shown in Table 2.6. The amplification efficiency is between 90 to 110%.

Table 2.6. Primers for real-time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’) Forward</th>
<th>Sequence (5’→3’) Reverse</th>
<th>Tm (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-ACTIN</td>
<td>taggcaccagggtgtgatgg</td>
<td>catggctggggtttgaagg</td>
<td>58</td>
<td>323</td>
</tr>
<tr>
<td>K20</td>
<td>ctgaaataagacctagctctcctcaa</td>
<td>tgtgcctagctgtggtg</td>
<td>68</td>
<td>76</td>
</tr>
<tr>
<td>VILLIN</td>
<td>ttcctggctgggatccctt</td>
<td>ccacctgggcttgac</td>
<td>68</td>
<td>121</td>
</tr>
</tbody>
</table>
2.14 Western blotting

Cell lysate preparation
Cells which were 90-100% confluent in the well of 12-well plate were washed with cold PBS 3 times. Cells were then lysed in 100 µl lysis buffer (Table 2.9) on ice for 15 min. Cell lysate was transferred to a 0.5ml tube and was stored at -20 °C.

Protein concentration measurement
To measure protein concentration, 5-10 µl protein was mixed with 100 µl protein assay dye (BioRad) and ddH₂O to a total volume of 500 µl. Bovine Serum Albumin (BSA) (Sigma) standard solution was prepared by mixing 0, 2, 5, 10, 15 and 20 µl of 1 mg/ml BSA with 200µl protein assay dye and ddH2O to the total volume of 1000µl. After 5 minutes incubation, 200 µl samples and standards solution were transfer to a 96-well plate. The absorbance of each standard or sample was measured at 595nm on an spectrophotometer (Tecal Spectra). The standard curve was obtained by plotting absorbance against BSA concentration. Protein concentration equation was then calculated and the protein concentration was obtained.

SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis)
BioRad SDS-PAGE system was used for protein electrophoresis. The cell lysate was mixed with 4x sample buffer and boiled at 95°C for 5 minutes. Cell lysate was then loaded into the well of the 8 or 10% SDS-PAGE gel. The gel was run in running buffer (Table 2.9) at 80V for 30 min and then 100V for 2 to 3 hours.
To transfer protein to the membrane, 5 x 9 cm PVDF membrane (BioRad) was rinsed with 100% methanol (Sigma) and was then immersed in transfer buffer (Table 2.9). Transfer stack was placed following the order from the top to the bottom - filter paper, gel, membrane and filter paper on a semi-dry transfer machine. The protein transfer step was performed at 25 volt for 1 hour.

After the transfer step, the membrane was incubated in the 5% non-fat milk prepared in PBST for 1 hour at room temperature on a rotator. The membrane was incubated with primary antibody (detailed in Table 2.7) diluted in 3% non-fat milk in PBST overnight at 4 °C. The membrane was washed with PBST 3 times, 10 minute each. Membrane was then incubated for 1 hour at room temperature with horseradish
peroxidase (HRP) conjugated secondary antibody (Table 2.8) diluted in 3% non-fat milk in PBST. The membrane was washed with PBST for 3 times, 10 minute each. To detect the signal, the membrane was incubated with enhanced chemiluminescence (ECL) solution (Thermo) for 1 minute. The membrane was then placed between two transparency films in a film cassette. Autoradiography film (Amersham) was exposed by placing on the top of the membrane in the dark room. Film was processed in an X-ray film processor (Protec).

### Table 2.7. Primary antibodies used in western blotting

<table>
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<tr>
<th>Name</th>
<th>Species</th>
<th>Supplier</th>
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<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Ambion</td>
<td>1:40000</td>
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<tr>
<td>ppERK (MAP kinase)</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:1000</td>
</tr>
<tr>
<td>Phopho-NF-κB p65 (Ser536)(93H1)</td>
<td>Rabbit</td>
<td>Cell signaling</td>
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### Table 2.8. Secondary antibodies used in Western blotting

<table>
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<tr>
<td>HRP conjugated Anti-mouse</td>
<td>Horse</td>
<td>Vector</td>
<td>1:3000</td>
</tr>
<tr>
<td>HRP conjugated Anti-rabbit</td>
<td>Goat</td>
<td>Vector</td>
<td>1:3000</td>
</tr>
<tr>
<td>Name</td>
<td>Supplier</td>
<td>Preparation methods</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------</td>
<td>----------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>1m</td>
<td></td>
<td>10mM in DMSO, stored at -20°C</td>
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</tr>
<tr>
<td>Dextran</td>
<td>Sigma</td>
<td>Dissolved in H₂O to 1mg/ml, filtered, stored at -20°C.</td>
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<tr>
<td>5’Azacytidine (5’Aza)</td>
<td>Sigma</td>
<td>Dissolved in PBS to 1mM, stored at -20°C.</td>
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<tr>
<td>Deoxycholic acid (DCA)</td>
<td>Sigma</td>
<td>Dissolved in ethanol to 200mM, store at 4°C.</td>
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<tr>
<td>Cholic acid (CA)</td>
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<td>Dissolved in ethanol to 400mM, stored at 4°C.</td>
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<tr>
<td>Taurocholic acid (TCA)</td>
<td>Sigma</td>
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<tr>
<td>Glycocholic acid (GCA)</td>
<td>Sigma</td>
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<tr>
<td>DAPT (γ-secretase inhibitor IX)</td>
<td>Calbiochem</td>
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<tr>
<td>BMP4</td>
<td>Peptotech</td>
<td>Reconstituted in 5mM HCl, pH3.0 to 100µg/ml, stored at -20°C</td>
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<tr>
<td>IL1β</td>
<td>Peptotech</td>
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<tr>
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<tr>
<td>Ampicillin</td>
<td>Sigma</td>
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<tr>
<td>Name</td>
<td>Supplier</td>
<td>Preparation methods</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>LB agar</td>
<td>Sigma</td>
<td>Dissolved 0.8g LB agar and 10g LB in 500 ml H₂O, autoclaved</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>Sigma</td>
<td>Dissolved 10g LB in 500mL H₂O, autoclaved</td>
<td></td>
</tr>
<tr>
<td>4% PFA</td>
<td>Fisher</td>
<td>Add 20g paraformaldehyde into 400ml PBS, heated at 60°C till dissolved. Adjust pH to 7.4 with 1M NaOH. Add PBS to 500 ml and stored at 4°C.</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Sigma</td>
<td>Dissolved 477.5g PBS in H₂O to 5L as 10X PBS stock. Dilute 10X PBS to 1X PBS with H₂O</td>
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</tr>
<tr>
<td>DAPI (4',6-Diamidino-2-phenylindole)</td>
<td>Sigma</td>
<td>Dissolved in H₂O, stored in dark at 4°C</td>
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<td>2% blocking buffer</td>
<td>Roche</td>
<td>50g blocking buffer powder dissolved in Maleic acid buffer (100mM Maleic acid, 150mM NaCl, pH 7.5) to 500ml as 10% blocking buffer. Further diluted in PBS to make 2% blocking buffer, stored at -20°C</td>
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<tr>
<td>Transfer buffer</td>
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<td>24.8mM Tris, 192mM Glycine, 20% methanol</td>
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<td>4x protein sample buffer</td>
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<td>200mM Tris-Cl (pH6.8), 400mM dithiothreitol (DTT), 2% (w/v) SDS, 0.4% bromophenol blue, 10% (v/v) glycerol, stored at -20°C</td>
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<td>PBST</td>
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<td>PBS with 0.1% Tween-20</td>
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<td>SDS-PAGE Running buffer</td>
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<td>24.8mM Tris, 192mM Glycine, 3.5mM SDS</td>
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<td>Cell lysis buffer</td>
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<td>75mM NaCl, 0.5mM EDTA, 3mM DTT, 10mM HEPES-KOH pH7, 0.5% Triton X-100, with freshly added 0.1mM Na₃VO₄, 50mM β-Glycerophosphate, 50mM NaF and 100x protease inhibitor cocktail</td>
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Chapter 3
The role of HNF4α in Barrett’s metaplasia
3.1 Introduction

3.1.1 Intestinal transcription factor CDX2 involved in BM development

The induction of intestinal transcription factors is associated with the development of intestinal metaplasia. For example, CDX2 is an intestinal-specific transcription factor and whose expression can be found induced in tissue displaying intestinal metaplasia. It has been demonstrated that Foxa3-Cdx2 transgenic mice (in which CDX2 is ectopically expressed in the murine gastric mucosa) display an intestinal metaplasia phenotype (Silberg et al., 2002). In BM, CDX2 has also been considered as a key marker and a master transcription factor for BM (Eda et al., 2003; Moons et al., 2004; Vallböhmer et al., 2006). However, in contrast, in vitro studies have suggested that CDX2 overexpression in reconstituted oesophageal epithelium cultures did not induce a columnar phenotype (Clemons et al., 2012). In addition, in vivo studies on K14-Cdx2 transgenic mice in which CDX2 is expressed in the basal squamous epithelial cells, also did not develop an intestinal phenotype. Interestingly, when DNA methylation was inhibited by treating the K14-Cdx2 mice with 5’Aza, BM-like phenotype was observed with the increase expression of K18 and Cdx1 genes (Kong et al., 2011b). These findings imply that CDX2 alone is not sufficient to induce BM and that further epigenetic modification might be involved in promoting CDX2-related BM formation.

3.1.2 HNF4α is a candidate transcription factor for the development of BM

In addition to CDX2, HNF4α is a potential intestinal transcription factor candidate for the development of BM. In the intestine, HNF4α is expressed at the crypt region (Babeu et al., 2009). HNF4α in the intestinal epithelium regulates the cytodifferentiation, homeostasis, cell architecture and barrier functions (Babeu et al., 2009; Cattin et al., 2009). Conditional knockout of Hnf4α in adult mice (Villin-Cre-ERT2; Hnf4α<sup>loxP/loxP</sup>) demonstrated that the cell growth rate in the crypt area was increased (Cattin et al., 2009). Hnf4α deletion also affected cell differentiation of the intestinal epithelium as goblet cell numbers were increased. The maturation of enterocytes and enteroendocrine cells was affected. There was a decrease in the enterocyte gene expression including microsomal triglyceride transfer protein (Mttp), apolipoprotein B (ApoB) and ApoA-IV and intestinal fatty acid binding protein (iFabp). There was also a reduction in the gene expression of
enteroendocrine cell markers such as gastric inhibitory polypeptide (Gip) and Somatostatin. In addition, Hnf4a deletion disturbed cell structure and cell-cell contact of the intestinal epithelium. The intercellular space of the tight junction is distended and the paracellular permeability is increased. Conditional deletion of Hnf4a in embryonic mice (Villin-CRE; Hnf4a^{loxP/loxP}), between E12.5 and E13.5 has been reported (Babeu et al., 2009). The results showed that compared with the wild type mouse, the number of goblet and enteroendocrine cells is increased in transgenic mice. The results also demonstrated a minor reduction in gene expression of different cell lineage markers, such as Si (enterocytes) and Lysozyme (goblet cells), but Chromogranin A (enteroendocrine cells) expression was slightly increased. These results suggest that HNF4α regulates cell proliferation and cytodifferentiation of the intestinal epithelium. In addition, transgenic mice in which Hnf4a (Foxa3-CRE; Hnf4a^{loxP/loxP}) was deleted from the E8.5 stage of development lacked crypts in the colon region and goblet cell maturation was also diminished (Garrison et al., 2006). The number of the immature goblet cells was increased and the number of the mature goblet cells decreased in the Hnf4a null mice. These results from transgenic mice studies all point to the fact that HNF4α is required for normal intestine function.

Since HNF4α is essential for normal intestinal development it is possible that ectopic expression of the transcription factor might play a role in the initiation of intestinal metaplasia. It has been shown that forced HNF4α expression in NIH-3T3 cells (mouse embryonic fibroblasts) and Mia PaCa-2 cells (human pancreatic carcinoma cell line) induced intestinal gene expression including VILLIN, APOA-IV and TFF3 (Babeu et al., 2009). These observations combined with the fact that HNF4α is expressed in BM (Piessen et al., 2007) and that ectopic expression of HNF4α induces VILLIN protein expression in adult mouse oesophageal epithelial explants (Colleypriest and Tosh personal communication) suggests that HNF4α may be involved in initiation of BM. However, whether HNF4α is a key driver in the development of BM remains to be discovered.
3.1.3 Methods of ectopic gene expression

Viral vectors have been used to deliver and express gene in the host cells (Benihoud et al., 1999). In this thesis, lentiviral and adenoviral vectors were utilized for ectopic gene expression in oesophageal epithelial cells. I will now briefly discuss the use of each type of expression system.

**Lentiviral vectors**

Lentivirus is derived from human immunodeficiency virus type 1 (HIV-1). Lentivirus transduces genes into both dividing and not-dividing cells. The transgene integrates into the host genome, so the transgene is stably expressed long-term. In the present study I utilized a third generation of lentiviral packing system (Tiscornia et al., 2007; Iacob et al., 2011). In this system, lentiviral vectors contain four plasmids: one transfer plasmid, two packaging plasmids and one envelope plasmid. The transfer vector contains the transgene. The packaging plasmid pMDLg/pRRE encodes the Gag, Pol and RRE proteins. Gag is required as a viral structural protein for viral particle production. Pol is necessary for reverse transcriptase and integrase activity. The second packaging plasmid pRSV-REV contains the REV protein which binds to the RRE (Rev-responsive element) sequence on the pMDLg/pRRE plasmid to facilitate viral RNA export. Envelope plasmid pVSV-G is required for the envelope protein which integrates into viral membrane for the transduction of a broad range of cell types. The four plasmids are transfected into HEK-293T cell to produce lentiviral particles which can then be recovered prior to infection of target cells (Tiscornia et al., 2007; Dropulicé, 2011).

**Adenoviral vectors**

Human adenovirus serotype 5 derived adenoviral vector system (in which the viral transcription units E1 and E3 were deleted) was used in the present study. E1 ablation results in the infectious virus no longer being able to be propagated in the target cells, but adenoviral particles still can be produced in the E1-expressing HEK293 cells. The E3 unit is necessary for evading host immunity and is not required in this system. Adenovirus infects both proliferating and non-dividing cells and adenovirus targets a wide variety of cell types. Adenoviral vector remains epichromosomal in the host cell, and thus the transgene is only transiently expressed.
(Graham et al., 1977; He et al., 1998; Benihoud et al., 1999; Luo et al., 2007).

3.1.4 Aim

We propose that HNF4α might drive the conversion of oesophageal stratified squamous epithelial cells to columnar intestinal cells. In the first part of this chapter, the HET-1A cell line was used as the study model. The HET-1A cell line originates from human oesophageal epithelium tissue and was immortalized by introduction of simian virus 40 (SV-40) large T-antigen sequence (Stoner et al., 1991). HET-1A cells have previously been used for the research into Barrett’s metaplasia or oesophageal adenocarcinoma (Liu et al., 2007; Kong et al., 2009; Goldman et al., 2010).

In order to observe the effects of HNF4α ectopic expression in the oesophageal epithelial cells, a lentiviral vector which carries the Hnf4α gene (and hereafter referred to as Lenti-HNF4α) was utilized to ectopically express HNF4α in HET-1A cells. To observe the long-term effects of HNF4α and also to obtain the homogenous HNF4α-expressing cell population, stable HNF4α-expressing HET-1A clones were established and gene expression profiles were determined.
3.2 Results

3.2.1 Characterisation of HET-1A cells

During the progress of this research we characterized the phenotype of the HET-1A cell line. Somewhat surprisingly, immunostaining results showed that HET-1A cells did not express the squamous transcription factor p63 (Fig. 3.1). However, the transcription factor was expressed in the mouse oesophageal epithelial explants (Fig. 3.1A, B). Immunostaining results also demonstrated that HET-1A cells did not express K14 a marker for basal squamous epithelial cells, but K14 was expressed in the mouse oesophageal epithelial explants when cultured in BME medium (Fig. 3.1C, D). Interestingly, HET-1A cells expressed the columnar marker K18 and mesenchymal marker Vimentin (Fig. 3.1E, F). In addition, the epithelial adhesion protein E-Cadherin was not detected in HET-1A cells, but was robustly detected in mouse oesophageal epithelial explants (cultured in BME medium) (Fig. 3.2A, B). We also confirmed using immunostaining that the intestinal transcription factors HNF4α and CDX2 were not expressed in either HET-1A cells or the mouse oesophageal explant model (Fig. 3.2C, D, E, F). Confirmation of the HNF4α and CDX2 antibodies was carried out in Caco-2 cells (a colorectal adenocarcinoma cell line).

3.2.2 Ectopic HNF4α expression in HET-1A cells

We initially used the lentivirus-mediated method to achieve ectopic HNF4α expression in the HET-1A cells. Hnf4α expression can be detected 2 days after lentiviral-HNF4α (lenti-HNF4α) infection (Fig. 3.3A). Examination of gene expression at day14 showed that forced HNF4α expression did not initiate the transcription of intestinal genes including CDX1, CDX2 and SI, but HNF4α induced intestinal VILLIN gene expression (Fig. 3.3B).

3.2.3 Establishment of Hnf4α-expressing HET-1A clones and the examination of gene expression

In order to establish HNF4α-expressing HET-1A cells, HET-1A cells were infected with lenti-HNF4α for 24 hours and then the medium was replaced with fresh medium. Two days after infection, cells were sub-seeded into 5 x 96-well plates. Single cells from lenti-HNF4α infected cultures were selected and expanded. Five
clones were obtained. The further expansion of HNF4α clone 2, 3 and 4 was not successful due to the poor proliferation and cell death. Finally, HNF4α clone 1 (HNF4α-c1) and HNF4α clone 5 (HNF4α-c5) were obtained. Immunostaining results confirmed that HNF4α-c1 and HNF4α-c5 expressed HNF4α (Fig. 3.4A). There was no morphological change following HNF4α ectopic expression in both HNF4α-c1 and HNF4α-c5 (Fig. 3.4B). However, the growth rate of both HNF4α-c1 and HNF4α-c5 was lower than the control HET-1A cells (Fig. 3.4C).

The lenti-HNF4α vector as well as encoding the sequence for HNF4α also encodes a sequence for green fluorescence protein (GFP). GFP was co-expressed with the transgene and allows the real-time indication of the transgene expression. Surprisingly, the green fluorescent signal from GFP protein was not visible in the HNF4α-c1 and HNF4α-c5 HET-1A cells under epifluorescence microscopy (Fig. 3.5A). However, immunostaining for GFP confirmed that GFP was indeed expressed albeit weakly (Fig. 3.5B). The absence of green fluorescence from GFP might be due to a low expression level of GFP protein. Immunostaining results also showed that HNF4α did not induce E-cadherin expression (Fig. 3.5C).

RT-PCR analysis technique was used to examine whether HNF4α ectopic expression of HNF4α induced intestinal genes (Fig. 3.6). The results showed that VILLIN gene expression was induced, but CDX2 expression was not detected in the HNF4α-c1 and HNF4α-c5 HET-1A cells. Moreover the expression of the squamous epithelial cell marker K14 was slightly down-regulated, but the columnar cell marker K18 was unchanged in both HNF4α-c1 and HNF4α-c5 HET-1A cells.

3.2.4 Mutant HNF4α does not induce intestinal VILLIN gene expression

As well as testing the ectopic expression of a lentiviral vector containing a normal version of Hnf4α, we also created one lentiviral vector which carried both N and C-terminal truncated Hnf4α transgene. Lenti-mutant HNF4α or Lenti-HNF4α viral particles were prepared and used to infect HET1-1A cultures. RT-PCR results showed that the conserved Hnf4α sequence region between Hnf4α and the mutant Hnf4α can be amplified by the Hnf4α-1 primer set, but only normal Hnf4α can be amplified by the Hnf4α-2 primer set (Fig. 3.7A,B). RT-PCR results also confirmed
that mutant HNF4α was unable to induce VILLIN gene expression (Fig. 3.7B). Furthermore, immunostaining results demonstrated that the wild type HNF4α can be recognized by the HNF4α antibody which bound to the 295-465 amino acid at the C-terminal of HNF4α (Fig. 3.7C). However, the truncated HNF4α was not detected by the HNF4α antibody.
**Figure 3.1. Characterisation of the HET-1A cell line**

Immunostaining was carried out in order to characterise the phenotype of the HET-1A cells. (A, C) p63 and K14 were not detected in HET-1A cells. (B, D) p63 and K14 expression in mouse oesophageal epithelial explants were used as positive controls. (E, F) HET-1A cells are positive for K18 and Vimentin. Nuclei were stained in blue with DAPI. One of two experiments is shown.
Figure 3.2. Characterisation of epithelial and intestinal markers in the HET-1A cell line

Immunostaining was performed to characterise the HET-1A cells for epithelial and intestinal markers. E-cadherin was not expressed in HET-1A cells (A), but was detected in the mouse oesophageal epithelial explants (B). Intestinal transcription factors CDX2 and HNF4α were not expressed in HET-1A cells (C, D) but were expressed in Caco-2 cells which were used as the positive controls (E, F). Nuclei were stained in blue with DAPI. One of two experiments is shown.
Figure 3.3. Effects of ectopic expression of HNF4α in HET-1A cells

(A) HET-1A cells were infected with Lenti-HNF4α virus. Twenty-four hours after infection, the culture medium was replaced and cells were fixed at day 2. Immunostaining results showed that HNF4α can be detected 2 days after Lenti-HNF4α infection. No virus infected cells (control) were negative for HNF4α.

(B) RT-PCR was performed on RNA isolated from HET-1A cells 14 days after Lenti-HNF4α infection. The expression of intestinal genes including CDX1, CDX2, SI and VILLIN were examined. Note that Hnf4α transgene is of mouse origin. β-ACTIN was used as a loading control. Controls include HT-29 cells (positive, +) and reaction without sample (negative, -). One of two experiments is shown.
A. DAPI/HNF4α

B. Phase contrast

Cell growth rate

C. Cell count vs. Day
**Figure 3.4. Creation of stable HNF4α-expressing Het1a clones**

HET-1A cells were infected with Lenti-HNF4α virus for 24 hours and then the medium was replaced. At day 2, single-cells were clonally expanded in a 96 well format. (A) HNF4α expression was analysed by immunostaining. Two out of 5 HNF4α-expressing stable clones were obtained (termed HNF4α clone 1 (HNF4α-c1) and HNF4α clone 5 (HNF4α-c5)). (B) Phase contrast images of HNF4α-c1, HNF4α-c5 and control HET-1A cells. (C) At day 0, cells were seeded at 30000 cells in a 35 mm dish and cultured. Cells were trypsinized and the cell number was calculated at the time points indicated (n=3).
**Figure 3.5. Phenotypic characterisation of the stable HNF4α-expressing Het-1A clones**

HNF4α-c1 or HNF4α-c5 cells were seeded on a glass coverslip in a 4-well plate. At day 3, cells were fixed. (A) Lenti-HNF4α construct contains a green fluorescence protein (GFP) gene, but the green fluorescence was not visible under the fluorescence microscopy. (B) Immunostaining for GFP demonstrated that GFP is expressed in the HNF4α-c1 and HNF4α-c5 cells. (C) HNF4α-c1 and HNF4α-c5 cells did not express E-Cadherin, as visualised by immunostaining. Nuclei were stained in blue with DAPI. One of two experiments is shown.
Figure 3.6. Examination of intestinal gene expression in Hnf4α-Het1a clones
HNF4α-c1 or HNF4α-c5 cells were seeded into 6-cm dishes. Seven days later, cells were harvested. RT-PCR result shows that CDX2 mRNA expression is not detected. Intestinal marker VILLIN expression is founded in both HNF4α-c1 and HNF4α-c5 cells. Expression of the squamous cell marker K14 was slightly decreased in HNF4α-c1 and HNF4α-c5 cells, but the columnar cell marker K8 was unchanged. β-ACTIN was used as a loading control. The experiments were performed in triplicate.
A. Primer design

HNF4α

Mutant HNF4α (50FCD2)

B. RT-PCR

<table>
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<tr>
<th></th>
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<th>GFP</th>
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<th>Lenti-mutant HNF4α</th>
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<td>mHNF4α-1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>mHNF4α-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VILLIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-ACTIN</td>
<td></td>
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</table>

C. Immunofluorescence

Ctrl  Lenti-HNF4α  Lenti-mutant HNF4α
Figure 3.7. RT-PCR and HNF4α immunostaining for HET-1A cells infected with Lenti-HNF4α or Lenti-mutant HNF4α

5’ and 3 truncated HNF4α lentiviral constructs were created and the viral particles were produced. HET-1A cells were infected with Lenti-HNF4α or Lenti-mutant HNF4α viruses. The medium was replaced with fresh medium 24 hours later. Fourteen days after infection, HET-1A cells were harvested for RT-PCR. (A) Figure displays the primers used for Hnf4α PCR (B) RT-PCR results showed that HNF4α-1 primer set amplified both the truncated and wild type Hnf4α, whilst the HNF4α-2 primer set only amplified the wild type Hnf4α. VILIN expression was only found in the Lenti-HNF4α infected cultures. (C) HNF4α antibody which recognises the 295-465 amino acids at the C-terminus of HNF4α was used for immunostaining. Lenti-HNF4α infected cells were positive for HNF4α staining, but Lenti-mutant HNF4α infected cells were negative for HNF4α staining. One of two experiments is shown.
3.3 Discussion

3.3.1 Characterisation of HET1-A cells

We initially examined the phenotype of the HET-1A oesophageal cell line. This cell line has been used extensively in studies on Barrett’s oesophagus (Liu et al., 2007; Vaninetti et al., 2009; Rafiee et al., 2009; Goldman et al., 2010)

Examination of HET-1A cells by immunostaining showed that the HET-1A cells did not express either p63 or K14. p63 is the master transcription factor for stratified squamous epithelium and is expressed in the basal region of some epithelial tissue including epidermis, oesophagus, cervix, urothelium and prostate (Yang et al., 1998).

In the oesophageal epithelium, p63 is expressed in the cell nucleus at the basal or parabasal region of the epithelium (Yang et al., 1999; Chen et al., 2008). K14 is a marker for differentiated squamous epithelial cells and is expressed in the cytoplasm of the basal stratified squamous epithelium (Yu et al., 2005; Chen et al., 2008).

Although the HET-1A cells did not express p63 and K14, they did express K8. K8 is a columnar epithelial marker and can be detected in the intestinal epithelium. Studies showed that in mouse, K8 is expressed in the oesophageal epithelium at embryonic and postnatal stages, but K8 is not detected in the oesophageal epithelium at adult stage (Yu et al., 2005). Interestingly, the epithelial adhesion protein E-cadherin is not expressed in HET-1A cells. Instead, the mesenchymal marker Vimentin was expressed. Vimentin is also considered as an epithelial-mesenchymal-transition (EMT) marker in the progression of cancer metastasis (Zeisberg & Neilson, 2009).

These results suggest that HET-1A cells might have lost some of the characteristics of oesophageal squamous epithelial cells and gained a columnar phenotype.

It has been reported that HET-1A cells are SV-40 large T-antigen transformed oesophageal epithelial cells (Stoner et al., 1991). In previous studies, HET-1A cells were tested in an organotypic culture system in which cells were co-cultured with fibroblasts (Underwood et al., 2010). Unlike the normal human primary oesophageal epithelial cells which were able to develop the stratified structure with a basal layer, resembling the in vivo oesophageal squamous epithelium, HET-1A cells developed a disordered dysplastic phenotype (Underwood et al., 2010). At present there is currently a lack of a cell line which fully represents the normal oesophageal epithelial cells. Additionally, the HET-1A cell line has been widely used for BM
studies (Liu et al., 2007; Kong et al., 2009; Goldman et al., 2010). There are therefore limitations when using HET-1A cell line as a BM study model. However, these limitations were found at a late stage of my PhD. Therefore, the HET-1A cell line was used as a model for BM research in this thesis.

NES (normal esophageal squamous) cell line has also been used as an in vitro model for BM research (Jaiswal et al., 2006; Zhang et al., 2008; di Pietro et al., 2012). NES cells were derived from normal human esophageal epithelial cells and are immortalized by introducing human telomerase reverse transcriptase (hTERT) (Morales et al., 2003; Zhang et al., 2008). It was shown that NES cells maintain the squamous epithelial cell characteristics with the expression of squamous epithelial marker such as K13 and K4 (Morales et al., 2003; di Pietro et al., 2012). However, whether NES cells represent the normal esophageal epithelial cells and whether NES cells are suitable for the studies of BM development remains to be investigated. For example, one report showed that NES cells do not express columnar cell marker K8 (di Pietro et al., 2012). However, another report indicated that NES cells are positive for K8 expression (Morales et al., 2003). The characteristics of NES cells therefore still need to be investigated. In future, the development of an esophageal epithelial cell line which maintains esophageal epithelial characteristics will be of great benefit to BM research.

3.3.2 Ectopic HNF4α expression in HET-1A cells

We initially tried overexpressing Hnf4α in HET-1A cells using a lentiviral expression system. The purpose of generating stable clone was to obtain sustained and long term transgene expression in a genetically homogenous cell population. HNF4α-expressing HET-1A clones were successfully created and the ectopic Hnf4α expression suppressed cell proliferation. In the liver, HNF4α is an important transcription factor for hepatocyte differentiation (Li et al., 2000; Watt et al., 2003). HNF4α regulates liver transcription factors such as HNF1α and pregnane-x-receptor (PXR) (Li et al., 2000). HNF4α also regulates some genes which are important for normal hepatocyte functions such as ApoCIII, transthyretin (TTR) and cytochrome P450 3A4 (CYP3A4) (Sladek et al., 1990; Tirona et al., 2003). In addition, it has been reported that the deletion of Hnf4a in mice liver (Hnf4a$^{Flox/Flox}$)
Albumin-ERT2cre) increased both hepatocyte proliferation and liver-body mass ratio. These changes were due to the upregulation of mitogenic genes including c-Myc, cyclin dependent kinase 1 (Cdk1), and cyclin D1 and the reduction of basal apoptotic activity (Bonzo et al., 2012; Walesky et al., 2013). The inhibition of cell proliferation by HNF4α has also been reported in cell types such as HET293 (human embryonic kidney cells) and F9 cells (murine embryonic carcinoma cell) (Lucas et al., 2005; Chibaa et al., 2005). Therefore, it would be interesting to investigate whether the suppression in cell proliferation is linked to the decreased expression of mitogenic genes in the HNF4α-expressing HET-1A clones. Cyclins, CDKs and e-MYC of mitosis-related genes expression cloud be examined.

VILLIN is an actin-binding protein and is the key component of the brush border of absorptive cells in the intestine and renal proximal tubule epithelium (Maunoury et al., 1992; Maclennan et al., 1999). VILLIN is considered as a marker for BM. Two studies in which VILLIN expression was assessed by immunohistochemistry method demonstrated that 81% or 100% of BM biopsy samples were positive for VILLIN staining (Maclennan et al., 1999; Shi et al., 2008), but VILLIN was not detected in the normal oesophageal epithelium or gastric columnar cells (Maclennan et al., 1999). In this chapter, although more intestinal and BM markers such as TFF3, K20 and MUC2 need to be examined (Lao-Sirieix et al., 2009; di Pietro et al., 2012), the induction of VILLIN expression by HNF4α implicated the development of intestinal metaplasia. In fact, the induction of VILLIN gene expression by HNF4α has been observed in NIH-3T3, Mia PaCa-2 and HEK293 cells (Lucas et al. 2005; Babeu et al. 2009), but the mechanism of induction has not yet been explored. VILLIN expression might be directly regulated by HNF4α. We identified two potential HNF4α binding sites (−26 ~ −14 bp GGGACAAAGGTCG and −1248 ~ −1260 bp GGGTCAGGGGTCA) in the −2 kb to +1 bp region of the VILLIN promoter by using web-based tool (HNF4 Motif Finder, http://nrmotif.ucr.edu) developed by the Sladek lab (Bolotin et al., 2010). In the future, the interaction between HNF4α and VILLIN expression could be examined by using a luciferase reporter assay. The luciferase reporter assay could be potentially performed in HET-1A cells or NES cells by co-transfecting HNF4α expression vector and the luciferase reporter vector in which the luciferase expression is driven by the wild type or the HNF4α-binding
site mutated \textit{VILLIN} promoter. The luminescence signal intensity will positively reflect the interaction strength between HNF4\textalpha protein and \textit{VILLIN} promoter.
Chapter 4
The role of candidate transcription factors in Barrett’s metaplasia
4.1 Introduction

The development of BM is linked to the ectopic expression of candidate transcription factors (particularly those of the intestine). In Chapter 3, it was shown that forced HNF4α expression in adult mouse oesophageal epithelium induced a partial squamous-to-columnar cell type change (based on induction of K8 and VILLIN and loss of K14 and p63). While HNF4α alone was able to bring about this phenotypic change, it did not result in a mature intestinal phenotype. It is therefore possible that intestinal transcription factors other than HNF4α may be involved in development of BM. These candidates include CDX2, HNF1α, PDX1 and FOXA2. The role of CDX2 in the intestine and BM has been reviewed in the introduction to Chapter 3.1.1 and therefore will not be examined here.

4.1.1 HNF1α

HNF1α is expressed along the crypt-villus axis in small intestinal epithelium (Escaffit et al., 2005; D’Angelo et al., 2010). The study of Hnf1α knockout mice demonstrated that Hnf1α is important for Paneth, enterocyte and Goblet cell differentiation. Hnf1α deletion reduced the gene expression of enterocyte differentiation marker dipeptidyl peptidase IV (DppIV) (D’Angelo et al., 2010). Hnf1α deletion also decreased the gene expression of Cdx2 which is a key regulator for enterocyte maturation (Suh & Traber, 1996; D’Angelo et al., 2010). In addition, knockout of Hnf1α moderately reduced the expression of genes that are associated with Goblet cell differentiation (such as Gob5) and Paneth cell differentiation (such as Lysozyme) (D’Angelo et al., 2010). In another study of Hnf1α knockout mice, the authors observed abnormalities such as the intestinalomegaly and increased cell proliferation in the crypt region. Hnf1α deletion also caused the disruption in Paneth cell differentiation and in enteroendocrine cell number (Lussier et al., 2010). Furthermore, HNF1α directly regulates intestinal genes such as Solute carrier family 26 member 3 (Slc26a3), Lactase and DppIV (Erickson et al., 1999; van Wering et al., 2002; Piessen et al., 2007; D’Angelo et al., 2010; Benoit et al., 2010). These data all point to the possibility that HNF1α is important for normal cell function and cytodifferentiation of the intestine epithelium. In terms of BM, HNF1α is not expressed in the normal oesophageal epithelium, but is detected in BM tissue and oesophageal adenocarcinoma (Piessen et al., 2007). Therefore, HNF1α might
potentially play an important role in the development of BM.

4.1.2 FOXA2
In the intestine, FOXA2 is coexpressed with MUC2. MUC2 is the mucus component of intestinal epithelium and a Goblet cells cell marker. FOXA2 regulates Muc2 transcription and is essential for normal expression (van der Sluis et al., 2008). Mice in which Foxa2 is deleted die by E10.5 due to the failure in forming the organizer node and notochord (Ang & Rossantt, 1994; Weinstein et al., 1994). The effects of conditional Foxa2 deletion in the intestine have not been reported. However, a report showed that the deletion of both Foxal and Foxa2 (Foxal/a2) in mice (Foxa1loxP/loxP, Foxa2loxP/loxP; Villin-Cre) affected goblet cell differentiation with the reduction of goblet cell number and mucin granules. Foxal/a2 deletion also reduced the number of differentiated enteroendocrine cells (Ye & Kaestner, 2009).

Importantly, although FOXA2 expression was not detected in the mouse oesophageal epithelium in adult life, FOXA2 expression has been investigated in E15.5 and E18.5 mouse oesophageal epithelium and FOXA2 was found to be expressed (Besnard et al., 2004). The expression of FOXA2 before the E15.5 stage of development remains to be investigated. Because K8 was also expressed in the oesophageal epithelium in the embryonic stage, but not in adult life, this may implicate a potential link between FOXA2 expression and the columnar phenotype.

4.1.3 PDX1
Pancreatic and duodenal homeobox 1 (PDX1) is a transcription factor expressed in the anterior part of the intestine, pancreas and posterior part of the stomach (Miller et al., 1994; Larsson et al., 1996). PDX1 is expressed along the villi and crypt region in the duodenum where it is believed to regulate intestinal cell differentiation. Overexpression of PDX1 in the undifferentiated rat intestinal IEC-6 cells drives cell differentiation towards the enteroendocrine cell type (Yamada et al., 2001). Conditional knockout of Pdx1 in mouse intestine (Pdx1loxP/loxP, Villin-Cre) disrupted the gene expression profile of enterocytes and enteroendocrine cells in the proximal small intestine. The genes which are decreased by Pdx1 deletion include alkaline phosphatase 3 (Akp3) of the enterocytes, and Gip and somatostatin of the enteroendocrine cells (Chen et al., 2009; Chen & Sibley, 2012). Furthermore, it was
shown that PDX1 regulates enterocyte gene transcription such as *Si*, fatty acid binding protein 1 (*Fabp1*) and *Lactase*, suggesting a regulatory role for PDX1 in nutrient metabolism (West & Oates, 2005; Chen et al., 2012b; Wang et al., 2004). Indeed, Pdx1 conditional deletion mice (*Pdx1*fl/fl; Villin-Cre) were born underweight and weight gain was delayed (Chen et al., 2012a). More importantly, globally repressing Pdx1 in adult mice (*Pdx1*TA/TA; *Tg*Pdx1) (*tTA*: tetracycline transactivator; *Tg*Pdx1: tTA-responsive Pdx1 transgene) showed hematoma formation with the intercalary regeneration in which the ectopic stratified squamous epithelium was integrated between the columnar epithelium at the gastro-duodenal junction. This suggests a pivotal role of PDX1 in the maintenance of positional identity in the gut (Holland et al., 2013).

**4.1.4 Network of intestinal transcription factors**

Intestinal gene expression is regulated by a combinatorial network of intestinal transcription factors (Traber & Silberg, 1996). For example, HNF4α interacts with the *CDX2* and *HNF1α* promoter regions in Caco2 cells and mouse small intestinal mucosa according to ChIP-chip (Chromatin Immunoprecipitation-chip) and ChIP-qPCR results respectively (Boyd et al., 2009). CDX2 partners with GATA-6 to interact with chromatin regions during proliferation, but during differentiation, CDX2 is accompanied by HNF4α to co-occupy the different chromatin regions (Verzi et al., 2010). Ectopic expression of HNF1α or CDX2 alone in human embryonic intestinal crypt (HIEC) cells does not induce *SI* gene expression, but the combination of HNF1α and CDX2 induced *SI* gene expression. HNF1α and CDX2 co-expression initiated intestinal differentiation, but only with the addition of GATA-4, did HIEC cells develop the differentiated features typical of intestinal cells such as microvilli (Benoit et al., 2010). These observations indicate that the transcription factor network is essential for modulating intestinal differentiation and intestinal cell function.

**4.1.5 Aims**

Firstly, given that HNF1α, PDX1 and FOXA2 are involved in intestinal differentiation they may act as potential drivers of BM. I therefore set out to determine the effects of ectopic expression of these intestinal transcription factors in
the HET-1A cell line. Secondly, because the network of master transcription factors is required for normal intestinal epithelial development and function, it is likely that multiple transcription factors are required for initiating BM. The expression of intestinal genes after ectopic expression of the multiple intestinal transcription factors was also investigated in the HET-1A cells.
4.2 Results

4.2.1 Determination of a suitable adenoviral MOI in HET-1A cells
To determine the amount of adenoviral particles required for transgene overexpression, different MOI (multiplicity of infection) of an adenoviral vector expressing Green fluorescent protein (Ad-GFP) was tested in HET-1A cells for 24 hours (Fig. 4.1). At day 3, no green fluorescence was observed in the absence of Ad-GFP. At an MOI of 5, about 60% of cells expressed green fluorescence following Ad-GFP exposure and with an MOI of 10, around 80% of cells expressed green fluorescence. Although nearly 100% of cells expressed intense green fluorescence at an MOI of 20, the cell viability was lowest among all the tested cultures. Therefore, an MOI of 10 was chosen for adenoviral infection experiments in the HET-1A cells.

4.2.2 Forced CDX2 and HNF4α expression induced intestinal gene expression
To investigate the effect of CDX2 and HNF4α ectopic overexpression, HET1-A cells were treated with Ad-CDX2 and Ad-HNF4α (MOI 10 in total) for 24 hours, and cells were harvested at different time points after infection up to day 6. RT-PCR reveals that the CDX2 reached the highest expression level by day 2 and decreased thereafter whereas HNF4α, expression appeared relatively stable over the time course (Fig. 4.2). Intestinal gene expression was also evaluated. VILLIN gene expression was induced in Ad-HNF4α-infected cultures either in the absence or presence of Ad-CDX2. VILLIN expression showed similar levels at day 2, 4 and 6 of culture. SI expression was provoked by Ad-CDX2, and both Ad-CDX2 and Ad-HNF4α infection and SI expression level is higher at day 2 and day 4. There was no significant change in K14 and K18 expression following overexpression of either CDX2 or HNF4α.

4.2.3 Forced PDX1 expression did not provoke intestinal gene expression
To understand whether ectopic PDX1 overexpression induces intestinal gene expression, HET-1A cells were infected with Ad-PDX1, Ad-CDX2, Ad-HNF4α alone or in combination (Fig. 4.3). Results from the RT-PCR showed that PDX1 did not provoke or enhance intestinal gene expression.
4.2.4 Ectopic CDX2, HNF1α and HNF4α expression induced intestinal gene expression

We further refined the experimental approach to investigate the effects of forced HNF1α expression in HET1-A cells. RT-PCR result showed that HNF1α, CDX2 and HNF4α alone or in combination all weakly induced intestine-specific homeobox (ISX) expression (Fig. 4.4). ISX encodes ISX intestinal transcription factor and is exclusively expressed in the intestinal epithelium in the adult (Choi et al., 2006). ISX regulates β,β-carotene absorption and vitamin A production (Lobo et al., 2010). Interestingly, SI and K20 were induced by the CDX2 alone or the transcription factor combinations which contained CDX2. In contrast, VILLIN was induced by the HNF4α alone or the transcription factor combinations including HNF4α. LGR5 was increased in CDX2 or the CDX2 containing combinations. In the intestine, LGR5 is expressed in the crypt base columnar cells and is considered as an intestinal stem cell marker (Barker et al., 2007). The CDX2 transgene in Ad-CDX2 originates from the mouse. We can therefore use different primers to distinguish the exogenous mouse Cdx2 gene from the endogenous human CDX2 gene expression. The expression of endogenous human CDX2 was not provoked nor was MUC2 induced. There was also no significant change in K14 and K18 expression level following CDX2 ectopic expression.

We extended this study to perform quantitative PCR (qPCR) analysis for VILLIN and K20 mRNA following overexpression of CDX2, HNF4α and HNF1α (Fig. 4.5). VILLIN qPCR measurements showed that Ad-CDX2 and Ad-HNF4α co-infection induced the highest VILLIN level followed by Ad-HNF4α infection alone. In addition, the K20 qPCR result demonstrated that the K20 level is highest in cultures infected with both Ad-CDX2 and Ad-HNF4α and the second highest level is in cultures infected with Ad-CDX2, Ad-HNF4α and Ad-HNF1α together. Immunostaining for VILLIN and HNF4α showed that CDX2, HNF4α and HNF1α ectopic expression did not provoke VILLIN protein expression in HET-1A cells (Fig. 4.6) and K20 and SI protein were also not detected in any of the cultures (Fig. 4.7, Fig. 4.8).

We wished to understand whether ectopic expression of HNF1α and CDX2 could
enhance intestinal gene expression in the stable HNF4α-expressing HET-1A clones (HNF4α-c1 and HNF4α-c5). To address this possibility, HET-1A cultures were infected with Ad-CDX2 or Ad-HNF1α alone and in combination. Results from RT-PCR analysis demonstrated that in both the HNF4α-c1 and HNF4α-c5 stable cell lines CDX2 in the absence and presence of HNF1α increased LGR5 and ISX transcription, and also induced K20 and SI transcription (Fig. 4.9). ISX was also weakly induced by HNF1α in HNF4α-c1, but not in HNF4α-c5. Results from qPCR analysis revealed that the level of the VILLIN gene was increased in HNF4α-c1 cells compared to the control HET-1A cells (Fig. 4.10). The K20 level was also increased in HNF4α-c1 cells infected with Ad-CDX2 in the absence and presence of Ad-HNF1α. VILLIN protein expression was also determined by immunostaining but was not induced (Fig. 4.11).

4.2.5 FOXA2, CDX2, HNF1α and HNF4α induces intestinal gene expression
To study the role of FOXA2 in BM development, HET-1A cells were infected with Lenti-FOXA2. Immunostaining result showed that FOXA2 protein was detected in Lenti-FOXA2 infected cultures, but not in control cultures (Fig. 4.12A). Because the Foxa2 transgene was of mouse origin, the mouse Foxa2 gene was expressed after Lenti-FOXA2 infection (Fig. 4.12B). Human type FOXA2 was very weakly expressed in both Lenti-GFP and Lenti-FOXA2 treated cells. RT-PCR results also showed that FOXA2 ectopic expression did not provoke intestinal gene expression. FOXA2 overexpression also did not change K14 and K18 expression in HET-1A cells.

We wanted to know whether ectopic expression of CDX2, HNF1α and HNF4α could induce intestinal gene expression in FOXA2-expressing cells. Lenti-FOXA2 infected HET-1A cells were further incubated with Ad-CDX2, Ad-HNF1α, Ad-HNF4α alone and in combination. Results from RT-PCR analysis showed that VILLIN was weakly induced by the transcription factor combination which contained HNF4α (Fig. 4.13). Additionally, SI, MUC2 and K20 were induced by CDX2 or the transcription factor combination which included this transcription factor. The endogenous human CDX2 expression was also examined. The results showed that the endogenous CDX2 expression was weakly detected in all the cultures which were incubated with
Ad-CDX2, Ad-HNF1α or Ad-HNF4α alone and in combination in the FOXA2-expressing cells.

4.2.6 Lenti-HCH infection did not induce intestinal gene expression
As it is not possible to control the relative levels of transcription factors in HET-1A cells we used the Lenti-HNF4α-2A-CDX2-2A-HNF1α (Lenti-HCH) viral particles to infect the cells. Immunostaining for CDX2 and HNF4α showed that the co-expression of CDX2 and HNF4α were observed in Lenti-HCH treated HET-1A cells (Fig. 4.14A). However, VILLIN and HNF4α co-staining results demonstrated that VILLIN protein was not induced by Lenti-HCH infection (Fig. 4.14B). The expression of intestinal genes in HET-1A cells infected with Lenti-HCH was also assessed by RT-PCR (Fig. 4.15). CDX2, HNF1α and HNF4α mRNA were ectopically expressed. However, intestinal gene expression was not induced by Lenti-HCH infection. In addition, there is no alteration in K14 and K18 expression.
Figure 4.1. Defining the optimal adenoviral multiplicity of infection in HET-1A cells

HET-1A cells were seeded the day prior to adenoviral infection. Different volumes of adenoviral GFP particles (Ad-GFP), corresponding to the required Multiplicity of infection (MOI), were added to the cell culture. Twenty-four hours after viral infection, the culture medium was replaced with fresh medium (but containing no virus). Green fluorescence images were taken at day 3 following infection. One of two experiments is shown.
Figure 4.2. Time course of CDX2 and HNF4α expression in HET-1A cells

HET-1A cells were incubated with Ad-Null, Ad-GFP, Ad-CDX2, Ad-HNF4α, or Ad-CDX2 and Ad-HNF4α viruses for 24 hours and then the medium was replaced with fresh medium. Cells were collected at day 2, 4 or 6 and RT-PCR was performed. Controls include HT-29 or mouse intestine (positive, +) and PCR reaction without sample (negative, -). β-ACTIN was used as a loading control. One experiment is shown.
**Figure 4.3. Ectopic CDX2, HNF4α and PDX1 expression induce intestinal gene expression in HET-1A cells**

HET-1A cells were treated with Ad-Null, Ad-CDX2, Ad-HNF4α or Ad-PDX1 alone or in combination for 24 hours. The medium was then replaced with fresh medium. Cells were harvested at day 4 and RT-PCR was performed on the isolated RNA. β-ACTIN was as a loading control. One of two experiments is shown.
**Figure 4.4. Induction of intestinal gene expression by CDX2, HNF4α and HNF1α in HET-1A cells**

(A) HET-1A cells were incubated with Ad-Null, Ad-CDX2, Ad-HNF4α, Ad-HNF1α or with the combinations shown for 24 hours. Medium was then replaced with fresh medium. At day 4, cells were collected, RNA extracted and RT-PCR was performed. \( \beta\)-ACTIN was as a loading control. The experiments were performed in triplicate.
Figure 4.5. Intestinal gene expression induction following overexpression of CDX2, HNF4α and HNF1α in HET-1A cells

HET-1A cells were incubated with Ad-Null, Ad-CDX2, Ad-HNF4α, Ad-HNF1α or with the combinations shown for 24 hours and the medium was refreshed. At day 4, cells were collected and RNA was extracted. VILLIN and K20 mRNA levels were quantified by qPCR and were normalised to β-ACTIN mRNA level. Bar chart expressed the relative mRNA levels of VILLIN compared with Ad-HNF4α infected control or mRNA levels of K20 compared with Ad-CDX2 infected control. Error bars represent standard error (SE) of three replicates. Statistical analysis performed with student paired t test. *p = 0.0714; **p = 0.0939.
Figure 4.6. Forced CDX2, HNF4α and HNF1α did not induce VILLIN protein expression in HET-1A cells

HET-1A cells were treated with Ad-Null, Ad-CDX2, Ad-HNF4α, Ad- HNF1α or various adenovirus combinations for 24 hours and the medium was replaced with fresh medium. Cells were fixed at day 4 and immunostaining was performed for VILLIN (green) and HNF4α (red). DAPI (blue) was counterstained for nuclei. Caco2 cells were used as a positive control for VILLIN. One of two experiments is shown.
Figure 4.7. K20 protein was not induced by forced CDX2, HNF4α and HNF1α expression in HET-1A cells

HET-1A cells were infected with Ad-Null, Ad-CDX2, Ad-HNF4α, Ad- HNF1α or different adenovirus combinations for 24 hours. Medium was then replaced with fresh medium. At day 4, cells were fixed and immunostaining for K20 (green) was performed. DAPI (blue) staining was for nuclei. Embryonic intestine isolated from E12.5 mouse embryos and cultured for 10 days was used as the positive control. One of two experiments is shown.
Figure 4.8. SI protein was not detected following forced expression of CDX2, HNF4α and HNF1α in HET-1A cells

HET-1A cells were incubated with Ad-Null, Ad-CDX2, Ad-HNF4α, Ad- HNF1α or the combinations for 24 hours and then the medium was changed. Cells were fixed at day 4. Immunostaining was performed for SI (green). DAPI (blue) was counterstained for nuclei. Embryonic day 12.5 intestine cultured for 10 days was used as the positive control. One of two experiments is shown.
Figure 4.9. CDX2 and HNF1α induced intestinal gene expression in HNF4α-expressing HET1A clones

(A) HNF4α-HET1A clone 1 and clone 5 (HNF4α-c1 and HNF4α-c5) were incubated with Ad-Null, Ad-CDX2, Ad-HNF1α alone or in combination for 24 hours. At day 4, cells were harvested, RNA extracted and RT-PCR performed. β-ACTIN was used as a loading control. The experiments were performed in triplicate.
Figure 4.10. Intestinal gene expression was induced by Cdx2 and Hnf1α in Hnf4α-expressing HET1A clones

HNF4α-HET1A clone 1 (HNF4α-c1) was incubated for 24 hours with the Ad-Null, Ad-CDX2, Ad- HNF1α adenoviral vectors either alone or in combination and then medium was replaced with fresh medium. At day 4, cells were harvested and RNA was extracted. VILLIN and K20 mRNA levels were quantified by qPCR and were normalised to β-ACTIN mRNA levels. Bar chart showing the relative mRNA levels of VILLIN compared with Ad-Null infected HNF4α-c1 or mRNA levels of K20 compared with Ad-CDX2 infected HNF4α-c1. Error bars represent standard error (SE) of three replicates. Statistical analysis was performed with student paired t test. *p = 0.1615; **p = 0.0385.
Figure 4.11. Cdx2 and Hnf1α did not induce Villin protein in Hnf4α-expressing HET-1A clones

HET-1A cells were treated with Ad-Null, Ad-CDX2, Ad-HNF1α alone or in combination for 24 hour and the medium was replaced with fresh medium. (A) At day 4, cells were fixed for VILLIN (green) and HNF4α (red) immunostaining. DAPI (blue) was counterstained for nuclei. One of two experiments is shown.
(A) Ctrl DAPI/FOXA2

(B) Ctrl Lenti-GFP Lenti-FOXA2

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Figure 4.12 – Forced FOXA2 expression did not induce intestinal gene expression in Het-1A cells

HET-1A cells were incubated with or without Lenti-FOXA2 virus for 24 hours and medium was replaced with fresh medium. (A) Cells were fixed at day 3 following infection for FOXA2 (green) immunostaining. DAPI (blue) was counterstained for nuclei. (B) Cells were harvested at day 7 following infection and RT-PCR was performed to examine gene expression. β-ACTIN was used as a loading control. One of two experiments is shown.
Figure 4.13. Intestinal gene expression was induced by CDX2, HNF4α and HNF1α in FOXA2-expressing HET-1A cells

HET-1A cells were infected with lenti-FOXA2 virus and medium was changed 24 hours later. At day2, cells were sub-cultured. At day3, Ad-CDX2, Ad-HNF1α, Ad-HNF4α or the combinations were added into cell culture and at day 4, medium was replaced with fresh medium. Cells were harvested at day 7 and RT-PCR was performed to examine gene expression. β-ACTIN was used as a loading control. One of three experiments is shown.
Figure 4.14. Lenti-HCH (HNF4α-2A-CDX2-2A-HNF1α) infection did not induce Villin protein

HET-1A cells were incubated with the Lenti-HCH virus for 24 hours and the medium was replaced with fresh medium. Three days after Lenti-HCH virus infection, HET-1A cells were fixed for immunostaining. Results showed that (A) CDX2 (green) and HNF4α (red) were co-localized in Lenti-HCH infected HET-1A cells (B) VILLIN (green) was not detected in HNF4α-positive cells (red) in Lenti-HCH culture. DAPI (blue) was counterstained for nuclei. The experiments were performed in duplicate.
Figure 4.15. Intestinal gene expression was not provoked following Lenti-HCH infection

HET-1A cells were infected with lenti-HCH virus for 24 hours and then medium was changed. Cells were harvested at day3 following infection. RNA was extracted and RT-PCR was performed to analyse gene expression levels. \( \beta\text{-ACTIN} \) was used as a loading control. The experiments were performed in triplicate.
4.3 Discussion

4.3.1 Ectopic expression of CDX2 and HNF4α in HET-1A cells

The aim of the work described in the present chapter was to investigate the role of candidate transcription factors in the conversion of stratified squamous epithelium to intestinal type columnar epithelium. Initially we tested different MOIs using a control reporter virus Ad-GFP to optimise adenoviral-mediated gene overexpression in HET-1A cells (Fig. 4.1). Based on the cell viability and GFP expression an MOI 10 was chosen for the adenoviral-mediated gene delivery experiment. In addition to MOI, the time course of transgene expression was also assessed (Fig. 4.2). The highest level of transgene CDX2 and HNF4α expression was between 2 and 4 days after initial adenoviral infection. Considering that downstream genes might only be provoked at the later stage, in the following adenoviral infection experiments, cells were harvested at day 4 post-infection for immunostaining or RT-PCR analysis.

We wished to explore whether HNF4α and CDX2 ectopic expression induced intestinal gene expression in HET-1A cells. RT-PCR results showed that forced HNF4α expression induced VILLIN gene expression in HET-1A cells. This is consistent with the results shown in Chapter 3 in which ectopic HNF4α expression (mediated by lenti-HNF4α infection) induced VILLIN expression. Forced CDX2 expression induced SI expression in the HET-1A cells (Fig. 4.2). SI is only expressed in differentiated enterocytes (Traber, 1990). In the intestinal epithelium, SI is a CDX2 target gene. CDX2 binds to the SI promoter region and activates SI transcription (Suzuki et al., 2008; Suh et al., 1994). Previous studies have shown that CDX2 is able to induce SI expression during intestinal epithelial differentiation (Suh & Traber, 1996). It has also shown that stable transfection of CDX2 in HET1A cells led to a 1.5-fold increase in SI mRNA expression according to qPCR analysis (Liu et al., 2007). These observations may explain why SI can be found in BM tissue (Wu et al., 1993).

We wished to determine whether ectopic expression of CDX2 could provoke K20 and MUC2 expression (Fig. 4.2). In the intestinal epithelium, K20 is mainly detected in the villus region (Zhou et al., 2003; Moll et al., 1990). The expression of K20 can also be found in BM tissue (van Baal et al., 2008). However, the role of CDX2 in
K20 expression remains unclear. MUC2 is a goblet cell marker (Gambus et al., 1993; Chang et al., 1994) and MUC2 expression is detected in BM tissue (Arul et al., 2000; Tamagawa et al., 2012). It was reported that CDX2 directly activates MUC2 expression via binding to the MUC2 promoter region in the intestinal cells (Yamamoto et al., 2003; Mesquita et al., 2003). However, ectopic expression of CDX2 in HET-1A cells did not induce MUC2 expression (Fig. 4.2). The fact that CDX2 did not induce MUC2 expression might due to the DNA methylation and Notch inhibition. First, regulation of MUC2 promoter by methylation is important for regulating MUC2 gene expression (Gratchev et al., 2001; Vincent et al., 2007; Okudaira et al., 2010). It was shown that the higher level of MUC2 promoter methylation corresponds to a lower MUC2 expression level in colorectal cancer tissues (Okudaira et al., 2010). Therefore, the reason that MUC2 was not induced by CDX2 might be due to the highly methylated status of the MUC2 promoter. Second, apart from DNA methylation, the Notch signalling pathway might be involved in regulating MUC2 expression. It is known that MUC2 is a marker for secretory goblet cells (Gambus et al., 1993; Chang et al., 1994) and the inhibition of Notch signalling drives the intestinal stem cells differentiating towards the secretory cell fate (Stanger et al., 2005; van Es et al., 2005a). The activated Notch signalling might negatively regulate MUC2 expression. More importantly, it was shown that in the OE19 (human adenocarcinoma from gastric cardia and oesophageal junction) and OE33 (human oesophageal adenocarcinoma) cell lines, that inhibition of Notch signalling by treating cells with \( \gamma \)-secretase inhibitor

\[
\text{N-}[N-(3,5\text{-Difluorophenacetyl})\text{-L-alanyl}]\text{-S-phenylglycine t-butyl ester (DAPT)}
\]

increased CDX2 as well as MUC2 gene and protein expression in a dose dependent manner (Tamagawa et al., 2012). Therefore, the induction of MUC2 by CDX2 might also require the inhibition of Notch signalling in the HET-1A cells. Taken together, it would be interesting to study whether the inhibition of DNA methylation with the DNA methyltransferase inhibitor 5’Aza and whether the inhibition of Notch signalling (with \( \gamma \)-secretase inhibitor DAPT), could induce MUC2 expression in the Ad-CDX2 infected HET-1A cells.
4.3.2 PDX1 ectopic expression in HET-1A cells

It was reported that Pdx1 deletion in adult mice causes anterior transformation in the gastro-duodenum junction and also shows the presence of foci of stratified squamous epithelium. It was also suggested that PDX1 might be involved in the pathogenesis of BM (Holland et al., 2013). However, forced PDX1 expression in HET-1A cells did not induce intestinal gene transcription (Fig. 4.3). Co-expression of HNF4α with PDX1 showed a slightly higher VILLIN mRNA level than HNF4α expression alone, but the co-expression of PDX1, CDX2 and HNF4α only showed weak VILLIN expression. This implies that PDX1 may not play a crucial role in the early development of BM. However, the expression of the PDX1 target genes (such as AKP3) could be checked to understand whether the ectopic PDX1 protein in HET-1A cells is functional. Complementary work going on in the lab using this transgene to convert hepatocyte-like cells to pancreatic beta-cells suggests the protein is functional.

4.3.3 Ectopic expression of CDX2, HNF4α and HNF1α in HET-1A cells

HNF1α regulates terminal differentiation and cell growth in the intestinal epithelium (Lussier et al., 2010; D’Angelo et al., 2010) and HNF1α is expressed in BM tissue (Piessen et al., 2007), suggesting a link between HNF1α expression and BM development. From the RT-PCR results, the ectopic expression of HNF1α in HET-1A cells showed weak ISX induction, but HNF1α did not induce VILLIN, SI or K20 (Fig. 4.4). The ectopic expression of both HNF1α and CDX2 increased ISX, K20 and SI transcription compared with CDX2 ectopic expression alone. The regulation of ISX expression by CDX2 has previously been reported. The deletion of Cdx2 in mouse gut (Cdx2loxP/loxP, Foxa3-Cre) reduced Isx mRNA level in both adult and embryonic intestine (Gao et al., 2009). The expression of ISX in BM has not been previously reported. From my results, the RT-PCR data suggest that in addition to CDX2, HNF1α and HNF4α also induce ISX expression. Further work is necessary to test whether ISX is expressed in BM tissue. LGR5 is a putative intestinal stem cell marker (Barker et al., 2007). LGR5 protein was found in BM tissue, but was not detected in normal oesophageal epithelium (von Rahden et al., 2011). The relationship between CDX2 and LGR5 is still not clear. From the RT-PCR results, ectopic expression of HNF1α, HNF4α and CDX2 transcription factor combinations
which included CDX2 increased LGR5 expression level in HET-1A cells (Fig. 4.4). It is possible that CDX2 may direct the differentiation of HET-1A cells into an intestinal stem cell lineage. However, the LGR5 protein expression and the expression of other intestinal stem markers such as BMI1 and MUSASHI-1 could be explored in the future (Barker, 2013). Whether the ectopic expression of CDX2 leads oesophageal epithelial cells to be in a progenitor/stem state before differentiating into SI and K20 expressing cells, remains to be investigated. It would also be useful if primary oesophageal epithelial cells or transgenic mice can be used as models to address this issue. RT-PCR results also showed that K14 and K18 level was not altered by the ectopic expression of HNF4, CDX2 or HNF1α (Fig. 4.4). This might due to the fact that HET-1A cells show the columnar cell characteristics with the expression of K8 protein, but not K14 protein (Chapter 3.2.1). HNF4, CDX2 and HNF1α which are expressed in the columnar intestinal cells would not change columnar phenotype in the HET-1A cells. Therefore, cells which show the squamous phenotype such as the primary cultured oesophageal epithelial cells, should be used to test the effects of HNF4, CDX2 or HNF1α on K18 and K14 expression.

The VILLIN qPCR results demonstrated that the highest VILLIN induction was by CDX2 and HNF4α, and followed by HNF4α alone (Fig.4.5). Similarly, the highest K20 expression was induced by CDX2 and HNF4α, but the second highest K20 induction was by CDX2, HNF1α and HNF4α ectopic expression. These results suggest that CDX2 and HNF4α together trigger intestinal differentiation in the oesophageal epithelial cells, and HNF1α might not play such a key role. However, according to the statistical analysis results, the addition of Ad-CDX2 in Ad-HNF4α infected cells did not significantly increase the VILLIN and K20 gene expression (p>0.05). In the qPCR analysis, due to the variation in adenoviral infection efficiency among the three independent experiments, the increase level of VILLIN and K20 expression varies widely. The variation in adenoviral infection efficiency might mainly be due to the cell dissociation and cell seeding step before infection. The presence of clumping cells in the culture may lead to the lower viral infection efficiency, because cells in clumps reduce the contact area between virus and cells. Therefore the improvement of cell dissociation and seeding techniques as well as more repeated experiments could provide more reliable results.
VILLIN, K20 or SI protein was not detected according to the immunostaining results (Fig.4.6, 4.7, 4.8). The absence of protein expression might be due to the short period of ectopic CDX2, HNF1α and HNF4α expression. In addition, the cell line used might also be an issue, as VILLIN protein was induced by HNF4α in primary oesophageal epithelial explants (more details will be showed in Fig 6.6A, B and Chapter 6.2.2). In primary oesophageal epithelial explant model, cells express p63 and K14 (in low Ca\(^2+\) condition, Ca\(^2+\) [60µM]), but HET-1A cells do not express p63 and K14. This might implicate p63 and K14-expressing basal cells have greater plasticity to convert to BM cells. In addition, it is also possible that another transcription factor(s) required for VILLIN protein expression is missing in the HET-1A cells.

The ectopic expression of CDX2 and HNF1α were also tested in HNF4α-c1 and HNF4α-c5 stable cell lines (Fig. 4.9). The addition of CDX2 expression induced ISX, K20, LGR5 and SI expression in both HNF4α-c1 and HNF4α-c5. HNF1α also increased LGR5 expression in HNF4α-c1 and HNF4α-c5 and increased ISX in HNF4α-c1. These results reveal that CDX2 and HNF1α may enhance the intestinal gene expression in HNF4α-expressing cells. VILLIN and K20 qPCR analysis further reveals that VILLIN and K20 level was highest in the culture with Ad-CDX2 infection (Fig. 4.10). However, results of statistical analysis revealed that there is no significant difference (p>0.05) in VILLIN mRNA level between Ad-CDX2 infected HNF4α-c1 cells and HNF4α-c1 control. Because the experiments were only performed in triplicate, more replication may need to be performed to obtain more reliable results. Finally, VILLIN protein expression was not detected in the HNF4α-c1 and HNF4α-c5 (Fig. 4.11). Overall, these results are consistent with the results of Ad-CDX2, Ad-HNF1α or Ad-HNF4α infection (Fig. 4.4, 4.6).

4.3.4 Ectopic expression of FOXA2, CDX2, HNF4α and HNF1α in HET-1A cells

Previous studies reported that FOXA2 in the intestinal epithelium regulates Goblet cell and enteroendocrine cell differentiation (Ye & Kaestner, 2009). However, further details of the function of FOXA2 in intestinal epithelium and the role of FOXA2 in the development of BM have not been explored. RT-PCR results showed that control Lenti-GFP infection induces very weak human type FOXA2 expression (Fig. 4.12A),
but this might be due to the non-specific binding of GFP to the FOXA2 promoter. RT-PCR results also showed that FOXA2 expression in HET-1A cells did not induce intestinal gene expression (Fig. 4.12B). Interestingly, CDX2, HNF1α and HNF4α ectopic expression induces the endogenous CDX2 gene expressing in FOXA2-expressing cells. MUC2 expression was also induced by CDX2 in FOXA2-expressing cells. Previous studies identified MUC2 as the target of both CDX2 and FOXA2. CDX2 or FOXA2 directly binds to MUC2 promoter and activates MUC2 transcription (van der Sluis et al., 2008; Yamamoto et al., 2003). In my studies, CDX2 or FOXA2 alone was not able to induce MUC2 expression (Fig. 4.4, 4.12B), but CDX2 and FOXA2 together provoke MUC2 expression in HET-1A cells (Fig. 4.13). This might reflect the fact that intestinal transcription factors work in synergy to regulate intestinal gene expression (Boudreau et al., 2002; van der Sluis et al., 2008).

4.3.5 Forced expression of CDX2, HNF4α and HNF1α by using Lenti-HNF4α-2A-CDX2-2A-HNF1α virus in HET-1A cells

In 4.2.4 section, ectopic CDX2, HNF1α and HNF4α expression was carried out by using the separate viral vectors and each vector only carries one transgene. However, there are several disadvantages to this approach. For example, firstly, the expression level might not be comparable between the different transgenes. Secondly, all the transgenes might not be co-expressed in the same cell. Thirdly, using multiple viral vectors might mean using higher total amounts of virus in order to reach the optimized expression level, but this may decrease cell viability. Therefore, a polycistronic Lenti-HNF4α-2A-CDX2-2A-HNF1α (Lenti-HCH) vector was created. In this vector, CDX2, HNF1α and HNF4α were linked by self-cleaving 2A peptides. CDX2, HNF1α and HNF4α can be expressed from a single promoter at the comparable level by using only one lentiviral vector. Using 2A peptides in polycistronic vectors for expressing multiple transgenes has been successful applied in various cell reprogramming studies (Akinci et al., 2012; Szymczak & Vignali, 2005; Carey et al., 2009)

In the Lenti-HCH infection experiment, CDX2 and HNF4α were co-expressed in the same HET-1A cell according to the immunostaining results (Fig.4.14A), but the
co-expression of CDX2, HNF1α and HNF4α was unable to be confirmed due to the availability of a suitable HNF1α antibody. RT-PCR results demonstrated that CDX2, HNF1α and HNF4α transgene were all expressed in HET-1A cells. However, the expression of intestinal genes was not detected (Fig. 4.15). The failure of inducing intestinal genes might due to the poor infection efficiency (~25%). This could be improved by concentrating lentiviral supernatant to increase the viral titre. In addition, single cell clones could be selected and expanded for analyzing the changes of gene expression profile. Finally, it is also important to examine whether transgenes are properly translated into protein and this could be checked by Western blotting. The induction of the protein downstream targets could also be evaluated to understand whether the protein is functional.
Chapter 5
The role of exogenous factors and DNA methylation in the development of BM
5.1 Introduction
In Chapters 3 and 4, I examined the ability of key transcription factors to induce the reprogramming of stratified squamous epithelial cells to intestinal cells. While changes in gene expression may induce the cell type transformation seen in Barrett’s, the question also arises as to what are the extracellular stimuli for these alterations. Changes in DNA methylation, release of inflammatory mediators, bile acid exposure, and the role of the Wnt, Notch and BMP pathways have all been implicated in the development of BM. In this chapter, I therefore investigated the role of these exogenous factors in the development of BM. Before discussing the results I will briefly discuss the role of each factor in BM.

5.1.1 DNA methylation in BM
DNA methylation is the mechanism by which a methyl group is added to the C5 position of cytosine to form 5’-methylcytidine. The process of DNA methylation is referred to as an epigenetic modification. The methylation process is catalyzed by a family of DNA methyltransferases (DNMTs) including DNMT1, DNMT3a, and DNMT3b. DNMT1 maintains DNA methylation status during DNA replication, whereas DNMT3a and DNMT3b methylate unmethylated DNA de novo. DNA methylation results in the inhibition of transcription factor binding to DNA. DNA methylation also inhibits the recruitment of accessory proteins required for gene transcription (Taby & Issa, 2010; Moore et al., 2013). It is well established that DNA methylation is involved in embryonic development, gene imprinting, X-chromosome inactivation as well as development of cancer (Taby & Issa, 2010). In studies of BM development, a number of reports showed the “DNA methylation signature” alters during the progression from normal oesophagus to BM (Eads et al., 2001; Kaz et al., 2011; Agarwal et al., 2012; Alvi et al., 2013). For example, the promoter region of the tumour suppressor gene p16 and adenomatous polyposis coli (APC) in BM biopsy material has a higher prevalence of hypermethylation (p16=30%; APC=50%) compared with the human normal oesophageal epithelium (p16=0%; APC=0%) (Wang et al., 2009). It was also demonstrated that the CDX1 promoter is hypomethylated in BM compared with the normal human oesophageal epithelium in which the CDX1 promoter is predominately methylated (Wong et al., 2005). In addition, research in which a transgenic mouse model was used to study the role of
CDX2 in the development of BM also suggested that epigenetic regulation might be involved in the development of BM. Ectopic Cdx2 expression in mice oesophageal epithelium using the cytokeratin 14 promoter to drive transcription factor expression (K14-Cdx2) did not induce intestinal metaplasia. However, after treating the K14-Cdx2 mice with the DNA methylation inhibitor, 5’Aza, the expression of BM-associated proteins CDX1 and K18 was induced (Kong et al., 2011b). Therefore, the inhibition of DNA methylation may promote the pathogenesis of BM.

5.1.2 Inflammation and BM

BM follows a sequence in terms of oesophagitis-BM-dysplasia-oesophageal-adenocarcinoma. BM development is linked to chronic inflammation caused by GORD. It is known that intrinsic (genetic alterations) and extrinsic (inflammation or infection) stimuli can lead to the activation of inflammation-related pathways such as NF-κB and STAT3. The activation of the NF-κB pathway results in the production of inflammatory mediators such as IL-1β and TNFα which in turn can further stimulate the NF-κB pathway. Overall this can lead to even more inflammatory mediators being secreted and creates a cancer-related inflammatory microenvironment (Mantovani et al., 2008). Activation of the NF-κB pathway is associated with cell differentiation, cell survival and cancerogenesis (Ben-Neriah & Karin, 2011). In the context of BM, the expression level of NF-κB is increased in BM and oesophageal adenocarcinoma (O’Riordan et al., 2005). It is therefore possible that the cell type transformation observed in BM could potentially be linked to the activation of the NF-κB pathway. In addition, the CDX2 gene is a target of the NF-κB pathway and the CDX2 promoter has two putative binding sites for NF-κB further suggesting that NF-κB might contribute to the development of BM (Kim et al., 2002; Kazumori et al., 2006).

IL-1β

Interleukin-1β (IL-1β) is a cytokine produced by activated macrophages. It was reported that the level of IL-1β level is up-regulated in oesophagitis tissue (Fitzgerald et al., 2002a; Hamaguchi et al., 2003; O’Riordan et al., 2005; Rieder et al., 2007). One report showed that the level of IL-1β is significantly increased in BM (O’Riordan et al., 2005). In contrast, no difference in the total amount of IL-1β
expression has been observed in BM (Fitzgerald et al., 2002a). However, the Fitzgerald group further suggested that the IL-1β level is in fact higher at the squamous-columnar junction at the proximal end of BM and above the inflamed squamous epithelium compared with the distal end of BM (Fitzgerald et al., 2002b). More importantly, a recent experimental animal model supports the concept that aberrant IL-1β expression is able to induce a BM-like phenotype in the mouse oesophagus (Quante et al., 2012). In the study, \textit{L2-IL-1β} transgenic mice were used in which the Epstein-Barr virus (ED-L2) promoter drives IL-1β expression in the tongue, oesophagus, and squamous stomach (Nakagawa et al., 1997; Quante et al., 2012). The oesophageal overexpression of IL-1β in \textit{L2-IL-1β} mice initiated and mimicked disease progression from oesophagitis, BM, dysplasia to oesophageal adenocarcinoma (Quante et al., 2012). Taken together, these data suggest that elevation of IL-1β is potentially linked to the development of BM.

**IL-6**

Interleukin 6 is a cytokine secreted by macrophages and T cells and functions in both a pro- and anti-inflammatory capacity. It has been reported that the levels of IL-6 are raised in oesophagitis and BM tissue compared with normal oesophageal epithelium (Rieder et al., 2007). IL-6 signalling primarily involves the initial binding of IL-6 to the IL-6R (IL-6 receptor)/gp130 (glycoprotein 130) receptor complex which leads to the phosphorylation of JAK (Janus kinase). The phosphorylated JAK then phosphorylates STAT (signal transducers and activators of transcription). The phosphorylated STAT moves to the nucleus and activates the downstream target genes including genes regulating cell survival, proliferation, differentiation and angiogenesis. In addition, the binding of IL-6 to the IL-6/gp130 receptor could also activate the MAPK (mitogen-activated protein kinase) cascade to activate target gene expression (Heinrich et al., 2003; Dvorak & Dvorak, 2013). The IL-6 pathway activation may play an important role in BM carcinogenesis. For example, results from the examination of BM biopsies by immunohistochemistry staining and the analysis of a transformed BM cell line (BAR-T) in which p53 expression is inhibited and H-RasG12V is overexpressed, all revealed that IL-6/STAT3 pathway is linked to apoptosis resistance (Dvorakova et al., 2004; Zhang et al., 2011; Dvorak & Dvorak, 2013). Furthermore, in the \textit{L2-IL-1β} transgenic mice, IL-1β-induced BM and
oesophageal adenocarcinoma phenotypes thought to be mediated through the IL-6 signalling pathway. Firstly, IL-6 expression is elevated in L2-IL-1β mice. Secondly, knock out of IL-6 (L2-IL-1β/IL-6−/− mice) reduced BM phenotype and also abolished dysplasia and development of adenocarcinoma. Therefore, IL-6 may be involved in the development and the progression of BM (Quante et al., 2012).

**IL-17**

IL-17 is a proinflammatory cytokine and is a key mediator of both innate and adaptive immune responses (Korn et al., 2009). It has been reported that IL-17 upregulates proinflammatory cytokine secretion such as TNFα, IL-1β and IL-6 by macrophages (Jovanovic et al., 1998). IL-17 expression is associated with chronic inflammatory and autoimmune diseases (Miossec et al., 2009; Korn et al., 2009). IL-17 is also implicated in the development of cancer (including skin cancer, breast cancer and non-small cell lung cancer (NSCLC)) (Zhu et al., 2008; Chen et al., 2010; Yusuf et al., 2012). Related to this observation is the fact that the proportion of IL17-producing cells is increased in oesophageal adenocarcinoma (Chen et al., 2012c). The expression of IL-17 is also increased in BM, dysplasia and oesophageal adenocarcinoma of human samples in comparison with the oesophageal stratified squamous epithelium. The increase of IL-17 level is therefore potentially linked to disease progression in BM (Bannister et al., 2012).

**TNF-α**

Tumour necrosis factor α (TNF-α) is involved in both innate and adaptive immune response and there is extensive evidence in the literature suggested that TNF-α participates in chronic inflammation and inflammation-associated cancer progression (Balkwill, 2006). In BM research, it has been shown that TNF-α levels are raised in oesophagitis tissue, BE, dysplasia and oesophageal adenocarcinoma compared with normal oesophageal epithelium (Tselepis et al., 2002; Hamaguchi et al., 2003). Importantly, the increase in TNF-α is positively associated with the stage of BE-dysplasia-oesophageal adenocarcinoma sequence (Tselepis et al., 2002).
**IFN-γ**

Interferon γ (IFN-γ) is a proinflammatory cytokine and has important immunoregulatory functions. IFN-γ promotes the activity of neutrophils, macrophages and natural killer cells (Schroder et al., 2004; Zhong et al., 2011). IFN-γ expression is up-regulated in oesophagitis tissues compared to normal stratified squamous oesophageal epithelium (Fitzgerald et al., 2002a; Zhong et al., 2011). In BM studies, two publications investigated the relationship of IFN-γ between BM and normal oesophageal epithelium. One report in which IFN-γ expression was examined by RT-PCR showed that there is no significant difference between BM and normal tissues (Fitzgerald et al., 2002a). However, in the second report using immunohistochemistry to assess IFN-γ expression, Zhong et al suggested that the level of IFN-γ was increased in BM samples (Zhong et al., 2011). The difference between these two observations might be due to the different sampling position and examination methods (RT-PCR versus immunohistochemistry) (Zhong et al., 2011).

**LPS**

Lipopolysaccharide (LPS) is the main component of outer membrane of Gram negative bacteria. LPS treatment stimulates the secretion of the inflammatory mediator IL-8 in BAR-T cells (Verbeek et al., 2013). The mechanism involves in the stimulation of Toll-like receptor 4 (TLR4) by LPS, which then activates the NF-κB pathway leading to the transcription of the downstream target IL-8 (Kunsch & Rosen, 1993; Beyaert, 2011; Verbeek et al., 2013). In addition, a study investigated the microbiomes of the human distal oesophageal region showing that the microbiome population can be classified into two groups depending on the hierarchical clustering (genetic distance). The result implies that the major microbiomes found in normal oesophagus belong to type1 which are mainly Gram positive bacteria. Conversely, the dominant microbiomes in oesophagitis and BM are mainly type2 which are mainly Gram negative bacteria (Yang et al., 2009, 2012). Therefore, microbial LPS may contribute to BM via activating NF-κB pathway.
Bile salts and acid

GORD is the main risk factor for BM pathogenesis and bile acids are the key components of gastro-oesophageal refluxate (Badreddine & Wang, 2010). Bile acids induce ROS and NO production and causes DNA damage in oesophageal epithelial cells (Table 5.1). Therefore, bile acid exposure is associated with genetic alternations which may lead to development of BM development (Jenkins et al., 2007; Dvorak et al., 2007; Jolly et al., 2009; Goldman et al., 2010; McAdam et al., 2012).

Bile salts or acid might initiate BM via activation of the NF-κB pathway. Previous studies showed that bile salts or acid activated NF-κB pathway in the oesophageal epithelial cells (Table 5.2) (Jenkins et al., 2004; Duggan et al., 2006; Wu et al., 2008; Cronin et al., 2010a). NF-κB in turn may regulate CDX2 expression by binding to the promoter and enhancing transcriptional activity. The increase in CDX2 promoter activity brought about by bile salt or acid exposure can be inhibited by mutating the CDX2 promoter’s putative NF-κB binding sites or by using an NF-κB inhibitor Bay11-7085 (blocking inhibitor κB (IκB) phosphorylation) or IKK (IκB kinase) inhibitor (Kazumori et al., 2006; Huo et al., 2010). Therefore, bile acid might act through the NF-κB pathway to induce CDX2 expression. Indeed, it was observed that bile acid induced or increased CDX2 expression level in oesophageal epithelial cells and BM cells respectively (Table 5.3) (Marchetti et al., 2003; Kazumori et al., 2006; Huo et al., 2010). Apart from CDX2, a bile salt mixture (cholic acid, glycocholic acid and taurocholic acid) can induce Cdx1 and increase Klf4 expression in primary cultured rat oesophageal epithelial cells (Kazumori et al., 2009, 2011). In addition, DCA exposure at pH5 decreased p63 expression in primary cultured human oesophageal epithelial cells (Roman et al., 2007). These results implicate that bile acids might drive the BM phenotype by inducing intestinal transcription factors and down-regulating the expression of key transcription factor for stratified squamous epithelium.
<table>
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<th>Bile salt/acid treatment</th>
<th>Results</th>
<th>Cell type</th>
<th>References</th>
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<td>DCA 100uM, pH6 ~ 7</td>
<td>↑ROS</td>
<td>OE33</td>
<td>Jenkins et al. 2007</td>
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<tr>
<td></td>
<td>DNA damage</td>
<td>KYSE</td>
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<tr>
<td>0.5 mM bile acid cocktail of GCA, TCA, GDCA, GCDCA, DCA, pH4</td>
<td>↑Mitochondria superoxide</td>
<td>HET-1A</td>
<td>Dvorak et al. 2007</td>
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<tr>
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<td>DNA damage</td>
<td>CP-D</td>
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</tr>
<tr>
<td>0.5mM bile acid of GCA, TCA, GDCA, GCDCA, DCA, and/or pH5.5</td>
<td>↑NO</td>
<td>CP-A</td>
<td>Goldman et al., 2010</td>
</tr>
<tr>
<td></td>
<td>DNA damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCA, pH5</td>
<td>DCA and/or acid → ↑iNOS, ↑NO</td>
<td>OE33</td>
<td>McAdam et al. 2012</td>
</tr>
<tr>
<td></td>
<td>DCA → ↑NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>↑NO</td>
<td>HET-1A</td>
<td>Jolly et al. 2009</td>
</tr>
<tr>
<td></td>
<td>DNA damage</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 5.1. The induction of NO, ROS or DNA damage by bile salts and/or acid

The Table summarises some examples where the induction of NO, ROS or DNA damage is induced by bile salts and/or acid in different oesophageal cell types. DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; ROS, reactive oxygen species; NO, Nitric oxide; iNOS, inducible nitric oxide synthase,
<table>
<thead>
<tr>
<th>Bile salt/acid treatment</th>
<th>Cell type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 –300 µM DCA</td>
<td>OE33</td>
<td>Jenkins et al. 2004; Jenkins et al. 2008; McAdam et al. 2012</td>
</tr>
<tr>
<td>pH6.5</td>
<td>SKGT-4 Esophageal adenocarcinoma</td>
<td>Duggan et al. 2006</td>
</tr>
<tr>
<td>DCA 300 µM, pH 5 ~7</td>
<td>OE33, KYSE, squamous, BM’s biopsies</td>
<td>Cronin, Alhamdani, et al. 2010</td>
</tr>
<tr>
<td>DCA 100 µM</td>
<td>SEG-1</td>
<td>Wu et al. 2008</td>
</tr>
<tr>
<td>400 µM bile salts cocktail of GCA, TCA, GCDCA, TCDCA, GDCA, TDCA (20:3:15:3:6:1 in molar concentration) and/or pH5.5</td>
<td>NES cells originated from BM biopsy specimens</td>
<td>Huo et al. 2010</td>
</tr>
</tbody>
</table>

**Table 5.2. Examples of the activation of NF-κB pathway by bile salts and/or acid**

Table showing examples of NF-κB pathway activation by bile salts and/or acid in the different types of oesophageal epithelial cells. DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid.
<table>
<thead>
<tr>
<th>Bile salt/acid treatment</th>
<th>Cell type</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µM CA or 100 µM DHCA, 24 hours</td>
<td>Primary rat oesophageal epithelial cells</td>
<td>Induce CDX2 protein</td>
<td>Kazumori et al. 2006</td>
</tr>
<tr>
<td>400 µM bile salts cocktail of GCA, TCA, GCDCA, TCDCA, GDCA, TDCA (20:3:15:3:6:1 in molar concentration) and/or pH4, 10 min, 3 times per day for 7 days</td>
<td>NES cells originated from BM biopsy specimens</td>
<td>Increase CDX2 gene</td>
<td>Huo et al. 2010</td>
</tr>
<tr>
<td>pH3.5, 18 days</td>
<td>Primary mouse oesophageal epithelial cells</td>
<td>Induce CDX2 protein</td>
<td>Marchetti et al. 2003</td>
</tr>
<tr>
<td>DCA 300 or 1000 µM, 1 ~24 hours</td>
<td>HET-1A, SEG-1, HKESC-1, HKESC-2</td>
<td>Induce or increase CDX2 gene</td>
<td>Hu et al. 2007</td>
</tr>
</tbody>
</table>

**Table 5.3. The induction of CDX2 expression by bile salts and/or acid**

Table lists the details of CDX2 induction or up-regulation by bile salts and/or acid in various oesophageal epithelial cell types. CA, cholic acid; DHCA, dehydrocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GDCA, glycodeloxycholic acid; TDCA, taurodeoxycholic acid; DCA, deoxycholic acid; HKESC-1 and HKESC-2 human squamous cell carcinoma cell lines.
5.1.3 Notch, Wnt and BMP4 in BM

Notch signalling
The Notch signalling pathway is important for cell growth, differentiation and also cell death. Notch is a heterodimeric transmembrane receptor protein composed of an extracellular domain and a transmembrane domain. The Notch extracellular domain interacts with Notch ligand on adjacent cells. Once the ligand binds to the receptor, the intracellular part of the Notch receptor the transmembrane domain is cleaved to yield a free Notch intracellular domain (NICD) which then translocates into the nucleus and regulates gene expression (Andersson et al., 2011; Kopan, 2012). In intestinal epithelium, Notch signalling maintains the stem cells in a proliferative and undifferentiated state. Notch signalling also controls cell differentiation in the intestine. Indeed inhibition of Notch signalling leads to a commitment into secretory lineage in the intestinal epithelium (van Es et al., 2005a; Fre et al., 2005; van der Flier & Clevers, 2009; van Es et al., 2010).

The link between Notch signalling and BM has been studied previously. It was reported that bile acids can inhibit Notch signalling in the oesophageal adenocarcinoma cells (OE19 and OE33) and inhibition of Notch signalling by the DAPT increased CDX2 and MUC2 expression. It has also been also confirmed that bile acid exposure up-regulated CDX2 and MUC2 expression in OE19, OE33 and HET-1A cells. Therefore, it was proposed that bile acids can act through inhibition of Notch signalling thereby inducing CDX2 expression (Morrow et al., 2009; Tamagawa et al., 2012). Furthermore, in the L2-IL-1β transgenic BM mouse model, inhibiting Notch signalling by treating the L2-IL-1β mice with the γ-secretase inhibitor dibenzazepine (DBZ), increased the population of Goblet-like cells (Quante et al., 2012). Therefore, inhibition of Notch signalling may be important for the development of BM.

BMP4
Bone morphogenetic protein 4 (BMP4) is a member of the transforming growth factor β (TGF-β) family which regulates cell differentiation, proliferation and migration (Badreddine & Wang, 2010). The link between activation of the BMP4 pathway and development of BM has been studied. BMP4 expression was enhanced in the L2-IL-1β transgenic mice of BM animal model. Additional treatment of the
bile acid DCA can also enhance BMP4 expression level (Quante et al., 2012).

*In vitro* studies showed that bile acids can induce BMP4 expression in human primary oesophageal epithelial cells (Zhou et al., 2009). BMP4 protein has been detected (and indeed the BMP4 pathway is activated) in oesophagitis and BM, but not in the normal stratified squamous epithelium. Additionally, BMP4 treatment in the human primary oesophageal squamous epithelial cells induced a columnar phenotype with the expression of CK7 and CK20 (Milano et al., 2007). Importantly, it has been suggested that stromal BMP4 plays an important role in development of BM. BMP4 expression is detected in the adjacent mesenchymal cells in the embryonic oesophagus and at this stage embryonic oesophageal epithelial columnar in phenotype (Yu et al., 2005; Que et al., 2006; Milano et al., 2007). BMP4 is not expressed in the adult oesophagus, but BMP4 is re-expressed in BM, particularly in the mesenchymal region (Milano et al., 2007; Wang et al., 2010). Additionally, BMP4 can induce SOX9 expression and ectopic expression of SOX9 may induce a columnar phenotype in HET-1A cells. Taken together, BMP4 secreted from mesenchymal cells may induce a squamous-to-columnar cell type conversion in the development of BM (Wang et al., 2010; Gibson et al., 2013).

**Wnt**

The Wingless (Wnt) pathway is activated by the binding of Wnt ligand to Frizzled and low-density lipoprotein receptor-related protein (LRP) receptors. Binding of the ligand to the receptor prevents β-catenin from being bound and phosphorylated by the complex of APC, glycogen synthase kinase 3 (GSK3), axin and casein kinase I (CKI). The non-phosphorylated β-catenin will not be sent to the proteasome for degradation, so β-catenin can accumulate and translocate into the nucleus wherein it binds to the TCF/β-catenin complex to activate the transcription of Wnt target genes (van der Flier & Clevers, 2009). In the intestine, Wnt signalling is important for embryonic intestinal development, Paneth cell terminal differentiation and crypt stem cell proliferation (Gregorieff & Clevers, 2005; van Es et al., 2005b; Barker et al., 2007; Garcia et al., 2009; Sato et al., 2011; Schuijers & Clevers, 2012). In addition, evidence shows that the aberrant Wnt activation is highly linked to colorectal carcinogenesis (Korinek et al., 1997; Clevers, 2006; Vermeulen et al.,
It has been reported that activation of Wnt signalling, assessed by nuclear β-catenin accumulation, is positively associated with neoplastic progression of BM (Bian et al., 2000; Osterheld et al., 2002). In addition, the increase of Wnt2 expression, and the promoter methylation in both the APC and the Wnt antagonist, secreted frizzled-related protein 1 (SFRP1) genes, is observed in BM tissue (Clément et al., 2006). Moreover, studies also showed that the introduction of dominant-active Wnt effector CatCLef in which an amino-terminally truncated β-catenin is fused to a full-length LEF1, initiated BM marker Na1/H1 exchanger 2 (NHE2) expression in a 3D organotypic model of oesophageal epithelium (Kong et al., 2011a). This suggests that the development of BM or the progression of BM to adenocarcinoma may be associated with the Wnt signalling activation.
5.2 Results

5.2.1 Inhibition of DNA methylation did not induce intestinal gene expression
In Chapter 3.2.3, I showed that ectopic expression of HNF4α induces intestinal VILLIN gene expression in HET-1A cells. To test whether the inhibition of DNA methylation can further induce or enhance intestinal gene expression, 5’Aza treatment was performed in HET-1A cells which have been infected with Ad-CDX2 and Ad-HNF4α. RT-PCR results demonstrated that 5’Aza did not induce or enhance intestinal gene expression in either control or CDX2 and HNF4α expressing cells (Fig. 5.1) and there was no change in K14 and K18 expression under all conditions.

5.2.2 Inflammatory factors did not induce CDX2 or HNF4α expression in HET-1A cells
In order to test whether HET-1A cells could respond to inflammatory factors we initially treated the cells with 10 or 100 ng/ml of IL-1β, TNF-α, LPS, IL-17 or IFNγ for 15 or 60 minutes after 24-hour serum starvation I then determined the expression of pp65 or ppERK to investigate whether these inflammation-related exogenous factors could stimulate NF-κB or ERK signalling respectively. The results showed that pp65 and ppERK levels are enhanced by IL-1β, TNF-α, LPS and IL-17. The level of ppERK is only moderately increased and pp65 level was not increased by IFNγ, while the level of pp65 was elevated by IL-1β, TNF-α and IFNγ mixture (Fig. 5.2). STAT3 signalling activation by IL-6 was determined in HET-1A cells. After 24-hour serum starvation HET-1A cells were treated with 10 or 100 ng/ml of IL-6 for 15 or 60 minutes. Western blotting showed that pSTAT3 level is slightly enhanced following 15 minutes of treatment with IL-6 (Fig. 5.3).

Next, to evaluate whether inflammation-related exogenous factors contribute to BM development, HET-1A cells were treated with IL-1β, IL-6, IL-17 or TNF-α at 10 - 100 ng/ml or LPS at 10 - 100 µg/ml for varying lengths of time (1, 4 or 7 days) (Fig. 5.4). RT-PCR analysis showed that CDX2 or HNF4α expression was not induced and K14 or K18 gene expression was not altered. The combination of IL-1β and TNF-α also did not induce CDX2 or HNF4α expression (Fig. 5.5). To test whether IFN-γ is potentially involved in the pathogenesis of BM, HET-1A cells were treated with 10 - 100 ng/ml of IFN-γ for 1, 4 or 7 days. Neither CDX2 nor HNF4α
expression was induced by IFN-\(\gamma\) treatment of HET-1A cells (Fig. 5.6A). The effect of combining IFN-\(\gamma\), IL-1\(\beta\) and TNF-\(\alpha\) was also tested. Cells were treated with 100 ng/ml of IFN-\(\gamma\), IL-1\(\beta\) and TNF-\(\alpha\) but unfortunately the cells did not survive (data not shown). However, cells treated with 10 ng/ml of IFN-\(\gamma\), IL-1\(\beta\) and TNF-\(\alpha\) were viable, but even under these conditions, the expression of \(CDX2\) and \(HNF4\alpha\) was not induced (Fig 5.6B).

We extended the experiments on inflammatory mediators to test whether the combination of cytokines and bile salts could induce intestinalisation of HET-1A cells. To address this possibility, HET-1A cells were treated for 4 days with 100 ng/ml of either IL-6 or IL-1\(\beta\) alone, or in the presence of 50 \(\mu\)M DCA. \(CDX2\), \(HNF4\alpha\) and \(VILLIN\) expression were not induced. \(K14\) and \(K18\) expression levels were not altered (Fig.5.7).

### 5.2.3 Bile acid and BM

To test the effect of DCA on intestinal gene expression in different oesophageal cell types, four different models were used: 7-days cultures of adult mouse oesophageal epithelial explants, 24-hour \textit{ex vivo} cultures of E11.5 mouse oesophagus, HET-1A and OE33 cell lines. All four models were treated with 100 - 400 \(\mu\)M of DCA for 24 hours. We examined the expression of two intestinal transcription factors \(CDX2\) and \(HNF4\alpha\). Neither were expressed by adult oesophageal epithelial explants following treatment with 400 \(\mu\)M DCA (Fig. 5.8A). Surprisingly, weak \(HNF4\alpha\) expression was detected in the control \textit{ex vivo} cultures of E11.5 mouse oesophagus. However, the expression of \(HNF4\alpha\) and \(CDX2\) were not detected following treatment with either 100 or 400 \(\mu\)M DCA. Control HET-1A cells did not express \(CDX2\) and \(HNF4\alpha\), but 400 \(\mu\)M DCA treatment did appear to induce weak \(CDX2\) expression (Fig. 5.8B). \(CDX2\) and \(HNF4\alpha\) were expressed in OE33 cells, but remarkably DCA treatment reduced \(CDX2\) and \(HNF4\alpha\) expression level.

The effects of cholic acid (CA), glycocholic acid (GCA) and taurocholic acid (TCA) bile acids on gene expression were evaluated. HET-1A cells were cultured with 200 or 400 \(\mu\)M CA, GCA, TCA or mixture of CA, GCA and TCA for 48 hours. Cells treated with 400 \(\mu\)M of CA, GCA and TCA mixture did not survive. RT-PCR showed
that CA, GCA, TCA or the combination did not induce \textit{CDX2} or \textit{HNF4}\textalpha expression (Fig. 5.9).

\textbf{5.2.4 BMP4, Notch and Wnt signalling and BM}

In Chapter 3, I showed that stable Hnf4\textalpha-expressing HET-1A clones (HNF4\textalpha-c1 and HNF4\textalpha-c5) could be established. To further test the potential roles of BMP4, Notch and Wnt pathways in BM pathogenesis, HET-1A and HNF4\textalpha-c1 and HNF4\textalpha-c5 stably-expressing HET-1A cells were treated with 10 or 100 ng/ml of BMP4, 5 or 25 \textmu M DAPT or 0.5 or 1 \textmu M 1m for 7 days. DAPT is a \textgamma-secretase inhibitor and is used to block Notch signalling activation (Dovey et al., 2001). 1m is a novel GSK3 inhibitor and has been used to activate the Wnt/\textbeta-catenin pathway in cell differentiation studies (Bone et al., 2009, 2011). Treatment with BMP4, DAPT and 1m did not provoke intestinal gene transcription in HET-1A, HNF4\textalpha-c1 and HNF4\textalpha-c5 HET-1A cells (Fig. 5.10). There was also no change in the expression of \textit{K14} and \textit{K18}.

\textbf{5.2.5 BMP4 and DCA did not induce CDX2 or HNF4\textalpha expression}

To assess the effect of BMP4 and DCA exposure in BM pathogenesis, HET1-A, HNF4\textalpha-c1 and HNF4\textalpha-c5 HET-1A cells were treated with 100 ng/ml of BMP4, 100 or 400 \textmu M of DCA or both BMP4 and DCA for 24 hours. RT-PCR results showed that \textit{CDX2} and \textit{HNF4}\textalpha expression were not detected in the cultures (Fig. 5.11).
Treatment with the DNA methyltransferase inhibitor 5-Azacytidine did not increase intestinal gene expression in Ad-HNF4α and Ad-CDX2 infected HET-1A cells

HET-1A cells were infected with Ad-Null or Ad-HNF4α plus Ad-CDX2. After 24-hour hours following infection, cells were treated with 1 or 4 µM 5’-Azacytidine, or DMSO for 3 days. Cells were harvested at day 4 and RT-PCR was performed. β-ACTIN was used as a loading control. Controls include HT-29 cells or mouse intestine (positive, +) and reaction that did not contain sample (negative, -).

Experiments were performed in triplicate.
Figure 5.2. Cytokine treatment activated ppERK or NF-κβ pathway in HET-1A cells

After 24-hours of serum starvation, HET-1A cells were treated with 10 or 100 ng/ml of IL-1β, TNFα, LPS, IL-17, IFN-γ, or the mixture of IL-1β, TNFα and IFN-γ for 15 or 60 minutes. Western blotting was performed to determine the level of ppERK and pp65 expression. GAPDH was used as a loading control. Experiments were performed in duplicate.
Figure 5.3. IL-6 activated pSTAT3 pathway in HET-1A cells

Twenty-four hours after serum starvation, HET-1A cells were incubated with 10 or 100 ng/ml of IL-6 for 15 or 60 minutes. Cells were harvested and the pSTAT3 level was determined by western blotting. GAPDH was used as a loading control. Experiments were performed in duplicate.
Figure 5.4. IL1β, IL-6, TNFα, LPS or IL17 treatment did not induce CDX2 and HNF4α expression in HET-1A cells
HET-1A cells were treated with 10 or 100 ng/ml IL-1β, IL-6, TNFα or IL-17, or 10 or 100 µg/ml of LPS for 1, 4 or 7 days. RNA was extracted and RT-PCR was performed. β-ACTIN was as a loading control. These experiments were repeated 3 times. Typical results are shown.
Figure 5.5. IL1β and TNFα did not induce CDX2 and HNF4α expression in HET-1A cells

HET-1A cells were treated with IL1β, TNFα either alone or in combination (at a concentration of 100 ng/ml) for 7 days and cells were harvested. RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown.
Figure 5.6. IFNγ, IL-1β and TNF did not induce CDX2 and HNF4α expression in HET-1A cells in HET-1A cells

(A) HET-1A cells were treated with IFNγ at a concentration of either 10 or 100 ng/ml. After IFNγ treatment for 1, 4 or 7 days, cells were harvested and RT-PCR was performed. (B) HET-1A cells were treated with 10 ng/ml of IFNγ, IL-1β and TNF for 7 days and RT-PCR was then performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown. Experiments were performed in triplicate.
**Figure 5.7. IL-1β, IL-6 and DCA treatment did not induce intestinal gene expression in HET-1A cells**

HET-1A cells were treated with 100 ng/ml of IL-1β or IL-6 along, or with 50 µM DCA for 4 days. Cells were harvested and RT-PCR was performed. \(\beta\)-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown. Experiments were performed in triplicate.
Figure 5.8. DCA treatment in different oesophageal epithelial cell types

(A) Adult mouse oesophageal explants which has been cultured in BME medium for 7 days were treated with 400 µM DCA for 24 hours. Mouse embryonic oesophagus isolated from E11.5 embryos was cultured for 24 hours and then was treated with 100 or 400 µM DCA for 24 hours. (B) HET-1A or OE33 cells were incubated with 100 or 400 µM DCA for 24 hours. Cultures were then harvested and RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown.
Figure 5.9. Cholic acid, glycocholic acid, and taurocholic acid treatment did not induce CDX2 and HNF4α expression in HET-1A cells

HET-1A cells were treated with 200 or 400 µM bile salt cholic acid (CA), glycocholic acid (GCA) and taurocholic acid (TCA) or the 200 µM mixture of cholic acid, glycocholic acid and taurocholic acid for 48 hours. Cells were harvested and RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown.

<table>
<thead>
<tr>
<th>µM</th>
<th>Ctrl</th>
<th>CA 200</th>
<th>CA 400</th>
<th>GCA 200</th>
<th>GCA 400</th>
<th>TCA 200</th>
<th>TCA 400</th>
<th>CA+GCA 200</th>
<th>+</th>
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</thead>
<tbody>
<tr>
<td>CDX2</td>
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Figure 5.10. BMP4, DAPT or 1m treatment did not induce intestinal gene expression in HET-1A cells

HET-1A cells were treated with 10 or 100 ng/ml of BMP4, 5 or 25 µM of DAPT, or 0.5 or 1 µM of 1m for 7 days. Cells were harvested and RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown.
Figure 5.11. BMP4 and DCA treatment did not induce CDX2 and HNF4α expression in HET-1A cells

HET-1A cells were treated with 100 ng/ml of BMP4, 100 or 400 µM of DCA, or the mixture of BMP4 and DCA for 48 hours. Cells were harvested and RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were repeated 3 times. Typical results are shown.
5.3 Discussion

5.3.1 DNA methylation and BM

Previous studies showed that epigenetic regulation could potentially be a key step in the development of BM. The inhibition of DNA methylation by 5’Aza treatment in retrovially-infected CDX2 oesophageal cells increased BM marker levels, including K20 and DRA/SLC26A3 in immortalized human esophageal keratinocytes (EPC-hTERT). Similar observations of BM marker induction, such as CDX1 and K18 was seen in K14-Cdx2 transgenic mice treated with 5-Aza (Kong et al., 2009, 2011b). In HET-1A cells, 5’Aza treatment did not increase or induce the expression of the selected intestinal genes in Ad-HNF4α and Ad-CDX2 infected cells (Fig. 5.1). In fact, HET-1A cell line was immortalized by introducing SV-40 large T antigen (Stoner et al., 1991). It was found that SV-40 large T antigen works with Ras protein to activate DNA methyltransferase, DNMT3B. Therefore, SV-40 large T antigen could result in aberrant DNA methylation profile (Soejima et al., 2003; Li et al., 2005). Therefore, it was suggested that if HET-1A cells were used as a study model, the effects of DNA methylation inhibition in development of BM might be different from the normal oesophageal cells (Kong et al., 2009). Primary oesophageal epithelial cells will be a more suitable model for the study of epigenetic effects on the initiation of BM and the related results is shown in Chapter 6.2.3.

5.3.2 Inflammation-related exogenous factors and BM

Bile acid can also induce ROS and NO production which in turn leads to DNA damage, and therefore activate the intrinsic pathway of inflammation (Dvorak et al., 2007; Jenkins et al., 2007, 2008; Jolly et al., 2009; Goldman et al., 2010; McAdam et al., 2012). In this chapter, several key inflammatory factors were added to the HET-1A cell cultures to understand whether they play a role in BM development. Exogenous factors IL-1β, TNF-α, LPS, IL-17, IL-6 and IFN-γ were tested. Previous studies showed that the combination of cytokines (TNF-α and IFN-γ) resulted in the synergistic effect of prostaglandin E2 (PGE2) synthesis in lung epithelial A549 cells (Wright et al., 2004) and PGE2 has been known to be involved in inflammation and carcinogenesis (Kalinski, 2012; Nakanishi & Rosenberg, 2013). Therefore, the effects of treating HET-1A cell with the combinations of IL-1β and TNF-α, or IL-1β, TNF-α and IFN-γ were also investigated. Additionally, DCA has been shown to
accelerate IL-6 and IL-1β induced BM phenotype in L2-IL1β transgenic mice (Quante et al., 2012). Thus, the effects of IL-6, IL-1β and DCA in HET-1A were also tested. However, RT-PCR results showed that BM-related genes were not induced by the treatment of all the exogenous factors tested (Fig. 5.4-5.7). The failure to induce BM-related genes such as CDX2 and HNF4α, might be due to the relatively short period of cytokine treatment. Indeed the development of BM is linked to chronic inflammation (Dvorak & Dvorak, 2013). In the L2-IL1β transgenic mice model, L2-IL1β mice displayed moderate inflammation 6 months after birth and 90% developed a BM-like phenotype at 12-15 month old, but only 20% of L2-IL1β mice had high grade dysplasia or oesophageal adenocarcinoma at 20-22 months old (Quante et al., 2012). Therefore, 7 days cytokine treatment may not be sufficient to induce BM phenotype. It might be possible to treat the cell line for longer time periods if the cells could be passaged while treating with the cytokines. Alternatively, a promoter luciferase vector for intestinal transcription factors such as CDX2 and HNF4α genes could be created. A screening test in which the oesophageal epithelial cells were transfected with promoter luciferase vector and then were treated with the different inflammation-related exogenous factors could be performed. Through observing the luminescence signal intensity which is related to the amount of luciferase produced, this screening test will demonstrate the induction of different exogenous factors on the promoter activity of the intestinal transcription factors.

5.3.3 Bile acid in BM development

The exposure of the oesophagus epithelium to bile salts and/or acid has been considered as a major factor for BM pathogenesis (Spechler, 2002; Souza, 2010). It has also been reported that oesophageal aspirates from the GORD patients contained higher concentration of bile acid than aspirates from the non-GORD patients (Kauer et al., 1997). The purpose of the studies on bile salt and acid treatment in this chapter were, firstly, to test whether bile salt and acid can induce intestinal transcription factors CDX2 and HNF4α expression and, secondly, to determine whether bile salt and acid can enhance HNF4α-induced intestinal gene expression. The examination of the effects of 24-hour DCA treatment showed different responses according to the origins of the oesophageal epithelial cells (Fig. 5.8). The results of the CDX2 induction of by DCA in HET-1A cells is consistent with a previous report (Hu et al.,
2007). However, in OE33 cells, CDX2 and HNF4α expression is reduced by DCA treatment. The result of CDX2 reduction by DCA is contradictory to previously published material. In previous reports, the CDX2 level in OE33 cells was slightly up-regulated by 8 or 12 hour DCA treatment (50-200 µM) (Tamagawa et al., 2012). It was demonstrated that bile salt mixture CA, TCA and GCA triggered Cdx1 and Klf4 expression in primary rat oesophageal epithelial cells (Kazumori et al., 2011, 2009). However, in my studies, CA, TCA and GCA did not induce Cdx2 and HNF4α transcription in HET-1A cells (Fig.5.9). This might be due to the difference in cell types used. Additionally, the different time course, the different bile salt concentrations and the lower pH condition (< pH7) could be tested.

Our original hypothesis was that HNF4α is a key driver of the switch from stratified squamous epithelium to intestinal epithelium. One possibility for HNF4α is that bile acids might induce the transcription factor as occurs in the liver and intestine (Jung et al., 2007; Hylemon et al., 2009; Lefebvre et al., 2009). Whether the ectopic HNF4α expression in BM is directly induced by bile acid has not been reported previously. My data showed that HNF4α expression was not induced by DCA in all cell types examined. A more extensive panel of conditions could be tested including different exposure periods, reducing the pH further from pH 6 and testing the addition of exogenous factors such as cytokines IL-1β and TNFα. In addition, other bile salts which have been shown to induce or increase CDX2, such as DHCA or bile salts cocktail of GCA, TCA, GCDCA, TCDCA, GDCA, TDCA (as listed in table 5.3) could be tested. It has been reported that different types of bile salt have different optimized pH condition to reach their maximum activity (Cronin et al., 2010b). In addition, it is also possible that ectopic expression of HNF4α is not directly up-regulated by bile acid, but by other intestinal transcription factors, particularly CDX2. It has been reported that HNF4α promoter contains CDX2 binding sites (Gao et al., 2009) and indeed CDX2 positively regulates HNF4α transcription through binding to HNF4α promoter and the 3’ enhance region during intestinal differentiation in Caco2 cells (Boyd et al., 2010). Therefore, the ectopic expression of HNF4α in BM might be linked to the ectopic expression of CDX2.
5.3.4 Notch, Wnt and BMP4 in BM

Notch
In both HET-1A and HNF4α-expressing cells, the inhibition of Notch pathway by 5 or 25 µM DAPT treatment for 7 days did not induce or enhance intestinal gene expression (Fig. 5.10). Previous studies using 20µM DAPT treatment of HET-1A cells for 48-hour increased CDX2 and MUC2 mRNA levels by 2- and 3-fold respectively (Tamagawa et al., 2012). In this report, the mRNA level was evaluated by real-time PCR method which would be more subjective than the traditional PCR used in this chapter. Therefore, real-time PCR could be utilized in experiments in the future to detect whether DAPT increase or induce intestinal gene expression in HNF4α-expressing cells.

Wnt
Several studies have indicated that aberrant activation of the Wnt/β-catenin signalling pathway is involved in the neoplastic progression of BM (Osterheld et al., 2002; Clément et al., 2006). However, it remains to be investigated whether Wnt/β-catenin signalling activation also participates in the development of BM. In my study, treating HET-1A cells or HNF4α-expressing HET-1A cells with the GSK3 inhibitor, 1m (which activates the Wnt/β-catenin pathway) did not provoke or enhance intestinal gene expression (Fig. 5.10). A previous study showed that the activation of Wnt signalling by introducing a dominant-active Wnt effector CatCLeF caused intestinalized of immortalized primary human oesophageal cells (Kong et al., 2011a). Nevertheless, the intestinalization was only observed when cells were cultured in the 3D organotypic model. This might implicate that cell-cell contact and polarization plays a role in Wnt-mediated BM development (Kong et al., 2011a). Therefore, a 3D organotypic culture system might be needed for the investigation of the role of Wnt/β-catenin signalling in the induction of intestinal transcription factor including HNF4α and CDX2.

BMP4
BMP4 either alone or in combination with DCA did not trigger or enhance intestinal gene transcription in either control HET-1A cells or in HET-1A cells expressing HNF4α (Fig.5.10, 5.11). Previous reports showed that BMP4 induced a
columnar-type cytokeratin expression in human primary oesophageal epithelial cultures (Milano et al., 2007). BMP4 was also shown to induce Sox9 expression in HET-1A cells (Wang et al., 2010). However, there is no direct evidence showing that BMP4 alone is sufficient to drive intestinal metaplasia in the oesophageal epithelial cells. Therefore, BMP4 might promote columnar phenotype in the early development BM, but other exogenous factors or master transcription factors are probably required to drive intestinal differentiation.
Chapter 6
Using mouse oesophageal explant culture as a BM model
6.1 Introduction

6.1.1 Issues of using oesophageal cell lines as a model for BM

In the Introduction (section 1.7), I described the use of oesophageal cell lines in BM research. These cell lines include those derived from normal oesophageal epithelium, BM or oesophageal adenocarcinoma. However, two main issues arise when using cell lines for BM research. Firstly, the phenotype of several cell lines has been questioned. For example, BIC-1, SEG-1, SK-GT-5 and TE-7 are in fact not oesophageal adenocarcinoma cell lines, but represent the colon adenocarcinoma (BIC-1), lung carcinoma (SEG-1), gastric fundus adenocarcinoma (SK-GT-5) or squamous carcinoma (TE-7) respectively (Boonstra et al., 2007; Alvarez et al., 2008; Boonstra et al., 2010).

Secondly, during the cell line generation process, cells may undergo genetic transformation in order to become immortalized. Therefore, the genetic transformation may change the original characteristics of the cells (Kong et al., 2010). For example, the HET-1A cell line was immortalized by the ectopic expression of the viral SV-40 gene (Stoner et al., 1991). SV-40 introduction results in the inactivation of p53 protein. Nevertheless, in BM and adenocarcinoma, p53 mutation (inactivation) is not usually observed until the stage of high-grade dysplasia (Weaver et al., 2014). Furthermore, SV-40 ectopic expression has also been shown to alter epigenetic states through activating DNMT3a (Li et al., 2005). Indeed epigenetic mechanisms have been suggested to be involved in the development of BM (di Pietro et al., 2012). Lastly, BM is the condition in which the lining of stratified squamous type of oesophageal epithelium is replaced by an intestinal-type columnar epithelial monolayer. The ideal cell model for BM model should display the characteristics of squamous epithelial cells. Nevertheless, the cell lines current widely used for BM research, particularly HET-1A cells, displays columnar epithelial phenotype as shown in Figure 3.1 in Chapter 3. Therefore, there may be some bias when using HET-1A cells to recapitulate the BM in terms of the conversion of squamous toward intestinal, columnar cell type.
6.1.2 3D culture models for human oesophageal epithelial epithelium
It has been suggested that the optimum in vitro model for BM studies should preserve the characteristics of the in vivo oesophageal cells, display the three dimensional (3D) organotypic architecture and can survive long-term (Colleypriest, 2010). Because tissue and organs exist in 3D in the physiological environment, cells cultured in a 3D organotypic system offers some advantages over the 2D cell culture system. For example, cells cultured in the 3D system mimics a more physiologically relevant tissue environment than the 2D cell culture. The 3D culture system not only supports the interaction between cells and extracellular matrix (ECM) but also cells cultured in the 3D model may represent the different differentiated cell morphology and function.

A number of reports have described the establishment of 3D human oesophageal epithelial culture models which mimic the features of the mature oesophageal epithelium (Underwood et al., 2010; Green et al., 2010; Maghsoudlou et al., 2014). It was shown that when cultured with the oesophageal fibroblasts, that primary human oesophageal epithelial cells are able to develop the stratified squamous structure. Additionally, a proliferative p63-expressing basal layer can be observed in this 3D organotypic culture (Underwood et al., 2010). Another report also has shown the success in developing a 3D culture system in which human oesophageal epithelial cells were cultured with fibroblasts on a porcine oesophagus-derided matrix (Green et al., 2010). In this system, a mature, stratified epithelium was produced and the epithelium resembled the similar expression pattern to the mature oesophageal epithelium including the K14 basal marker and differentiated markers K4 and Involucrin (Green et al., 2010).

6.1.3 Primary mouse oesophageal epithelial explants as a model for studying BM
As human oesophageal tissue is in limited supply we sought to develop from an alternative source. The use of mouse oesophageal epithelial cells as a model for studying BM has been described (Colleypriest, 2010). In the study, mouse oesophageal epithelium was stripped from the muscular layer and the epithelium was cut into small pieces (1-2 mm). The epithelial pieces were then placed on a scratched
plastic cover slide in a 35mm dish supplied with culture medium. Because some mesenchymal cells remains attached to the oesophageal epithelium, mesenchymal cells will grow as a feeder layer under the epithelial cells when cultured in BME medium. Results from the histological examination showed that this explant culture model not only recapitulates the \textit{in vivo} oesophageal stratified structure but also preserves the expression of oesophageal epithelial markers. Oesophageal epithelial cells at different differentiation stages were observed including basal cells (p63 / K14-expressing) and suprabasal/differentiating cells (K4 / Involucrin-expressing). Furthermore, in this mouse oesophageal explant culture technique, the expansion of oesophageal epithelial cells can be manipulated by using different culture medium. When oesophageal explants were cultured in the low calcium Epilife medium ($\text{Ca}^{2+} [60\mu M]$) instead of BME medium ($\text{Ca}^{2+} [1.8mM]$), the oesophageal explant grew into a monolayer cell sheet. Cells cultured in the Epilife medium contained a homogenous population of p63- and K14-expressing cells which represent the immature oesophageal cell population of the basal layer (Colleypriest, 2010).

6.1.4 Aim
The aim of this chapter was to utilise the primary mouse oesophageal epithelial cultures as a model to explore the mechanisms underlying the development of BM. In the first part of this chapter, adenoviral-mediated gene delivery was performed to induce HNF4α expression in the oesophageal epithelial cells. The effects of ectopic HNF4α expression on intestinal gene expression were then examined. In the second part of the chapter, mouse oesophageal explants were exposed with bile salt and/or acid to mimic the GORD condition. The expression of HNF4α, CDX2 and other intestinal genes was analysed.
6.2 Results

6.2.1 Mouse oesophageal epithelial explants as model for BM studies
Previous studies in the Tosh lab showed that mouse oesophageal epithelial explants can be maintained in either of two culture media: BME medium and Epilife medium. Mouse oesophageal epithelial explants cultured in the BME high calcium medium ([Ca\(^{2+}\)] 1.8mM) developed into a stratified structure resembling the in vivo oesophageal epithelial structure, whilst in the low calcium Epilife medium ([Ca\(^{2+}\)] 60µM), cells expanded into single-layer cell sheet (Colleypriest 2010). The morphology of explants which were cultured under either condition for 7 days is demonstrated in Fig. 6.1A. Immunostaining results showed that the explants cultured in the BME medium were positive for K14. Cells located near the original explant were positive for p63, but cells at the periphery of the cultures were negative for p63 (Fig. 6.1B). By contrast, cells cultured in the Epilife medium were all positive for both K14 and p63 (Fig. 6.1B).

6.2.2 Ectopic HNF4α expression in mouse oesophageal epithelial explants induces squamous-to-columnar cell type switch and VILLIN expression
In order to understand whether forced HNF4α expression in the mouse oesophageal explants induces intestinal gene expression, an adenoviral vector which carries the HNF4α gene (Ad-HNF4α) or a control adenoviral vector (without any transgene and referred to as Ad-Null) was tested in mouse oesophageal epithelial explants. The explants were maintained in culture in Epilife medium (as these allow better access for the viral particles) for 7 days prior to infection. Following HNF4α expression (but not Ad-Null) there was a morphological change and the epithelial cells enlarged in culture 3 days after infection (Fig. 6.2).

We next determined the expression of K8 and K14 in oesophageal explants following Ad-Null and Ad-HNF4α infection. Ad-Null infected cells did not express the columnar marker K8, but approximately 8% of cells in Ad-HNF4α infected cultures expressed K8 (Fig. 6.3A and Table 6.1A). All cells in Ad-Null infected cultures were positive for K14. However, in Ad-HNF4α treated cultures, around 25% lost K14 expression (Fig. 6.3B and Table 6.1B). Furthermore, we performed co-staining for both K8 and K14. Immunostaining for both K8 and K14
demonstrated that some K8-positive cells lost K14 expression, indicating that the squamous cells had undergone reprogramming to columnar-like cells (Fig. 6.4). It can also be observed that in some cells, the ectopic HNF4α expression inhibited p63 expression (Fig. 6.5). Finally, the expression of intestinal VILLIN protein was checked. Forced HNF4α expression initiated VILLIN expression (Fig. 6.6A). Approximately 15.9% of cells in Ad-HNF4α infected cultures expressed VILLIN (Table 6.2). Finger-like protrusions expressing VILLIN can also be observed in higher magnification images (Fig. 6.6B).

6.2.3 Inhibition of DNA methylation did not significantly induce intestinal gene expression
In Chapter 5.2.1, I showed that 5’Aza did not induce or enhance intestinal gene expression in either control or CDX2 and HNF4α expressing cells. To further test the effect of the inhibition of DNA methylation on mouse oesophageal epithelial explants, explants were initially infected with Ad-HNF4α or Ad-Null virus for 24 hours prior to treatment with 5’Aza. Explants treated with 2µM 5’Aza did not survive (data not shown). However, explants treated with 1µM 5’Aza did survive for up to 4 days. RT-PCR results showed that intestinal gene expression was not induced by this treatment in Ad-Null culture. In Ad-HNF4α culture, TFF3 was weakly induced by 5’Aza and TFF3 was not detected in cultures without 5’Aza treatment (Fig. 6.7).

6.2.4 Bile acid and BM
In the Chapter 5.2.3, I analysed the effects of DCA treatment on CDX2 and HNF4α expression in different oesophageal cell types. To further obtain informative studies, we decided to test the effects of bile acid treatment on intestinal gene expression in adult mouse oesophageal epithelial explants infected with Ad-HNF4α. We therefore cultured explants for 7 days prior to infection with either Ad-Null or Ad-HNF4α adenoviruses at $2.55 \times 10^7$ IU/ml and at day 5, explants were treated with medium containing 300µM DCA at pH 6 for 24 hours. There was no induction of intestinal genes in the Ad-Null infected cells treated with or without DCA (Fig. 6.8). In the Ad-HNF4α infected cultures, VILLIN and TFF3 were detected, but surprisingly, VILLIN and TFF3 levels were reduced with DCA treatment. The expression of other
selected intestinal genes was not detected.

We considered that the DCA might require a longer duration of exposure to induce a change in gene expression. We therefore extended the period of DCA treatment for up to 3 days. Mouse adult oesophageal epithelial explants were infected with Ad-HNF4α. At day 3, explants were incubated with 50 or 100 μM DCA for a further 3 days. To mimic GORD, some cultures which were incubated with DCA containing medium were further treated with DCA containing medium at pH 6 for 10 minutes, 3 times a day for 3 days. The results suggest there was no increase in VILLIN or TFF3 gene expression (Fig. 6.9).
Figure 6.1. Characterization of mouse oesophageal epithelial culture

Mouse oesophageal epithelium was isolated and cut into small pieces and then cultured on scratched plastic slides before being placed in 35mm dishes supplied with different culture medium. (A) Phase contrast images displayed the oesophageal epithelial cells maintained in either BME or Epilife medium after 7 days of culture. (B) Immunostaining result displayed that in BME medium, cells near the central original explant expressed p63 and most of the cells expressed K14. By contrast, cells cultured in Epilife medium all co-expressed p63 and K14. One of two experiments is shown.
Figure 6.2. Ectopic Hnf4α expression in primary mouse oesophageal epithelial explants

After 7 days of culture in Epilife medium, mouse oesophageal epithelial explants were treated with either Ad-HNF4α or Ad-Null virus (5.1 x 10^5 IU) for 24 hours and then medium was changed. Phase contrast images showed the morphology of oesophageal epithelial explants with Ad-Null or Ad-HNF4α infection at day3. Enlarged cells (arrow) are found in the Ad-HNF4α treated cultures.
Figure 6.3. The induction of K8 and the loss of K14 following Hnf4α expression in mouse oesophageal epithelial explants

Mouse oesophageal epithelial explants were cultured in Epilife medium for 7 days and then Ad-HNF4α or Ad-Null virus was added. Twenty-four hours later, the medium was replaced with fresh medium. Following 2 days of further culture, cells were fixed for immunostaining. (A) K8 is not detected in the Ad-Null infected cultures, but K8 is found in the Ad-HNF4α cultures. (B) All cells in the Ad-Null treated culture expressed squamous cell marker K14, but a proportion of the cells in Ad-HNF4α treated culture no longer expressed K14 (arrow). The experiments were performed in triplicate.
Table 6.1. The number of HNF4α/K8 and HNF4α/K14 expressing cells in mouse oesophageal epithelial explants following HNF4α expression

Mouse oesophageal epithelial explants were cultured in Epilife medium for 7 days. Explants were infected with Ad-HNF4α or Ad-Null virus for 24 hours before the medium was changed. Following 2 days the explants were fixed for immunostaining. Table shows the number of (A) HNF4α and K8 and (B) HNF4α and K14 expressing cells based on the immunostaining results Figure 6.3A and 6.3B. The experiments were performed in triplicate.

<table>
<thead>
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<th>Cell types</th>
<th>Ad-Null</th>
<th>Ad-HNF4α</th>
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<td>44.1%±11.2%</td>
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<td>46.4%±8.4%</td>
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<table>
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<th>Ad-HNF4α</th>
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<td>K14⁺/HNF4α⁻</td>
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<td>6.4%±3.7%</td>
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<td>0%</td>
<td>68.0%±7.3%</td>
</tr>
<tr>
<td>K14⁻/HNF4α⁻</td>
<td>0.4%±0.5%</td>
<td>4.5%±5.4%</td>
</tr>
<tr>
<td>K14⁻/HNF4α⁺</td>
<td>0%</td>
<td>20.5%±5.3%</td>
</tr>
</tbody>
</table>
Figure 6.4. Ectopic expression of HNF4α in adult mouse oesophageal epithelium induces a squamous-to-columnar switch in phenotype

Seven days after culture in Epilife medium, mouse oesophageal epithelial explants were infected with Ad-HNF4α or Ad-Null virus for 24 hours and then the medium was replaced with fresh medium. The explants were fixed after 2 days of further culture. Immunostaining was performed to examine the expression of K8 and K14. Merged images of K8 and K14 demonstrate that explants infected with Ad-Null continue to express K14. However, some cells (arrow) in the Ad-HNF4α culture were negative for K14 and positive for K8, indicating that the cells may have undergone a squamous-to-columnar cell type switch. The experiments were performed in triplicate.
Figure 6.5. The down-regulation of p63 by HNF4α ectopic expression in oesophageal epithelium

After 7 days of maintenance in Epilife medium, Ad-Null or Ad-HNF4α virus was added into mouse oesophageal epithelial cultures and medium was replaced 24 hours later. After 2 days of further culture, the explants were fixed. (A) Immunostaining results showed that some HNF4α-expressing cells down-regulated the expression of p63 (arrow), while in Ad-Null infected cells were all positive for p63. The experiments were performed in triplicate.
Figure 6.6. The induction of VILLIN expression by ectopic HNF4α expression in adult mouse oesophageal epithelium

Mouse oesophageal epithelial explants were cultured in Epilife medium for 7 days and then were treated with either Ad-HNF4α or Ad-Null virus (5.1 x 10⁵ IU) for 24 hours. The explants were fixed after 2 days of further culture. VILLIN and HNF4α immunostaining results displayed that (A) intestinal protein VILLIN is not expressed in the Ad-Null-infected cells, but ectopic HNF4α expression induces VILLIN expression. (B) Microvilli protrusions can be observed in the high magnification images. The experiments were performed in triplicate.
Table 6.2. The number of HNF4α/VILLIN expressing cells in mouse oesophageal epithelial explants following HNF4α expression

Mouse oesophageal epithelial explants were cultured in Epilife medium for 7 days. Explants were infected with Ad-HNF4α or Ad-Null virus for 24 hours before the medium was changed. Following 2 days the explants were fixed for immunostaining. Table shows the number of HNF4α and VILLIN expressing cells based on the immunostaining results Figure 6.6A. The experiments were performed in triplicate.
Figure 6.7. Treatment with the DNA methyltransferase inhibitor 5’-Azacytidine enhanced intestinal gene expression in Ad-HNF4α-infected oesophageal epithelial explants

Mouse oesophageal epithelial explants were initially cultured in BME medium for 7 days and then treated with either Ad-Null or Ad-HNF4α. After 24-hours of viral infection, the cells were treated with 1µM 5’-Azacytidine or DMSO for a further 4 days. Cells were harvested at day 5 and RT-PCR was performed. Controls include mouse intestine (positive, +) and reaction without sample (negative, -). β–ACTIN was used as a loading control. The experiments were performed in duplicate.
Figure 6.8. DCA treatment did not enhance intestinal gene expression in HNF4α-expressing mouse oesophageal epithelial explants

Mouse oesophageal epithelial explants were cultured in BME medium for 7 days and then infected with Ad-HNF4α or Ad-Null for 24 hours. The medium was then changed. At day 5, cells were treated with medium containing 300 µM DCA, at pH 6 for 24 hours. Cells were then harvested and RT-PCR performed. β-ACTIN was used as a loading control. The experiments were performed in duplicate.
Figure 6.9. DCA and low pH treatment did not enhance intestinal gene expression in HNF4α-expressing mouse oesophageal epithelial explants

Mouse oesophageal epithelial explants which have been cultured in BME medium for 7 days were incubated with Ad-HNF4α virus for 24 hours, and medium was changed. At day 3, cultures were treated with medium containing 50 or 100 µM DCA. DCA treated cultures were further treated with DCA containing medium at pH 6 for 10 minutes, 3 times per day for 3 days. Cultures were harvested at day 6 and RT-PCR was performed. β-ACTIN was used as a loading control. The experiments were performed in duplicate.
6.3 Discussion
6.3.1 Ectopic HNF4α expression in primary mouse oesophageal explants
Primary mouse oesophageal explants can be cultured in the serum-containing BME medium or under serum-free conditions in Epilife medium. Oesophageal epithelial explants in BME medium formed a stratified structure resembling in vivo oesophageal epithelium structure. Additionally, p63-expressing cells were observed near the original epithelial explant, but not at the edge of the outgrowth of cells. By contrast, oesophageal epithelial explants in Epilife medium formed a single layer and homogenously expressed p63 and K14 of basal cell phenotype. Because explants in Epilife medium formed a single layer, this greatly improves the virus infection efficiency compared with the virus infection efficiency in explants cultured in BME medium. The difference between the media is mainly due to the difference in calcium concentration. BME medium contains 1.8mM Ca²⁺, but Epilife medium contains 60µM Ca²⁺. Ca²⁺ is an import regulator for human epidermal keratinocyte differentiation. Epidermal keratinocyte stratified and showed terminal differentiation when cultured in the high Ca²⁺ concentration (1.0mM), but epidermal keratinocyte remained in undifferentiated state and grew in single layer when maintained in lower Ca²⁺ concentrations (0.3mM) (Boyce & Ham, 1983).

We tested the ability of HNF4α to induce a phenotypic switch in the K14/p63-positive cells maintained in Epilife medium. Following ectopic expression of HNF4α, the columnar marker K8 expression was induced and squamous marker K14 expression was decreased in the primary mouse oesophageal epithelial cells. Furthermore, K8 and K14 dual staining showed the presence of K14 negative and K8 positive cells. Additionally, HNF4α ectopic expression resulted in the loss of p63 and induction of VILLIN protein expression. Taken together, this evidence suggests that HNF4α ectopic expression may induce a squamous-to-columnar cell type conversion and may also induce intestinal differentiation. It will be interesting to investigate whether HNF4α induces morphological features of intestinal epithelial cells such as microvilli as has been observed in NIH-3T3 cells (Babeu et al., 2009). It will also be important to investigate the expression of other intestinal genes, and understand the mechanisms and signalling pathway underlying the development of BM. For example, the p63 gene expression could be checked by RT-PCR and p63 protein
level could be examined by Western blotting in a time and dose (Ad-HNF4α amount) dependent manner. It is also important to understand how HNF4α inhibits p63 expression. According to the search results from web-based tool (HNF4 Motif Finder, http://nrmotif.ucr.edu) (Bolotin et al., 2010), there is no potential HNF4α binding site found in the −2 kb to +1 bp region of the p63 promoter. Therefore, HNF4α might indirectly inhibit p63 expression. It is also possible that the loss of p63 is the indirect effect of the intestinal differentiation.

Finally, it has been suggested that HNF4α might regulate intestinal function and differentiation through regulating the Wnt/β-catenin signalling pathway (Cattin et al., 2009). In the study, conditional knockout of Hnf4α in adult mice (Villin-Cre-ERT2; Hnf4αloxP/loxP) increased the β-catenin level in the nucleus revealing the activation of Wnt/β-catenin pathway. In vitro studies also showed that HNF4α inhibited β-catenin/TCF-dependent luciferase activity in human colorectal cancer cells HCT116, and HNF4α interacts with TCF-4 protein in HCT116 and Caco-2/TC7 cells. These results suggest that HNF4α inhibits Wnt/β-catenin pathway in the intestinal epithelium. Wnt/β-catenin pathway has been known to play a critical role in the regulation of cell proliferation and differentiation balance, and the maintenance of stem cells and progenitor cells in the intestinal epithelium (Pinto & Clevers, 2005). Therefore, it is important to investigate whether Wnt/β-catenin signalling pathway contributes to the HNF4α-induced intestinal differentiation in normal oesophageal epithelial cells.

6.3.2 DNA methylation mechanism and BM

Epigenetic regulation could be an important factor in the development. Studies in both EPC-hTERT cell line and K14-Cdx2 transgenic mice showed that the inhibition of DNA methylation by 5’Aza treatment induce BM marker expression, but BM marker expression was not observed in non-treatment control groups (Kong et al., 2009, 2011b). In Chapter 5.2.1, I investigated the effects of 5’Aza treatment in HNF4α-expressing HET-1A cells. Results showed that the expression of BM markers was not induced by 5’Aza treatment. In this chapter, I further used mouse oesophageal epithelial explant culture as a model to understand whether 5’Aza treatment could induce the expression of BM markers. My study results showed that
5’Aza treatment weakly enhanced *TFF3* expression level in mouse oesophageal epithelial explants infected with Ad-HNF4α compared to Ad-HNF4α infected explants alone (Fig. 6.7). The inhibition of methylation by 5’Aza might open the inactivated promoter regions and enhance transcriptional activity (Kong et al., 2011b). Indeed, the DNA methylation mechanism is involved in the development of BM. It was observed that there are differences in the promoter methylation profile between normal oesophageal epithelium and BM (Eads et al., 2001; Wang et al., 2009; Kaz et al., 2011; Agarwal et al., 2012; Alvi et al., 2013). In the future it will be interesting to investigate the effects of DNA methylation inhibition by treating 5’Aza on CDX2, and both CDX2 and HNF4α-expressing oesophageal epithelial explants.

### 6.3.3 Bile acid/salt and BM

It has been known that patients with GORD have increased risk of BM and bile salt and acid are the potential toxic component of gastric contents (Dvorak et al., 2007; Souza, 2010). Therefore, it has been considered that the exposure of bile salt and acid at the lower end of the oesophageal epithelium are the key factors for BM development. The aim of exposure of bile salt and acid on oesophageal epithelial explants was to understand whether bile salt and acid could (i) induce intestinal gene expression and (ii) enhance HNF4α-induced intestinal genes. My results showed that bile salt and acid stimulation did not enhance intestinal gene expression in mice adult oesophageal epithelial explants which has been infected with Ad-HNF4α (Fig. 6.8, 6.9). The failure of intestinal gene induction could be due to exposure time, pH, and concentration and types of bile salts. The effects of bile salt and acid on CDX2 expression in normal primary oesophageal epithelial cells have been described (Marchetti et al., 2003; Kazumori et al., 2006; Huo et al., 2010). Study in primary mouse oesophageal epithelial cells showed that exposure to pH3.5 for 18 days induced CDX2 protein expression (Marchetti et al., 2003). Another report in which rat oesophageal epithelial cells were used as the study model demonstrated that 50 µM CA or 100µM DHCA treatment for 24 hours induced CDX2 protein expression (Kazumori et al., 2006). However, acid (pH4) and/or bile salts cocktail 10 minutes, 3 times a day for 7 days treatment did not induce CDX2 expression in oesophageal epithelial cells derived from GORD patients without BM (Huo et al., 2010). Therefore, a more extensive panel of conditions including different exposure
periods, different pH, and different types and different concentration of bile salts could be tested in the future.
Chapter 7
Final discussion
7.1 Limitations of the study

In this thesis, three cell models were used for studying the development of BM: HET-1A cells, mouse oesophageal epithelial explants cultured in BME medium and explants cultured in Epilife medium. HET-1A cells were the most widely used cell model in my thesis. Culturing HET-1A cells was relatively less labour intensive and expanding HET-1A cells was relatively robust when compared with culturing the primary mouse oesophageal epithelial explants. However, HET-1A cells might not be a suitable model for studying BM, because HET-1A cells do not represent the normal oesophageal squamous characteristics. HET-1A cells do not show the typical squamous markers such as K14 and p63, but express instead the columnar marker K8 and mesenchymal marker Vimentin (Figure 3.1 in Chapter 3). Therefore, as an alternative model I utilised mouse oesophageal epithelial explants.

Mouse oesophageal epithelial explants cultured in BME medium displayed the 3D multilayer structure resembling the in vivo oesophageal stratified squamous epithelium (Colleypriest, 2010). However, the major problem of using this culture system as a model is the poor infection efficiency with the adenoviral-mediated gene delivery. Because of the thickness of the 3D epithelial multilayer, only cells at the explant periphery were able to be infected with the adenovirus. Therefore, to improve the adenoviral infection efficiency, mouse oesophageal epithelial explants cultured in low calcium (Ca^{2+} [60µM]) Epilife medium were used.

Mouse oesophageal epithelial cells cultured in Epilife medium grew as a monolayer and showed the characteristics of the basal oesophageal epithelial cells with the expression of K14 and p63. The efficiency of the adenoviral infection was significantly improved under these culture conditions. However, the reproducibility of the establishment of the explants cultured in the low calcium medium is not ideal. There are difficulties in expanding cells and cells were also only able to survive for up to two weeks in the low calcium media. In addition to Eplife medium (Invirtogen), different types of low calcium keratinocyte media and medium from difference sources including MCDB153 (Sigma) and MCDB153 kit (Biochrom) were tested. Furthermore, to create low calcium medium, a method for removing calcium from the BME medium was also evaluated. A low calcium environment was created by
supplementing the BME medium with either of the calcium chelators EDTA or BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). However, the poor consistency and reproducibility of culturing oesophageal explant cultures in the low calcium medium did not improve. Therefore, the model of oesophageal explant cultured in under low calcium conditions was not used extensively in this thesis. In the future, other culture methods could be tested. For example, oesophageal epithelium could be dissociated into single cells before culture in the low calcium medium. If the culture conditions could be optimised, the experiments performed in HET-1A cells in this thesis could be re-evaluated including the ectopic expression of intestinal transcription factors and the screening of exogenous factors.

7.2 The role of master intestinal transcription factors in BM

As shown in Chapter 4, in addition to the intestinal markers K20, SI, ISX, ectopic CDX2 expression in HET-1A cells up-regulated LGR5, a marker for stem cells in several types of organs including the intestine and the stomach. Although LGR5 protein expression should be determined, the up-regulation of LGR5 gene expression raised the possibility that, if CDX2 is involved in the early stage of BM, the oesophageal squamous cell may turn into a stem cell state and then differentiate into intestinal-like cells. This can be investigated by overexpressing CDX2, and for example, real-time tracking of LGR5-GFP and VILLIN-mCherry expression at the single cell level.

Overexpression of CDX2 and HNF4α (either alone or in combination) in HET-1A cells induced expression (at least at the mRNA level) of VILLIN and K20. Indeed the levels of VILLIN and K20 were the highest when both transcription factors were overexpressed. The improvement of the consistency of the viral infection efficiency and more repeated experiments will provide the more reliable results of whether there is a synergistic effect of CDX2 and HDX4α on inducing intestinal gene expression. Although K14-CDX2 transgenic did not display a BM phenotype (Kong et al., 2011b), it will be interesting to investigate whether BM phenotype can be induced by double ectopic CDX2 and HNF4α expression in the K14-CDX2; K14-HNF4α transgenic mice model. Another possible synergistic effect was also observed in the ectopic expression of FOXA2 with the addition of CDX2. FOXA2
and CDX2 together induced MUC2 expression which was not induced by either FOXA2 or CDX2 alone. Given that the possible synergistic effect of intestinal gene expression by CDX2 and HNF4α or by CDX2 and FOXA2, a polycistronic construct of FOXA2, CDX2 and HNF4α could be created and tested in oesophageal epithelial squamous cells to understand whether the coexpression of FOXA2, CDX2 and HNF4α induces a more robust BM phenotype.

In this thesis, the induction of the intestinal phenotype was assessed by intestinal gene and protein expression. In fact, the intestinal phenotype can also be evaluated through electron microscopy method. The presence of intestinal epithelial ultrastructural features including microvilli and mucin granules are important characteristics of the intestinal epithelium (Fre et al., 2005; Quante et al., 2012).

7.3 Regulatory network of CDX2, HNF1α and HNF4α in BM development
Gene expression is complex and finely regulated. The gene expression is controlled by the combination of transcription factors rather than the individual transcription factor. Indeed, in the intestinal epithelium, the transcription factor network regulates intestinal cell growth and cell fate decisions (Phillips & Hoopes, 2008; Olsen et al., 2012). The potential regulatory network in the intestinal epithelium includes CDX2, HNF1α and HNF4α and has been described in Chapter 1.5.4. It possible that the development of BM is also controlled by the regulatory network of intestinal transcription factors. My results on CDX2, HNF1α and HNF4α ectopic expression in the HET-1A cells (Fig 4.5, 4.10) showed that VILLIN and K20 mRNA levels were highest in Ad-HNF4α and Ad-CDX2 co-infected cells. Additionally, the mRNA levels of VILLIN and K20 in Ad-HNF4α, Ad-CDX2 and Ad-HNF1α infected cells were lower than the levels in cells infected with Ad-HNF4α and Ad-CDX2. It remains to be investigated that whether HNFα and CDX2 work cooperatively to transcribe K20 and VILLIN in HET-1A cells and whether HNF1α may have inhibitory effects on K20 and VILLIN expression when HNF4α and CDX2 are also present. It is known that transcription factors could influence each other’s binding to the DNA (Phillips, 2008). Therefore, the negative regulation of gene expression by one transcription factor may occur through impeding the binding of other transcription factors to the DNA.
Hierarchical role of intestinal transcription factors in BM development

The combinational control of transcription factors could exhibit a hierarchical and dynamic pattern. For example, it was found that the dynamic pattern of C/EBPα, C/EBPβ and C/EBPδ expression is different between C/EBPs and cell types (adipocyte and granulocyte) during the differentiation process (Calkhoven & Ab, 1996; Cao et al., 1991; Scott et al., 1992). Similarly, it is likely that intestinal gene expression may be controlled by the hierarchical and dynamic transcription factors network. In this context, CDX2 interacts with intestinal DNA with GATA-6 in cell proliferation, while CDX2 partners with HNF4α during cell differentiation (Verzi et al., 2010). In addition, CDX2 enables the binding of HNF4α to the chromatin in the mouse adult intestinal epithelium (Verzi et al., 2013). Finally, the “two-hit hypothesis” has been proposed to explain the development of BM. In the hypothesis, the ectopic expression of HNF4α first leads to oesophageal squamous cell converting into columnar cell type and in the next step, CDX2 differentiates columnar cell into intestinal-like cell (Colleypriest, 2010). This hypothesis might also be supported by the fact that HNF4α is also expressed in the stomach which is lined by the columnar-type cells, but CDX2 expression is only limited to the intestine (Gao et al., 2009; Takano et al., 2009). Additionally, CDX2 ectopic expression in transgenic mice (Fox3-Cdx2) developed intestinal metaplasia in the gastric mucosa (Silberg et al., 2002).

By contrast, it is also possible that CDX2 firstly induced by NF-κB activation due to the bile acid exposure or inflammatory response caused by GORD (Kim et al., 2002; Jenkins et al., 2004; Kazumori et al., 2006). CDX2 ectopic expression may then induce HNF4α expression and the combination of CDX2 and HNF4α expression leads to BM. Especially it was observed that in the developing mice, HNF4α expression is largely increased during the intestinalisation process between E14.5-E16.5 (Li et al., 2009). During this period, the intestinal epithelium is reorganised and villus structure is formed. Therefore, it was speculated that HNF4α plays an important role in the final stabilisation of intestinal epithelium in the embryonic stage (Li et al., 2009).
Therefore, the specific order and the dynamic expression of the master intestinal transcription factors may be important when investigating the effects of the ectopic expression of intestinal transcription factors in BM development. For example, the sequential expression could be examined by comparing the presence of intestinal transcription factors in the BM tissue and the adjacent normal or oesophagitis squamous tissue. The relative levels of the different intestinal transcription factors expressed in the BM tissue could also be investigated. Additionally, in the in vitro experiment, CDX2 could first be introduced for a period of time and then followed by the HNF4α introduction in the oesophageal cells or vice versa. The effects of intestinal gene induction could then be investigated.

**Threshold level of transcription factor in cell fate determination**

The expression level of transcription factor might be a major mechanism that regulates cellular differentiation. For example, studies in hematopoietic stem cell differentiation revealed that the concentration of transcription factor PU.1 is a critical factor that alters the threshold level of cell fate. The high expression level of PU.1 shows normal hematopoietic development. However, when the expression level of PU.1 is low and meets a threshold, with other mutation such as c-MYC, hematopoietic stem cells could undergo malignant transformation and this leads to leukemia (Rosenbauer et al., 2005). In BM, whether the initiation of intestinal differentiation requires the expression of intestinal transcription factor to reach a threshold level remains to be investigated. In particular, because multiple intestinal transcription factors may be involved, it will be interesting to understand whether the development of BM requires the expression of the particular intestinal transcription factors which are all required to meet their individual threshold level.

**Post-translational modification and epigenetic mechanism in BM**

Besides the threshold level and dynamic pattern of transcription factors, the modulation of transcription factor activity itself may also be important for the temporal and spatial specificity of the gene expression. Transcription factor function and activity could be affected by the post-translational modifications such as phosphorylation, glycosylation, sumoylation, acetylation and ubiquitination (Tootle & Rebay, 2005). For example, studies showed that HNF4α activity could be
modulated by phosphorylation. Phosphorylation at HNF4α Ser158 facilitates the recruitment of transcriptional co-activator, PC4, and enhances the target gene transcription (Guo et al., 2007). However, phosphorylation at HNF4α Ser78 results in the failure of HNF4α binding to the DNA and therefore inhibits the HNF4α transcription activity (Sun et al., 2007). In addition to post-translational modification, DNA methylation, imprinting and conceding DNA of epigenetic mechanisms also exert a finer level of control over gene expression (Phillips, 2008).

An epigenetic mechanism has been suggested to be involved in the BM development (Kong et al., 2011b). In addition to DNA methylation, histone modification might also play a role in the BM pathogenesis. It was demonstrated that the ectopic expression of HOXB5, 6 and 7 were associated with BM development. Analysis in human specimens of BM-related oesophageal adenocarcinoma revealed that Histone 3 lysine 27 trimethylation (H3K27me3) level was reduced, and histone 3 acetylation (AcH3) level was increased near the transcription start region of HoxB5, 6 and 7, compared with the normal oesophageal epithelium. The degree of chromatin decomposition in HOXB locus was positively associated with the progression of BE and oesophageal adenocarcinoma (di Pietro et al., 2012). Therefore, it will be interesting to investigate the importance of histone modification in the development of BM.

In summary, the mechanisms underlying BM development may not only involve the ectopic expression of intestinal transcription factors, but mechanisms such as the threshold and dynamic regulation of intestinal transcription factors, epigenetic mechanism and post-translational modification could also be critical. More importantly, in addition to intestinal transcription factors, the transcriptional control from squamous oesophageal transcription factors such as p63 should also be taken into consideration. It will be important to know when and how the expression of p63 is inhibited and what the effects of p63 inhibition are in terms of the loss of squamous phenotype in the BM.
7.4 Model for BM research
In this thesis, the forced expression of HNF4α in the mouse oesophageal epithelial explants induces VILLIN protein expression. However, HNF4α only induces VILLIN mRNA but not VILLIN protein expression in HET-1A cells. Given that epithelial explants (in low Ca\(^{2+}\) condition) are p63\(^+\)/K14\(^+\) basal cell type, but HET-1A cells lack p63 and K14 expression, this implied that BM cells may be arise from the oesophageal epithelial cells at stem or progenitor lineage. In addition, it has also been observed the oesophageal epithelial cells cultured in 2D or 3D culture system showed a different response to Wnt signalling activation (Kong et al., 2011a). The activation of Wnt pathway only provoked BM gene expression in cells which are cultured under the 3D culture environment. This suggests that the mechanism involved in the cell-cell contact may be important for BM development. Therefore, the chosen cell models and culture conditions are important for BM research.

7.5 Cell origin of BM
LGR5, Musashi-1 and doublecortin-like kinase-1 (DCLK-1) are intestinal stem cell markers and have also been suggested as cancer stem cell markers. Recent studies show that that Musashi-1, LGR5 and DCLK-1 expression were increased in BM and oesophageal adenocarcinoma (Bobryshev et al., 2010; Becker et al., 2010; Todaro et al., 2010; Vega et al., 2012; Wu et al., 2012). These findings may support that BM cells are originated from stem cell population. It is therefore the different research strategy and study models may be required to understand the mechanism of BM development. For example, it would be important to identify the location of Musashi-1, LGR5 or DCLK-1 stem cell population in the oesophagus. Lineage tracing of stem cells in animals and observing the phenotype changes of stem cells following exposure to exogenous factors, such as bile acid, will help to the understanding of the molecular mechanism underlying BM pathogenesis.

7.6 Conclusion
The aims of my PhD project were (i) to understand the role of candidate intestinal transcription factors on BM development and (ii) to investigate whether the exposure of candidate exogenous factors initiate BM. The results from the HET-1A cell model showed that intestinal transcription factors including HNF4α, CDX2 and HNF1α
induced the transcription of intestinal genes including $K20$, $VILLIN$ and $SI$. Additionally, intestinal transcription factor FOXA2 may also play a role in intestinalisation, but further investigation is required. Results from the model of the oesophageal epithelial explants revealed that HNF4α ectopic expression induces squamous-to-columnar cell type conversion and VILLIN protein expression. The work presented in this thesis would suggest that the ectopic expression of intestinal transcription factors including HNF4α, CDX2 and HNF1α and FOXA2 may have a potential link to the development BM.

Results from the treatment of various candidate exogenous factors did not show the effects on intestinal differentiation in the HET-1A cell or HNF4α-expressing HET-1A cells. However, this may due to the experimental design and the cell model used. In the future, longer exposure period and the use of suitable cell models such as primary oesophageal epithelial culture could be tested to understand the role of exogenous factors in BM development.
References


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Appendix

Production of recombinant adenoviral virus by using Ad-Easy™ Adenoviral vector system

Source: Ad-Easy™ Adenoviral vector system instructional manual
Shuttle vectors for production of recombinant adenoviral virus

1. Ad-HNF4α, Ad-HNF1α, Ad-PDX1

2. Ad-CDX2

Source: Ad-Easy™ Adenoviral vector system instructional manual
**Lentiviral vector**

**Lenti-FOXA2**, plasmid/vector pL-S-FOX2A-I-puro  
**Lenti-HNF4α**, plasmid/vector pL-S-HNF4α-I-EGFP  
**Lenti- mutant HNF4α**, plasmid/vector pL-S-HNF4α mutant-I-puro  
**Lenti-HCH**, plasmid/vector pL-S-HCH-I-puro  

(mutant HNF4α: HNF4α 50F-CD2; HCH: HNF4α-2A-CDX2-2A-HNF1α)

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**SIN**: Self-Inactivating 3’ Long Terminal Repeat (LTR)  
**PRE**: post-transcriptional regulatory element  
**IRES**: EMCV internal ribosome entry site  
**SFFV**: strong internal promoter of SFFV (Spleen focus forming virus)  
**PPT**: central polypurine tract  
**RRE**: Rev responsive element  
**dGag**: portion of the HIV-1 gag gene with a closed reading frame  
**PBS**: primer binding site  
**RU5**: 5’-HIV-1 repeat and U5-region of the LTR  
**RSV**: rous sarcoma virus promoter

Source (Iacob et al., 2011)

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216
Sequence: Lenti-HNF4a

**HNF4α**

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Mutant HNF4α (50F-CD2)

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FOXA2

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