The effects of enteropathogenic *Escherichia coli* on the classic genetic pathways of colorectal cancer, using *in vitro* and *ex vivo* human models.

Oliver David Kenneth Maddocks

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Declaration

I declare that this thesis is my own work, and has not been accepted in any previous applications for a degree at this or any other university. I have performed all of the work described herein, except where explicitly stated otherwise, and all sources of information have been acknowledged.

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Abstract

Despite high prevalence and mortality, and an excellent knowledge of the aetiologic genetic changes of sporadic colorectal cancer, the causes of this disease are not well defined. DNA mismatch repair and Wnt signalling (via β-catenin) are classic genetic pathways altered during colorectal carcinogenesis, currently there is little evidence to suggest how gene-environment interactions could influence these pathways. Recent studies have found that adherent *Escherichia coli* are associated with colonic adenocarcinomas, leading to speculation that in similarity to gastric cancer, bacterial infection has a central role in colonic tumourigenesis. The attaching and effacing (AE) bacterium enteropathogenic *E. coli* (EPEC) intimately attaches to the intestinal epithelium and is found in 2.5-10% of healthy children and adults in developed countries. When attaching to host cells, EPEC secretes effector proteins that have wide ranging effects on host molecular biology. The aim of this study was to test the hypothesis that EPEC infection causes molecular changes in host epithelial cells that predispose to neoplastic transformation. Model systems for EPEC infection were successfully established using *in vitro* co-culture with human colorectal cancer cell lines and co-culture with *ex vivo* human colonic mucosa; human adenocarcinomas were also probed for the presence of AE *E. coli.* Immunofluorescence identified mucosa associated AE *E. coli* in 5/20 (25%) adenocarcinomas. When co-cultured with normal human colonic mucosa, EPEC entered 10.6% of crypts, and closely associated with cells in the proliferative progenitor compartment. Mass spectrometry and microarray analysis validated the *in vitro* model and revealed a range of proteomic and transcriptomic effects in EPEC infected cells. Western blots and quantitative immunofluorescence demonstrated that EPEC downregulated the expression of key DNA mismatch repair proteins MSH2 and MLH1 and the Wnt signalling / adhesion protein β-catenin *in vitro*. Disruption of DNA mismatch repair is a causative factor in the development of many hereditary and sporadic colorectal cancers, and disruption of cell-cell adhesion has the potential to subvert normal colonic crypt homeostasis. These novel findings therefore suggest that chronic EPEC infection can predispose to cancer development by increasing the susceptibility of colonic epithelial cells to mutation by dietary or other carcinogens, and by altering expression of cytoskeletal and cell attachment proteins.

Abbreviations

AE = attaching and effacing

Bfp = Bundle forming pili

C. rodentium = *Citrobacter rodentium*

CFU = colony forming unit

D10 = DMEM media with 10% fetal calf serum

DBS100 = wild-type *C. rodentium*

DBS255 = non-intimately attaching mutant *C. rodentium*

DMH = 1,2-dimethylhydrazine

E. coli = *Escherichia coli*

E2348/69 = wild-type EPEC

EAF = *E. coli* attaching and effacing (plasmid)

EHEC = Enterohemorrhagic *Escherichia coli*

EPEC = Enteropathogenic *Escherichia coli*

FAP = familial adenomatous polyposis

Fitc = fluoroescein isothiocyanate

h = hours

H. pylori = *Helicobacter pylori*

HNPCC = hereditary non-polyposis colorectal cancer

LB broth = Luaria Bertani broth

LEE = locus of enterocyte effacement

LOH = loss of heterozygosity

min = minutes

MOI = multiplicity of infection

MMR = mismatch repair

MSI = microsatellite instability

mut = mutant

n = number of experimental replicates

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PhIP = 2-amino-1-methyl-1-6-phenolimidazo[4,5-b]pyridine

PVDF = polyvinylidene difluoride

R5 = RPMI media with 5% fetal calf serum

SAM = significance analysis of microarray

UMD864 = non-intimately attaching mutant EPEC

VSN = variance stabilisation normalisation

wt = wild-type

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Introduction

1.1. Cancer

Cancer is a worldwide disease, estimated to be responsible for over 6.2 million deaths per year (Parkin *et. al.,* 2001). Clinically, cancer is characterised by the growth of tumours – large masses of cells that have no physiological function. Tumours which do not affect surrounding tissues are classed as benign and do not cause an immediate risk to health. Tumours that invade surrounding tissues or spread to secondary sites around the body are classed as malignant. Malignant tumours subvert the normal function of the tissues and organs that they invade, eventually causing organ failure and death. Over the last 60 years, the development of molecular biology has confirmed that genetic abnormalities are responsible for the changes that transform a normal cell into a cell capable of forming a tumour. Studies on genetics and the molecular biology of cancer cells have demonstrated that a wide range of genetic changes (mutations) can contribute to cellular transformation.

The term "oncogene" was introduced in 1969 to describe genes that when expressed, promote the transformation of normal cells into those capable of forming tumours (Huebner & Todaro, 1969). Oncogenes such as *ras* and *c-myc* encode proteins that promote cell cycle progression and therefore cell division. In cancer, mutations that cause over expression of oncogenes such as *H-ras* and *c-myc* lead to over stimulation of cell growth and proliferation, therefore promoting tumourigenesis (Sinn *et. al.,* 1987). Activation of oncogenes therefore provides a simple explanation for the initiation of cancer. However, experimental activation of these genes in normal cells does not lead to proliferation, as mechanisms exist to prevent excessive cell division (Alberts *et. al.,* 2002). Hence the action of no oncogene or combination of oncogenes can cause tumourigenesis unless the pathways that suppress oncogenic activity are also inactivated (Harris, 2005).

Studies in which a tumourigenic and normal cell were fused together revealed that genes expressed in the normal cell had the ability to suppress tumourigenicity (Harris *et. al.,* 1969). Genes with this property are termed "tumour-suppressor" genes, and their continual expression is thought to be required in healthy cells to prevent

uncontrolled growth. The first tumour suppressor gene to be characterised was *RB*, (Friend *et. al.,* 1986), retinoblastoma protein (Rb), the product of *RB* regulates the cell cycle, acting as a brake on proliferation. In retinoblastoma (an inherited form of eye cancer), *RB* mutation results in loss of Rb function and increased cell division (Alberts *et. al.,* 2002). Another well-known tumour suppressor gene is *TP53* (Finlay *et. al.,* 1989), which is mutated in many forms of cancer. The protein product of TP53 (p53) has been extensively studied and is involved in the cell cycle, DNA repair and programmed cell death (apoptosis). Via these pathways, p53 inhibits cell proliferation and promotes cell death in response to stress. In cancer, mutation of *TP53* prevents normal p53 expression and therefore increases cell division, while also decreasing apoptosis, hence facilitating tumour growth (Knudson, 2001).

The identification of tumour suppressor genes and oncogenes and the functional pathways that they influence has furthered the understanding of cancer development. Mutations of genes that control cell cycle and apoptosis leading to uncontrolled cell division are therefore capable of promoting tumour growth. However, the question of whether genetic aberrations in these pathways are either the critical factor in tumour initiation or transition from a benign to malignant state remains unclear. It has been argued that chromosomal abnormalities initiate tumour formation, and that the factor limiting tumour growth is the blood supply a tumour can obtain (by angiogenesis). Recently it has been argued that none of these pathways provides an adequate explanation for the origination of malignant tumours, and that cancer should be seen as a disease of cell differentiation rather than cell division (Harris, 2004). This model for tumourigenesis is explained by the contention that exponential multiplication rather that rest is the normal state for cells, and therefore the key step in determining normal cell behaviour is differentiation. By this argument, the initiating event in the formation of a malignant tumour could be a mutation that inactivates a specific gene involved in the process of normal differentiation (Harris, 2005).

The process by which cancerous gene mutations are acquired and how these mutations affect gene function (i.e. protein expression) are also key features in cancer development. The traditional "two-hit" model proposed by Alfred Knudson

was based on epidemiological studies of retinoblastoma (Knudson *et. al.,* 1971). This study determined that both (maternal and paternal) alleles of the *RB* gene must be mutated in order for cancer to develop. The author noted that the first mutation was often inherited; but that inheritance of a mutated gene did not necessarily mean the carrier would develop cancer, thus confirming the recessive nature of the mutation. Therefore, in order for cancer to develop in an individual with an inherited mutation, another (second) mutation must occur during childhood. Hence, the model dictates that children who develop retinoblastoma in the absence of an inherited a mutation in *RB,* must have sustained two mutations during their lifetime.

This model also illustrates that mutations may be inherited within the genetic information from parents (germline mutations) or generated during the life of the organism (somatic mutations). Somatic mutations may arise spontaneously (e.g. due to errors in DNA replication), or be caused by exogenous environmental factors that damage DNA (e.g. ultraviolet light).

Though it has been suggested that oncogene mutations could be genetically dominant, it is now accepted that they are generally recessive in nature (Cavanee *et. al.,* 1989), supporting the need for multiple mutations. Despite providing a convenient model for retinoblastoma development, the two-hit model cannot account for the development of most cancers. It is now accepted that cancer occurs as a consequence of several somatic mutations (Knudson, 2001). For example, colon cancer development is a multi-stage process, which occurs over a number of years. In this case a genetic "five-hit" hypothesis (including one to two mutations in *APC*, *RAS* and *TP53*) has been proposed, whereby each mutation confers clonal growth advantage (Fearon & Vogelstein, 1990). Mutation of genes other that those classed as oncogenes and tumour suppressor genes can also promote tumourigenesis. Genes that have a function in mechanisms that maintain overall genomic stability (such as DNA repair) are also affected in cancer.

Aside from gene mutations there are a number of other mechanisms that contribute to cancer development at a genetic level. Chromosomal abnormalities (e.g. abnormal

number and structure) are very often found in cancers (Knudson, 2001). Whether aneuploidy is a primary or secondary event in cancer development has received much debate (Harris, 2005). An accepted consequence of chromosomal changes is the loss of genetic material, which is the most frequently observed genetic abnormality in solid tumours (Kufe *et. al.,* 2003). Gene deletion often results in the loss of either the maternal or paternal allele of a gene. This loss of heterozygosity (LOH) occurs frequently, with tumour suppressor genes such as *APC* and *TP53* often affected (Fearon & Vogelstein, 1990). Once LOH has occurred, a single mutation in the remaining allele can lead to loss of function.

Epigenetic phenomena, such as chromatin remodelling do not affect the primary DNA sequence, but can regulate the expression of oncogenes and tumour suppressor genes. Chromatin alterations can be caused by changes in DNA methylation and by altered patterns of histone modifications (reviewed by Baylin & Ohm, 2006). Methylation of CpG islands within gene regulatory regions occurs throughout the genome. Widespread hypomethylation has been observed in colon adenomas and adenocarcinomas, and is thought to cause increased chromosomal instability (Rodriguez *et. al.,* 2006). Hypermethylation of CpG islands reduces the expression of the genes that the island precedes and can result in the silencing of tumour suppressor genes. In colon cancer, hypermethylation of the *MLH1* promoter correlates with a lack of MLH1 expression in sporadic tumours and in repair defective tumour cell lines (Kane *et. al.,* 1997).

In summary, cancer at cellular level is a genetic disease promoted by gene mutations, the key consequences of which are changes in the function or expression of proteins. Mutations may be inherited or somatic, with exogenous factors having the potential to cause somatic mutations. The development of malignancy depends on accumulation of several mutations over time, and is therefore a feature of the influence of environment on the genome.

1.2. Colorectal Cancer

1.2.1. Epidemiology and clinical background

Each year over 940,000 new cases of colorectal cancer are diagnosed worldwide, and colorectal cancer is responsible for nearly half a million deaths. Though relatively rare in developing countries, colorectal cancer is the second most common form of cancer in terms of incidence and mortality in affluent societies (IARC, 2003). Hence it was estimated that in 2006 there were 412,900 cases and 207,400 deaths attributable to colorectal cancer in Europe (Ferlay *et. al.*, 2007). This represents an increase in the number of deaths caused by colorectal cancer of 1.8% since estimates only 2 years earlier. With the increasing age of the European population and an expected reduction in lung cancer cases (due to a reduction in smoking) colorectal cancer may soon become the most prevalent form of cancer in Europe. Furthermore, the link between colorectal cancer and affluence suggests that economic growth in developing countries is likely to increase the rates of colorectal cancer worldwide.

Cases of colorectal cancer can be crudely classified as being either familial or sporadic. Patients with familial disease have inherited germline mutations that predispose to colorectal cancer, in sporadic colorectal cancer genetic mutations are caused by exogenous factors. The genes involved in the overall development of both sporadic and familial colorectal cancer are often the same, thus it may be difficult to assess whether a cancer is purely sporadic or whether inherited genetic factors are involved. A review of genetic predisposition to colorectal cancer suggests that the "textbook" estimate for the proportion of familial disease is 20-25%, however, the author points out that this varies according to the definition of "familial" (de la Chapelle, 2004). The World Health Organisation states that colorectal cancer is inherited in only 5% of cases (IARC, 2003). Whether defined as familial or sporadic, the long-term development of most colorectal cancers is likely to depend on interplay between genetic and environmental factors.

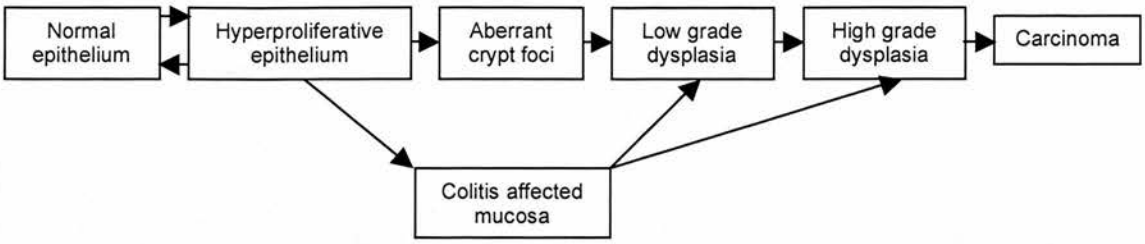


Figure 1.1. The adenoma carcinoma sequence. Flow diagram representing the clinico-pathological features (including ulcerative colitis associated neoplasia) that occur in the adenoma to carcinoma sequence. Adapted from Hardy *et. al.,* 2000, BMJ 321: 886-889.

The clinical development of colorectal cancer has long been known to involve the transformation of normal mucosa into adenomatous polyps, of which one or more eventually progresses to carcinoma (Muto *et. al.,* 1975). This "adenoma to carcinoma" sequence is a multi-stage process that occurs over several years (Figure 1.1). Hence the possibility to apply medical (generally surgical) interventions to slow or halt the progression to carcinoma is good; therefore colorectal cancer is theoretically a preventable disease. Unfortunately, the vast majority of patients with early forms of colorectal cancer are asymptomatic; the eventual presentation of symptoms (such as bleeding) usually indicating advanced disease. This lack of symptoms severely impairs the diagnosis of colorectal cancer when it is at a stage early enough for medical intervention to be efficacious. A potential solution to the problem of diagnosis is screening. However, screening via endoscopy is costly (although it may prove cost effective long term) and often inaccurate (Lieberman *et. al.*, 2001; Winawer *et. al.,* 2000). At present procedural screening for colorectal cancer is only performed on higher risk individuals i.e. those over the age of 50 or younger individuals who have familial predisposition. Development of improved diagnostic techniques using less invasive tests (e.g. biochemical markers) therefore has the potential to greatly enhance the diagnosis and treatment of colorectal cancer.

1.2.2. Genetics

The orderly pathological progression of colorectal cancer has facilitated research into the genetic events underlying colorectal tumourigenesis and the discovery of

mutations in a number of genes (Figure 1.2). The existence of familial colorectal cancer syndromes has also promoted the understanding of cancer genetics. Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) are well-defined inherited syndromes that confer direct genetic predisposition to colorectal cancer. Accounting for approximately 20% of familial colorectal cancer, these syndromes have been extensively studied, providing a vital insight into distinct molecular pathways involved in colorectal cancer.

FAP is dominantly inherited and carries a 100% risk for the development of colorectal cancer if untreated (Fearnhead *et. al.,* 2002). The main clinical feature of FAP is the appearance of hundreds to thousands of adenomatous polyps in the colon and rectum. The key genetic characteristic of FAP is a germline mutation of the adenomatous polyposis coli (*APC*) gene. LOH or somatic mutation of the homologous *APC* allele triggers colorectal cancer development and is followed by activation of oncogenes such as *K-ras* and *c-myc* (Jessup *et. al.,* 1992). This sequence of events creates a state of chromosomal instability, promoting the occurrence of further LOH, particularly in the tumour suppressor gene *p53* and the *deleted in colorectal cancer* (*DCC*) gene (Vogelstein *et. al.,* 1988). This mechanism of colorectal cancer development is classically termed the suppressor or chromosomal instability (CIN) pathway (Lengauer *et. al.,* 1998).

HNPCC is also dominantly inherited, carriers having an 80% lifetime risk of developing colorectal cancer (Lynch & de la Chapelle, 2003). The key genetic changes underlying HNPCC were first identified by Ionov and co-workers (1993). This group reported thousands of somatic mutations in simple repeated sequences in colorectal cancer cells, terming them ubiquitous somatic mutations. These findings supported the earlier hypothesis of Loeb (1991) who postulated that a mutator phenotype (created by genomic instability) was implicated in colorectal tumourigenesis. Thibodeau and colleagues (1993) observed a similar pattern of mutations in colon tumours (noting that LOH was not present) and introduced the term microsatellite instability (MSI) to describe this phenomenon.

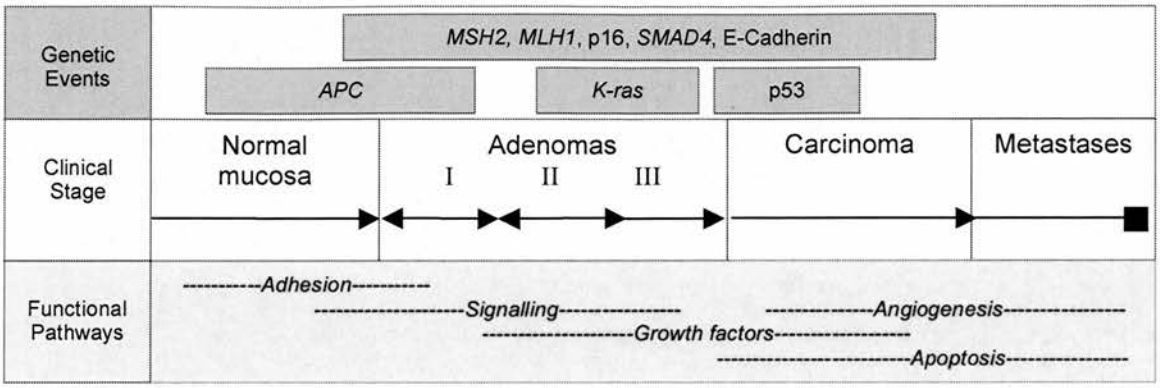


Figure 1.2. Temporal outline of colorectal cancer development and progression. A schematic diagram including common genetic mutations (sporadic / familial), the corresponding functional pathways affected by these mutations and the likely clinical stage. Adapted from Fearnhead *et. al.*, 2002 and Jessup *et. al.*, 2002.

As germline mutations of the *APC* gene are the catalyst for chromosomal instability in FAP, so germline mutations of DNA mismatch repair (MMR) genes are chiefly responsible for the mutator phenotype and hence MSI in HNPCC. The human MMR genes produce two sets of proteins, the MutS (*MSH2, MSH3 and MSH6*) and MutL (*MLH1, PMS1, PMS2* and *MLH3*) homologues (Jiricny, 2006). The MMR genes can be described as indirect tumour suppressor genes. Their role is to correct DNA base pair mismatches acquired from replication, recombination and chemical damage (Figure 1.3). Failure of this DNA repair system confers significant genomic instability, enabling a cell to rapidly accumulate mutations most easily identified as MSI. Progression of HNPCC tumours is thought to involve mutation of *APC* and the tumour-suppressor gene *TGFβRII,* activation of *K-ras* and mutation of *p53* (Jessup *et. al.*, 1992). In addition to its presence in the majority of HNPCC cases, MSI is also found in 12-15% of sporadic cases of colorectal cancer (Liu *et. al.,* 1995).

APC mutations are present in up to 70-75% of *all* colorectal carcinomas (Kinzler & Vogelstein, 1996). Mutation of the *APC* gene is one of the earliest genetic changes observed in colorectal cancer and is potentially the critical step in the initiation of neoplastic transformation in colorectal epithelial cells (Powell *et. al.,* 1992). Wild-type APC protein is a key component of the Wingless/Wnt signal transduction pathway. In the absence of Wnt stimulation, *APC* protein forms a functional complex with axin and glycogen synthase kinase-3β (GSK-3β) that phosphorylates

cytoplasmic β-catenin. Phosphorylation of β-catenin is rapidly followed by ubiquitinylation and proteosomal degradation.

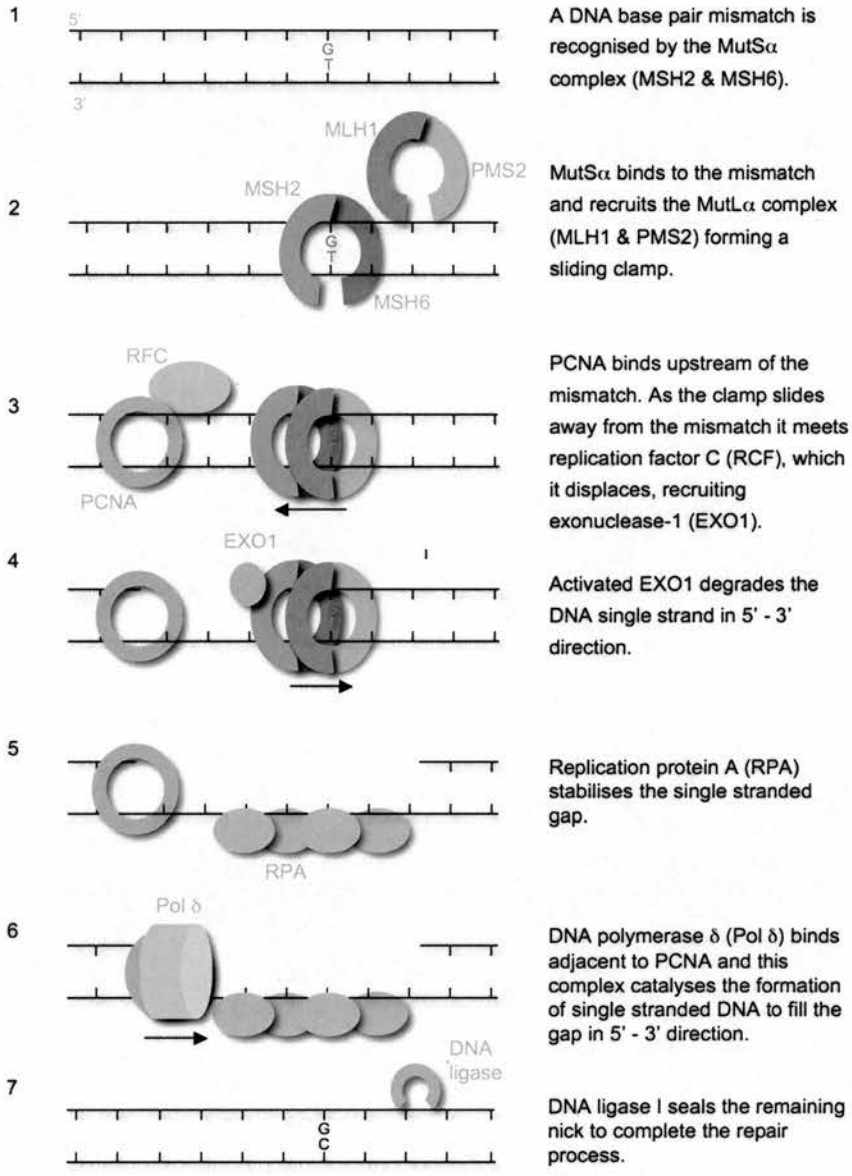


Figure 1.3. Schematic diagram representing human DNA MMR system. The MutS-MutL sliding clamp may slide up or downstream of the base-pair mismatch, here it is shown moving upstream. Adapted from Jiricny, 2006.

However, when Wnt binds to cell surface 'frizzled' receptors, GSK-3β is inactivated and β-catenin escapes phosphorylation and subsequent degradation. When this occurs, β-catenin molecules accumulate within the cytoplasm and translocate to the nucleus where they bind to TCF/LEF (T-cell factor/lymphoid enhancer factor). This

complex then activates the transcription of Wnt target genes including the oncogenes *c-myc* and *cyclin D1*, extracellular proteases such as MMP-7, and nuclear receptor factors such as the peroxisome proliferator-activator receptor δ (PPARδ). The protein products of these genes are thought to promote cell growth, proliferation and/or inhibition of apoptosis. In colorectal cancer, and particularly FAP, mutations of the *APC* gene promote neoplastic transformation by preventing formation of functional *APC* protein-axin-GSK-3β complexes, leading to elevated cytoplasmic β-catenin levels and activation of Wnt target genes.

In addition to its function as a signal transduction protein in the Wnt pathway, β-catenin has an important role in cell-cell adhesion (Figure 1.4). The majority of cellular β-catenin is bound to cadherins (transmembrane adhesion molecules) at adherens junctions. Here, β-catenin helps anchor E-cadherin to the actin cytoskeleton (Alberts *et. al.,* 2002), facilitating strong cell-cell adhesion. Disruption of the catenin-cadherin-cytoskeleton complex is often found in cancer and confers an invasive phenotype (Mareel *et. al.,* 1997).

Epigenetic gene silencing via DNA methylation is an additional mechanism involved in colorectal cancer development (reviewed by Baylin & Ohm, 2006). Hypermethylation of the *MLH1* gene promoter (Herman *et. al.,* 1998; Wheeler *et. al.,* 1999) and of the promoter region of the tumour suppressor gene encoding p16 (Burri *et. al.,* 2001) have been reported in colorectal cancer. Presently, silencing of *MLH1* by hypermethylation is the most prevalent genetic aberration found in cases of sporadic colorectal cancer, and MLH1 silencing is thought to be an early event in carcinogenesis (Giovannucci & Ogino, 2005).

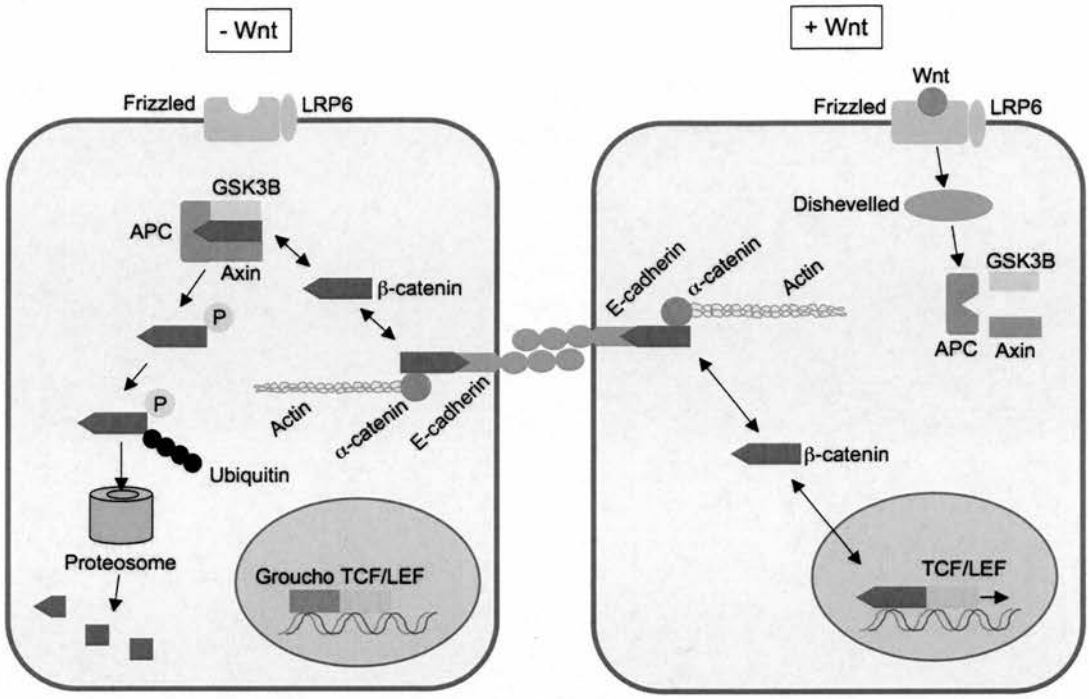


Figure 1.4. Schematic diagram illustrating the role of β-catenin in the Wnt signalling pathway. At the cell periphery, β-catenin helps anchor cell-cell adherens junctions to the actin cytoskeleton by binding to E-cadherin and α-catenin. In the absence of Wnt, unbound cytoplasmic β-catenin is phosphorylated by the APC / GSK-3β / Axin complex, leading to ubiquitination and proteosomal degradation. Binding of Wnt to cell surface Frizzled receptors leads to inhibition of the APC / GSK-3β / Axin complex via Dishevelled. Subsequently, cytoplasmic β-catenin is not degraded and translocates to the nucleus, here β-catenin interacts with TCF/LEF (T-cell factor/lymphoid enhancer factor), promoting the transcription of Wnt target genes. Adapted from Nathke, 2006.

1.2.3. Aetiology and risk factors

Currently, the main exogenous factors thought to be responsible for causing sporadic colorectal cancer are so called "lifestyle factors" such as diet, obesity, exercise, smoking and alcohol intake. In an ongoing prospective study of 478,040 men and women across 10 European countries, it was found that colorectal cancer risk was positively associated with high consumption of red and processed meat, and inversely associated with fish intake (Norat *et. al.,* 2005). Within this trial (the European Prospective Investigation into Cancer and Nutrition) body size was also studied as a risk factor. Indicators of abdominal obesity (such as waist-height-ratio and waist circumference) were strongly associated with colorectal cancer risk in men and women (Pischon *et. al.,* 2006). Somewhat inevitably, a diet rich in calories and

animal fat, but low in vegetables and fibre is associated with increased colorectal cancer risk (IARC, 2003).

There is evidence to support the role of alcohol as a causative factor in number of forms of cancer, with alcohol metabolites such as acetaldehyde thought to be the cancer causing agents (reviewed by Seitz & Stickel, 2007). A recent study (using pooled data equating to over 480,000 participants) on the link between alcohol consumption and colorectal cancer found that high levels of alcohol intake correlated with a modest relative elevation in cancer rate (Cho *et. al.,* 2004). Smoking tobacco, the main risk factor for lung cancer, has also been strongly linked to colorectal cancer development. It has been reported that smoking over a pack of cigarettes per day can cause an increase in colon cancer risk of up to 50% (Slattery *et. al.,* 1997).

Factors thought to reduce colorectal cancer risk include physical exercise and increased vitamin D and calcium intake (IARC, 2003). Folic acid, originally thought to reduce colorectal cancer risk (Song *et. al.,* 2000), may in fact increase risk (Cole *et. al.,* 2007). Chronic use of non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin was first identified as a protective factor for colorectal cancer development nearly 20 years ago (Kune *et. al.,* 1988). Currently, NSAIDS are accepted as effective chemopreventive agents against colorectal cancer. However, this effect seems only to occur in a genetically defined subset of the population, suggesting it is a pharmacogenetic effect (reviewed by Ulrich *et. al.,* 2006). Another pharmacological intervention that may reduce colorectal cancer risk (in women) is hormone replacement therapy (HRT) (Pischon *et. al.,* 2006).

Ulcerative colitis, an inflammatory disease of the bowel is a strong pre-disposing factor to colorectal cancer development. Colitis associated cancers are clinically and genetically distinct from other forms of colorectal cancer (Figure 1.1), evolving from microscopic dysplasia rather than from adenomas (Hardy *et. al.,* 2000). Bacteria have a major influence on the environment of the colon and potentially on colonic diseases such as ulcerative colitis; their role in colorectal cancer development is discussed below.

Overall there is a lack of convincing evidence as to how these risk factors can promote tumourigenesis at a molecular level. It is highly likely that carcinogens are contained within the diet (especially in developed countries) and it is probable that risk factors work in combination to increase susceptibility to mutation. However, cell turnover in the colonic epithelium is high and cells that acquire mutations should, in theory be shed before they have the chance to cause cancer. The following statement succinctly summarises current understanding of the causes of colorectal cancer:

"Gene-gene and gene-environment interactions have a significant influence on the susceptibility to colorectal cancer. Our current understanding of these interactions is limited, and concerted research efforts in this area will be important for a full understanding of predisposition to this cancer."

Albert de la Chapelle, Nature Reviews Cancer: Vol 4; 770. 2004.

1.2.4. Bacteria as a risk factor for cancer and colorectal cancer

It has been estimated that in 1990 15.6% of all cancers worldwide were attributable to some form of infection, including viruses, bacteria, schistosomes and liver flukes (Pisani *et. al.,* 1997). The bacteria *Salmonella typhi* is associated with gallbladder cancer and *Chlamydia pneumoniae* with lung cancer (reviewed by Mager, 2006). A precedent for tumourigenesis in the gastro-intestinal tract due to bacterial infection is provided by *Helicobacter pylori* (*H. pylori*) within the stomach. It was concluded by The International Agency for Research on Cancer in 1994 that there is a causal relationship between *H. pylori* infection and gastric cancer (IARC, 1994). Subsequent analysis of data from epidemiological studies supports this conclusion (Huang *et. al.,* 1998), and Graham (2000) claims that *H. pylori* infection is the primary cause of gastric cancer. Despite a wealth of research into the interaction between *H. pylori* and the gastric mucosa, the underlying mechanisms responsible for cancer development remain unclear. Oxidative DNA damage-induced gastric inflammation has been identified as a potential contributing factor (Hahm *et. al.,* 1997; Farinati *et. al.,* 1998). *H. pylori* infection has also been shown to impair DNA mismatch repair via reduction in MSH2 and MLH1 protein levels in gastric epithelial

cells (Kim *et. al.,* 2002; Park *et. al.,* 2005). It has also been found that patients with MSI-positive tumours were more likely to have active *H. pylori* infection than patients with MSI-negative tumours (Leung *et. al.,* 2000).

Within the colon, a number of bacteria have been linked to increased susceptibility to cancer, including *streptococcus sanguis* (Siegert & Overbosch, 1995), group G streptococcus (Kim *et. al.,* 2002), and particularly *Streptococcus bovis* (Gold *et. al.,* 2004). The presentation of frequent colon neoplasia in patients with *Streptococcus bovis* associated endocarditis has lead to further investigation of this bacterium. Potential mechanisms of *S. bovis* carcinogenesis include chemokine and prostaglandin release corresponding to cyclooxygenase-2 (COX-2) over-expression, activation of mitogen activated protein kinases, increased inflammation, reduced apoptosis and increased proliferation (reviewed by Mager, 2006).

In light of multiple studies focussed on *H. pylori* infection (relating to gastric cancer), Swidsinski *et. al.* (1998) set out to study mucosa-associated bacteria in colorectal cancer patients. Of control individuals (without adenomas or carcinomas) bacteria were identified in biopsies from 1/31 (3%) asymptomatic patients and 10/34 (31%) patients with GI symptoms. However, high levels of bacteria (1,000-10,000 CFU/μl) were identified in 27/29 (93%) adenoma patients, and 28/31 (90%) carcinoma patients. *E. coli* were the predominant bacteria species identified in 62% and 77% of adenoma and carcinoma patients respectively compared to 3% and 12% of asymptomatic and symptomatic controls respectively (Swidsinski *et. al.,* 1998).

A recent study analysing the presence of bacteria in tissue removed from colon cancer patients revealed that over 70% of patients had mucosa-associated bacteria. A significant proportion of the bacterial isolates were *Escherichia coli* strains that demonstrated *in vitro* adhesion to embryonic intestinal (I407) and colon adenocarcinoma (HT29) cells (Martin *et. al.,* 2004). Isolated *E. coli* also caused release of interleukin-8 (IL-8) from I407 cells. The authors suggest that mucosa-associated bacteria might express adhesins with binding specificity for the "oncofetal" carbohydrate antigens that are over-expressed by mucosal

glycoconjugates on adenocarcinoma cells. Furthermore, the authors contend that colon cancer might be primarily a bacterial disease, proposing a central role for mucosally adherent bacteria in the pathogenesis of colon cancer. This thesis will further explore the role of mucosally adherent *E. coli* in colon cancer development.

1.3. Enteropathogenic *Escherichia coli* (EPEC)

1.3.1. Diarrheagenic *E. coli*; characterisation of EPEC

Escherichia coli are gram negative, anaerobic bacteria found in abundance within the human intestine. Most *E. coli* strains are non-pathogenic, only causing illness in susceptible individuals or when infection escapes from the intestine. However, several highly adapted pathogenic *E. coli* strains are able to cause disease in healthy individuals, included in this category are the diarrheagenic *E. coli* strains. These strains share an enhanced ability to adhere to and colonise the intestinal epithelium, but derive pathogenicity by specific mechanisms that differ between pathotypes. The virulence factors that confer these adaptations (e.g. toxin secretion, intimate adherence, aggregation, invasion) are encoded on virulence related plasmids and in chromosomal pathogenicity islands that are absent in non-pathogenic *E. coli* (Nataro & Kaper, 1998). Hence each pathotype has distinct pathogenic mechanisms, resulting in varying clinical symptoms (Table 1.1).

The term 'enteropathogenic *E. coli*' was initially used to describe all *E. coli* causing pathological intestinal infection (Neter, 1965). Subsequently, the strains releasing toxins (ETEC, EHEC) or with invasive ability (EIEC) were characterised and separately categorised. At this point the pathogenicity of bacteria remaining within the EPEC group was poorly understood. The ability of EPEC strains to intimately attach to epithelial cells (in the ileum and colon of pigs and rabbits), causing effacement of microvilli, led to the characterisation of EPEC as "attaching and effacing" (AE) *E. coli* (Moon *et. al.,* 1983). The authors also noted "considerable animal-animal variation in response to the same strain of EPEC", and drew attention to the ability of other bacteria to cause AE lesions i.e. *Citrobacter freundii* in mice.

Currently, the only bacteria known to induce AE lesions in man are EPEC, EHEC, and some *Hafnia alvei* strains (Nataro & Kaper, 1998).

Pathotype	Pathogenesis	Virulence determinants	Symptoms
Enteropathogenic (EPEC)	Intimate attachment with AE lesions on epithelial cells	LEE pathogenicity island, encoding intimin (*eae*) and EPEC secreted proteins (*espA,D,B*); ~60-MDa EAF plasmids, encoding bundle forming pili (*bfp*)	Profuse watery diarrhoea
Enterotoxigenic (ETEC)	Epithelial cell surface adhesion & toxin delivery	Heat-stable toxins (*STa, STb*), Heat-labile toxins (*LT-I, LT-II*)	Watery diarrhoea
Enteroinvasive (EIEC)	Epithelial cell invasion & intracellular multiplication	Chromosomal pathogenicity island (*virR, kcpA*), 140-MDa plasmid (*virB,G,F,K*)	Watery diarrhoea, dysentery syndrome
Enterohemorrhagic (EHEC)	Intimate attachment with AE lesions on epithelial cells & toxin delivery	LEE pathogenicity island encoding intimin (*eae*) and secreted proteins (*espA,D,B*); chromosomal shiga toxins (*stxA,B*); 60-MDa plasmid encoding enterohemolysin (*ehx*)	Watery & bloody diarrhoea, hemorrhagic colitis, haemolytic uremic syndrome
Enteroaggregative (EAEC)	Form mucus biofilm with aggregative adherence, deliver cytotoxin	60-MDa plasmid encoding aggregative adherence fimbriae (AAF/I,II); 108-kDa cytotoxin	Persistent watery, mucoid diarrhoea, often bloody
Diffusely adherent (DAEC)	Adhere diffusely to epithelial cells, elongate microvilli	Chromosome and plasmid encoded F1845 fimbriae	Watery diarrhoea

Table. 1.1. Summary of diarrheagenic *E. coli* pathotypes. Other symptoms associated with diarrheagenic *E. coli* infection include fever and vomiting. Compiled from Nataro & Kaper, 1998.

Since the initial characterisation of the AE phenotype, considerable research has been undertaken into how intimate attachment is achieved and how this process relates to EPEC's pathogenicity. The ability to identify EPEC based on pathogenic characteristics has also facilitated the identification of EPEC as an important category of diarrheagenic *E. coli*, responsible for human diarrhoeal infections worldwide.

1.3.2. EPEC Clinical background and epidemiology

Transmission of EPEC is generally faecal-oral, via contamination of hands and food. Acute EPEC infection is characterised by severe, profuse watery diarrhoea, which may be accompanied by vomiting and fever, symptoms are usually acute, but may last for weeks. Infants are far more susceptible to EPEC infection than adults. The major danger to the health of a patient is dehydration, which is compounded by malabsorption of nutrients due to effacement of absorptive enterocytes. Parenteral rehydration and nutrition may therefore be required (especially in infants), without which death often results (Nataro & Kaper, 1998). In developing countries the lack of rapid, appropriate medical care for EPEC induced diarrhoea often results in death. For example, in a recent study of children under 5 years old admitted to hospital with acute diarrhoea in Brazil, over 30% of patients had EPEC, and of the 17 patients who died, over half were infected with EPEC (Fagundes-Neto & Andrade, 1999).

With improved treatment of acute diarrhoea and hospital hygiene, the incidence of EPEC induced diarrhoeal infections in developed countries has considerably decreased since the mid 1900's. Consequently, EPEC is not viewed as a major pathogen in the developed world, and routine screening is no longer performed in hospitals (Nataro & Kaper, 1998). However, outbreaks of EPEC induced diarrhoea (affecting children and adults) still occur in developed countries (Wight *et. al.,* 1997; Viljanen *et. al.,* 1990). Furthermore, epidemiological studies continue to reveal the presence of EPEC in symptomatic and asymptomatic children in Europe, the United States and Australia (Table 1.2).

The reservoir of EPEC infection is thought to be asymptomatic and symptomatic children, and asymptomatic adult carriers; the inability to identify certain strains of EPEC in animals supports the conclusion that man is a natural reservoir for EPEC (Levine & Edelman, 1984). It is difficult to estimate the carriage of EPEC amongst healthy adults in the developed world; this is due to the lack of routine screening and the fact that epidemiological studies focus on children. It is certainly possible for healthy adults (and children) to carry EPEC without displaying any symptoms.

Overall there is strong evidence that a significant proportion of the healthy population in developed countries harbour EPEC as part of their normal gastro-intestinal micro-flora.

Location	Subjects (age)	Healthy controls	Symptomatic patients	Reference
Norway	Children < 5 years	21/210 (10.0%)	37/251 (15.1%)	Afset *et. al.*, 2004
Australia & Germany	Children < 2 years	26/389 (6.7%)	-	Beutin *et. al.*, 2003
Australia & Germany	Children < 1 year	12/450* (2.7%)	-	Bettelheim *et. al.*, 2003
Australia	Adults and children	12/489 (2.5%)	91/696 (13.1%)	Robins-Browne *et. al.*, 2004
America	Children < 11 years	-	20/455 (4.4%)	Bokete *et. al.*, 2003
Finland	1 month – 98 years	0/92 (0%)	19/603 (3.2%)	Keskimaki *et. al.*, 2001
Switzerland	Children < 16 years	15/137 (10.9%)	30/187 (16.0%)	Pabst *et. al.*, 2003
Denmark	Children < 5 years	6/866 (0.7%)	10/424 (2.4%)	Jensen *et. al.*, 2007

Table 1.2. Summary of recent EPEC studies in developed countries. *In this study the number of individuals from which bacterial strains were obtained is not stated, only the total number of strains that were analysed.

1.3.3. Genetic basis of the EPEC AE phenotype

The defining characteristic of EPEC is its ability to form AE lesions on host intestinal epithelial cells. AE lesion formation is characterised by the effacement of microvilli and formation of cup-like pedestal structures on which EPEC sits in intimate contact with the apical membrane of the host cell (Moon *et. al.*, 1983). The virulence factors on which this process depends are encoded by the EPEC Attaching and Effacing (EAF) plasmid, and the 35.6b kb chromosomal pathogenicity island entitled the Locus of Enterocyte Effacement (LEE). The genes encoded by LEE and the EAF have specific roles and work in combination to achieve AE lesion formation and intimate attachment.

1.3.3.1. Locus of enterocyte effacement (LEE)

The first chromosomal gene necessary for AE lesion formation to be identified was *eae* (*E. coli* attaching and effacing) in 1990 (Jerse *et. al.,* 1990). The LEE pathogenicity island (containing *eae*) was identified five years later (McDaniel *et. al.,* 1995) and eventually cloned (McDaniel & Kaper, 1997). The genes encoded by LEE were initially separated into three groups; the first containing *esc* genes, encoding structural proteins that form the type III secretion system (TTSS); the second group containing *eae* and *tir*, encoding intimin and the translocated intimin receptor respectively; and the third group including the *esp* genes, which encode the EPEC secreted proteins (EspA, B, D, F) (Elliot *et. al.,* 1998; reviewed by Frankel *et. al.,* 1998). Further investigation has revealed the function of other LEE encoded genes, summarised in Table 1.3.

Protein (s)	Gene (s)	Function
EPEC secreted proteins (Esp's)	*espA, B, D*	Secreted translocator proteins – extracellular components of TTSS
	espF, G, H	Secreted effector proteins
Secretin proteins (Esc's)	*escC, D, F, J, N, R, S, T, U, V*	Intracellular TTSS structure & machinery
Intimin (Int280)	*eae*	Bacterial outer membrane adhesin
Translocated intimin receptor (Tir)	*tir*	Translocates to host membrane, binds intimin
Secreted effector proteins (Sep's)	*sepD, Q, L, Z*	Secreted effector proteins
Chaperone proteins (Ces's)	*cesAB, D, D2, F, T*	Aid secretion and translocation of secreted proteins
Mitochondrion associated protein (Map)	*map*	Effector protein acting on mitochondria and tight junctions
-	*ler, grlA, R*	Regulate LEE gene expression

Table 1.3. Genes encoded by the EPEC (E2348/69) Locus of Enterocyte Effacement (LEE) and protein functions. Compiled from Garmendia *et. al.,* 2005.

The EPEC TTSS shares homology with other bacteria, including *Yersinia*, *Shigella* and *Salmonella.* Assembly of this multi-component organelle opens a physical channel between bacteria and host cell allowing rapid, one-step translocation of bacterial proteins (Garmendia *et. al.,* 2005). The Esc proteins form the basal body of

the TTSS, penetrating the inner and outer bacterial membranes; at the bacterial outer membrane polymerised EspA proteins form a hollow filament, at the end of which are EspD/B proteins, which form a pore in the host plasma membrane (Garmendia *et. al.*, 2005) Figure 1.5.

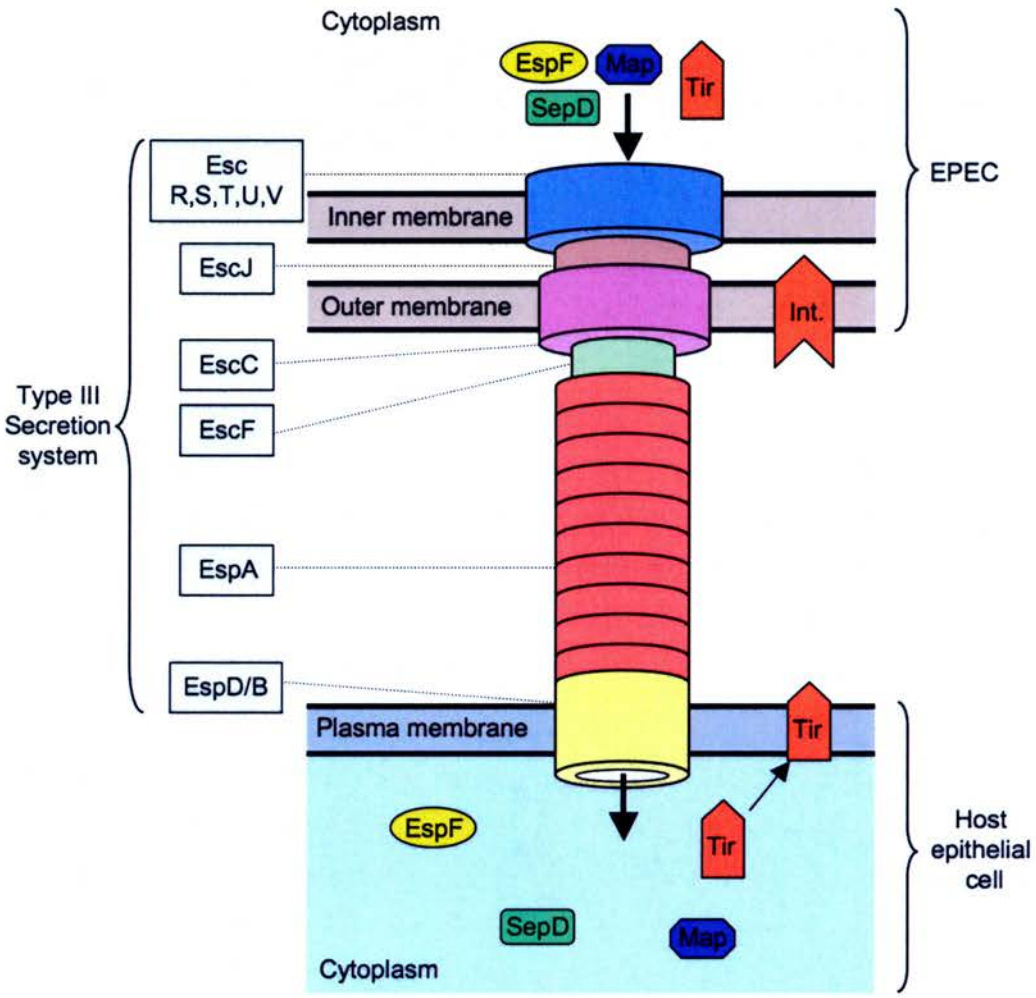


Figure 1.5. Schematic representation of EPEC type III secretion system (TTSS). Diagram shows structural assembly of Esc & Esp proteins required for a functional TTSS and examples of secreted effector proteins (EspF, Map, SepD) including Tir, which localises to the host apical plasma membrane in readiness to bind the bacterial adhesin intimin (Int.). Adapted from Garmedia *et. al.*, 2005.

1.3.3.2. EPEC adherence factor plasmid (EAF/pMAR2)

Prior to identification of the LEE chromosomal pathogenicity island, it had been deduced that EPEC adhesion and pathogenicity could be mediated by plasmid

encoded virulence factors (Baldini *et. al.,* 1983; Nataro *et. al.,* 1985). EPEC adherence factor (EAF) was suggested as a name for the putative plasmid encoded factor necessary for adhesion and virulence in humans (Levine *et. al.,* 1985). A subsequent study revealed that EPEC strains cured of the EAF plasmid were unable to grow as adherent colonies due to the absence of surface filaments, which the authors termed bundle forming pili (Bfp) (Giron *et. al.,* 1991). Bfp provide a means for bacteria-bacteria adherence by forming an intertwined mesh in which bacteria become embedded. This mechanism is responsible for the "localised adherence" pattern of EPEC on epithelial cells whereby bacteria adhere in clusters (microcolonies). The *bfp* gene cluster contains 14 genes required for bfp biogenesis (Stone *et. al.,* 1996).

The role of bfp in bacteria-cell interaction is contentious, particularly whether bfp mediate the initial attachement of bacteria to host cells. The use of EPEC strains with mutations in bfp genes has revealed that that loss of *bfpA* drastically reduces EPEC adhesion to cultured epithelial cells and prevents LA, but does not preclude adhesion, or prevent AE formation if intimin is expressed (Zhang & Donnenberg, 1996). Loss of intimin however, does prevent AE lesion formation but does not prevent localised adhesion to cultured cells (Donnenberg & Kaper 1991). Experiments co-culturing EPEC mutants with isolated human tissue reveal that loss of bfp does not prevent adherence, but that loss of intimin does, suggesting that bfp are not involved in initial adherence *in vivo* (Hicks *et. al.,* 1998). However, co-culturing *bfp* mutant EPEC and *C. rodentium* with mouse and human intestinal epithelial cells support a role for bfp in the initial adherence of AE bacteria and their species specificity (Tobe & Sasakawa, 2001 & 2002).

Though the EAF plasmid is not essential for AE lesion formation, its presence does enhance the efficiency of the AE process, possibly by increased expression of LEE genes due to regulatory (*per*) genes in the EAF plasmid (Nataro & Kaper, 1998). Alternative mediators of initial bacterial adherence have been suggested and include rod-like fimbriae and fimbrillae, a second adhesin molecule (in addition to intimin) and flagella (reviewed by Clarke *et. al.,* 2003).

The matter of bfp mediated adherence is particularly relevant to naturally occurring EPEC strains which do not contain the EAF plasmid. These so called "atypical EPEC" are often found in developed countries (Beutin *et. al.,* 2003; Robins-Browne *et. al.,* 2004). The pathogenicity of atypical EPEC has not been fully established and their clinical significance remains unresolved. In a recent study, atypical EPEC were found to be slightly more prevalent in patients with diarrhoea than controls (not statistically significant), however, a significant association was observed with diarrhoea lasting for 14 days or more, a finding that may indicate a role of atypical EPEC in prolonged disease (Afset *et. al.,* 2004).

1.3.4. The process of EPEC pathogenesis

The sequence of events by which EPEC induces the formation of AE lesions on host cell is complex, relying on initial attachment, the assembly of various multi-molecule complexes, secretion of effector proteins and significant changes within the host cell. Presently a four-stage model is proposed to describe this process and is outlined in figure 1.6 (Clarke *et. al.,* 2003).

1.3.5. Consequences of EPEC attachment

EPEC induced pedestals are composed of: Filamentous actin (F-actin), actin regulatory proteins [such as Arp2/3, neuronal Wiskott-Aldrich syndrome protein (N-WASP), calpactin, cofilin, cortactin, ezrin, gelsolin, lipoma preferred partner (LLP), p130cas, tropomyosin, vasodilator-stimulated phosphoprotein (VASP), and zyxin], adaptor proteins [including Nck, CrkII, Grb2 and Shc], focal adhesion associated proteins [e.g. α-actinin, talin and vinculin], lipid rafts [e.g. annexin 2 and CD44] and intermediate filaments (reviewed by Caron *et. al.,* 2006).

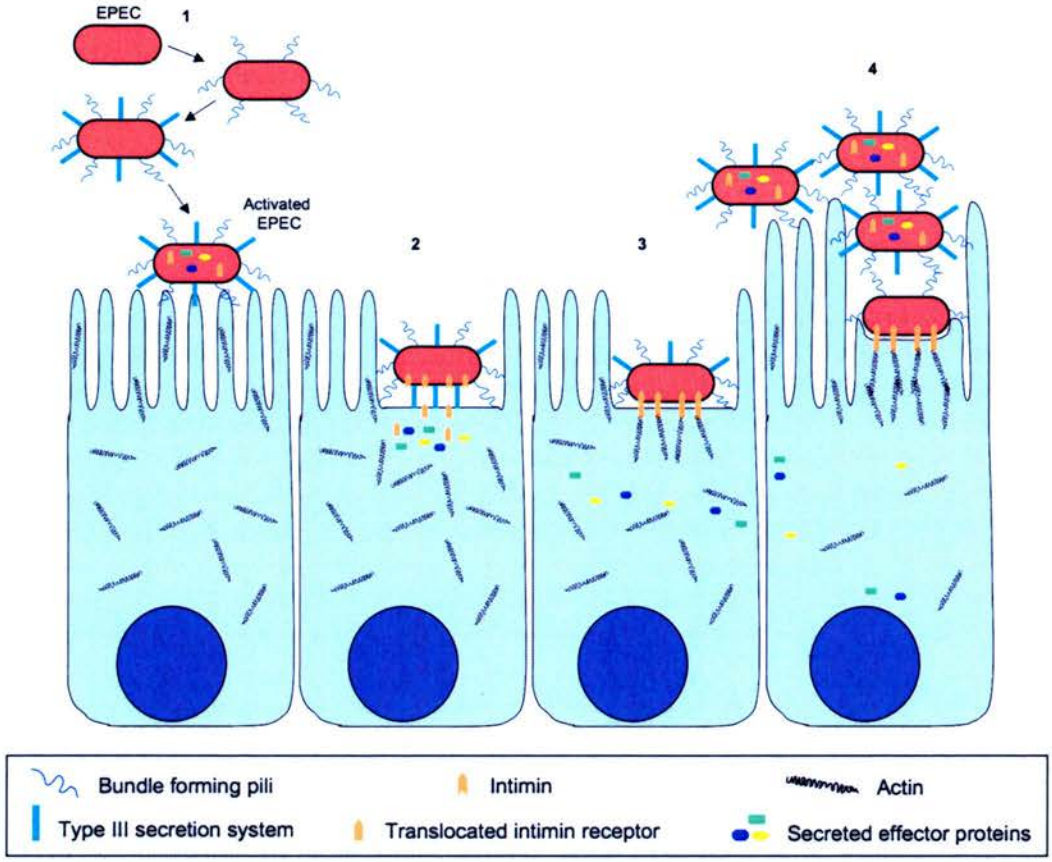


Figure 1.6. Schematic representation of four stage EPEC pathogenesis.

1. Bacterial expression of Bfp, intimin and EspA filaments (environment dependent)
2. Initial adherence via Bfp and EspA filaments, injection of Tir and effector molecules via TTSS, resulting in actin de-polymerisation and loss of microvilli, phosphorylation and insertion of Tir into apical membrane
3. Loss of EspA filaments, intimin binds to Tir, intimate attachment, accumulation of actin and other cytoskeletal proteins at site of adhesion
4. Massive accumulation of cytoskeletal elements, formation of pedestal structure, microvilli elongation, adhesion of other EPEC via Bfp to initiate microcolonies (localised adherence), translocated effector molecules precipitate a wide range of cellular events

Adapted from Clarke *et. al.*, 2003.

The process of intimate attachment, AE lesion formation and insertion of bacterial effector proteins has significant and wide-ranging effects on the host cell and surrounding tissue. The exact changes that occur, and the mechanisms that cause them are not fully understood, but they are interlinked and undoubtedly complex. EPEC induced changes will be crudely divided and discussed as follows:

(i) Cytoskeletal remodelling

(ii) Disruption of epithelial barrier

(iii) Mitochondrial damage

(iv) Cell cycle disruption

(v) Pro-apoptotic and anti-apoptotic responses

(vi) Inflammatory cell transmigration and activation

(vii) Diarrhoea

(viii) Signal transduction

(i) Cytoskeletal remodelling. EPEC attachment modulates all three host cell cytoskeletal networks – actin microfilaments, intermediate filaments and cytoskeletal microtubules (Caron *et. al.,* 2006). Clustering of Tir molecules within the host apical membrane initiates the downstream signalling events that lead to actin polymerisation and pedestal formation. After Tir is phosphorylated by host tyrosine kinases, it recruits the host adaptor protein Nck to initiate N-WASP-Arp2/3-mediated actin polymerisation (Schuller *et. al.,* 2007). In order for progression from this stage to full intimate attachment and pedestal formation, Tir must bind to intimin in the bacterial outer membrane, failure to do so results in the formation of immature AE lesions (Taylor *et. al.,* 1999). This is likely to be achieved by intimin dependent tyrosine kinase activation, as loss of intimin or addition of tyrosine kinase inhibitors prevents EPEC induced pedestal formation (Kenny & Finlay, 1997). Pedestal formation is a highly specialised strategy, which combined with microvilli elongation is likely to aid in the avoidance of immune cells by partially 'hiding' the adherent bacteria. Furthermore, the ability of EPEC to induce TTSS dependent cytoskeletal rearrangements protects it from phagocytosis by macrophages (Goosney *et. al.,* 1999).

(ii) Disruption of epithelial barrier. A result of EPEC attachment is a reduction in transepithelial electrical resistance (TER), indicating compromised epithelial barrier function (Simonovic *et. al.,* 2001). Epithelial membrane integrity depends on cell-cell adhesion junctions (e.g. adherens junctions and tight junctions) and cell-matrix adhesion junctions (e.g. focal adhesions and hemidesmosomes). These structures also help maintain cell polarity, by anchoring the base of the cell to the basal lamina membrane (Alberts *et. al.,* 2002). The benefit to EPEC of reduced barrier function

could be access to nutrients within the lamina propria (Shifrin *et. al.,* 2002), and also access to adhesion molecules that form the junctions (Muza-Moons *et. al.,* 2003).

Intercellular tight junctions provide a dynamic, regulated barrier to the movement of water, ions and immune cells across the intestinal epithelium, and control cell polarity by limiting diffusion of proteins between the basolateral and apical membranes (Shen & Turner, 2006). EPEC infection has been shown to disrupt tight junctions, causing redistribution of occludin (McNamara *et. al.,* 2001). EPEC induced phosphorylation of ezrin (a protein that links the plasma membrane to the cytoskeleton) causes this protein to become associated with the cytoskeleton, resulting in disruption of tight junction function (Simonovic *et. al.,* 2001). It has been suggested that Rho kinase may regulate phosphorylation of ezrin and mediate other EPEC induced protein changes that effect epithelial barrier function (Simonovic *et. al.,* 2001). EPEC induced tight junction disruption has also been related to phosphorylation of myosin light chain protein (Yuhan *et. al.,* 1997). EPEC infection also causes disruption of adherens junctions via PKC mediated effects on catenin redistribution (Malladi *et. al.* 2004).

In vitro experiments demonstrate that EPEC infection causes epithelial cells to detach from the substratum. This effect has been attributed to modification of focal adhesions via EPEC induced dephosphorylation of focal adhesion kinase (FAK) (Shifrin *et. al.,* 2002). EPEC infection has also been shown to disrupt epithelial cell polarity, enabling basolateral proteins (such as β_1–integrin), to migrate to the host apical membrane, potentially providing additional biding sites for intimin (Muza-Moons *et. al.,* 2003). EPEC induced migration of basolateral adhesion proteins, such as integrins from focal adhesions or hemidesmosomes would also reduce host cell adhesion to the basal lamina.

EPEC induced changes in both tight junctions and focal adhesions were dependent on a fully functioning TTSS system (Shifrin *et. al.,* 2002; Muza-Moons *et. al.,* 2003). This suggests that secreted effector molecules mediate these processes and translocated EspF has indeed been found to fulfil this function (McNamara *et. al.,*

2001). The secreted protein Map is also important in tight barrier disruption (Garmendia *et. al.,* 2005). A recent study has found that EspG dependent RhoA activation is related to increased paracellular permeability of epithelial cells consequent to EPEC infection (Matsuzawa *et. al.,* 2005).

(iii) Mitochondrial damage. In addition to functions in epithelial barrier disruption, Map and EspF are both targeted to host cell mitochondria via N-terminal targeting sequences (reviewed by Garmendia *et. al.,* 2005). Map interferes with mitochondrial membrane potential, leading to mitochondrial swelling and damage (Kenny & Jepson, 2000). EspF induces mitochondrial membrane permeabilisation, the release of the toxic protein cytochrome *c* from mitochondria and with caspase-3 cleavage, indicating a role in initiation of the mitochondrial death pathway (Nougayrede & Donnenberg, 2004) this will be discussed further under pro- and anti-apoptotic responses (**v**).

(iv) Cell cycle. Bacterial effectors that modulate the eukaryotic cell cycle are known as cyclomodulins. A recently discovered EPEC secreted effector known as Cif (cycle inhibiting factor) has been shown to block the cell cycle at G_2/M transition, causing cell cycle arrest, characterised by inactive phosphorylated Cdk1 (Marches *et. al.,* 2003). Cif also has a role in promotion of actin cytoskeleton rearrangement, and is the first EPEC effector molecule to be encoded by a pro-phage rather than in the LEE (Marches *et. al.,* 2003). The pro-phage is only found in some EPEC strains and is not present in the classic wild-type strain (E2348/69) or in *C. rodentium* (Garmendia *et. al.,* 2005).

(v) Pro-apoptotic and anti-apoptotic effects. The literature surrounding the effects of EPEC on cell death, and in particular apoptosis is somewhat tentative. For bacterial pathogens such as *Salmonella*, *Yersinia* and *Shigella,* apoptosis is a favourable mechanism of pathogenesis, advantageously stimulated by cytotoxic bacterial effector molecules (reviewed by Melo *et. al.,* 2005). Unlike these bacteria, EPEC does not invade cells; hence it depends on the host cell to provide a specialised binding site and commits considerable resources into influencing host

cell biology. Therefore it seems unlikely (certainly in the short term) that induction of cell death would profit an AE pathogen such as EPEC. Despite this logic it seems that most studies seek to study the induction of pro- rather than anti-apoptotic pathways in response to EPEC.

In a study on EPEC induced cell death it was found that EPEC induced comparably less cell death that *Salmonella*, *Yersinia* and *Shigella* (Crane *et. al.*, 1999). The authors also found that addition of a tyrosine kinase inhibitor to cells co-cultured with EPEC greatly increased apoptosis (suggesting that EPEC signalling suppresses apoptosis). Furthermore, this study potentially overestimated apoptosis by using dye permeability and lactate dehydrogenase (LDH) release assays. These are inappropriate choices considering EPEC carry a TTSS capable of creating pores (large enough for dye uptake or LDH release) in the host plasma membrane. The authors note that EPEC adherence has been shown to strongly activate anti-apoptotic pathways, such as (intimin dependent) protein kinase C activation (Crane & OH, 1997). Their own results support tyrosine kinase activation as one such pathway. Despite interesting speculation by the authors that EPEC has developed strategies to slow rather than stimulate apoptosis, the title of the paper only draws attention to the fact that 'Host cell death due to enteropathogenic *E. coli* infection has features of apoptosis.'

Another study intent on investigating 'induction of epithelial cell death including apoptosis by Enteropathogenic *E. coli*' establishes that Bfp expression is a key mediator of host cell apoptosis and necrosis (Abul-Milh *et. al.*, 2001). The link between Bfp and cell death is undoubtedly a valid one, however, careful analysis of the data, combined with consideration of the more recently described affects of EPEC reveals a potentially more interesting result. Using microscopic analysis of host cell nuclei as a measure of apoptosis and necrosis, this study finds that loss of Bfp expression attenuates EPEC induced cell death to non-pathogenic *E. coli* levels. Also, introduction of Bfp (but not intimin) into the non-pathogenic *E. coli* strain more than doubled its apoptotic response. Interestingly, in one cell line the non-pathogenic Bfp^{+}/$intimin^{-}$ strain induced more cell death than wild-type EPEC

(Bfp$^+$/intimin$^+$). This suggests that wild-type EPEC do promote anti-apoptotic pathways in the host cell, and that intimate attachment mediated by intimin is responsible.

A pathway by which intimin expression could promote an anti-apoptotic response is via tyrosine kinase activation. Intimin signalling (during intimate attachment) induces host cell tyrosine kinase activation (Kenny & Finlay, 1997) and the results of Crane *et. al.* (1999) show that attachment-dependent tyrosine kinase activation mediates an anti-apoptotic host response. Thus, mutant EPEC cured of *eae* (Bfp$^+$/intimin$^-$) would be expected to induce more apoptosis that wild-type EPEC (Bfp$^+$/intimin$^+$). However, EPEC lacking intimin induced *less* cell death than either wild-type EPEC (Bfp$^+$/intimin$^+$) or natural non-pathogenic *E. coli* (Bfp$^+$/intimin$^-$). This counter intuitive observation can be explained when the method of cell death detection is considered. In this assay, indicative changes in nuclear morphology were used to judge cell death (Abul-Milh *et. al.,* 2001). It is now known that EPEC secreted cyclomodulins (e.g. Cif) are able to induce cell cycle arrest, causing changes in nuclear morphology that do not necessarily indicate cell death (Marches *et. al.,* 2003). Following EPEC induced G_2/M arrest, the cytoskeleton attaches to the host cell membrane, a process that causes the inhibition of mitosis while DNA replication occurs unimpeded. Hence cellular and nuclear enlargement occurs with excess DNA within the nucleus (Marches *et. al.,* 2003). This could easily be mistaken for signs of cell death, hence cells infected with wild-type EPEC could be counted as apoptotic / necrotic when they were not.

Therefore it is possible that intimin signalling does mediate a specific anti-apoptotic response that opposes and balances the pro-apoptotic response to Bfp. Indeed when using an alternative (annexin labelling) method Abul-Milh *et. al.* (2001) found that infection with EPEC cured of *eae* (Bfp$^+$/intimin$^-$) induced higher levels of apoptosis than the wild-type EPEC (Bfp$^+$/intimin$^+$).

The initiation of the mitochondrial death pathway by EPEC via Map and EspF has already been introduced (**iii**). Nougayrede & Donnenberg (2004) demonstrated that

EPEC infection induced cytochrome *c* release and cleavage of caspase-3 and caspase-9 via EspF. It should be noted that cells were only infected with EPEC strains for 1 hour, giving little time for induction of anti-apoptotic pathways. Also, cleavage of caspase-9 was only identified in EPEC strains with transactivated EspF, which showed far higher EspF protein expression than wild-type EPEC. Hence the authors concede that over-expression of EspF may overwhelm EPEC induced anti-apoptotic signals in these experiments.

Other studies on EPEC induced apoptosis revolve around signalling by NF-κB, a nuclear factor that is activated by EPEC infection (Savkovic *et. al.,* 1997). NF-κB has pro- and anti-apoptotic effects in different cell types, but has been shown to promote cell survival in intestinal epithelial cells (Chen *et. al.,* 2000). EPEC induced NF-κB activation is however only a transient effect (Savkovic *et. al.,* 1997; Melo *et. al.,* 2005). Furthermore, a recent study has found that a compound suppressing NF-κB did not promote apoptosis on its own or influence EPEC induced apoptosis (Melo *et. al.,* 2005). The authors conclude that although Bfp expression was essential for EPEC apoptosis signalling, NF-κB signalling was not.

It is important to consider that *in vitro* studies of apoptosis do not necessarily well represent the *in vivo* situation. In a rare paper that declares in its title that EPEC is responsible for 'decreased apoptosis' an *in vivo* model (rabbit) was used. Heczko *et. al.* (2001) found that severe EPEC infection (with a rabbit specific strain) caused a significant decrease in apoptosis in Peyer's patches (using TUNEL assay), the tips of ileal absorptive villi (H&E staining morphology) and in whole ileal cell lysates (caspase-3 assay). Overall there is overwhelming evidence that in addition to pro-apoptotic mechanisms (probably induced non-specifically by Bfp), EPEC has evolved the ability to specifically induce anti-apoptotic pathways. Intimin dependent tyrosine kinase and protein kinase activation surely contribute to these anti-apoptotic pathways, providing a balance between pro- and anti-apoptotic mechanisms beneficial to the adherent bacteria.

(vi) Immune response. The initial immune response to pathogenic infection involves recruitment of acute inflammatory cells; EPEC has been shown to induce the recruitment of polymorphonuclear leukocytes (PMN) into the infected intestinal lumen (Moon *et. al.,* 1983). The pro-inflammatory cytokine interleukin-8 (IL-8) mediates PMN recruitment in intestinal epithelial cells and IL-8 transcription is governed by NF-κB. Early studies of EPEC induced PMN transepithelial migration found the process to be dependent on EPEC attachment (via intact TTSS) and cite EPEC induced NF-κB nuclear translocation as the mechanism (Savkovic *et. al.,* 1996 & 1997). However, a subsequent study found that although EPEC initially increased nuclear NF-κB, in the longer term (>1 hour) it was reduced. Furthermore, this study found that pre-infection of cells with EPEC prevented TNFα induced NF-κB nuclear accumulation (Hauf & Chakraborty, 2003). This study demonstrates that although there is an initial pro-inflammatory response, EPEC is eventually able to initiate an anti-inflammatory effect. This time frame suggests that insertion of secreted effector proteins into the host cell is responsible for the anti-inflammatory response.

A recent study has found that incubation of epithelial cells with sterile supernatant from EPEC culture caused higher IL-8 secretion than did EPEC itself; an effect dependent on an intact TTSS (Sharma *et. al.,* 2006). This and another study have found that EPEC flagella are potent stimulators of IL-8 secretion (Zhou *et. al.,* 2003). Hence it can be concluded that EPEC induced NF-κB activation is an initial non-specific effect initiated by contact of epithelial cell with EPEC flagella, and that once secreted effector proteins enter the host cell, NF-κB signalling is suppressed. It is notable that the studies that found NF-κB activation to be EPEC attachment specific effect used a growth medium (DMEM) that has been shown to both inhibit EPEC flagella expression and to reduce EPEC flagella induced IL-8 secretion (Zhou *et. al.,* 2003). Hence it may be concluded that the net effect of EPEC infection on epithelial cells is a balance between pro- and anti-inflammatory mediators (Sharma *et. al.,* 2006) based on active strategies to evade the host immune response, and is therefore analogous to apoptosis.

(vii) Diarrhoea. The exact mechanism(s) leading to EPEC induced diarrhoea are still unclear, disruption of the epithelial barrier and malabsorption (caused by microvilli effacement) are thought to contribute. Disrupted electrolyte transport is also thought to contribute to EPEC induced diarrhoea, support for this mechanism is provided by a study that demonstrated upregulation of galanin-1 receptors in response to EPEC (Hecht *et. al.,* 1999). Intestinal epithelial cells express galanin-1 receptors, which secret Cl^- on activation, leading to net loss of water from cells into the lumen. EPEC infection was shown to upregulate galanin-1 receptors via NF-κB mediated transcriptional activation (Hecht *et. al.,* 1999). Intimin dependent tyrosine phosphorylation of phospholipase C-γ1 (Kenny & Finlay, 1997) is thought to promote diarrhoea via inositol phosphate, calcium ion and diacylglycerol signalling resulting in activation of protein kinase C and 'brisk secretion of ions and fluids' (reviewed by Garmendia *et. al.,* 2005).

(viii) Signalling pathways. The ability of EPEC to induce signal transduction mechanisms is clear. The cellular consequences of EPEC infection previously discussed are a combination of; (a) signalling pathway effects (TTSS and intimin mediated intimate attachment dependent); (b) specific effects of secreted effector proteins (requiring TTSS but not necessarily intimin); (c) non-specific (generally toxic) effects of extracellular factors such as flagella and Bfp. However, many more EPEC induced effects on host cell biology are likely to be discovered as a result of these, and other pathways. A recent study in which the proteome of EPEC infected cells was analysed (Hardwidge *et. al.,* 2004) has revealed that changes in the expression of proteins within the following categories occurred: Actin binding, carbohydrate metabolism, cell cycle, cytochrome, cytoskeletal, DNA repair, DNA replication, extracellular matrix, general metabolism, G-protein signalling, immunity, ion transport, lipid metabolism, protein degradation, signal transduction, transcription and translation, as well as proteins with currently unclassified functions.

1.3.6. EPEC – a potential risk factor for colorectal cancer?

The finding that adherent *E. coli* are present on colonic adenocarcinomas has raised the possibility of a link between AE *E. coli* and carcinogenesis (Swidsinski *et. al.*, 1998; Martin *et. al.*, 2004). Perhaps surprisingly, all bacteria isolates found by Martin *et. al.* (2004) were negative for virulence factors of the classic *E. coli* pathotypes (aside from a gene encoding a cytotoxin), including *eae* (intimin), the EAF plasmid, *bfp* and *espB*. However, it is apparent that these studies do suggest a link between attaching *E. coli* strains and cancer. Whether this relationship is a cause of cancer or an effect of cancer remains to be seen.

In mice, evidence of a link between AE bacteria and carcinogenesis has been provided by *Citrobacter rodentium* (*Citrobacter freundii* biotype 4280). *C. rodentium* is the causative agent in Transmissible Murine Colonic Hyperplasia, a gross thickening of the colon in laboratory mice (Schauer *et. al.*, 1995). In mice *C. rodentium* infection has been shown to reduce the latent period in appearance of chemically induced tumours (Barthold & Jonas, 1977), and cause a three-fold increase in colonic adenomas in $Apc^{Min/+}$ animals (Newman *et. al.*, 2001). A recent literature review draws parallels between *H. pylori* and *C. rodentium* pathogenicity mechanisms, concluding that signals delivered to the colonic epithelium from infecting bacteria "might exacerbate defects in developmental or oncogenic pathways in the tissue" (Vogelmann & Amieva, 2007).

EPEC has effects on pathways such as cell cycle control (Marches *et. al.*, 2003), DNA repair (Hardwidge *et. al.*, 2004), apoptosis (Heczko *et. al.*, 2001) and cell adhesion (Simonovic *et. al.*, 2001) that are disrupted in the pathogenesis of cancer. Many of these properties are shared by the carcinogenic bacterium *H. pylori,* which also associates closely with gastric epithelial cells. *H. pylori* has been shown to efface microvilli and cause the formation of cup / pedestal structures containing polymerised actin while intimately attaching to epithelial cells (Smoot *et. al.*, 1993; Segal *et. al.*, 1996). Like EPEC, *H. pylori* have a secretion system (type IV secretion system) and translocate the effector protein CagA (Odenbreit *et. al.*, 2000), which is

tyrosine phosphorylated within host cells (Asahi *et. al.,* 2000). Also in similarity to EPEC, *H. pylori* induce disruption of host adherens junctions (via CagA) causing loss of epithelial cell adhesion (Suzuki *et. al.,* 2005), and interfere with host cell DNA repair protein expression (Kim *et. al.,* 2002).

1.4. Summary

- Colorectal cancer is a disease of high prevalence and mortality, caused by well defined genetic changes, including activating mutations of oncogenes and inactivating mutations of tumour suppressor genes.
- The genetic aberrations that contribute to colorectal tumourigenesis occur in a multiple-hit fashion and accumulate over decades. Concurrent with these genetic changes, the pathology of colorectal cancer follows a characteristic multi-stage adenoma-carcinoma sequence.
- Although it is accepted that gene-environment interactions play a crucial role in the generation of somatic mutations in sporadic colorectal cancer, there is a paucity of understanding in the nature and mechanism of these interactions. Hence the risk factors so far identified for colorectal cancer do not adequately explain the aetiology of this disease.
- Bacteria are a known cause of (gastric) cancer in man, and are abundant in the colon, furthermore adherent *E. coli* are very often associated with colonic adenocarcinomas.
- Enteropathogenic *E. coli* are carried by a significant percentage of healthy individuals and attach intimately to the colonic mucosa.
- EPEC shares many properties with the carcinogenic bacterium *H. pylori*, and influences apoptosis, cell cycle, cell-cell adhesion and DNA repair in host cells, pathways frequently disrupted in colorectal cancer.
- In mice AE bacteria are strongly linked to tumourigenesis.
- In combination these findings support the investigation of EPEC infection as a possible risk factor for colorectal cancer development.

1.5. Hypothesis

It is the study hypothesis that infection of colonic epithelial cells by enteropathogenic *E. coli* can increase the susceptibility of these cells to neoplastic transformation in man.

1.6. Aims & Objectives

This study aimed to establish the effects of EPEC infection on molecular genetic pathways involved in colorectal cancer development via the following objectives:

- Establish and validate *in vitro*, *in vivo* and *ex vivo* models for EPEC infection.
- Analyse the ability of AE *E. coli* to colonise the normal human colonic mucosa and colonic adenocarcinoma tissue.
- Analyse the effect of infection on DNA MMR repair, Wnt signalling (β-catenin) and cell adhesion pathways.

Chapter 2.

Materials and Methods

Unless otherwise stated, reagents were obtained from Sigma, Poole, UK.

2.1. *In vitro* co-culture model

2.1.1. General cell culture

The human colorectal cancer cell lines SW480, HT29, LS513 and the mouse colorectal cancer cell line CMT93 were obtained from the European Collection of Animal Cell Cultures (ECACC; Porton Down, Wiltshire, UK). Stocks of HT29, SW480 and LS513 were grown in Roswell Park Memorial Institute 1640 (RPMI) media containing L-glutamine (300g/L 2.05mM) (Gibco/Invitrogen, Paisley, UK) supplemented with 5% (v/v) foetal calf serum (Labtech/Biosera, Sussex, UK), referred to as R5. CMT93 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (Gibco/Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, referred to as D10. Cells were grown as adherent monolayers in T25 and T75 (25 cm^2 & $75cm^2$) cell culture flask (Costar/Corning, High Wycombe, UK) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Stock cells were grown to 90-95% confluency before passage. After removal of growth media, cells were washed with PBS and treated with warmed trypsin solution (Gibco/Invitrogen, Paisley, UK) and incubated at 37°C for 4-6 minutes. Trypsin was inactivated by the addition of RPMI / DMEM supplemented with foetal calf serum. New flasks were seeded from a 1 in 10 dilution of the trypsinised cell suspension. Frozen cell stocks were maintained in RPMI or DMEM supplemented with 5-10% foetal calf serum and Dimethyl sulphoxide (DMSO) 10% and stored in a liquid nitrogen freezer.

2.1.2. Bacterial strains

Wild-type (wt) EPEC, strain E2348/69 and mutant (mut) EPEC strain UMD864 (E2348/69 Δ48-759 *espB1*: Taylor *et. al.,* 1999) were kindly donated by Professor Michael Donnenberg, University of Maryland. Wild-type *Citrobacter rodentium* strain DBS100 and mutant *Citrobacter rodentium* strain DBS255 (DBS100 Δ*eae*) were kindly donated by Professor David Schauer, Massachusetts Institute of Technology.

2.1.3. Bacterial Glycerol Stocks

EPEC glycerol stocks were made by adding glycerol (final concentration 20%) to overnight bacterial cultures grown in Luria Bertani (LB) broth (Table 2.1) + Nalidixic acid 0.005% (50mg/L) at 37°C in an orbital shaker. 1ml aliquots of glycerol stock were stored at -70°C. *Citrobacter rodentium* glycerol stocks were made by adding 20% glycerol to overnight bacterial cultures grown in LB broth + Nalidixic acid 0.005% at 37°C in an orbital shaker. 1ml aliquots of glycerol stock were stored at -70°C.

2.1.4. Working bacterial cultures

Working stocks of bacteria were grown on nutrient agar. EPEC strains were grown on selective MacConkey agar and *Citrobacter rodentium* strains were grown on LB agar. Agar plates were set up by inoculating 100ul of sterile LB broth with an aliquot (~10-20ul) of glycerol stock obtained by a stab with a sterile pipette tip. The suspension was mixed, pipetted on to the agar and spread with a sterile spreading tool. Plates were incubated overnight at 37°C then stored at 4°C. Fresh plates were set up every eight weeks. Bacterial cultures used for co-culture experiments were grown from single colonies picked from agar plates. Colonies were transferred to 4ml sterile LB broth using a sterile pipette tip and incubated overnight at 37°C in an orbital shaker set to 180 revolutions per minute.

Working stocks of EPEC were maintained on MacConkey agar supplemented with crystal violet, sodium chloride, 0.15% bile and 0.005% Nalidixic acid (Table 2.2). Overnight cultures were grown from single colonies inoculated into LB broth and incubated at 37°C in an orbital shaker. *Citrobacter rodentium* working stocks were maintained on LB nutrient agar (Table 2.3), overnight cultures were grown from single colonies inoculated into LB broth and incubated at 37°C in an orbital shaker.

Component	Concentration
Tryptone (SLS, Nottingham, UK)	1.0%
Yeast extract (SLS, Nottingham, UK)	0.5%
NaCl (Fisher, Loughborough, UK)	0.5%
Dissolved in ddH_2O, pH adjusted to 7.2	

Table 2.1. LB Broth composition

Component	Concentration
Bacto Agar (BD Biosciences, Oxford, UK)	1.5%
Proteose Peptone	1.0%
Peptone Bacteriological	1.0%
Lactose	1.0%
Bile salts (ICN Biomedicals, Ohio, USA)	0.15%
NaCl (Fisher, Loughborough, UK)	0.5%
Neutral red	0.005%
Grams Crystal Violet Solution (BDH Lab supplies, Poole, UK)	0.001%
Nalidixic acid	0.005%
Dissolved in ddH_2O	

Table 2.2. MacConkey Agar composition

Components	Concentration
Bacto Agar (BD Biosciences, Oxford, UK)	1.2%
Dissolved in LB broth	

Table 2.3. Nutrient agar composition

2.1.5. Twelve-hour co-culture protocol with three-hourly washes, providing protein for western blot analysis.

HT29 cells were grown to 90-100% confluency in T75 cell culture flasks. At the start of the co-culture period medium was aspirated from all flasks, four flasks received 15ml media plus 150ul E2348/69 (wt) overnight bacterial culture, and four flasks received 15ml media plus 150ul UMD864 (mut) overnight bacterial culture (MOI ~10:1). Two

flasks were uninfected; one was washed by the same protocol as the infected flasks and one was not washed (Table 2.3). All flasks were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At intervals of three hours, flasks were removed from the incubator and washed by the following method: Medium was aspirated and approximately 10ml of fresh R5 media was added to each flask. Flasks were then gently tilted side-to-side by hand to ensure the media washed over the cells 4-6 times, medium was aspirated and the process repeated a further two times.

Once the cells had been washed three times they received 15ml of fresh R5 media. 150ul of overnight culture of UMD864 was re-added to the mutant-infected cells to replace the non-adherent bacteria that had been washed away. After washing, cells were returned to the incubator. When cells reached the designated time-point for protein isolation they were liberated from the flask by treatment with warmed trypsin and centrifuged to form cell pellets. Protein was immediately isolated from the cell pellets (section 2.3.1).

Flask Number	Contents	Wash time-points	Protein isolation time-point
1	HT29 cells	No wash	12h
2	HT29 cells	3, 6, 9, 12h	12h
3	HT29 cells & EPEC (wt)	3h	3h
4	HT29 cells & EPEC (wt)	3, 6h	6h
5	HT29 cells & EPEC (wt)	3, 6, 9h	9h
6	HT29 cells & EPEC (wt)	3, 6, 9, 12h	12h
7	HT29 cells & EPEC (mut)	3h	3h
8	HT29 cells & EPEC (mut)	3, 6h	6h
9	HT29 cells & EPEC (mut)	3, 6, 9h	9h
10	HT29 cells & EPEC (mut)	3, 6, 9, 12h	12h

Table 2.4. *In vitro* co-culture conditions for isolating protein at 3 hour time-points, where bacteria were first added to cells at 0 hours.

2.1.6. Nine- and twelve-hour co-culture time-course, with three-hourly wash, providing protein for western blot analysis and fixed cells for immunofluorescence.

Once it had been established that changes in protein expression in HT29 cells generally occurred after 9-12 hours of co-culture it was decided to further analyse these changes *in situ* by use of immunofluorescence. By growing cells in dishes containing cover-slips

it was possible to obtain cells for immunofluorescence and isolate protein from the same population of cells. A bacterial pre-culture step was also included to activate the bacteria prior to infection (Rosenshine *et. al.,* 1996).

Ten sterile 13mm glass cover-slips were placed into round 10cm diameter cell culture dishes and spaced out so that no overlapping occurred. Cells (HT29/SW480/LS513) were added to the dishes and grown to 90-100% confluency, whereby the cell monolayer covered both the cover-slips and the exposed surface of the dish. E2348/69 and UMD864 EPEC were prepared by diluting overnight cultures 1:50 in warmed (37°C) R5 media. This infected media was then incubated for 1 hour at 37°C (without shaking) in order to "activate" the bacteria (Rosenshine *et. al.,* 1996).

At the start of the co-culture period media was aspirated from all dishes, two dishes received 10ml media infected with wild-type EPEC and two dishes received 10ml media infected with mutant EPEC (MOI ~ 50:1). Two dishes were uninfected; one was washed by the same protocol as the infected flasks and one was not washed (Table 2.4). All dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At intervals of three hours dishes were removed from the incubator and washed as previously described (section 2.2.1). Once the cells had been washed three times they received 10ml of fresh R5. 100ul of overnight culture of mutant UMD864 EPEC was re-added to the mutant infected cells to replace the non-adherent bacteria that had been washed away. After washing, cells were returned to the incubator.

At the relevant time-points cover-slips were removed from the dish and placed into new $10cm^2$ cell culture dish where they were fixed for immunofluorescence (section 2.3.5.1). Cells that remained adherent to the original dish were washed with PBS, removed by treatment with trypsin and centrifuged to form cell pellets. Protein was immediately isolated from the cell pellets by the standard method (section 2.3.1).

2.1.7. Recovery from infection

HT29 cells were initially co-cultured with E2348/69 for 9 and 12 hours (as described in section 2.2.2) then washed three times with RPMI media and treated with R5 media supplemented with penicillin 200,000 u/L, streptomycin 200mg/L and gentamicin

100mg/L (all from Gibco/Invitrogen, Paisley, UK). Media was changed approximately every 12 hours. After 36 - 39 hours (12 hour and 9 hour prior infection respectively) monolayers were trypsinised and protein was isolated from cells.

Dish Number	Contents	Wash time-points	Protein isolation time-point
1	HT29 cells	No wash	12h
2	HT29 cells	3, 6, 9, 12h	12h
3	HT29 cells & EPEC (mut)	3, 6, 9h	9h
4	HT29 cells & EPEC (wt)	3, 6, 9h	9h
5	HT29 cells & EPEC (mut)	3, 6, 9, 12h	12h
6	HT29 cells & EPEC (wt)	3, 6, 9, 12h	12h

Table 2.5. *In vitro* co-culture conditions to provide protein and fixed cells after 9 & 12 hours.

2.2. Protein Analysis

2.2.1. Protein isolation

Cell pellets were re-suspended in RIPA buffer (NP-40, 1%; Sodium deoxycholate, 0.5%; SDS, 0.1%; in PBS, pH 8.8) containing Complete Protease Inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). The suspension was mixed using a vortex mixer at high speed for approximately 10 seconds then repeatedly drawn through a 25G (0.5mm diameter) needle attached to a sterile plastic 1ml syringe. The resultant lysate was centrifuged at 13,000 rpm for 15 minutes at 4°C to remove cell debris. The supernatant (comprising whole-cell protein extract) was removed and homogenised, aliquots were stored at -70°C.

2.2.2. Polyacrylamide gel electrophoresis

Whole cell protein extracts were resolved through pre-cast 4-12% gradient NuPAGE® gels (Invitrogen, Paisley, UK) using MOPS running buffer (Invitrogen, Paisley, UK). Gels were removed from their plastic cast, trimmed and washed three times with ddH_20. Protein bands were identified by staining overnight with Simplyblue Safestain® (Invitrogen, Paisley, UK). Gels were then destained with two 1-hour washes with

ddH_2O. Gels were either dried using the DryEase™ Mini-Gel Drying system (Invitrogen, Paisley, UK) or bands were cut out for mass spectrometry. Gels used for mass spectrometry were manipulated inside class II safety cabinets and stained, destained and stored in fresh, sterile plastic-ware.

2.2.3. Mass spectrometry

Whole cell protein extracts were resolved through pre-cast 4-12% gradient NuPAGE® Novex gels (Invitrogen, Paisley, UK). Gels were stained overnight with SimplyBlue® Safestain (Invitrogen, Paisley, UK) and destained in ddH_2O to reveal protein bands. Bands were cut from gels, washed with actonitrile 50%, dried and reduced with dithiothreitol (DTT) 10mM and EDTA 0.2% in ammonium bicarbonate 100mM, then alkylated with iodoacetamide 20mM in ammonium bicarbonate 100mM. After washing, proteins were digested with sequencing grade modified trypsin (Promega, Southampton, UK). Spectra were measured on a Voyager DE-STR Maldi-Tof Mass Spectrometer (Applied Biosystems, Foster City, CA). Protein matches were identified via the MS-Fit SwissProt. database:
http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm.

2.2.4. Western blot analysis

Whole cell protein extracts were resolved through pre-cast (4-12% gradient / 10%) NuPAGE Novex gels (Invitrogen, Paisley, UK) or 10% sodium dodecyl sulphate / polyacrylamide (SDS-PAGE) gels (Table 2.6) and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond P, GE Healthcare Biosciences, Buckinghamshire, UK). Protein levels could not be equalised via conventional densitometry due to the presence of bacterial protein in some samples. Therefore, levels were equalised via successive western blots with reference to beta-actin and proliferating cell nuclear antigen (PCNA) band intensities, coumassie blue staining was also used to aid equal protein loading.

Name	Composition / Product
10% SDS-PAGE stacking gel	Tris pH 6.8, 0.125M Sodium dodecyl sulphate, 0.1% Ammonium persulphate, 0.1% Acrylamide, 4% Tetramethylethylenediamine (TEMED), 0.1% In ddH_2O
10% SDS-PAGE resolving gel	Tris base pH 8.8, 0.375M Sodium dodecyl sulphate, 0.1% Ammonium persulphate, 0.1% Acrylamide, 10% TEMED, 0.1% In ddH_2O
SDS-PAGE running buffer	Tris base, 0.6% Glycine, 1.44% Sodium dodecyl sulphate, 0.05% In ddH_2O
SDS-PAGE transfer buffer	Tris base, 0.6% Glycine, 2.88% In ddH_2O
SDS-PAGE protein loading buffer	Tris base pH 6.8, 0.0625M Sodium dodecyl sulphate, 3.3% β-mercaptoethanol, 5% Glycerol, 12.5% Bromophenol blue, 0.01% In ddH_2O
SDS-PAGE protein marker	BenckMark prestained protein ladder (Invitrogen, Paisley, UK)
NuPAGE running buffer	NuPAGE MOPS SDS running buffer (20X) (Invitrogen, Paisley, UK)
NuPAGE transfer buffer	NuPAGE transfer buffer (20X) (Invitrogen, Paisley, UK)
NuPAGE protein loading buffer	NuPAGE LDS sample buffer (4X) (Invitrogen, Paisley, UK)
NUPAGE protein marker	SeeBlue Plus2 (Invitrogen, Paisley, UK)
Membrane washing solutions	TBS (Tris buffered saline) TBST (TBS with 0.1% Tween-20)
Blocking buffer	TBST with 5% Marvel powdered milk (Premier Int. Foods, Lincolnshire, UK)
Coumassie stain	SimplyBlue SafeStain (Invitrogen, Paisley, UK)

Table 2.6. Electrophoresis and Western blot solutions

Primary antibodies were used at a concentration of 1 in 250 to 1 in 5000, incubation time varied from 45 minutes at room temperature to 16-hours (overnight) at 4°C (Table 2.7). Peroxidase conjugated secondary antibodies were applied for 30 minutes – 2 hours

at room temperature (Table 2.8). Proteins were detected using ECL Plus chemiluminescence detection system and exposed to Hyperfilm MP (both from GE Healthcare Bio-Sciences, Buckinghamshire, UK), which was developed in a Hyperprocessor (Amersham Biosciences, Buckinghamshire, UK).

Antibody	Clone	Manufacturer	Raised in	Conc.	Time
Anti-MSH2	Ab-2 / FE11	Calbiochem / Merck (Nottingham, UK)	Mouse (monoclonal)	1 in 500	Overnight (4°C)
Anti-MLH1	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 1000	Overnight (4°C) or 2 hours RT
Anti-Beta-actin	AC-15	Sigma-Aldrich (Saint Louis, MI)	Mouse (monoclonal)	1 in 5000 – 10,000	45 minutes RT
Anti-PCNA	C-20	Santa Cruz Biotechnology (Santa Cruz, CA)	Goat (polyclonal)	1 in 1000	Overnight (4°C) or 2 hours RT
Anti-β-catenin (C-terminal)	E-5	Santa Cruz Biotechnology (Santa Cruz, CA)	Mouse (monoclonal)	1 in 500	Overnight (4°C)
Anti-β-catenin (non-phoshorylated)	8E4	Upstate (Lake Placid, NY)	Mouse (monoclonal)	1 in 500	Overnight (4°C)
Anti-E-cadherin	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 500	Overnight (4°C)
Anti-Gamma-catenin	H-1	Santa Cruz Biotechnology (Santa Cruz, CA)	Mouse (monoclonal)	1 in 1000	Overnight (4°C)
Anti-Ezrin	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 500	Overnight (4°C)

Table 2.7. List of primary antibodies used for probing Western blots.

Antibody	Manufacturer	Raised in	Concentration	Time
HRP conjugated Anti-mouse IgG	Diagnostics Scotland (Carluke, UK)	Sheep (polyclonal)	1 in 2000	45 min. – 2 hours RT
HRP conjugated Anti-goat IgG	Dako Cytomation (Glostrop, Denmark)	Rabbit (polyclonal)	1 in 2000	1 – 2 hours RT
HRP conjugated Anti-sheep IgG	Dako Cytomation (Glostrop, Denmark)	Rabbit (polyclonal)	1 in 2000	1 – 2 hours RT
HRP conjugated Anti-rabbit	Dako Cytomation (Glostrop, Denmark)	Swine (polyclonal)	1 in 2000	1 – 2 hours RT

Table 2.8. List of secondary antibodies used for probing Western blots.

2.2.5. Immunofluorescent staining of proteins and phalloidin staining of F-actin

2.2.5.1. Fixing cells

Cover-slips holding cell monolayers were placed into fresh sterile 10cm cell culture dishes and washed twice with 10ml sterile PBS. Cells were then fixed with 10ml paraformaldehyde in PBS (Table 2.14) for 5 minutes. The paraformaldehyde solution was aspirated and cells were washed three times with PBS. Finally, 15ml of PBS supplemented with 0.02% sodium azide was added, dishes were sealed with paraffin film and stored at 4°C.

2.2.5.2. Immunocytochemistry

- Cover-slips were placed cell-side up in individual wells in 12-well plastic plates (Costar/Corning, High Wycombe, UK) and washed in PBS for 5 minutes on an orbital shaker
- A solution of 0.1% v/v Triton-X100 in PBS was added (to enhance cell membrane permeability) and plates were placed on an orbital shaker for 30 minutes
- The Triton solution was aspirated and cells were washed in PBS for 5 minutes on an orbital shaker
- Blocking buffer (Bovine serum albumin, 0.3%; Tween-20, 0.2% in PBS) was added and plates were placed on an orbital shaker for 1 hour
- Blocking buffer was aspirated and cells were washed in PBS for 5 minutes on an orbital shaker
- Primary antibodies (Table 2.9) were diluted in PBST (Tween-20, 0.2% in PBS) and added to cells, plates were placed on the bench (stationary) for 1 hour
- Antibody solution was aspirated and cells were washed with PBST (three 5 minute washes) on an orbital shaker
- Secondary antibodies (Table 2.10) were diluted in PBST and added to cells, plates were covered in metal foil and placed on the bench (stationary) for 30 minutes
- Antibody solution was aspirated and cells were washed with PBST (three 5 minute washes) on an orbital shaker

- Cover-slips were removed from wells, blotted on tissue paper to remove excess PBST and mounted (cell-side down) onto glass microscope slides with Vectashield Mounting Medium with DAPI (Vector Labs, Burlingame, CA).
- Excess mounting medium was blotted away with tissue paper
- Slides were placed in slide holders, covered with metal foil and stored at 4°C.

Antibody	Clone	Manufacturer	Raised in	Conc.	Time
Anti-MSH2	Ab-2 / FE11	Calbiochem / Merck (Nottingham, UK)	Mouse (monoclonal)	1 in 200	1 hour
Anti-MLH1	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 200	1 hour
Anti-Beta-actin	AC-15	Sigma-Aldrich (Saint Lous, MI)	Mouse (monoclonal)	1 in 500	1 hour
Anti-PCNA	C-20	Santa Cruz Biotechnology (Santa Cruz, CA)	Goat (polyclonal)	1 in 500	1 hour
Cytochrome *c*	-	Abcam (Cambridge, UK)	Sheep (polyclonal)	1 in 500	1 hour
Anti-*E. coli*	-	Europa Bioproducts (Cambridge, UK)	Rabbit (polyclonal)	1 in 1000	1 hour
MTCO2	-	Abcam (Cambridge, UK)	Mouse (monoclonal)	1 in 1000	1 hour
Anti-E-cadherin	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 500	1 hour
Anti-Gamma-catenin	H-1	Santa Cruz Biotechnology (Santa Cruz, CA)	Mouse (monoclonal)	1 in 200	1 hour
Anti-β-catenin (C-terminal)	E-5	Santa Cruz Biotechnology (Santa Cruz, CA)	Mouse (monoclonal)	1 in 500	1 hour
Anti-β-catenin (non-phoshorylated)	8E4	Upstate (Lake Placid, NY)	Mouse (monoclonal)	1 in 500	1 hour

2.9. Table of primary antibodies used for Immunofluorescence immunocytochemistry

Antibody	Manufacturer	Raised in	Concentration	Time
Fitc conjugated Alexa-fluor 488 anti-mouse IgG	Invitrogen Molecular Probes, Oregon USA	Goat	1 in 2000	1 hour
Texas red conjugated Alexa-fluor 594 anti-sheep / goat IgG	Invitrogen Molecular Probes, Oregon USA	Donkey	1 in 2000	1 hour
Texas red conjugated Alexa-fluor 594 anti-rabbit IgG	Invitrogen Molecular Probes, Oregon USA	Goat	1 in 2000	1 hour

2.10. Table of secondary antibodies used for immunofluorescent immunocytochemistry

2.2.5.3. *In situ* cell death detection

Cells undergoing late stage apoptosis / necrosis were identified based on labelling of DNA strand breaks (TUNEL technology) using an *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers instructions, and visualised via fluorescence microscopy. Percentages of TUNEL positive cells were calculated by counting at least 400 cells from each experimental condition (n = 3). Positive control cells were treated with DNAse (Qiagen, Crawley, UK).

2.2.5.4. Early apoptosis detection

Early stage apoptosis was detected in cells by immunofluorescence staining of cytochrome *c* (Castedo *et. al.*, 2002), either in conjunction with the mitochondrial marker MTCO2 or MLH1 using the antibodies listed in Table 2.9. Positive control cells were treated with etoposide 1μM for 2 hours, followed by 6 hours in normal media, then fixed as described above.

2.2.6. Immunofluorescence image capture and quantitative image analysis

Fluorescent images were captured using either a Zeiss Axioskop 20 fluorescence microscope (Zeiss, Luton, UK) using Smartcapture software (Digital Scientific, Cambridge, UK) or a Zeiss Axioplan II fluorescence microscope using IPLab software (Scanalytics Corp., Fairfax, VA). Image analysis was performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp., Fairfax, VA). Software calculated the nuclear staining intensities for the proteins of interest. With reference to images of uninfected controls, cells were categorized as having either negative / very low (-), intermediate (+) or very strong (++) nuclear staining. The numerical staining intensity boundaries between these three cell populations were calculated for control cells and applied to the intensity data generated from each of the experimental conditions. The individual intensity values for over 300 cells were counted for each experimental condition (see appendix for example).

2.3. Mutation frequency analysis by inter-Alu PCR

2.3.1. Co-culture and DNA isolation

To demonstrate method reproducibility, uninfected, untreated SW480 cells were used. To analyse the effect of EPEC on mutation frequency, SW480 cells grown in T75 flasks were either uninfected or infected with mutant or wild-type EPEC. After 12 hours, cells were treated with antibiotics and allowed to recover from infection as previously described. Once cells reached 100% confluency, flasks were split into 10 new T25 flasks and grown to 100% confluency (this took longer for cells infected with wild-type EPEC). Cells were removed from flasks by treatment with trypsin and centrifuged to form cell pellets. After removal of the media supernatant, cell samples were incubated with lysis buffer (Table 2.11) for 2 hours at 37°C while rotating. DNA was isolated from the resulting homogenate by phenol / chloroform extraction (Phenol : Chloroform : Isoamyl alcohol solution, Ambion, Cambridge, UK).

Solution	Composition
TBE	Tris base, 1% Boric acid, 0.55% Ethylenediaminetetraacetic acid disodium (EDTA), 0.1% In ddH_2O, pH 8.3
Lysis buffer	Tris base pH 8, 10mM Sodium chloride, 400mM EDTA, 3mM Sodium dodecyl sulphate, 1% Proteinase K, 0.4mg/ml
Agarose gel for PCR	Biotechnology grade agarose (Amresco, Solon, OH), 3% Ethidium bromide ~1 x 10^{-6} % In TBE
Running buffer	TBE
Denaturing sequencing gel for inter-Alu PCR	Ammonium persulphate, 0.1% TEMED, 0.01% In Severn Super Sequencer; 6.5% Acrylamide, Urea, TBE solution (Severn Biotech, Worcestershire, UK)

Table 2.11. Gels and solutions for PCR

2.3.2. Inter-Alu PCR

The Alu-specific primer R12A/267 (AGCGAGACTCCG) spans 12 nucleotides at the 3′ end of the Alu consensus sequence, downstream from position 267 (Zietkiewicz *et. al.*,

1992). Reactions were performed using R12A/267 in the presence of redivue deoxyadenosine 5'-[alpha-33P] triphosphate (triethylammonium salt, 9.25 MBq, 250 uCi, GE Healthcare Biosciences, Buckinghamshire, UK). PCR products were resolved through denaturing 6.5% polyacrylamide gels (Table 2.11) and visualised by overnight exposure of dried gels to Kodac X-OMAT AR autoradiographic film (VWR International, Leicestershire, UK).

2.4. Microarray gene expression analysis

2.4.1. Co-culture and RNA isolation

HT29 cells were either uninfected or co-cultured with wild-type or mutant EPEC for 9 and 12 hours as described above (section 2.2.2). A total of three repeat co-culture experiments were performed on separate days. Cells were removed from dishes with trypsin and centrifuged to form cell pellets. Supernatant media was removed and pellets were stored at -70°C overnight. RNA was isolated from cell pellets using Qiagen Mini Prep kits with the addition of DNAse (Qiagen, Crawley, UK) according to the manufacturer's instructions. The concentration and stability (RIN number) of RNA samples was analysed on an Agilent Bioanalyser (Agilent Technologies, Berkshire, UK). RNA isolated from cells co-cultured with EPEC (especially wild-type EPEC) generally had lower RIN number and concentration. RNA concentrations were equalised prior to amplification.

2.4.2. RNA amplification and biotin tagging

RNA was amplified and biotin tagged using Illumina Total Prep RNA Amplification Kits (Ambion, Cambridge, UK) according to the manufacturer's instructions. Subsequent to amplification, the concentration and purity of samples was checked on an Agilent Bioanalyser (Agilent Technologies, Berkshire, UK) and sample concentrations were equalised before microarray analysis.

2.4.3. Microarray analysis

The microarray hybridisation procedure was carried out by staff at the Wellcome Trust Clinical Research Facility (Western General Hospital) in accordance with manufacturers instructions. Sentrix Human-6 v2 Beadchips were used in conjunction with the Illumina microarray platform (Illumina Inc., Cambridge, UK).

2.4.4. Microarray data output and analysis

A total of three Beadchips, each holding six individual arrays were used, hence each repeat experiment was performed on one Beadchip (Table 2.12). Each Human-6 v2 Beadchip generated expression data for over 46,000 gene probes, corresponding to approximately 26,000 characterised genes and 20,000 uncharacterised genes.

Experiment	Infection	Time-course	Abbreviation	Replicate	Array	Beadchip
1	-	0 h	- 0h	A	1	1
	-	12 h	-12h	B	2	
	mut EPEC	9 h	+mut 9h	C	3	
	mut EPEC	12 h	+mut 12h	D	4	
	wt EPEC	9 h	+wt 9h	E	5	
	wt EPEC	12 h	+wt 12h	F	6	
2	-	0 h	- 0h	A	7	2
	-	12 h	-12h	B	8	
	mut EPEC	9 h	+mut 9h	C	9	
	mut EPEC	12 h	+mut 12h	D	10	
	wt EPEC	9 h	+wt 9h	E	11	
	wt EPEC	12 h	+wt 12h	F	12	
3	-	0 h	- 0h	A	13	3
	-	12 h	-12h	B	14	
	mut EPEC	9 h	+mut 9h	C	15	
	mut EPEC	12 h	+mut 12h	D	16	
	wt EPEC	9 h	+wt 9h	E	17	
	wt EPEC	12 h	+wt 12h	F	18	

Table 2.12. Microarray sample list showing co-culture conditions and experimental replicates.

Raw gene expression data was collated by the proprietary Illumina Beadstudio software (Illumina Inc., Cambridge, UK). Raw data was uploaded to the IlluminaGUI software package (downloaded from http://illuminagui.dnsalias.org), running from the 'R' statistical computing programme (v-2.5.1 www.r-project.org/index.html) IlluminaGUI is a recently introduced graphical user interface for analysing gene expression data generated from the Illumina Beadchip platform (Schultze & Eggle, 2007). Analysis of the functional pathways to which differentially expressed genes belonged was performed using the DAVID (Database for Annotation, Visualisation and Integrated Discovery) 2007 bioinformatics database (http://david.abcc.ncifcrf.gov; Dennis *et. al.*, 2003).

Outline of data analysis workflow:

- Upload raw data to IlluminaGUI
- Output control probe data (validation of array performance, see appendix)
- Normalise data
- Validate normalisation method by comparing normalised and non-normalised (i.e. raw) data via box & whisker plots, MA plots and scatter plots
- Group replicate data
- Perform differential expression analysis, based on fold change and statistical significance
- Record number of differentially expressed genes and plot as 'volcano' scatter plots
- Examine gene ontology by clustering based on functional annotation and pathway enrichment (via DAVID Bioinformatics database)
- Examine expression of individual genes of interest

The IlluminaGUI data analysis package was chosen as it was designed specifically for Illumina Beadchip data and allows use of the powerful R statistical platform with a straightforward user interface. Normalisation techniques used were quantile/quantiles normalisation (Bolstad *et. al.*, 2003) and variance stabilisation normalisation (VSN) (Huber *et. al.*, 2002). The statistical tests used were conventional T-test and significance analysis of microarrays (SAM) test (Tusher *et. al.*, 2001).

2.5. *In vivo* mouse model of EPEC infection

2.5.1. Animal husbandry

All animal work was approved by the University of Edinburgh, under Home Office personal and project licences. C57Bl/6 mice and C3H/HeN mice were obtained from Charles River (Kent, UK). Mice were caged and housed in negative pressure isolators with a normal 12 hour light / dark cycle. Mice were fed standard solid diet and allowed drinking water *ad libitum*.

2.5.2. *In vivo* infection of C57Bl/6 mice with *Citrobacter rodentium*

Mature, male C57Bl/6 mice received 100ul (~1 x 10^8) of *Citrobacter rodentium* DBS100 (n = 3) or DBS255 (n = 3) bacterial suspension (grown overnight in LB broth) by gavage using a rigid gavage needle, negative controls (n = 3) were untreated.

After 14 days all animals were killed by cervical dislocation. The abdomen was opened and the entire colon (from just below the cecum to the rectum) was excised. The colon was cut longitudinally and opened, then thoroughly washed in sterile PBS to remove all faecal matter. The tissue was placed lumen side up onto a dry sterile Petri dish lid and forceps were used to fully unfold the tissue. The resulting strip of tissue was rolled around a small metal rod (3mm diameter) to form a compact roll. The roll was carefully removed from the rod with forceps and pierced with a metal sewing pin to hold it together. Tissue was fixed in 4% buffered formalin for 48 hours then embedded in paraffin (Histopathology Labs, QMRC, Little France, Edinburgh).

2.5.3. *In vivo* infection of C57Bl/6 mice with EPEC

This mouse model for EPEC infection is derived from that published by Savkovic *et. al.* (2005).

2.5.3.1. Inoculation of mice

EPEC E2348/69 were grown overnight in 4ml LB broth. The bacterial suspension was centrifuged (3500g for 10 minutes) and the LB broth was aspirated. The bacterial pellet

was re-suspended in 4ml PBS. Nine male 6-8 week old C57Bl/6 mice received 200ul of the EPEC E2348/69 bacterial suspension (~2 x 10^8 bacteria) by gavage using a rigid gavage needle and returned to their cages.

2.5.3.2. Infection time-course

At intervals of 24, 48 and 72 hours from the time of infection, animals (n = 3) were removed from the group and killed by cervical dislocation. The abdomen was opened and the gastrointestinal tract (ileum to rectum) was excised and placed into sterile PBS. The tissue was cut into ileum, cecum, proximal colon and distal colon sections. Each section was cut longitudinally, and thoroughly washed in a universal container containing sterile PBS (10ml for ileum, and colon sections, 20ml for cecum). The PBS-lumen contents suspensions were retained. The tissue was then washed a further three times in sterile PBS, placed in a universal container containing 5ml sterile PBS and transported to the laboratory.

2.5.3.3. Mechanical homogenisation of tissue

All tissues were weighed within their containers (the weight of each container + the relevant volume of PBS had previously been recorded). Tissues were removed from their containers and placed onto a sterile, dry, plastic Petri dish lid. Tissues were cut repeatedly with curved scissors (John Weiss, Buckinghamshire, UK) until all pieces were no bigger than $1mm^3$. Tissue was then transferred back into its original container (containing 5ml PBS) and repeatedly drawn (6-8 times) through a 21G (0.8mm x 40mm) needle attached to a sterile 10ml plastic syringe.

2.5.3.4. Bacteria counts

The number of EPEC adherent to gastrointestinal tissue sections was calculated by inoculating selective MacConkey agar plates with 100ul of the PBS-tissue homogenate. The number of non-adherent EPEC present in the lumen of the gut sections was calculated by inoculating selective MacConkey agar plates with 100ul of the PBS-lumen contents suspension. Three agar plates – each inoculated with 100ul – were grown from each tissue / lumen suspension. The agar plates were covered and placed

upside down in a 37°C incubator for 24 hours. EPEC colony forming units (CFU's) were identified as round pink colonies and counted manually. Average numbers of CFU's were calculated and the total number of adherent / non-adherent EPEC were calculated based on the dilution in PBS and size of the inoculum.

2.5.3.5. Longer-term infection

EPEC E2348/69 and UMD864 were grown overnight in 4ml LB broth. The bacterial suspensions were centrifuged (3500g for 10 minutes) and the LB broth was aspirated. The bacterial pellets were re-suspended in 4ml PBS. Male 6-8 week old C57Bl/6 mice received 200ul of either EPEC E2348/69 (n = 3) or UMD864 (n = 3) bacterial suspension (~2 x 10^8 bacteria) by gavage using a rigid gavage needle and returned to their cages. After 14 days animals were killed and tissue was prepared as described above (section 2.6.2.1).

2.5.4. *In vivo* infection of C3H/HeN mice with EPEC

The aim of this protocol was to improve on the C57Bl/6 mouse model for EPEC infection, i.e. to produce a mouse model that was colonized by higher numbers of EPEC for a greater period of time. C3H/HeN mice were used because of their increased susceptibility to infection by attaching and effacing bacteria (*Citrobacter rodentium*) (Vallance *et. al.,* 2003). Also, bacteria preparation was modified in order to activate bacteria (Rosenshine *et. al.,* 1996) before gavage.

2.5.4.1. Inoculation of mice

EPEC E2348/69 were grown overnight in 4ml LB broth. 1ml of the bacterial suspension was added to 20ml of LB broth and incubated until optical density was 0.5 at 600nm. This bacterial suspension was centrifuged (3500g for 10 minutes) and the LB broth was aspirated. The bacterial pellet was re-suspended in 3.5ml of sterile PBS. Fifteen male 6-8 week old C3H/HeN mice received 200ul of the EPEC E2348/69 bacterial suspension (~1 x 10^9 bacteria) by gavage using a rigid gavage needle and returned to their cages.

2.5.4.2. Infection time-course, homogenisation of tissue and bacteria counts

Infected mice (n = 9) were used for bacteria counts using the same protocol as previously described.

2.5.4.3. Longer-term infection

The remaining animals (n = 6) were kept under normal conditions for over 5 weeks post infection. Over this time their behaviour was observed to be normal, with no signs of diarrhoea. Three of these animals were killed 38 days post infection; numbers of adherent and non-adherent EPEC in the cecum and proximal colon were counted as previously described.

2.5.4.4. Fixed cecum and proximal colon tissue for H&E staining

A second group of C3H/HeN mice (n = 3) were infected with EPEC by the same method. All animals were killed 48 hours from the time of infection. The cecum and proximal colon were excised, lumen contents were collected, tissues were washed three times, rolled and fixed in 4% buffered formalin. Within 48 hours, fixed tissues were taken to the Histopathology Laboratories at Queens Medical Research Centre where they were embedded in paraffin, cut into 4um sections and placed onto glass microscope slides for immunohistochemistry. Cecum and proximal colon tissues from C3H/HeN mice not infected with EPEC were used as controls and prepared for staining in the same way.

2.6. Human tissue *ex vivo*

2.6.1. Ethics

Ethical approval for the use of human tissue was provided by the NHS Lothian Research Ethics Committee, under the title: Edinburgh Cancer Research Centre tissue and body fluids collection with linkage to pathological and clinical data; reference 06/S1101/16.

2.6.2. Patients

Tissue was removed from patients undergoing surgical resection of the large intestine at the Western General Hospital, Edinburgh. Only normal, 'healthy' tissue (removed from sites distant from any disease) was used in co-culture (Table 2.13).

Reference number	Age	Sex	Disease	Tissue sample
OMSA241238	69	F	Metastatic endometrial cancer	Transverse colon
OMSA290942	65	M	Rectal cancer	Transverse colon
OMSA040143	64	M	Rectal cancer	Transverse colon

Table 2.13. Patient details for colon mucosa tissue donors

2.6.3. Bacteria preparation

EPEC E2348/69 and UMD864 were prepared by diluting overnight cultures 1:50 in warmed (37°C) R5 media. This infected media was then incubated for 1-2 hours at 37°C (without shaking) in order to "activate" the bacteria (Rosenshine *et. al.*, 1996).

2.6.4. Co-culture

In vitro organ co-culture of *ex vivo* tissue with EPEC was based on the published *in vitro* organ culture (IVOC) protocols of Browning & Trier (1969), Knutton *et. al.* (1987) and Cleary *et. al.* (2004).

Full thickness sections of colon tissue were removed from surgical patients and washed thoroughly with sterile saline. The mucosal layer was separated from the muscularis, leaving strips of colonic mucosa with surface area of approximately 3.5 – 4.5cm^2. Mucosa strips were placed into universal containers containing R5 media (room temperature) supplemented with Nalidixic acid 0.005% and transferred to the laboratory in 10-20 minutes. Tissues were washed twice in warmed (37°C) sterile PBS then trimmed with scissors to produce roughly equally sized pieces of mucosa with surface area approximately 2 - 3cm^2. Each piece of mucosa was placed onto a flat rectangle of stainless steel mesh (United Wire, Edinburgh, UK) measuring approximately 4cm^2 and

manipulated with tweezers so that it was completely unfolded with the luminal side facing upward. The gauze was placed into a 6cm central well culture dish containing 2.5ml R5 media and Nalidixic acid 0.005%, warmed to 37°C. More media was added to the central well until the surface of the mucosa was covered to a depth of 1 – 2 mm.

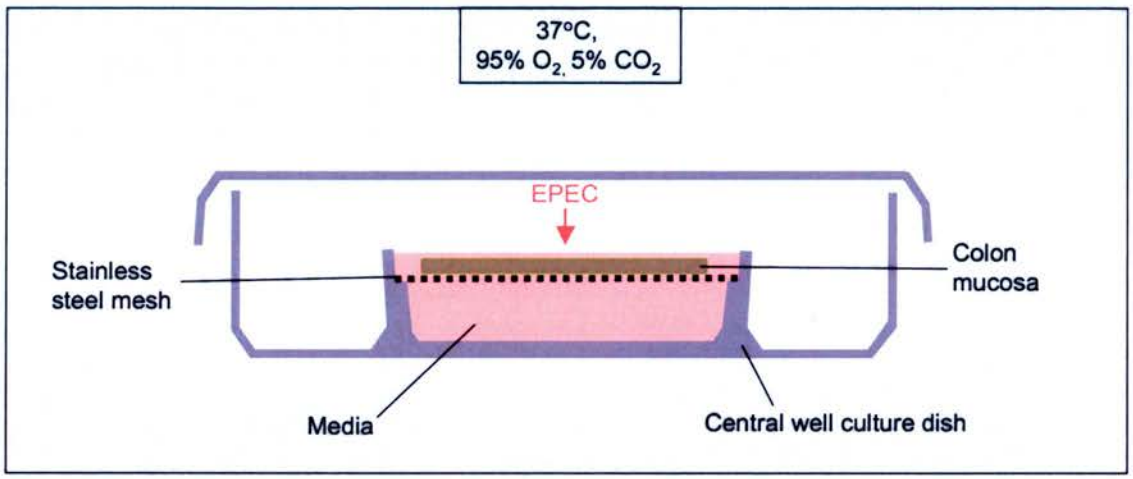


Figure 2.1. *In vitro* organ culture of *ex vivo* human colon mucosa.

Mucosa sections that were to be infected with EPEC were inoculated with 200ul of infected media directly onto the surface of the mucosa. Dishes were covered and placed into a modular incubator chamber (Billups-Rothenberg, CA) that was closed, gassed with 5% CO_2 in O_2 (BOC Medical Supplies, Guildford, UK) sealed and placed into a bench top oven set to 37°C. At three hour intervals tissues were removed from their dishes (by grasping metal gauze with forceps), washed twice in sterile PBS and transferred to a fresh 6cm central well culture dish containing 2.5ml R5 media supplemented with Nalidixic acid 0.005%, warmed to 37°C. More media was added to the central well until the surface of the tissue was covered to a depth of 1 – 2 mm.

2.6.5. Fixing and embedding tissue

At the relevant time-points the mucosa sections were removed from their culture dishes, washed twice in sterile PBS and submerged (whilst still attached to the metal gauze) in 4% buffered formalin (Table 2.14). Tissue was allowed to fix for 14-16 hours then separated from the gauze and transferred to 70% ethanol in ddH_2O. Within 48 hours, fixed tissues were taken to the Histopathology Laboratories at Queens Medical

Research Centre where they were embedded in paraffin, cut into 3μm sections and placed onto glass microscope slides for immunohistochemistry.

2.7. Identifying AE *E. coli* in human adenocarcinoma and normal colon

2.7.1. Tissue

Formalin fixed, paraffin embedded tissue sections were provided by the Histopathology Department, University of Edinburgh, New Royal Infirmary of Edinburgh. Sections of normal and adenocarcinoma tissue from 20 colorectal cancer patients were used. Sections were cut to 3μm for immunofluorescence staining and 20μm for PCR.

2.7.2. DNA isolation and PCR for *eae*

Paraffin embedded tissue sections were subjected to proteinase K digestion in lysis buffer (Table 2.11) for 48 hours at 55°C, then phenol chloroform extraction of DNA (Phenol: Chloroform: Isoamyl alcohol, Ambion, Cambridge, UK) followed by ethanol precipitation in the presence of yeast transfer RNA. Primers for *eae* were: forward 5' GTG ACG ATG GGG ATC GAT and reverse 5' GGC TCA ATT TGC TGA GAC CAC GGT T (product 110bp) or 5' ACG GCT GCC TGA TAA TGT T (150bp product). PCR cycle conditions: 94°C 2min, (94°C 30 sec, 58°C 30sec) x 30 cycles. PCR primers designed by Scott Bader.

PCR products were resolved through 3% agarose gels with ethidium bromide (Table 2.11). Products were visualised under UV light in a Bio-Rad Gel Doc (Bio-Rad Laboratories, Hercules, CA) in conjunction with Quantity One software version 4.6 (Bio-Rad Laboratories).

2.8. Immunofluorescence staining protocol

- Paraffin was removed and tissue was hydrated by washing slides sequentially in xylene (two 5 minute washes), 100% ethanol (two 5 minute washes), 70% ethanol (one 5 minute wash) and tap water (one 5 minute wash)
- Antigen retrieval was carried out by submerging slides in citrate buffer (Table 2.14), heating at full power in a 950 Watt microwave-oven for 25 minutes (buffer was topped up every 6-8 minutes) and allowing slides to cool for one hour at room temperature
- Slides were then rinsed in deionised water and placed onto plastic Sequenza clips (Shandon, Cheshire, UK) and slotted into a Sequenza immunostaining rack (Shandon, Cheshire, UK)
- A solution of 1% Triton-X100 was added (to enhance tissue permeability) for 20 minutes
- Slides were washed with PBS (two 5 minute washes)
- PBA blocking buffer was added for 1 hour
- Slides were washed with PBS (two 5 minute washes)
- Primary antibodies (Table 2.15) were diluted in PBS and added for the relevant time-course
- Slides were washed with PBS (three 5 minute washes)
- Secondary antibodies (Table 2.15) were diluted in PBS and added for 30 minutes (after addition of the secondary antibody slides were protected from prolonged exposure to light using metal foil)
- Slides were washed with PBS (three 5 minute washes)
- Slides were removed from the plastic clips, excess PBS was wiped away
- Cover-slips were mounted onto slides using Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA)
- Slides were placed into holders, covered in metal foil and stored at 4°C.

Solution	Composition
Paraformaldehyde (PFA)	Paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) 4% in PBS.
Buffered formalin	Formaldehyde solution, for molecular biology, 36.5% (Sigma, Poole, UK) diluted in PBS to 4%.
Citrate buffer	Citric acid, trisodium salt dihydrate, 99% (Arcos Organics, Geel, Belgium) 0.3% in ddH_2O, pH adjusted to 6.4.

Table 2.14. Immunocytochemistry and immunohistochemistry solutions

Antibody	Clone	Manufacturer	Raised in	Conc.	Time
Anti-*E. coli*	-	Europa Bioproducts (Cambridge, UK)	Rabbit (polyclonal)	1 in 2000	1 hour
Anti-Ki67	Mib1	Dako Cytomation (Glostrop, Denmark)	Mouse (monoclonal)	1 in 500	2 hours
Anti-Ezrin	-	BD Biosciences (Oxford, UK)	Mouse (monoclonal)	1 in 500	Overnight (4°C)
Fitc conjugated Alexa-fluor 488 anti-mouse IgG	-	Invitrogen Molecular Probes, Oregon USA	Goat	1 in 2000	1 hour
Texas red conjugated Alexa-fluor 594 anti-rabbit IgG	-	Invitrogen Molecular Probes, Oregon USA	Goat	1 in 2000	1 hour

Table 2.15. Primary and secondary antibodies used for immunofluorescence staining of human tissue

2.9. Haematoxylin and eosin (H&E) staining

- Paraffin was removed and tissue was hydrated by washing slides sequentially in xylene (two 5 minute washes), 100% ethanol (two 5 minute washes), 70% ethanol (two 5 minute washes) and tap water (one 5 minute wash)
- Slides were placed in Harris Haematoxylin for 5 minutes, then washed in water for 30 seconds
- Slides were then placed in acid alcohol solution (0.5% v/v HCl in 70% v/v ethanol) for 30 seconds, then washed in water for 30 seconds
- Next, slides were placed in Lithium carbonate solution (Li_2CO_3 0.5% in ddH_2O) for 30 seconds, then washed in water for 30 seconds

- Slides were then stained with eosin aqueous solution 1% (BDH Laboratory Supplies, Poole, UK) for 20 seconds, then washed in water for 30 seconds
- Finally, slides were dehydrated by reversing the alcohol gradient listed above and cover-slips were mounted onto slides with Pertex mounting solution (CellPath, Newtown, UK)

2.10. Bright-field image capture

Images of H&E stained tissue were captured using an Olympus BX51TF microscope (Olympus Optical, Japan) in conjunction with Olympus DP-Soft Software.

Chapter 3.

Development and validation of an extended time-course *in vitro* model for EPEC infection.

3.1. Introduction

3.1.1. *In vitro* EPEC co-culture models

An *in vitro* co-culture model provides a convenient means to study the molecular consequences of EPEC infection on human epithelial cells. Review of the literature describing *in vitro* co-culture of EPEC with human cells reveals two notable trends; firstly, the majority of reports use 3-4 hour co-culture protocols; and secondly, the effects of EPEC infection change with time and cell type. Good examples are studies analysing the effect of EPEC on NF-κB. Savkovic *et. al.* (1997) report on EPEC induced activation of NF-κB, showing a large increase in nuclear accumulation in T84 cells after 1 hour, however, after only 3 hours, nuclear NF-κB was significantly diminished. Melo *et. al.* (2005) co-cultured EPEC with HeLa cells for 1-4 hours and found that nuclear NF-κB signal was maximal after 2-3 hours, decreasing thereafter. The hypothesis that EPEC infection has the ability to increase susceptibility of colonic epithelial cells to neoplastic transformation is based on the assumption of chronic, asymptomatic intestinal colonisation. Hence the initial aim of this study was to establish an extended time-course *in vitro* co-culture model using a biologically relevant cell line.

The time-course of any experiment that co-cultures bacteria with mammalian cells is fundamentally limited by the non-specific toxicity eventually generated by bacterial overgrowth. Specific bacterial effects may also shorten the co-culture period before adherent mammalian cells undergo apoptosis / necrosis or other changes which result in loss of adhesion. EPEC infection has the potential to induce both specific and non-specific host cell alterations that would potentially limit the co-culture period. These include loss of adhesion due to focal adhesion / tight junction / adherens junction disruption (Shifrin *et. al.,* 2002; McNamara *et. al.,* 2001; Malladi *et. al.,* 2004) or possible induction of cell death (Abul-Milh *et. al.,* 2001; Melo *et. al.,* 2005). Therefore a key aspect of developing an extended time-course model was minimising non-specific toxicity.

Reference	Topic	Time-course	MOI	Cell type
Hardwidge *et. al.*, 2004	Cell death	4 h	50:1	Caco-2
Abul-Milh *et. al.*, 2001	Cell death	5 h	100:1	Caco-2, HEp-2, HeLa
Melo *et. al.*, 2005	Cell death	4 h	-	HeLa
Crane *et. al.*, 1999	Cell death	1-3 h	-	T84, HeLa
Nougayrede & Donnenberg, 2004	EspF signalling	1-3 h	100:1	Caco-2
Crane *et. al.*, 2004	PKC activation	30 – 60 min.	100-200:1	T84, HeLa
Kenny & Finlay 1997	PLC activation	2.5 – 3 h	100:1	HeLa
Cleary *et. al.*, 2004	Bfp effects	3-6 h*	-	HEp-2, Caco-2

Table 3.1. Representative sample of published protocols for co-culture of EPEC with adherent cell monolayers. Caco-2 & T84 derived from colon cancers, HeLa derived from cervical cancer and HEp-2 from laryngeal cancer. * In this study cells co-cultured with EPEC for 6 hours were washed after 3 hours. Where stated, the initial multiplicity of infection (MOI) (i.e. approximate number of bacteria per human cell) is included.

3.1.2. Cell lines

The literature demonstrates that EPEC can intimately attach to cells derived from the epithelia of different organs (Table 3.1). In order to achieve a biologically relevant model, cell lines derived from the human colon were used in this study. HT29 cells represent a good model as they are derived from human colonic (adenocarcinoma) cells and form adherent monolayers displaying tight junctions and a brush border (Chantret *et. al.*, 1988). In genetic terms HT29 cells also represent a good model as they express the proteins we hope to analyse i.e. DNA MMR repair (Wheeler *et. al.*, 1999), β-catenin and cell adhesion proteins (Efstathiou *et. al.*, 1998). However, like many colon cancer cell lines (and especially those that have wild-type MMR genes) HT29 cells carry mutations in *APC* (Rowan *et. al.*, 2000) that may effect the regulation of the proteins of interest (e.g. β-catenin). Repeat co-culture experiments were performed with SW480 (and in one instance LS513) cells to help confirm the effects of EPEC. SW480 cells were derived from a human colonic adenocarcinoma (Chantret *et. al.*, 1988) and LS513 cells were derived from a human mucin-secreting cecal tumour (Suardet *et. al.*, 1992). HT29, SW480 and LS513 cells are all classed as microsatellite stable, i.e. they have no mismatch repair defects and have no MSI (Gayet *et. al.*, 2001).

3.1.3. Bacterial strains

EPEC E2348/69 is a wild-type strain isolated from an outbreak of diarrhoea in Taunton in 1969 (Gross *et. al.,* 1976). It is the classic wild-type EPEC strain, and is commonly used experimentally. E2348/69 carries complete copies of both the EAF plasmid, encoding bundle forming pili, and the LEE pathogenicity island, encoding the TTSS, EPEC secreted effector proteins and *eae* (Cleary *et. al.,* 2004). EPEC UMD864 is an experimentally generated isogenic mutant of E2348/69, which does not express the LEE encoded EPEC secreted protein EspB (Taylor *et. al.,* 1998). UMD864 is therefore unable to form a functional TTSS, hence it cannot translocate effector proteins into host cells so is unable to achieve intimate attachment. UMD864 was used as a negative control, to help identify whether the effects of wild-type EPEC were specific (i.e. dependent on secreted effector molecules and / or intimin signalling) or non-specific (i.e. induced by alteration to cell environment / contact with Bfp, bacterial filaments or other factors).

3.1.4. FAS test

Initial validation of the *in vitro* model included evaluation of cell detachment and the ability of cells to survive post-infection. It was also important to ensure that EPEC strains were displaying their relative phenotypes *in vitro*. A convenient test for AE lesion formation by wild-type EPEC is the fluorescent actin-staining (FAS) test (Knutton *et. al.,* 1989). This test utilises the phallotoxin phalloidin, which binds with high affinity and specificity to filamentous actin *in vitro*. Hence fluorescein isothiocyanate (fitc) labelled phalloidin binds to the AE lesions, displaying intense spots of fluorescence indicating site-specific concentration of cytoskeletal actin below adherent bacterium. This is a highly sensitive diagnostic test for AE bacteria.

3.1.5. Mass spectrometry

Co-culture of wild-type EPEC with human colonic cells has a wide range of effects on the host cell proteome after 4 hours, causing the up-regulation and down-

regulation of hundreds of proteins (Hardwidge *et. al.,* 2004). As a further validation of our *in vitro* model, and to gain an insight into the molecular effects of EPEC, host cell protein expression was analysed subsequent to infection. Protein separation by electrophoresis followed by mass spectrometry represents a crude but rapid means of proteomic analysis. In the proteomic context, mass spectrometry was initially used to analyse protein structure and to detect errors in translation or post-translation changes in known proteins. This is achieved by measuring the mass of peptides cleaved from the protein by proteases, changes in peptide masses reflect protein structural or sequence changes. This approach has generated a database of accurate molecular weight 'fingerprints' for many proteins. Comparison of the molecular weight fingerprints of unknown sample proteins to this database has subsequently enabled the identification of proteins via mass spectrometry (Pappin *et. al.,* 1993). Matrix-assisted laser desorption time of flight (Maldi-tof) mass spectrometry is appropriate for analysing peptides as it utilises a 'soft' ionisation technique suitable for thermo-labile high molecular weight compounds.

3.1.6. Gene expression microarray

Further validation of our model, and insight into the effects of EPEC infection was gained by studying gene expression in host cells. Gene expression may be analysed by reference to cellular mRNA levels (indicating the level of gene transcription), microarray analysis is a recently developed technique that utilises this approach. Microarray analysis is achieved by exposing a fixed array of probe DNA nucleotides (corresponding to regions of all human genes) to tagged, amplified cDNA transcribed from mRNA isolated from the sample. Hybridisation of transcribed cDNA nucleotides to the array probes identifies the genes being expressed and the amount of corresponding mRNA. Comparison of mRNA levels between control and test samples allows relative changes in mRNA and hence gene expression to be quantified.

3.2. Results

3.2.1. HT29 cells were co-cultured with wild-type EPEC for 12 hours and survived post infection

The initial objective was to control bacterial growth in order to extend the co-culture period and hence analyse the longer-term molecular effects of EPEC infection on host cells. By washing cells three times every 3 hours to remove non-adherent bacteria, and adding fresh media it was possible to maintain co-culture conditions for 12 hours. Detachment of HT29 cells was observed, particularly after 12 hours co-culture and subsequent washing with PBS, however, a significant proportion of cells remained attached (Figure 3.1a). Post-infection antibiotic treatment of HT29 cells co-cultured with wild-type EPEC for 12 hours allowed them to recover and survive long term (Figure 3.1b).

It was notable that during the recovery period, cells temporarily continued to lose adherence from the culture flask. Hence, although most cells were still adherent after 12 hours infection, only ~50% of cells were still adherent after 84 hours treatment with antibiotics. It was also evident that subsequent to infection, cell division was inhibited, with cells not dividing until 60 hours post antibiotic treatment. Due to this effect, nine days of recovery were needed before cells returned to pre-infection confluency (~95%). These observations are likely to reflect the time taken to remove all of the bacteria from culture and for any inhibitory cellular changes within host cells to be reversed.

3.2.2. Co-culture with EPEC induced actin rearrangement in HT29 cells

Fluorescein isothiocyanate (fitc) labelled phalloidin was used to perform the fluorescent actin staining (FAS) test (Knutton *et. al.,* 1989) on HT29 cells co-cultured with wild-type EPEC (Figure 3.2). Images show the change in actin distribution brought about by intimate attachment of EPEC to the host cells. That is, the re-distribution of actin from the cell periphery (as seen in uninfected cells) to

discrete (bright green) clusters in the cytoplasm that co-localise with EPEC in the apical focal plane. This test confirms the formation of the actin rich pedestal structure that is characteristic of AE bacterial infection *in vitro*.

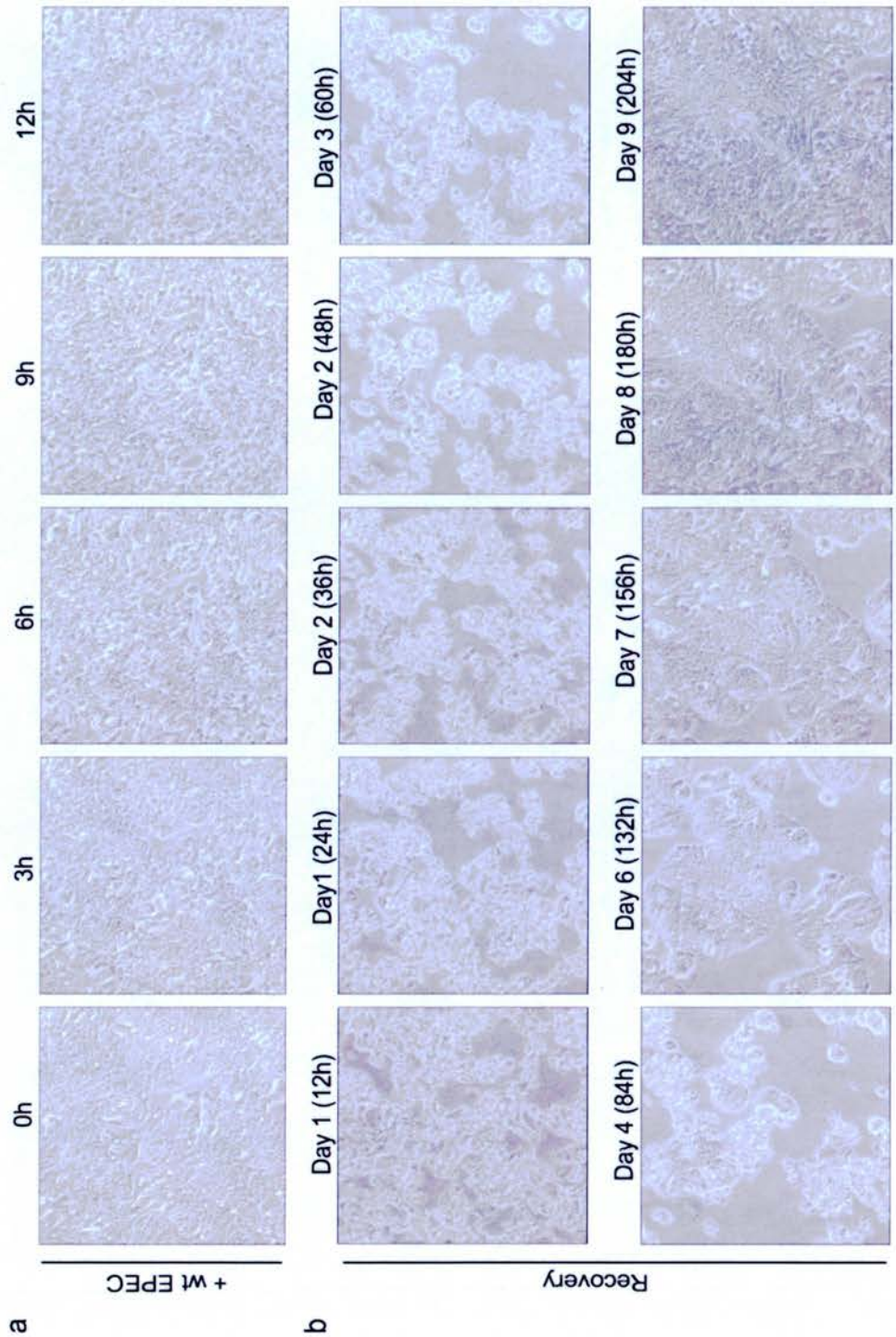


Figure 3.1. Co-culture of HT29 cells with EPEC and subsequent recovery from infection. HT29 cells were co-cultured with wild-type (E2348/69) EPEC for 12 h (**a**), then treated with antibiotics (Penicillin, Streptomycin & Gentamicin) for 9 days to remove EPEC (**b**). Time in brackets refers to hours of treatment with antibiotics i.e. exact recovery period. All photographs were taken of the same field.

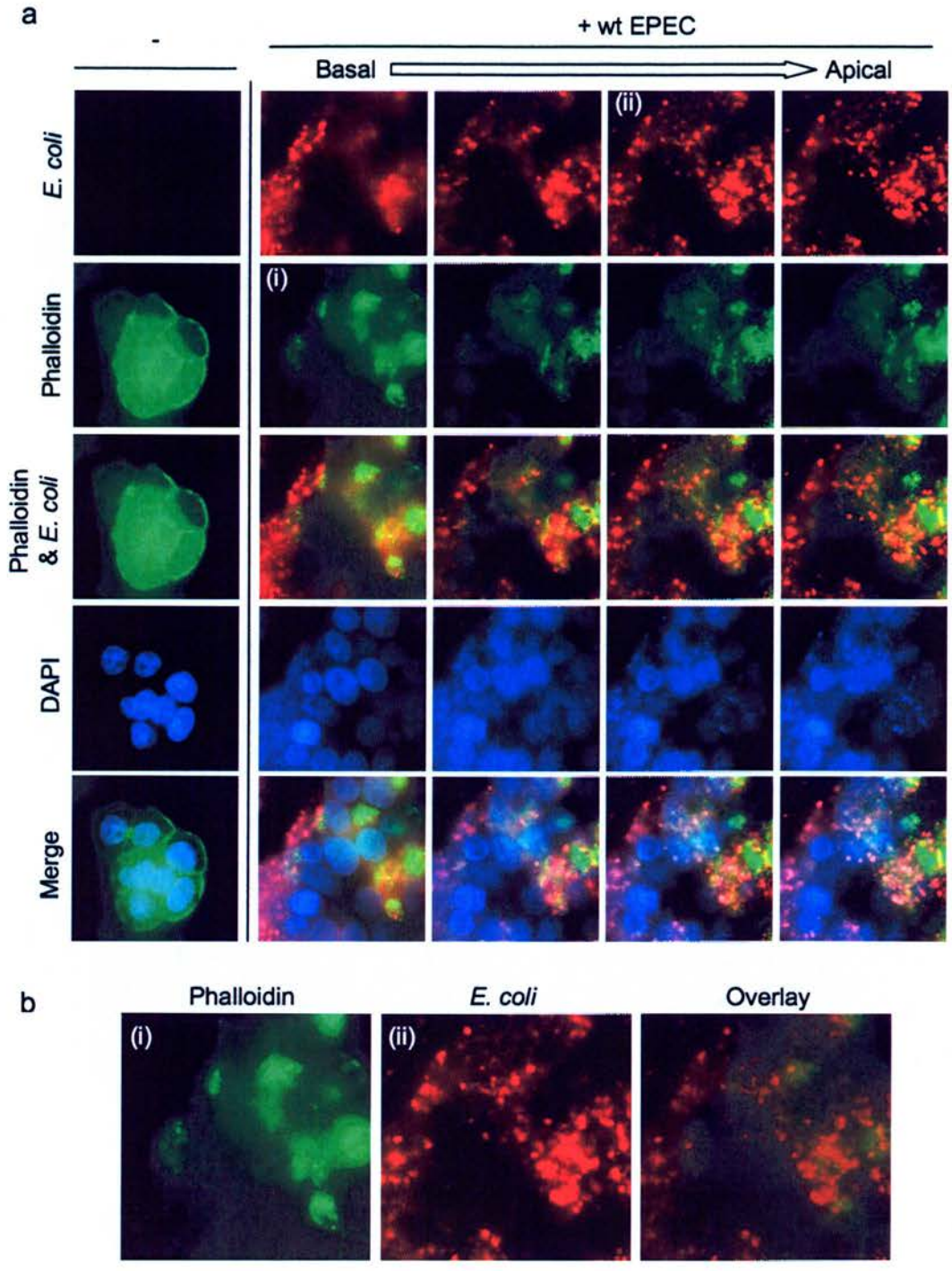


Figure 3.2. Fluorescent actin staining (FAS) test. HT29 cells were either uninfected (-) or co-cultured with wild-type EPEC for 12 hours (+ wt EPEC). Cells were stained with fitc conjugated phalloidin for F-actin (green) and immunostained for *E. coli* (red), nuclei were counter stained with DAPI (blue). Focal plane in infected cells moves from basal to apical planes (a). Different focal planes (i) & (ii) are required to observe actin and EPEC co-localisation (b). Original Magnification x 100.

3.2.3. Co-culture with EPEC for 12 hours caused up and down-regulation of HT29 cell proteins

Whole cell protein extracts of HT29 cells that were uninfected or co-cultured with wild-type EPEC for 12 hours were subjected to 1D gel electrophoresis followed by coumassie staining. A number of crude changes in protein band pattern after 12 hours co-culture were observed (Figure 3.3a). Changes included the apparent up- and downregulation of protein bands, in addition to bands showing relatively constant expression. The protein content of selected bands was analysed via mass spectrometry (Figure 3.3b).

Proteins belonging to a wide range of functional categories, including actin-binding, cytoskeletal, metabolic, cell-cell adhesion, DNA repair, signal transduction and protein synthesis were identified. A number of proteins that were identified in uninfected HT29 cells were not detected in matched bands from cells co-cultured with EPEC, suggesting that EPEC infection caused their downregulation (Table 3.2). Conversely, a range of proteins that were not detected in uninfected cells, were identified in matched bands from EPEC infected cells. Hence the expression of these proteins was potentially upregulated by EPEC infection (Table 3.3). Several proteins were also identified in matched bands taken from infected and uninfected cells, suggesting that the expression of these proteins remained relatively constant during infection (Table 3.4). Proteins identified from a number of bands that showed high expression in EPEC infected cells contained *E. coli* proteins (Table 3.5).

These results suggest that after 12 hours, EPEC continues to have differential effects on expression of host cell proteins with a wide range of functions. These results also demonstrate that the proteomic consequences of EPEC infection are complex, with proteins belonging to the same functional category (e.g. actin binding) showing upregulated, downregulated or unchanged expression simultaneously.

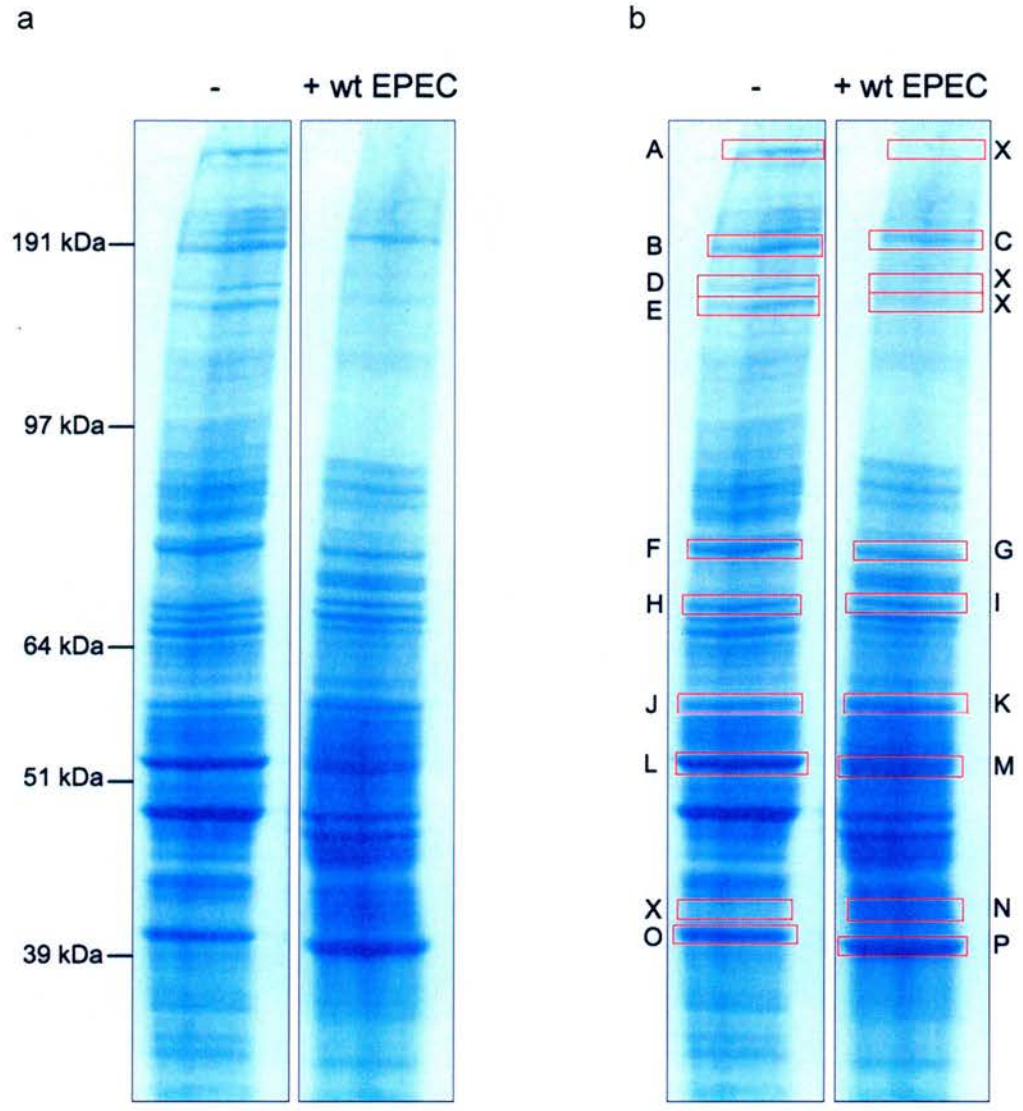


Figure 3.3. Effect of EPEC on protein expression in HT29 cells. Cells were either uninfected (-) or co-cultured with wild-type (E2348/69) EPEC for 12 h. Whole cell protein extracts were obtained by lysis with RIPA buffer and resolved through pre-cast 4-12% gradient Novex NuPAGE gels. Protein was stained non-specifically with coumassie blue stain (**a,b**). Bands enclosed with boxes (**b**) were subjected to mass spectrometry (see Tables 3.2 - 3.5). Protein bands were cut from gels, digested with trypsin and subjected to MALDI-TOF mass spectrometry. Protein matches were identified via the MS-Fit SwissProt. database (http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm). Information on protein function and processes downloaded from NCBI Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

Band	Matched proteins	Accession No.	MOWSE score	Masses matched	Protein function / processes
F	Heat shock protein HSP 90-beta (& alpha)	P08238	2.33e+16	26%	Molecular chaperone for signal transduction proteins and actin
D	Ras GTPase activating-like protein	P46940	6.15e+15	23%	Regulation of cell morphology & motility
A	Spectrin alpha chain, brain (& beta chain, brain1)	Q13813	5.54e+10	22%	Actin cross-linking molecular scaffold protein
E	Mitogen-activated protein kinase kinase kinase 4	Q9Y6R4	1.03e+6	11%	Environmental stress response
E	NEDD4-binding protein 2 / BCL-3 binding protein	Q86UW6	9.70e+5	9%	Possible role in transcription coupled DNA repair or genetic recombination
J	Serine/threonine-protein kinase 3	Q13188	4.41e+5	10%	Part of MAPK cascade; positive regulation of apoptosis
L	Tryptophanyl-tRNA synthase, cytoplasmic	P23381	3.87e+5	7%	Catalyses aminoacetylation of tRNA, negative regulation of proliferation
H	Calnexin precursor	P27824	3.60e+5	8%	Facilitates folding & assembly of newly formed proteins
F	BRCA1-associated RING domain protein 1	Q99728	2.84e+5	9%	Binds to BRCA1 aiding its tumour suppression properties

Table 3.2. Proteins potentially downregulated by EPEC infection. Proteins identified by mass spectrometry in protein bands from uninfected HT29 cells, but not in matched protein bands from HT29 cells infected with wild-type EPEC. Accession number refers to Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). Molecular weight search (MOWSE) score gives an indication of the validity of the protein match, the higher the score, the greater the probability of a correct match. The percentage of 'masses matched' represents the number of peptide masses in a sample that match peptide masses for a known protein held on the database.

Band	Matched proteins	Accession No.	MOWSE score	Masses matched	Protein function / processes
I	Heat shock 70kDa protein 5	P11021	4.67e+9	13%	Folding and assembly of proteins, protein transport monitoring
C	Zinc finger protein 106 homolog	Q9H2Y7	7.04e+6	11%	Metal ion and nucleic acid binding
C	Myosin IIIA	Q8NEV4	2.69e+6	11%	Actin & ATP binding, protein amino acid phosphorylation
C	Pleckstrin homology domain-containing family G member 1	Q9ULL1	2.40e+6	9%	Regulation of Rho protein signal transduction
C	Probable phospholipids-transporting ATPase IK	O60423	1.72e+6	7%	Phospholipid-translocating ATPase activity
M	Ectonucleotide pyrophosphatase / Phosphodiesterase 5 precursor	Q9UJA9	4.53e+5	5%	Integral to membrane, involved in nucleotide metabolic processes
G	Peptidylprolyl isomerase domain & WD repeat containing protein1	Q96BP3	3.12e+5	10%	RNA splicing, mRNA processing and protein folding
I	Propionyl-CoA carboxylase alpha chain, mitochondrial precursor	P05165	1.52e+5	9%	Subunit of mitochondrial enzyme involved in fatty acid metabolism
I	Ectodem-neral cortex 1 protein / p53-induced protein 10	O14682	1.02e+5	8%	A p53 induced protein (PIG), binds protein & actin

Table 3.3. Proteins potentially upregulated by EPEC infection. Proteins identified by mass spectrometry in protein bands from HT29 cells infected with wild-type EPEC, but not in matched protein bands from uninfected HT29 cells.

Bands	Matched proteins	Accession No.	MOWSE score	Masses matched	Protein function / processes
B & C	Myosin-9 (Non-muscle myosin heavy chain IIa)	P35579	3.00e+5 4.03e+11	13% 21%	Cell motility & migration, cell-cell adhesion, actin binding
H & I	Protein disulphide-isomerase A4 precursor	P13667	22793 1.20e+8	6% 13%	Cell redox homeostasis & protein secretion
L & M	ATP synthase subunit alpha, mitochondrial precursor	P25705	1.64e+6 1.47e+5	7% 7%	Catalyses ATP synthesis
J & K	60kDa heat shock protein, mitochondrial precursor	P10809	7.43e+10 8.60e+9	16% 13%	Folding & assembly of proteins newly imported into the mitochondria
O & P	Annexin A2	P07355	8.45e+9 4.09e+8	18% 15%	Regulation of cellular growth and signal transduction pathways
O & P	GAPDH	P04406	68971 1.21e+7	9% 7%	Carbohydrate metabolism

Table 3.4. Proteins whose expression is potentially unchanged by EPEC infection. Proteins identified by mass spectrometry in both protein bands from HT29 cells infected with wild-type EPEC, and in matched protein bands from uninfected HT29 cells (top row MOWSE score & masses matched refers to uninfected cells, lower row to EPEC infected cells).

Band	Matched proteins	Accession No.	MOWSE score	Masses matched	Protein function / processes
N	Elongation factor Tu	P0A6N1	2.86e+9	13%	Translation, response to osmotic stress
N	Enolase	P0A6P9	1.76e+8	10%	Anaerobic respiration; gluconeogenesis, glycolysis
G	*E.coli* elongation factor	P0A6M8	2.11e+13	22%	Translation, ribosomal structure & biogenesis
O	GAPDH-A	P0A9B2	1.02e+5	10%	Pyridoxine biosynthetic process, gluconeogenesis, glycolysis

Table 3.5. *E. coli* proteins identified in protein bands from HT29 cells co-cultured with EPEC. Proteins identified by mass spectrometry in protein bands from HT29 cells infected with wild-type EPEC, but not in matched protein bands from uninfected HT29 cells.

3.2.4. EPEC strains show different adherence patterns on HT29 cells

Immunofluorescent staining of EPEC revealed the crude pattern of wild-type and mutant EPEC adherence after 9 and 12 hours co-culture with HT29 cells (Figure 3.4). Wild-type EPEC shows widespread distribution after 9 hours co-culture, co-localising with virtually all cells. By 12 hours, wild-type EPEC had extensively colonised the surface of the HT29 cell monolayer. In contrast, after 9 hours, the mutant EPEC is distributed in small, discrete colonies. After 12 hours the colonies of mutant EPEC show increased size, however their distribution remains limited to discrete patches, covering no more than 1-4 cells.

These observations demonstrate the inability of the mutant EPEC to attach and colonise the host cells in the same way as the wild-type bacteria. This difference in adhesion is due to the absence of *espB*, the gene encoding EPEC secreted protein B (EspB), essential for the formation of the TTSS. These results indicate that without the TTSS this strain is only able to adhere to HT29 cells in a "localised adherence" pattern only. EspB expression and hence a functional TTSS allows widespread colonisation over a cell monolayer. For this reason UMD864 represents a good negative biological control for wild-type EPEC infection. Hence, differences in biological effects caused by mutant and wild-type EPEC infection represent changes brought about consequent to the insertion of EPEC secreted proteins and intimin signalling pathways.

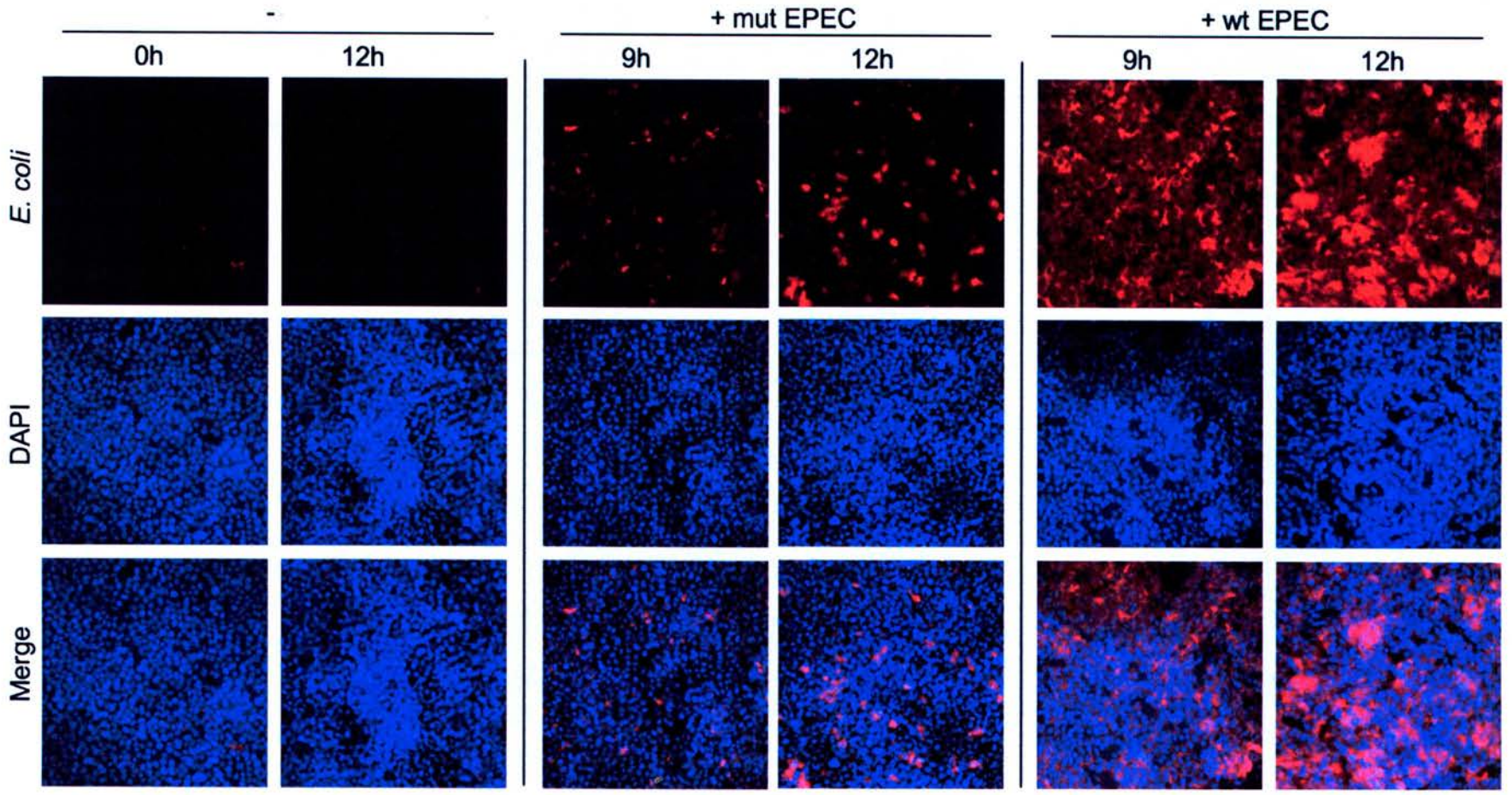


Figure 3.4. Immunofluorescent staining for *E. coli* in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC. Immunofluorescent staining of *E. coli* (red) and counterstaining of nuclei with DAPI (blue) identifies the pattern of EPEC infection. Original magnification x 16.

3.2.5. Co-culture with EPEC caused changes in gene expression in HT29 cells

Microarray analysis was performed on total mRNA extracts from HT29 cells that were either: uninfected and untreated (-0h), uninfected but washed in the same way as infected cells (-12h), infected with mutant EPEC for 9 hours (+mut 9h) or 12 hours (+mut 12h), or infected with wild-type EPEC for 9 hours (+wt 9h) or 12 hours (+wt 12h). Gene expression profiles from each condition were compared to the baseline condition (-12h) also; mutant and wild-type infected cells were compared directly (+mut 9h / +wt 9h and +mut12h / +wt 12h).

Internal microarray quality control measures validated the microarray analysis procedure (appendix). Differentially expressed genes were identified by comparing gene expression using conventional T-test and significance analysis of microarray (SAM) statistical tests (Figure 3.5). Tests were performed on raw data and on data that been normalised by quantiles normalisation or variance stabilisation normalisation (VSN). Choice of normalisation technique had a marked effect on the number of genes that were reported as up or downregulated after EPEC infection. VSN normalised data more closely resembled the raw data, where approximately 4-5 times as many genes were downregulated compared to those upregulated after wild-type EPEC infection. Quantiles normalised data showed that approximately equal numbers of genes were up and downregulated after wild-type EPEC infection. In either case, it is clear that these results support the ability of EPEC infection to have differential effects on gene expression after 9 – 12 hours infection. That is, while the expression of the vast majority of genes was not significantly changed by infection, many genes were upregulated or downregulated.

The effects of wild-type EPEC on gene expression were clearly greater (both in terms of up- and downregulated genes) than for mutant EPEC. This supports the role of EPEC secreted effector molecules and intimin signalling in influencing host cells gene expression and therefore host signalling pathways.

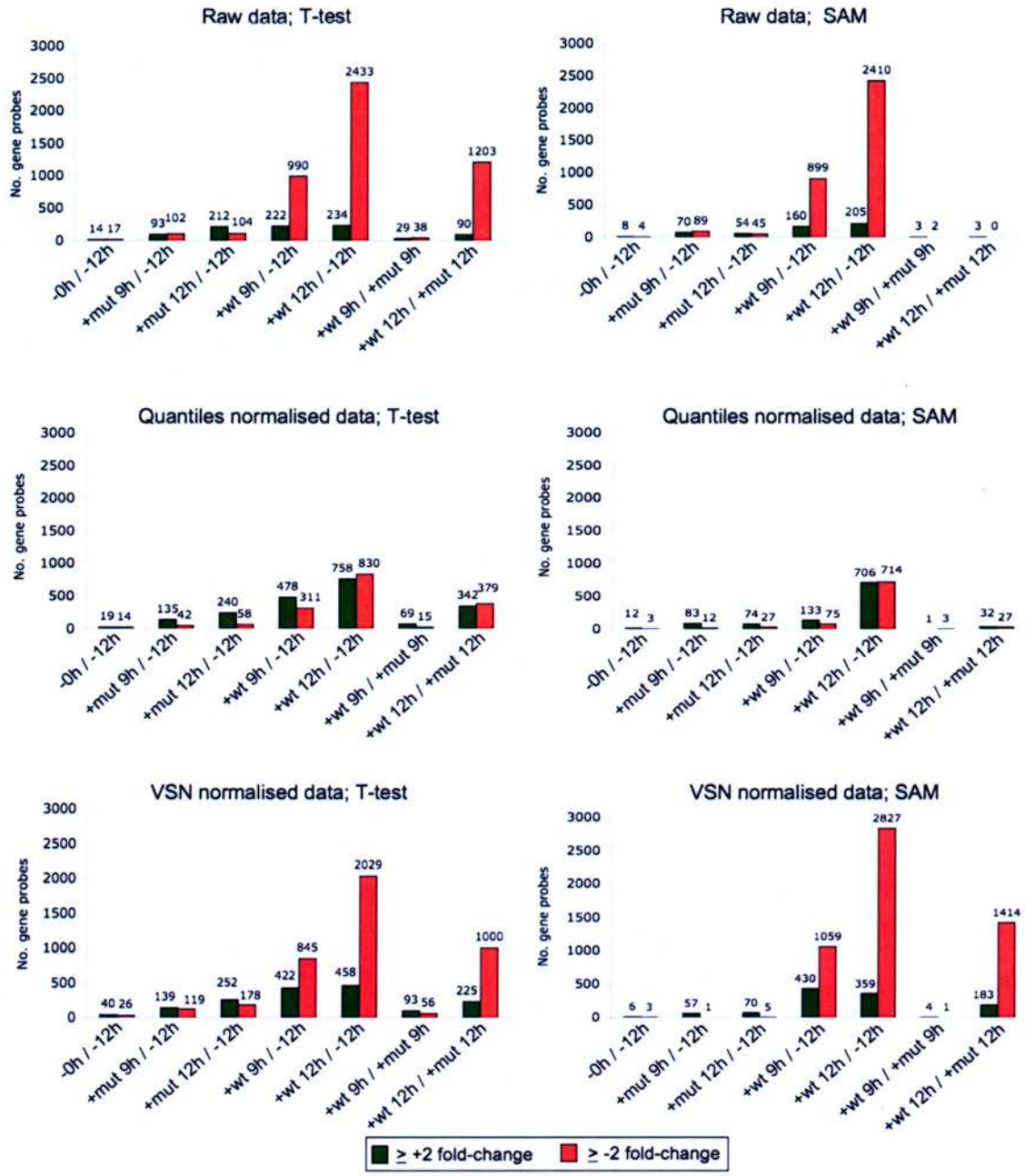


Figure 3.5. Quantification of differential gene expression in HT29 cells co-cultured with EPEC, using different statistical tests and normalisation methods. Data was either compared via a conventional T-test (P<0.05, ≥2 fold change, difference in expression ≥100) or significance analysis of microarray (SAM) test (false discovery rate = 0.1, ≥ 2 fold change). Raw data was normalised using Quantiles normalisation or VSN (Variance stabilisation normalisation) methods. Data includes characterised and uncharacterised genes.

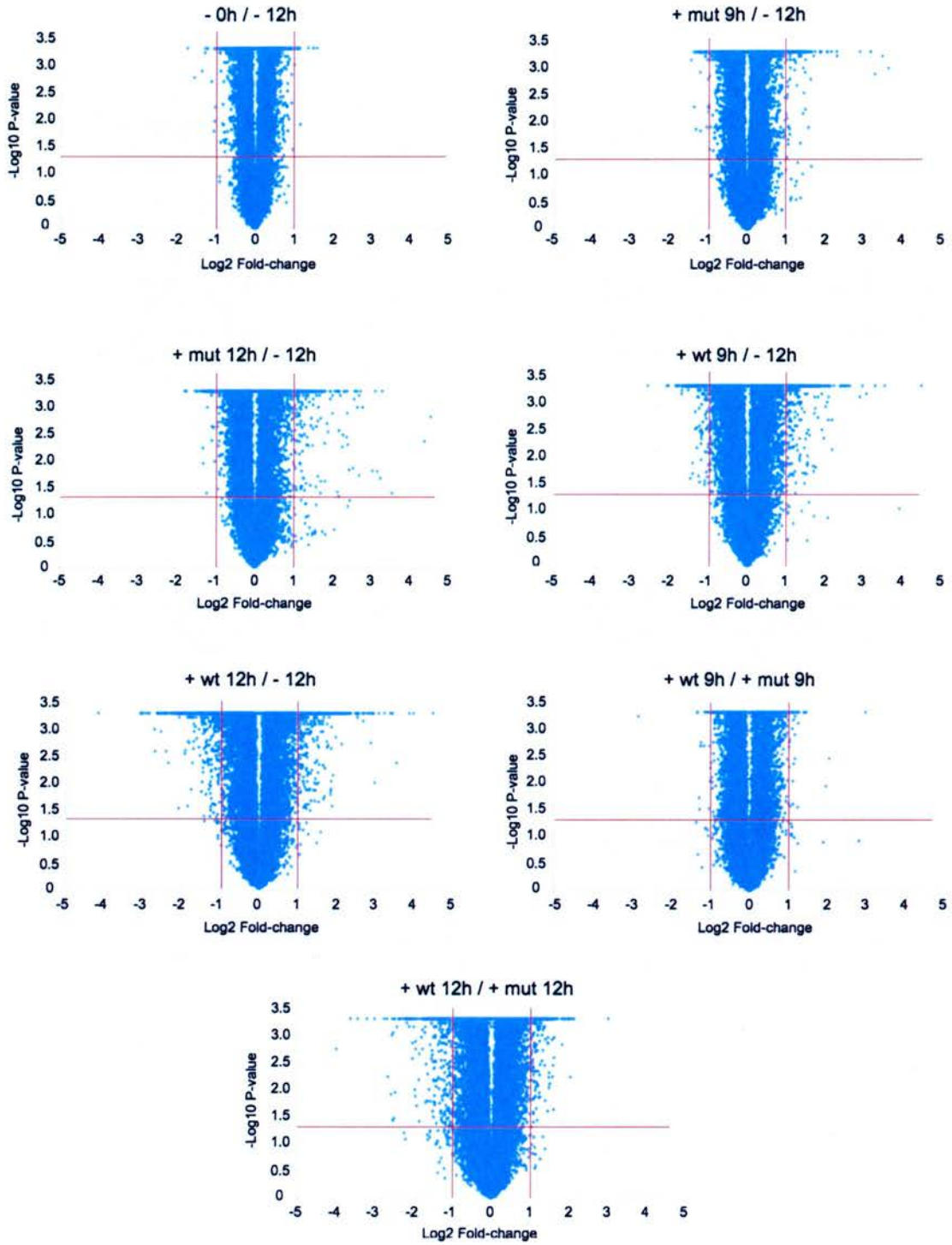


Figure 3.6. 'Volcano' scatter plots of gene expression in HT29 cells co-cultured with EPEC, using Quantiles normalisation and T-test. Genes above the horizontal red line have -Log10 P-value of > 1.3 (i.e. P-value < 0.05), genes to the left and right of corresponding vertical red lines have Log2 Fold-change (ratio) values > $\pm$ 1 respectively (i.e. fold-change > $\pm$2). Data includes characterised genes only, i.e. 25,503 gene probes. P-values below 0.0005 were rounded to 0.0005.

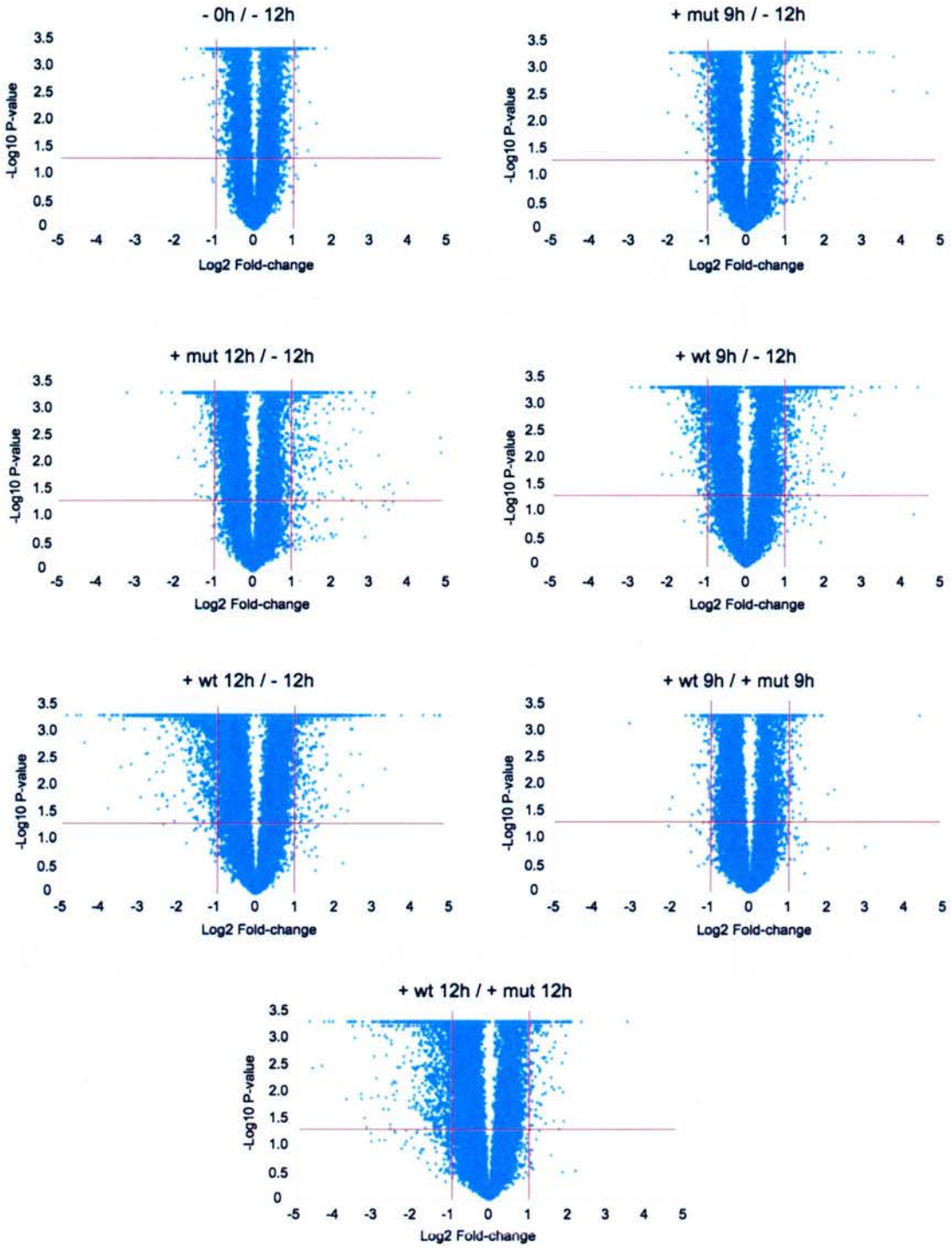


Figure 3.7. 'Volcano' scatter plots of gene expression in HT29 cells after co-culture with EPEC, using VSN normalisation and T-test. Genes above the horizontal red line have -Log10 P-value of > 1.3 (i.e. P-value < 0.05), genes to the left and right of corresponding vertical red lines have Log2 Fold-change (ratio) values > $\pm$1 respectively (i.e. fold-change > $\pm$2). Data includes characterised genes only, i.e. 25,503 gene probes. P-values below 0.0005 were rounded to 0.0005.

Volcano plots give an indication of the effect of EPEC infection on global gene expression in HT29 cells (Figures 3.6 and 3.7). Although it is clear that the expression of hundreds of genes is potentially altered subsequent to EPEC infection, it is also clear from these plots that expression of the overwhelming majority of genes was not significantly changed. It can be seen that the expression of a small number of genes was altered by the washing procedure (-0h / -12h), supporting the use of the uninfected, washed cells (-12h) as the baseline for differential expression analysis. It is also notable that when the mutant and wild-type EPEC infected cells are compared directly (e.g. +mut 12h / +wt 12h) there are differences in gene expression, demonstrating that effects of EPEC on the host cell can be intimate attachment specific.

Further analysis of the lists of differentially expressed genes was achieved by using bioinformatics database searches, based on enrichment of gene ontology terms. The results of these searches reveal a vast array of host pathways affected by both wild-type and mutant EPEC infection (Tables 3.6 and 3.7). Many pathways identified in the literature as being targets for EPEC induced effects (e.g. cell cycle, apoptosis, NF-κB, inflammation) were identified. Pathways involving proteins identified by mass spectrometry (section 3.2.3) in this study (e.g. MAP kinase, Ras, BRCA1) also showed altered gene expression. More detailed functional annotation clustering searches (see appendix) show more specific pathways influenced by EPEC infection. These include pro-and anti-apoptotic pathways and DNA metabolism, processing and repair.

Analysis of the effect of EPEC infection on individual mismatch repair genes (mutS and mutL homologues) identified specific changes due to EPEC infection (Tables 3.8 & 3.9). It was noticeable that wild-type EPEC consistently caused a 2-3.9 fold reduction in MSH2, MLH1 and MSH6 (the three MMR genes most commonly implicated in colorectal cancer development) gene expression. Although mutant EPEC also caused a general downregulation in the expression of these genes, the changes were lower and were not reported at all by the SAM test. Conversely, the expression of MSH3, MSH4, MSH5 and MLH3 was not significantly altered by

EPEC infection. The expression of the genes encoding the Wnt signalling and cell-cell adhesion proteins β- and γ-catenin show a similar pattern to MSH2, MLH1 and MSH6 (Tables 3.10 and 3.11). That is, wild-type EPEC infection induced a 2-3 fold reduction in the expression of these genes. Whereas, the genes encoding the functionally related molecules E-cadherin, α-catenin and ezrin remained relatively constant despite EPEC infection.

-0h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	-			
KEGG	ARGININE AND PROLINE METABOLISM	3	5.3	2.70E-02
+mut 9h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	CD40L Signaling Pathway	3	1.4	2.70E-02
BIOCARTA	TNFR2 Signaling Pathway	3	1.4	3.80E-02
BIOCARTA	p53 Signaling Pathway	3	1.4	3.80E-02
BIOCARTA	Influence of Ras and Rho proteins on G1 to S Transition	3	1.4	7.40E-02
BIOCARTA	NFkB activation by Nontypeable Hemophilus influenzae	3	1.4	7.40E-02
KEGG	CELL CYCLE	11	5	2.30E-05
KEGG	LIMONENE AND PINENE DEGRADATION	4	1.8	1.80E-02
KEGG	ETHYLBENZENE DEGRADATION	3	1.4	3.90E-02
KEGG	HISTIDINE METABOLISM	4	1.8	6.30E-02
KEGG	BUTANOATE METABOLISM	4	1.8	7.20E-02
KEGG	1- AND 2-METHYLNAPHTHALENE DEGRADATION	3	1.4	7.60E-02
KEGG	VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	4	1.8	7.90E-02
KEGG	TYROSINE METABOLISM	4	1.8	8.90E-02
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	4	1.8	9.90E-02
+mut 12h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Cadmium induces DNA synthesis and proliferation in macrophages	3	0.8	7.70E-02
BIOCARTA	p53 Signaling Pathway	3	0.8	8.60E-02
BIOCARTA	METS affect on Macrophage Differentiation	3	0.8	8.60E-02
BIOCARTA	Hypoxia-Inducible Factor in the Cardiovascular System	3	0.8	9.40E-02
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	6	1.7	2.30E-02
KEGG	CELL CYCLE	8	2.2	2.70E-02
KEGG	B CELL RECEPTOR SIGNALING PATHWAY	6	1.7	3.30E-02
KEGG	CYTOKINE-CYTOKINE RECEPTOR INTERACTION	13	3.7	3.30E-02
KEGG	ADIPOCYTOKINE SIGNALING PATHWAY	6	1.7	3.70E-02
KEGG	LIMONENE AND PINENE DEGRADATION	4	1.1	5.00E-02
KEGG	DITERPENOID BIOSYNTHESIS	2	0.6	7.60E-02
KEGG	ETHYLBENZENE DEGRADATION	3	0.8	7.90E-02
+wt 9h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	CDK Regulation of DNA Replication	4	0.4	3.80E-03
BIOCARTA	Cell Cycle	5	0.5	4.30E-02
BIOCARTA	ATM Signaling Pathway	4	0.4	5.40E-02
BIOCARTA	Oxidative Stress Induced Gene Expression Via Nrf2	4	0.4	6.80E-02
KEGG	CELL CYCLE	25	2.5	1.50E-08
KEGG	PYRIMIDINE METABOLISM	19	1.9	8.00E-06
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	11	1.1	3.00E-03
KEGG	HISTIDINE METABOLISM	9	0.9	9.40E-03
KEGG	RNA POLYMERASE	6	0.6	2.10E-02
KEGG	PURINE METABOLISM	17	1.7	2.30E-02
KEGG	ARGININE AND PROLINE METABOLISM	8	0.8	4.50E-02
KEGG	FRUCTOSE AND MANNOSE METABOLISM	7	0.7	4.80E-02
KEGG	SELENOAMINO ACID METABOLISM	6	0.6	4.90E-02
KEGG	NITROBENZENE DEGRADATION	4	0.4	6.20E-02

(Table 3.6. part 1)

+wt 12h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Cyclins and Cell Cycle Regulation	9	0.5	1.50E-03
BIOCARTA	Cell Cycle	9	0.5	6.50E-03
BIOCARTA	Oxidative Stress Induced Gene Expression Via Nrf2	7	0.4	1.40E-02
BIOCARTA	Hypoxia and p53 in the Cardiovascular system	7	0.4	3.10E-02
BIOCARTA	Regulation of p27 Phosphorylation during Cell Cycle Progression	5	0.3	3.70E-02
BIOCARTA	Spliceosomal Assembly	5	0.3	5.80E-02
BIOCARTA	Role of Mitochondria in Apoptotic Signaling	6	0.3	6.00E-02
BIOCARTA	BTG family proteins and cell cycle regulation	4	0.2	6.10E-02
BIOCARTA	AKAP95 role in mitosis and chromosome dynamics	4	0.2	6.10E-02
BIOCARTA	Apoptotic Signaling in Response to DNA Damage	6	0.3	7.00E-02
BIOCARTA	Cell Cycle	6	0.3	7.00E-02
BIOCARTA	Activation of Src by Protein-tyrosine phosphatase alpha	4	0.2	7.90E-02
BIOCARTA	p53 Signaling Pathway	5	0.3	8.40E-02
KEGG	CELL CYCLE	40	2	6.50E-12
KEGG	PYRIMIDINE METABOLISM	27	1.4	6.30E-06
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	19	1	6.90E-05
KEGG	FRUCTOSE AND MANNOSE METABOLISM	13	0.7	2.70E-03
KEGG	SELENOAMINO ACID METABOLISM	11	0.6	2.90E-03
KEGG	PURINE METABOLISM	30	1.5	3.30E-03
KEGG	NITROBENZENE DEGRADATION	7	0.4	4.70E-03
KEGG	AMINOPHOSPHONATE METABOLISM	8	0.4	4.80E-03
KEGG	RNA POLYMERASE	9	0.5	7.50E-03
KEGG	HISTIDINE METABOLISM	13	0.7	7.70E-03
KEGG	PYRUVATE METABOLISM	11	0.6	1.60E-02
KEGG	ONE CARBON POOL BY FOLATE	6	0.3	2.30E-02
KEGG	GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	7	0.4	2.50E-02
KEGG	OXIDATIVE PHOSPHORYLATION	24	1.2	2.60E-02
KEGG	GLYCEROPHOSPHOLIPID METABOLISM	14	0.7	7.70E-02
KEGG	ARGININE AND PROLINE METABOLISM	11	0.6	8.00E-02
KEGG	GLYCOSPHINGOLIPID METABOLISM	9	0.5	9.40E-02
+wt 9h / +mut 9h				
Database	Pathway	Count	%	P_value
BIOCARTA	IL 17 Signaling Pathway	2	2	9.00E-02
KEGG	NITROBENZENE DEGRADATION	2	2	9.30E-02
+wt 12h / +mut 12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	8	0.9	2.10E-02
BIOCARTA	Role of Mitochondria in Apoptotic Signaling	5	0.6	3.20E-02
BIOCARTA	Hypoxia and p53 in the Cardiovascular system	5	0.6	4.70E-02
BIOCARTA	p53 Signaling Pathway	4	0.5	6.80E-02
BIOCARTA	Chaperones modulate interferon Signaling Pathway	4	0.5	7.80E-02
BIOCARTA	Cell Cycle	5	0.6	8.10E-02
BIOCARTA	Regulation of BAD phosphorylation	4	0.5	8.80E-02
BIOCARTA	ATM Signaling Pathway	4	0.5	8.80E-02
BIOCARTA	BTG family proteins and cell cycle regulation	3	0.3	9.70E-02
KEGG	CELL CYCLE	16	1.8	2.40E-03
KEGG	PYRIMIDINE METABOLISM	14	1.6	4.30E-03
KEGG	PURINE METABOLISM	19	2.1	6.30E-03
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	10	1.1	1.10E-02
KEGG	APOPTOSIS	12	1.4	2.00E-02
KEGG	GLYCOSPHINGOLIPID METABOLISM	7	0.8	4.30E-02
KEGG	AMYOTROPHIC LATERAL SCLEROSIS (ALS)	4	0.5	7.60E-02
KEGG	ANDROGEN AND ESTROGEN METABOLISM	7	0.8	9.80E-02

(Table 3.6 part 2)

Table 3.6. Gene ontology: Pathways with altered gene expression in HT29 cells co-cultured with EPEC, using VSN normalisation and T-test. Genes with fold-change > ±2 (with P<0.05) subjected to functional annotation pathway enrichment search via DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/tools.jsp). Results of both BIOCARTA and KEGG functional pathway databases included. 'Count' signifies the number of differentially expressed genes found in the pathway, and as a percentage (%) of the total number of differentially expressed genes. P_value = modified Fisher Exact P-value for gene enrichment analysis; the smaller the value the more enriched the pathway, 0 represents perfect enrichment.

-0h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	-			
KEGG	-			
+mut 9h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Adhesion and Diapedesis of Granulocytes	3	5.2	4.60E-03
BIOCARTA	Cells and Molecules involved in local acute inflammatory response	3	5.2	4.60E-03
BIOCARTA	Cytokines and Inflammatory Response	3	5.2	9.90E-03
BIOCARTA	SODD/TNFR1 Signaling Pathway	2	3.4	6.00E-02
BIOCARTA	Free Radical Induced Apoptosis	2	3.4	6.60E-02
BIOCARTA	Adhesion and Diapedesis of Lymphocytes	2	3.4	8.20E-02
BIOCARTA	Regulation of hematopoiesis by cytokines	2	3.4	8.20E-02
KEGG	CYTOKINE-CYTOKINE RECEPTOR INTERACTION	7	12	1.70E-03
KEGG	ADIPOCYTOKINE SIGNALING PATHWAY	3	5.2	5.40E-02
KEGG	MAPK SIGNALING PATHWAY	5	8.6	5.50E-02
KEGG	APOPTOSIS	3	5.2	8.60E-02
KEGG	TOLL-LIKE RECEPTOR SIGNALING PATHWAY	3	5.2	9.10E-02
KEGG	T CELL RECEPTOR SIGNALING PATHWAY	3	5.2	9.30E-02
+mut 12h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	NFkB activation by Nontypeable Hemophilus influenzae	4	5.6	1.50E-03
BIOCARTA	CD40L Signaling Pathway	3	4.2	8.00E-03
BIOCARTA	Cadmium induces DNA synthesis and proliferation in macrophages	3	4.2	1.00E-02
BIOCARTA	TNFR2 Signaling Pathway	3	4.2	1.20E-02
BIOCARTA	Cells and Molecules involved in local acute inflammatory response	3	4.2	1.30E-02
BIOCARTA	Adhesion and Diapedesis of Granulocytes	3	4.2	1.30E-02
BIOCARTA	NF-kB Signaling Pathway	3	4.2	2.00E-02
BIOCARTA	Cytokines and Inflammatory Response	3	4.2	2.70E-02
BIOCARTA	Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	3	4.2	8.60E-02
BIOCARTA	Erythropoietin mediated neuroprotection through NF-kB	2	2.8	9.90E-02
BIOCARTA	SODD/TNFR1 Signaling Pathway	2	2.8	9.90E-02
KEGG	EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	4	5.6	3.50E-03
KEGG	TOLL-LIKE RECEPTOR SIGNALING PATHWAY	5	6.9	3.60E-03
KEGG	T CELL RECEPTOR SIGNALING PATHWAY	5	6.9	3.80E-03
KEGG	CYTOKINE-CYTOKINE RECEPTOR INTERACTION	7	9.7	6.30E-03
KEGG	MAPK SIGNALING PATHWAY	7	9.7	9.20E-03
KEGG	ADIPOCYTOKINE SIGNALING PATHWAY	4	5.6	1.20E-02
KEGG	APOPTOSIS	4	5.6	2.40E-02
KEGG	B CELL RECEPTOR SIGNALING PATHWAY	3	4.2	7.90E-02
+wt 9h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	CDK Regulation of DNA Replication	4	0.3	6.90E-03
BIOCARTA	Cyclins and Cell Cycle Regulation	5	0.4	4.20E-02
BIOCARTA	Role of BRCA1	5	0.4	5.30E-02
BIOCARTA	Cell Cycle	5	0.4	8.10E-02
BIOCARTA	ATM Signaling Pathway	4	0.3	8.80E-02
KEGG	CELL CYCLE	25	2	1.00E-07
KEGG	PYRIMIDINE METABOLISM	23	1.8	1.10E-07
KEGG	HISTIDINE METABOLISM	11	0.9	1.40E-03
KEGG	RNA POLYMERASE	8	0.6	1.50E-03
KEGG	FRUCTOSE AND MANNOSE METABOLISM	10	0.8	2.10E-03
KEGG	NITROBENZENE DEGRADATION	6	0.5	2.50E-03
KEGG	PURINE METABOLISM	21	1.7	2.90E-03
KEGG	SELENOAMINO ACID METABOLISM	8	0.6	5.80E-03
KEGG	AMINOPHOSPHONATE METABOLISM	6	0.5	9.10E-03
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	10	0.8	1.80E-02
KEGG	ANDROGEN AND ESTROGEN METABOLISM	9	0.7	2.00E-02
KEGG	TYROSINE METABOLISM	9	0.7	3.60E-02
KEGG	ARGININE AND PROLINE METABOLISM	8	0.6	7.00E-02
KEGG	DNA POLYMERASE	5	0.4	7.20E-02

(Table 3.7 part 1)

+wt 12h / -12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Role of BRCA1	11	0.4	1.10E-03
BIOCARTA	Role of Mitochondria in Apoptotic Signaling	10	0.4	1.20E-03
BIOCARTA	Cyclins and Cell Cycle Regulation	9	0.3	9.50E-03
BIOCARTA	Spliceosomal Assembly	7	0.3	1.10E-02
BIOCARTA	Cell Cycle	10	0.4	1.20E-02
BIOCARTA	Regulation of p27 Phosphorylation during Cell Cycle Progression	6	0.2	2.40E-02
BIOCARTA	Induction of apoptosis through DR3 and DR4/5 Death Receptors	9	0.3	3.50E-02
BIOCARTA	Role of Erk5 in Neuronal Survival	6	0.2	5.50E-02
BIOCARTA	Calcium Signaling by HBx of Hepatitis B virus	5	0.2	5.60E-02
BIOCARTA	p53 Signaling Pathway	6	0.2	6.80E-02
BIOCARTA	Trka Receptor Signaling Pathway	6	0.2	6.80E-02
BIOCARTA	CDK Regulation of DNA Replication	4	0.1	7.00E-02
BIOCARTA	EPO Signaling Pathway	7	0.3	7.50E-02
BIOCARTA	RB Tumor Suppressor/Checkpoint Signaling in response to DNA damage	5	0.2	9.20E-02
BIOCARTA	IL-2 Receptor Beta Chain in T cell Activation	10	0.4	9.80E-02
KEGG	CELL CYCLE	42	1.5	2.00E-09
KEGG	PYRIMIDINE METABOLISM	33	1.2	1.50E-06
KEGG	SELENOAMINO ACID METABOLISM	16	0.6	3.10E-05
KEGG	NITROBENZENE DEGRADATION	10	0.4	9.50E-05
KEGG	HISTIDINE METABOLISM	19	0.7	1.50E-04
KEGG	AMINOPHOSPHONATE METABOLISM	11	0.4	2.40E-04
KEGG	ANDROGEN AND ESTROGEN METABOLISM	19	0.7	2.60E-04
KEGG	RNA POLYMERASE	12	0.4	8.90E-04
KEGG	FRUCTOSE AND MANNOSE METABOLISM	16	0.6	1.10E-03
KEGG	OXIDATIVE PHOSPHORYLATION	34	1.2	1.80E-03
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	19	0.7	2.00E-03
KEGG	PURINE METABOLISM	36	1.3	4.90E-03
KEGG	TYROSINE METABOLISM	17	0.6	7.40E-03
KEGG	GLYCEROPHOSPHOLIPID METABOLISM	20	0.7	1.20E-02
KEGG	ONE CARBON POOL BY FOLATE	7	0.3	1.80E-02
KEGG	APOPTOSIS	21	0.8	3.30E-02
KEGG	SULFUR METABOLISM	6	0.2	3.70E-02
KEGG	PYRUVATE METABOLISM	12	0.4	3.80E-02
KEGG	GLYCOSPHINGOLIPID METABOLISM	12	0.4	3.80E-02
KEGG	VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	14	0.5	5.10E-02
KEGG	DNA POLYMERASE	8	0.3	5.20E-02
KEGG	LYSINE DEGRADATION	15	0.5	5.30E-02
KEGG	METHIONINE METABOLISM	6	0.2	6.30E-02
KEGG	TRYPTOPHAN METABOLISM	19	0.7	7.00E-02
KEGG	LIMONENE AND PINENE DEGRADATION	9	0.3	7.20E-02
KEGG	BUTANOATE METABOLISM	13	0.5	7.80E-02
KEGG	GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	7	0.3	7.90E-02
KEGG	GALACTOSE METABOLISM	9	0.3	8.30E-02
KEGG	ALKALOID BIOSYNTHESIS II	7	0.3	9.40E-02
KEGG	ETHYLBENZENE DEGRADATION	6	0.2	9.80E-02
+wt 9h / +mut 9h				
Database	Pathway	Count	%	P value
BIOCARTA	-			
KEGG	-			

(Table 3.7 part 2)

+wt 12h / +mut 12h				
Database	Pathway	Count	%	P_value
BIOCARTA	Trefoil Factors Initiate Mucosal Healing	8	0.4	1.20E-02
BIOCARTA	Role of Erk5 in Neuronal Survival	6	0.3	2.30E-02
BIOCARTA	Role of Mitochondria in Apoptotic Signaling	7	0.4	2.30E-02
BIOCARTA	PTEN dependent cell cycle arrest and apoptosis	7	0.4	2.80E-02
BIOCARTA	Cell Cycle	8	0.4	3.10E-02
BIOCARTA	Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)	11	0.6	3.60E-02
BIOCARTA	NFkB activation by Nontypeable Hemophilus influenzae	7	0.4	4.10E-02
BIOCARTA	Influence of Ras and Rho proteins on G1 to S Transition	7	0.4	4.10E-02
BIOCARTA	EGF Signaling Pathway	8	0.4	4.30E-02
BIOCARTA	Integrin Signaling Pathway	9	0.5	4.30E-02
BIOCARTA	Regulation of BAD phosphorylation	6	0.3	4.40E-02
BIOCARTA	Regulation of p27 Phosphorylation during Cell Cycle Progression	5	0.3	4.50E-02
BIOCARTA	Human Cytomegalovirus and Map Kinase Pathways	5	0.3	4.50E-02
BIOCARTA	Agrin in Postsynaptic Differentiation	8	0.4	4.90E-02
BIOCARTA	Acetylation and Deacetylation of RelA in The Nucleus	5	0.3	5.70E-02
BIOCARTA	Ceramide Signaling Pathway	6	0.3	6.40E-02
BIOCARTA	Multiple antiapoptotic pathways from IGF-1R signaling lead to BAD phos.	6	0.3	6.40E-02
BIOCARTA	p38 MAPK Signaling Pathway	8	0.4	6.40E-02
BIOCARTA	IL-2 Receptor Beta Chain in T cell Activation	9	0.5	6.90E-02
BIOCARTA	BTG family proteins and cell cycle regulation	4	0.2	7.20E-02
BIOCARTA	Transcription factor CREB and its extracellular signals	6	0.3	7.50E-02
BIOCARTA	Apoptotic Signaling in Response to DNA Damage	6	0.3	8.70E-02
BIOCARTA	NF-kB Signaling Pathway	6	0.3	8.70E-02
BIOCARTA	SODD/TNFR1 Signaling Pathway	4	0.2	9.20E-02
BIOCARTA	E2F1 Destruction Pathway	4	0.2	9.20E-02
KEGG	APOPTOSIS	22	1.1	8.20E-04
KEGG	NITROBENZENE DEGRADATION	7	0.4	5.10E-03
KEGG	AMINOPHOSPHONATE METABOLISM	8	0.4	5.30E-03
KEGG	GLYCOSPHINGOLIPID METABOLISM	12	0.6	6.30E-03
KEGG	PYRIMIDINE METABOLISM	20	1	7.70E-03
KEGG	SELENOAMINO ACID METABOLISM	10	0.5	1.10E-02
KEGG	ANDROGEN AND ESTROGEN METABOLISM	13	0.7	1.20E-02
KEGG	PURINE METABOLISM	28	1.4	1.40E-02
KEGG	GLYCOLYSIS / GLUCONEOGENESIS	14	0.7	1.70E-02
KEGG	CELL CYCLE	21	1.1	1.90E-02
KEGG	SULFUR METABOLISM	5	0.3	5.80E-02
KEGG	GLYCEROLIPID METABOLISM	11	0.6	7.20E-02
KEGG	GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	6	0.3	8.40E-02
KEGG	OXIDATIVE PHOSPHORYLATION	22	1.1	8.50E-02

(Table 3.7 part 3)

Table 3.7. Gene ontology: Pathways with altered gene expression in HT29 cells infected with EPEC, using VSN normalisation and SAM analysis. Genes with statistically significant fold-change (false discovery rate 0.1, $P<0.05$) were subjected to functional annotation pathway enrichment search via DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/tools.jsp). Results of both BIOCARTA and KEGG functional pathway databases included. The number of differentially expressed genes found in the pathway is displayed under 'Count', and as a percentage (%) of the total number of differentially expressed genes. P_value = modified Fisher Exact P-value for gene enrichment analysis; the smaller the value the more enriched the pathway, 0 represents perfect enrichment.

Expression comparison	MSH2	MSH3	MSH4	MSH5	MSH6	MLH1	MLH3
-0h / -12h	1.06	1.06	-1.47	-1.20	-1.12	-1.13	-1.23
+mut 9h / -12h	**-2.22**	-1.02	-1.05	1.01	-1.88	-1.82	-1.06
+mut 12h / -12h	-1.94	1.04	-1.08	-1.02	-1.72	-1.76	-1.18
+wt 9h / -12h	**-3.05**	-1.11	-1.26	1.21	**-2.74**	**-2.59**	-1.51
+wt 12h / -12h	**-3.90**	-1.29	-1.16	1.12	**-2.56**	**-3.02**	-1.56
+wt 9h / +mut 9h	-1.37	-1.09	-1.20	1.21	-1.46	-1.42	-1.42
+wt 12h / +mut 12h	**-2.02**	-1.35	-1.07	1.14	-1.49	-1.72	-1.32

Table 3.8. Mismatch repair gene expression in HT29 cells co-cultured with EPEC, using VSN normalisation and T-test. Table shows fold-change values calculated with T-test, numbers in bold have fold-change > 2, with $P<0.05$.

Expression comparison	MSH2	MSH3	MSH4	MSH5	MSH6	MLH1	MLH3
-0h / -12h	x	x	x	x	x	x	x
+mut 9h / -12h	x	x	x	x	x	x	x
+mut 12h / -12h	x	x	x	x	x	x	x
+wt 9h / -12h	**-3.05**	x	x	x	**-2.74**	**-2.59**	x
+wt 12h / -12h	**-3.90**	x	x	x	**-2.56**	**-3.02**	x
+wt 9h / +mut 9h	x	x	x	x	x	x	x
+wt 12h / +mut 12h	**-2.02**	x	x	x	x	x	x

Table 3.9. Mismatch repair gene expression in HT29 cells co-cultured with EPEC, using VSN normalisation and SAM. Table shows fold-change values calculated with significance analysis of microarray (SAM; false discovery rate 0.1), numbers in bold have fold-change > 2, with $P<0.05$. x = no statistically significant fold-change.

Expression comparison	β-catenin	α-catenin	γ-catenin	E-cadherin	Ezrin
-0h / -12h	-1.10	-1.02	-1.30	1.15	-1.32
+mut 9h / -12h	-1.14	1.16	1.46	1.24	1.23
+mut 12h / -12h	-1.31	1.21	1.43	1.27	1.24
+wt 9h / -12h	-1.37	1.12	1.74	1.23	1.36
+wt 12h / -12h	**-2.55**	1.11	1.55	1.04	1.37
+wt 9h / +mut 9h	-1.27	-1.03	1.19	-1.01	1.10
+wt 12h / +mut 12h	**-2.47**	-1.09	1.08	-1.22	1.11

Table 3.10. Cell-cell adhesion gene expression in HT29 cells co-cultured with EPEC, using VSN normalisation and T-test. Table shows fold-change values calculated with T-test, numbers in bold have fold-change > 2, with $P<0.05$.

Expression comparison	β-catenin	α-catenin	γ-catenin	E-cadherin	Ezrin
-0h / -12h	x	x	x	x	x
+mut 9h / -12h	x	x	x	x	x
+mut 12h / -12h	x	x	x	x	x
+wt 9h / -12h	x	x	x	x	x
+wt 12h / -12h	**-2.55**	x	-1.93	x	x
+wt 9h / +mut 9h	x	x	x	x	x
+wt 12h / +mut 12h	**-2.47**	x	**-3.04**	x	x

Table 3.11. Cell-cell adhesion gene expression in HT29 cells co-cultured with EPEC, using VSN normalisation and SAM. Table shows fold-change values calculated with significance analysis of microarray (SAM; false discovery rate 0.1), numbers in bold have fold-change > 2, with $P<0.05$. x = no statistically significant fold-change.

3.3. Discussion

Co-culture of wild-type EPEC with HT29 cells for 12 hours was made possible by washing away excess bacteria at 3 hour intervals. This procedure evidently prevented bacterial overgrowth (and hence toxicity) without preventing attachment to and colonisation of the cultured cells, as demonstrated by the FAS test and immunofluorescence staining of EPEC. Detachment of cells from the culture flask was expected and this phenomenon was observed, particularly after 12 hours co-culture. The literature suggests that this is a result of EPEC induced disruption of focal adhesions, adherens junctions and tight junctions (Shifrin *et. al.,* 2002; McNamara *et. al.,* 2001; Malladi *et. al.,* 2004). Other reports suggest that cell death could be responsible for detachment (Abul-Milh *et. al.,* 2001; Melo *et. al.,* 2005). However, a significant percentage of cells remained adherent after 12 hours infection, and many were able to survive long term. Hence, despite heavy infection, many cells did not undergo apoptosis or necrosis.

It was notable that in the initial recovery period (i.e. within 60 hours of starting antibiotic treatment) cells continued to detach from the culture flask, hence it is likely that the residual effects of EPEC (and residual EPEC themselves) continued to disrupt cell adherence. Interestingly, cell division was also disrupted during this period; cell division did not occur at noticeable levels until at least 4 days after infection. This is a significant inhibitory effect, as the normal population doubling time of HT29 cells is approximately 24 hours. This result supports the finding that EPEC secreted cyclomodulins (such as Cif) are able to cause cell cycle arrest (Marches *et. al.,* 2003). This work also demonstrates that this effect is reversible on removal of infection.

Proteomic analysis of intestinal epithelial cells co-cultured with EPEC for 4 hours has revealed differential effects on the expression of a wide range of host cell proteins (Hardwidge *et. al.,* 2004). It was hypothesised that 12 hours co-culture with EPEC would not result in non-specific toxicity for host cells, and that differential effects on host protein expression would continue to be seen after 12 hours infection.

Electrophoresis demonstrated that EPEC infection had a clear influence on global protein expression in HT29 cells. The effect of EPEC on protein expression were differential i.e. while the expression of some proteins was either upregulated or suppressed, the expression of many others remained relatively constant. Mass spectrometry provided a convenient means to identify some of the proteins revealed by electrophoresis. Proteins within diverse functional groups were identified, and proteins within the same functional groups (e.g. actin binding and protein-processing) were subject to increased / reduced or unchanged expression simultaneously. It should be noted that mass spectrometry does not provide unequivocal protein identification. Hence, further analysis by amino acid sequencing / western blot / immunocytochemistry would be required to confirm the identity of these proteins and their expression after EPEC infection. It would also be desirable to compare protein changes induced by wild-type EPEC to those caused by UMD864.

UMD864 adhered to HT29 cells in a localised adherence pattern, demonstrating that a functional TTSS is required not only for intimate attachment, but also for the widespread epithelial cell colonisation characteristic of the wild-type EPEC. Adherence of the UMD864 is likely to be the result of Bfp binding only; hence washing removed the majority of UMD864 but not the intimately attached E2348/69. It was also observed that cells infected with UMD864 showed less detachment after 12 hours co-culture, and recovered post infection quicker than E2348/69 (not shown). These observations suggest that the ability of E2348/69 to cause detachment and slow cell division are TTSS and / or intimin signalling specific. Hence UMD864 represents a useful negative control that will help to distinguish between specific effects (i.e. due to TTSS or intimin signalling), and non-specific effects (i.e. due to contact with bacterial surface antigens, changes in media nutrient composition or pH) caused by *in vitro* infection.

This study presents a genome-wide analysis of the effect of EPEC infection on host cell gene expression, potentially the first study of this type performed with EPEC. As with the proteomic analysis, gene expression analysis demonstrated differential effects due to EPEC infection, i.e. up- and downregulated expression of some genes,

while the expression of others was unaffected. Small changes in gene expression profile were seen due to the experimental washing process, supporting the use of uninfected, washed cells as an experimental control. Also, many changes in gene expression were observed due to UMD864 infection, these effects are likely to be due to contact with surface antigens including bfp, and the changes in the cell culture environment due to infection. Direct comparison of gene expression in UMD864 and E2348/69 infected cells revealed that the expression of many genes was influenced differently by these types of EPEC, revealing that TTSS / intimin signalling dependent effects occurred.

EPEC induced changes in gene expression within a number of functional pathways were observed. Notable amongst these pathways were the cell cycle (which was strongly represented), this observation potentially correlates with cell cycle effects observed in this study and in the literature. In addition, both wild-type and mutant EPEC infection influenced apoptotic pathways, notably, wild-type EPEC had effects on both pro- and anti-apoptotic signalling pathways. These observations also correlate well with the effects of EPEC on apoptosis reported by the literature and discussed earlier in this report. Furthermore, pathway analysis also reflected novel changes due to wild-type EPEC infection that corresponded to protein expression results (from mass spectrometry analysis) e.g. effects on the BRCA1 pathway.

Of specific interest was the effect of wild-type EPEC infection on genes encoding DNA MMR and Wnt signalling / cell-cell adhesion proteins. E2348/69 caused the downregulation of MSH2, MSH6 and MLH1 genes, while the expression of other mutS and mutL homologue genes remained relatively constant. E2348/69 also induced the downregulation of β- and γ-catenin. These findings therefore suggest that EPEC infection may influence the classic genetic pathways of colorectal cancer. Although microarray analysis has provided many interesting results, it is acknowledged that microarray techniques are not definitive in the quantification of gene expression. These results therefore represent a preliminary study into the transcriptomic effects of EPEC and identify many target genes that could be investigated further. Confirmation and validation of gene expression results could be

achieved with quantitative real-time PCR, the translation of transcriptomic effects into protein expression could be analysed by western blot and immunocytochemistry.

Overall the objective of developing an extended time-course EPEC co-culture model was achieved. The model was validated by a number of methods, which consistently produced results in line with previously published data on the *in vitro* effects of EPEC. This model therefore represents a reliable, repeatable and relevant model for EPEC infection of the human colonic epithelium.

Chapter 4.

Development and validation *in vivo* and *ex vivo* models of EPEC infection and the prevalence of AE *E. coli* infection in cancer patients.

4.1. Introduction

4.1.1. *In vivo* mouse models for EPEC infection

In parallel to establishing a reliable *in vitro* model for EPEC infection, an aim of this study was to develop a relevant *in vivo* model. The aim in establishing this model was to provide a system in which to analyse both the molecular and pathological effects of EPEC infection. Thus making it possible to determine whether any effects observed *in vitro* also occurred in a whole animal, and most importantly whether these changes incurred long-term consequences.

Citrobacter rodentium (formerly *Citrobacter freundii* biotype 4280) is a mouse pathogen responsible for transmissible murine colonic hyperplasia (TMCH) and provides a well-established, convenient model for infection by AE bacteria (Schauer *et. al.,* 1995). In laboratory mice *C. rodentium* attaches intimately in large numbers to the surface mucosa of the descending colon within 4 days of inoculation. Hyperplasia of the descending and sometimes the transverse colon rapidly develops, reaching maximal levels after approximately 16 days (Barthold, 1980). Hence mice infected with *C. rodentium* have colonic crypts up to 3 times longer than uninfected animals (Barthold, 1979). EPEC and *C. rodentium* share a number of genetic and phenotypic traits; *C. rodentium* carry a chromosomal copy of the *eae* gene and form AE lesions when attaching to the colonic mucosa (Schauer & Falkow, 1993), *C. rodentium* also secrete a translocated intimin receptor (Tir), which is tyrosine phosphorylated in host cells (Deng *et. al.,* 2003). In similarity to EPEC, acute *C. rodentium infection* induces diarrhoea, though this not generally profuse or watery (Barthold, 1980).

Experiments using mice infected with *C. rodentium* have helped to establish a link between AE bacteria and tumourigenesis. A report published 30 years ago analysed the time taken for tumours to occur in mice injected with the carcinogen DMH (1,2-dimethylhydrazine) and infected with *C. rodentium* (Barthold & Jonas, 1977). It was found that *C. rodentium* infection reduced the latent period for appearance of early

DMH tumours from 2 months to 1 month, the percentage incidence of tumours was also more than double in infected mice compared to controls after 2 months. In a more recent study, $Apc^{Min/+}$ mice (heterozygous for the multiple intestinal neoplasia [Min] allele, which is a germline mutation in the *Apc* tumour suppressor gene) were infected with *C. rodentium* (Newman *et. al.,* 2001). Five months after infection, significantly more adenomas were found in infected $Apc^{Min/+}$ mice (average 2.8 tumours / animal) compared to uninfected $Apc^{Min/+}$ mice (0.8) or infected wild-type mice (0.0).

A recently published report establishes an alternative *in vivo* model by infecting mice directly with EPEC (Savkovic *et. al.,* 2005). By using EPEC, this model is a potentially more relevant system for studying EPEC pathogenesis in man. Savkovic *et. al.* (2005) report that in C57Bl/6 mice, EPEC E2348/69 colonises and adheres to the intestinal epithelium, causing the effacement of microvilli, actin rearrangement and the recruitment of immune cells. These are interesting results considering EPEC E2348/69 is generally accepted as a purely human pathogen. *In vitro* studies analysing the ability of EPEC to adhere to mouse colorectal (CMT-93 & Colon-26) cells found that adherence was remarkably low compared to human colonic (Caco-2) cell adhesion (Tobe & Sasakawa, 2002). The authors attributed the species specificity of EPEC to selective binding by bundle forming pili.

4.1.2. *Ex vivo* models for EPEC infection

The availability of fresh human colon tissue from surgical patients presented the opportunity to develop an *ex vivo* model for EPEC infection. The aim of developing this model was to gain an understanding of the behaviour of EPEC within the human colon. As colon cancer is generally a disease of middle age, adult colon tissue represents the most biologically relevant tissue for this model. As with the *in vitro* model, a long time-course of infection was used. A number of published *in vitro* organ culture (IVOC) experiments have been carried out to analyse the pathogenesis of EPEC in association with human tissue. These studies generally use small intestine biopsy tissue removed from children and are specifically concerned with the

AE process itself. These studies show that wild-type EPEC (E2348/69) colonise the surface of human small intestine mucosa, producing AE lesions after 6-8 hours incubation (Table 4.1).

Reference	Tissue	Donors	Time-course
Knutton *et. al.*, 1987	Duodenal mucosal biopsies	Adult volunteers	Up to 12 h (2-3 h media changes)
Hicks *et. al.*, 1996	Jejunum, distal ileum & transverse colon mucosal biopsies	Children 5 months-14 years	8 h (2 h media changes)
Hicks *et. al.*, 1998	Duodenum & terminal ileum biopsies	Children 1-15 years	8 h (2 h media changes)
Phillips & Frankel, 2000	Duodenum, terminal ileum, Peyer's patches transverse colon mucosal biopsies	Children 6 month-18 years	8 h (2 h media changes)
Phillips *et. al.*, 2000	Duodenal & ileal mucosal biopsies / surgery	Children 3-15 years	8 h (2 h media changes)
Cleary *et. al.*, 2004	Duodenal mucosal biopsies	Children	1.5 h
Schuller *et. al.*, 2007	Proximal small intestine (D4), ileal & Peyer's patch mucosal biopsies	Children	8 h (2 h media changes)

Table 4.1. Published reports using *in vitro* organ culture (IVOC) methods for co-culture with EPEC. Studies generally used small intestine tissue and in all but one case this tissue came from children.

Hicks *et. al.* (1996) used IVOC of paediatric transverse colon to investigate the adhesion properties of EAEC, finding that EAEC adhered to colonic mucosa and that colon tissue incubated with EAEC had larger crypt openings than uninfected tissue. EPEC strain KH1/8 (that displayed localised adherence to Hep-2 cells) was used as a control and did not adhere to the colon mucosa (Hicks *et. al.*, 1996). A study by Phillips & Frankel (2000) included the use of paediatric colon tissue to evaluate the effect of intimin sub-type expression on tissue binding specificity. Using EPEC strain CVD206 (E2348/69 *eae*$^-$) transformed to express intimin-α or intimin-γ it was found that intimin subtype did influence tissue specificity. Hence EPEC expressing intimin-α adhered to small intestine (100% of samples), Peyer's patch (80%) and colon (25%), whereas EPEC expressing intimin-γ did not adhere to the colon, but did adhere to the small intestine (14%) and Peyer's patch (83%). Hence EPEC have the

ability to bind to paediatric colonic mucosa, depending on intimin type. This therefore suggests that host cells display other surface receptors that bind intimin (aside from Tir), and that intimin subtypes interact differently with these receptors.

The literature therefore provides little evidence on the normal behaviour of EPEC within the adult colon. Hence a key aspect of this investigation was to analyse whether EPEC attached to this tissue and if there were preferred binding sites. The function and therefore structure of the human colon differs significantly from the small intestine. The principal role of the colon is the absorption of salt and water from the lumen contents. Unlike the small intestine mucosa, which is characterised by villi that project into the lumen, the colonic mucosa is flat, and pitted with tubular crypts. The gut epithelium is a constitutively developing tissue, constantly differentiating from stem cells throughout the life of the organism (de Santa Barbara *et. al.,* 2003). Stem cells lie near the base of colonic crypts, newly formed undifferentiated cell are classed as proliferative progenitors, these cells migrate from the crypt base towards the lumen. When proliferative cells reach the top third of the crypt they differentiate terminally and are eventually shed from the epithelium due to apoptosis or physical processes (Sancho *et. al.,* 2004) (Figure 4.1). Colorectal cancer is thought to arise from transformation of undifferentiated proliferative cells within the crypts (van der Wettering *et. al.,* 2002). It was therefore an aim of this study to investigate whether EPEC could influence to biology of these cells by entering colonic crypts.

4.1.3. *E. coli* in colorectal cancer patients

Previous studies have identified the presence of tumour-associated *E. coli* in colorectal cancer patients, but did not identify *E. coli* of the AE phenotype (Swidinski *et. al.,* 1998; Martin *et. al.,* 2004). The central hypothesis of this study is based on the theory that chronic EPEC infection would help to initiate tumourigenesis in the healthy colon, and not necessarily affect the development of an *in situ* tumour. Hence the presence of adherent *E. coli* on adenocarcinomas could indicate a residual infection that contributed to the initial formation of the tumour. A

further aim was therefore to investigate whether tumour-associated *E. coli* of AE phenotype were present in a sample of colorectal cancer patients. A high incidence of EPEC infection in cancer patients would also suggest a link with tumourigenesis. However, it is difficult to know what the baseline EPEC-infection rate in the healthy population is, as routine screening is no longer performed in hospitals.

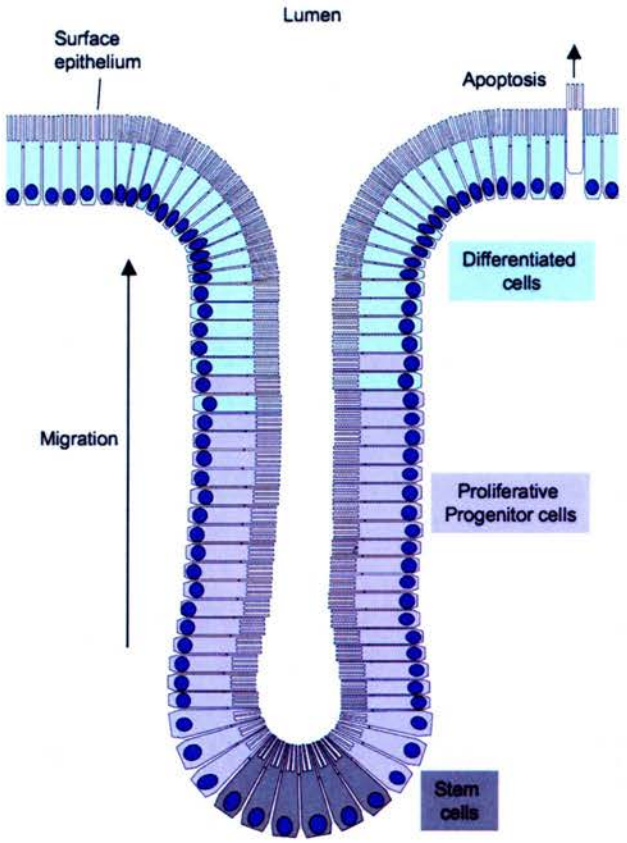


Figure 4.1. Schematic diagram representing cell compartments within the colonic crypt. Proliferative progenitors are amplified by constant division along the bottom two-thirds of the crypts, cell cycle arrest and differentiation occur when progenitors reach the top third of the crypts. Crypt progenitors divide every 12-16 h, generating 200 cells per crypt per day. Three mechanisms maintain epithelial homeostasis; (i) shedding of differentiated cells from the surface epithelium, (ii) continuous upward migration of proliferative and differentiated cells, (iii) proliferative and differentiated compartments are maintained as cells transit along the crypt axis (hence proliferation is not a cell autonomous feature). Adapted from Sancho *et. al.*, 2004.

4.2 Results

4.2.1 *In vivo* mouse model for EPEC infection

4.2.1.1. Infection of C57Bl/6 mice with *Citrobacter rodentium* and EPEC

As expected, C57Bl/6 mice infected with wild-type *C. rodentium* developed a gross thickening of the colon due to hyperplasia manifest by increased crypt length. Whereas, uninfected and mutant *C. rodentium* infected mice showed no hyperplasia, demonstrating the necessity of *eae* in initiating the pathological response (Figure 4.2). By comparison, C57Bl/6 mice infected with EPEC did not display hyperplasia, despite receiving a higher bacterial inoculum (Figure 4.3).

To further validate the EPEC mouse model and to quantify the infection present within the lower GI tract bacterial counts were performed. The number of adherent and non-adherent EPEC present in the lower intestine were counted by growing homogenised tissue and gut contents on selective agar. In C57Bl/6 mice, low average levels of adherent EPEC were counted in the ileum (1,917), cecum (752), proximal (2,637) and distal colon (97), 24 hours post infection. After 48 hours, similar numbers of adherent EPEC were counted in the cecum (1,505) and proximal colon (1,956), but none were present in the ileum or distal colon. After 72 hours, no adherent EPEC were detected in any of the tissues (Figure 4.4a). Non-adherent bacteria showed a similar time-course of infection, peaking after only 24 hours with an average of 89,367 EPEC in the cecum. After 72 hours, only the cecum contained any non-adherent EPEC, but numbers were low (4,667) (Figure 4.4b).

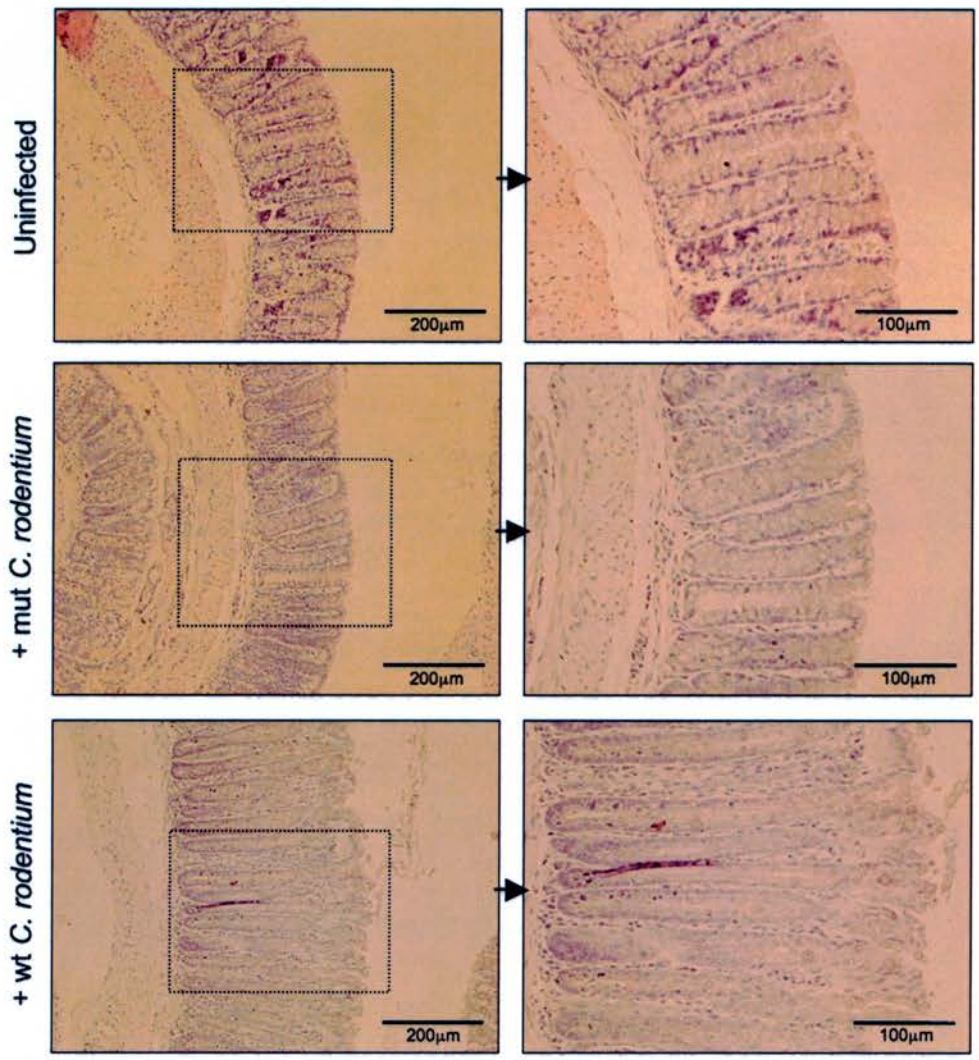


Figure 4.2. Effect of *Citrobacter rodentium* on colon pathology in C57Bl/6 mice. C57Bl/6 mice were either uninfected or received mutant (DBS255) or wild-type (DBS100) *Citrobacter rodentium* (~1 x 10^8) via gavage. After 14 days animals were killed, the colon was excised, rolled, fixed and then embedded in paraffin. Immunosections were stained with haematoxylin and eosin. Original magnification of panels on left, x 20; on right x 40.

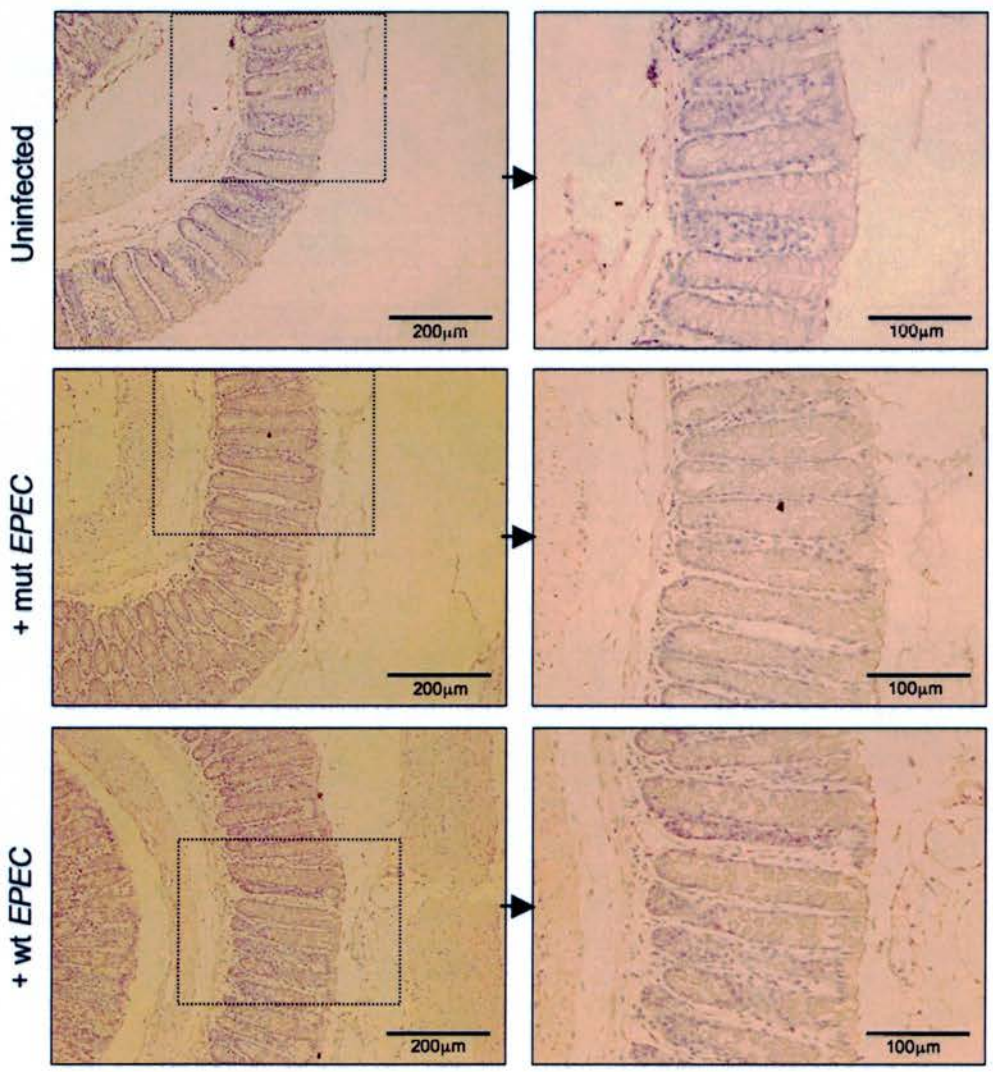


Figure 4.3. Effect of EPEC on colon pathology in C57Bl/6 mice. C57Bl/6 mice were either uninfected or received mutant (UMD864) or wild-type (E2348/69) EPEC (~2 x 10^8) via gavage. After 14 days animals were killed, the colon was excised, rolled, fixed and then embedded in paraffin. Immunosections were stained with haematoxylin and eosin. Original magnification of panels on left, x 20; on right x 40.

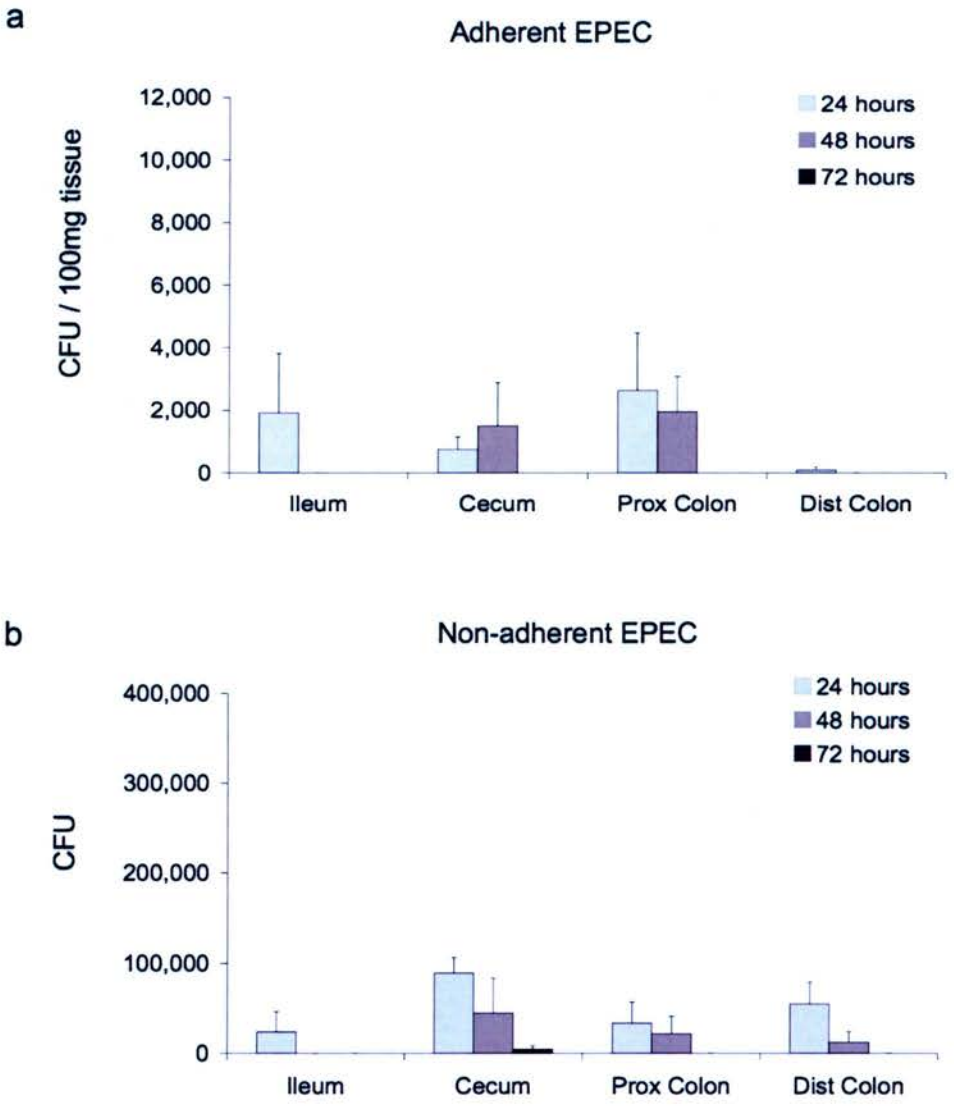


Figure 4.4. Quantification of bacterial load in C57Bl/6 mice infected with EPEC. Mice (n = 9) received wild-type (E2348/69) EPEC (~2 x 10^8) via gavage. At 24 h intervals post-infection animals (n = 3) were killed, the lower GI tract was removed and cut into sections, the homogenised tissue and lumen contents from each section was plated onto selective MacConkey agar to count EPEC colony forming units (CFU). EPEC colonies grown from tissue homogenates (**a**) were classified as representing the number of adherent EPEC, and colonies grown from lumen contents (**b**), non-adherent EPEC.

In comparison with the published results, and considering the number of bacteria the C57Bl/6 mice received (~2 x 10^8), the numbers of adherent and non-adherent bacteria were low. Evidently, the time-course of infection was also short, with no adherent bacteria identified after 72 hours. It was hypothesised that other strains of mice would be more susceptible to EPEC infection and hence provide a better model. C3H/HeN mice are highly susceptible to *C. rodentium* infection, which is potentially fatal to these animals (Vallance *et. al.*, 2003).

4.2.1.2. Infection of C3H/HeN mice with EPEC

Higher numbers of adherent and non-adherent EPEC were counted in the cecum of C3H/HeN mice compared to EPEC infected C57Bl/6 mice. Hence, an average of 9,203 adherent EPEC were counted in the cecum after 48 hours infection. However, numbers dropped rapidly, and after 72 hours only 284 EPEC were counted in the cecum. Less EPEC were counted in the proximal colon after 24 hours (1,090) or 48 hours (572) than in C57/Bl6 mice, however at 72 hours numbers were higher (445) in the C3H/HeN animals. Very few adherent EPEC were detected in the ileum or distal colon of C3H/HeN mice at any time-point. In contrast to C57Bl/6 mice, infection persisted (at low levels) for at least 72 hours in the cecum and proximal colon. In summary, more non-adherent EPEC were counted in C3H versus C57 mice, but the majority of adherent EPEC were confined to the cecum, and overall numbers remained relatively low, particularly after 72 hours (Figure 4.5a).

C3H/HeN mice were infected with a higher inoculum of EPEC than the C57 mice, and this translated into greater average numbers of non-adherent EPEC in the cecum, and colon. Hence, after 24 hours the cecum (157,767), proximal (38,000) and distal colon (142,100) of C3H mice contained more EPEC than C57 mice (cecum 89,367; proximal colon 33,567; distal colon 55,067). In the C3H mice, non-adherent EPEC were also present in higher numbers after 72 hours in the cecum (50,067) proximal (25,533) and distal colon (15,867) versus C57 mice (cecum 4,667; proximal colon 0; distal colon 0) (Figure 4.5b).

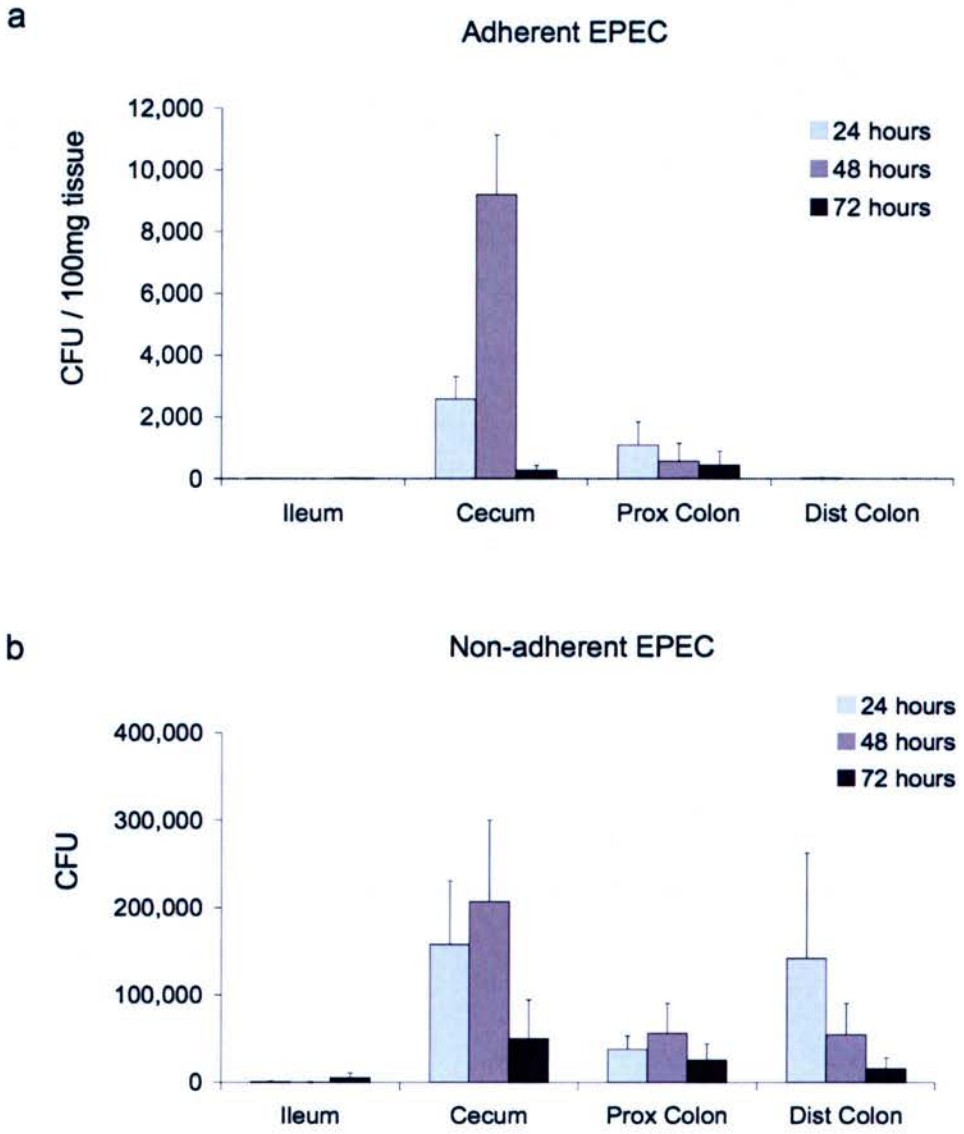


Figure 4.5. Quantification of bacterial load in C3H/HeN mice infected with EPEC. Mice (n = 9) received wild-type (E2348/69) EPEC (~1 x 10^9) via gavage. At 24 h intervals post-infection animals (n = 3) were killed, the lower GI tract was removed and cut into sections, the homogenised tissue and lumen contents from each section was plated onto selective MacConkey agar to count EPEC colony forming units (CFU). EPEC colonies grown from tissue homogenates (**a**) were classified as representing the number of adherent EPEC, and colonies grown from lumen contents (**b**), non-adherent EPEC.

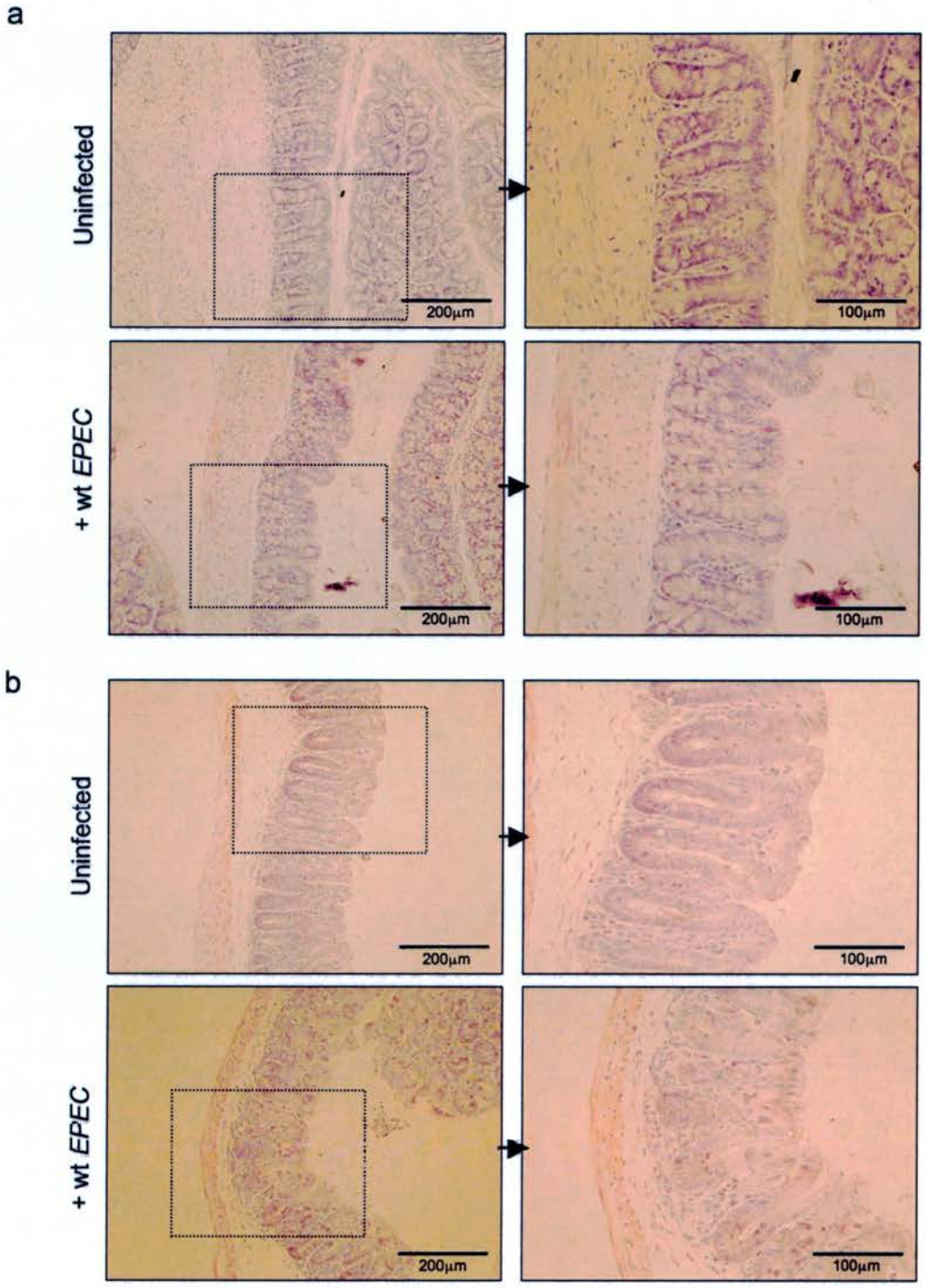


Figure 4.6. Effect of EPEC on colon pathology in C3H/HeN mice. C3H/HeN mice were either uninfected or received wild-type (E2348/69) EPEC (~1 x 10^9) via gavage. After 2 days animals were killed, the colon (**a**) and cecum (**b**) were excised, rolled, fixed and then embedded in paraffin. Immunosections were stained with haematoxylin and eosin. Original magnification of panels on left; x 20, on right; x 40.

To test whether EPEC infection was able to persist in C3H/HeN mice long term, cecum and proximal colon tissue and contents were analysed for EPEC 5 weeks after infection (Table 4.2). No EPEC were identified, and tissue did not display the crude signs of hyperplasia that were clearly visible in C57Bl/6 mice infected with *C. rodentium.* No symptoms or changes in behaviour were observed in the C3H/HeN mice during the 5-week period. Haematoxylin and eosin staining of C3H/HeN cecum and colon tissue after short-term (48 hours) infection with EPEC also revealed normal pathology (Figure 4.6).

	Adherent EPEC (CFU)		Non-adherent EPEC (CFU)	
	Cecum	Prox. colon	Cecum	Prox. Colon
Animal 1	0	0	0	0
Animal 2	0	0	0	0
Animal 3	0	0	0	0

Table 4.2. Quantification of bacterial load in C3H/HeN mice 5 weeks after infection with EPEC. Mice received wild-type (E2348/69) EPEC (~2 x 10^9) via gavage. After 5 weeks animals were killed, the cecum and proximal colon were removed. Homogenised tissue and gut contents were plated onto selective MacConkey agar to count EPEC colony forming units (CFU). Colonies grown from tissue homogenate were classified as adherent EPEC, and colonies grown from gut contents as non-adherent EPEC.

4.2.1.3. Co-culture of mouse colorectal cells with *C. rodentium* and EPEC

As a further test for the validity of a mouse model for EPEC infection, EPEC was co-cultured with the mouse derived intestinal epithelial cell line CMT93. Co-culture experiments with human cell lines in this study revealed that EPEC infection downregulated the expression of certain cell-adhesion and DNA repair proteins (Chapter 5). It was hypothesised that EPEC or *Citrobacter rodentium* infection would have the similar effects on protein expression in CMT93 cells.

Co-culture of EPEC with CMT93 cells for 12 hours did not cause downregulation of cell adhesion (beta-catenin) and DNA repair (MSH2 and MLH1) proteins (Figure 4.7a). By viewing co-cultured cells under a light microscope, it was noticeable that far fewer EPEC were adherent to CMT93 cells than to human (HT29 cells) after 12

hours co-culture (not shown). Despite the fact that *Citrobacter rodentium* is a murine AE pathogen, no change in the expression of MSH2 or MLH1 expression was observed after co-culture with CMT93 cells (Figure 4.7b). These results demonstrate not only the species specificity of AE bacteria adherence, but also the potential species specificity of the molecular effects of AE bacteria.

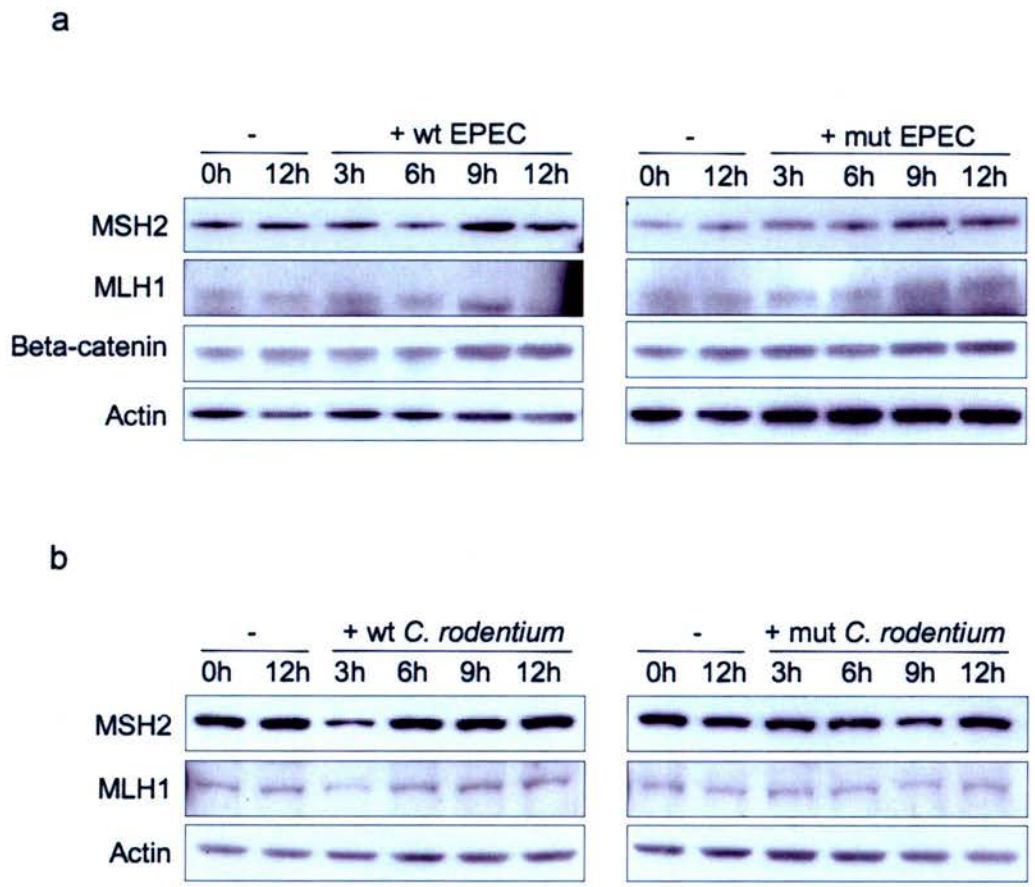


Figure 4.7. Co-culture of CMT93 cells with EPEC or *Citrobacter rodentium*. Western blots of CMT93 cells that were either uninfected (-) or co-cultured with wild-type (E2348/69) or mutant (UMD864) EPEC (**a**), or with mutant (DBS255) or wild-type (DBS100) *Citrobacter rodentium* for 12 hours (**b**).

4.2.2. *Ex vivo* human tissue model for EPEC infection

The architecture of uninfected tissue (fixed soon after removal from the patient) was compared with adjacent tissue (from the same patient) that had been co-cultured with EPEC for 12 hours. Despite the co-culture period, general mucosa structure appeared normal, the surface epithelial layer remained intact and crypt structure was maintained (Figure 4.8).

It was hypothesised that EPEC could enter human colonic crypts, with the potential to influence to molecular biology of proliferative progenitor cells. Due to the lack of a commercial antibody to specifically identify EPEC, a generic anti-*E.coli* antibody was used. Immunofluorescent staining of fixed sections of colon mucosa co-cultured with EPEC frequently revealed the presence of *E. coli* in crypts (Figure 4.9). Images show that the *E. coli* closely associated with the surface epithelium (marked by ezrin staining). It can also be seen that the bacteria were found in the mid-region of the crypts. Immunofluorescent staining of Ki67, a marker for cells capable of proliferation (Sancho *et. al.*, 2004) shows that *E. coli* were identified in the region of the crypt containing proliferative progenitor cells (Figure 4.10).

In order to quantify the frequency of crypt infection, and to confirm that *ex vivo* tissue did not harbour endogenous *E. coli* prior to co-culture, the number of crypts containing *E. coli* was counted. In tissue co-cultured with EPEC for 12 hours, 10.6% of crypts contained *E. coli*, in uninfected tissue *E. coli* was not identified anywhere within the tissue (Figure 4.11). This result helps to confirm that the human tissue used did not harbour endogenous *E. coli* prior to co-culture, and that observations were based on identification of EPEC. PCR amplification for *eae* confirms that AE *E. coli* were absent in uninfected *ex vivo* tissue, but were present in tissue co-cultured with EPEC (Figure 4.14). Therefore, results strongly support the conclusion that EPEC is able to enter a significant proportion of crypts in normal, adult human colon tissue.

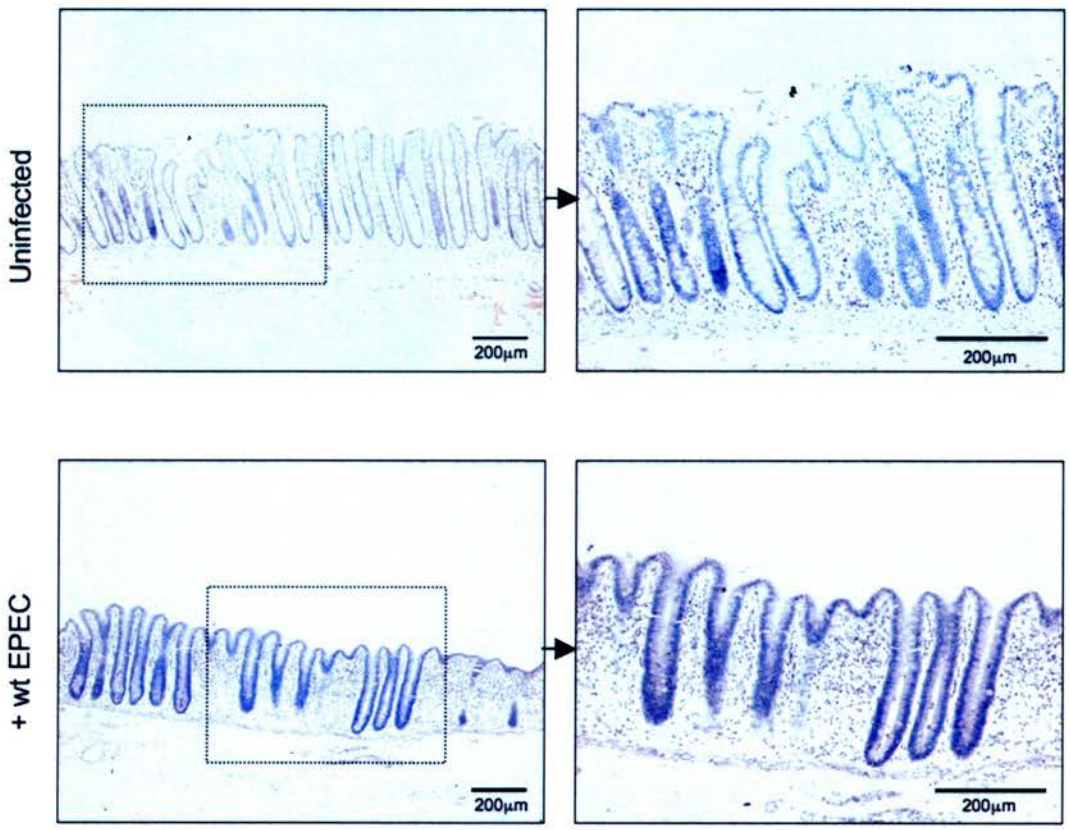


Figure 4.8. Co-culture of human *ex vivo* colon mucosa with EPEC.
Normal, full thickness colon tissue was removed from surgical patients, the mucosa was separated from the muscularis and washed several times. Pieces of mucosa were either fixed in formalin immediately (Uninfected, 0h), or co-cultured with wild-type EPEC for 12 h (+ wt EPEC, 12h) then washed and fixed in formalin. Fixed tissue was embedded in paraffin, immunosections were stained with haematoxylin and eosin. Broken black boxes delineate areas of magnification shown in subsequent images.

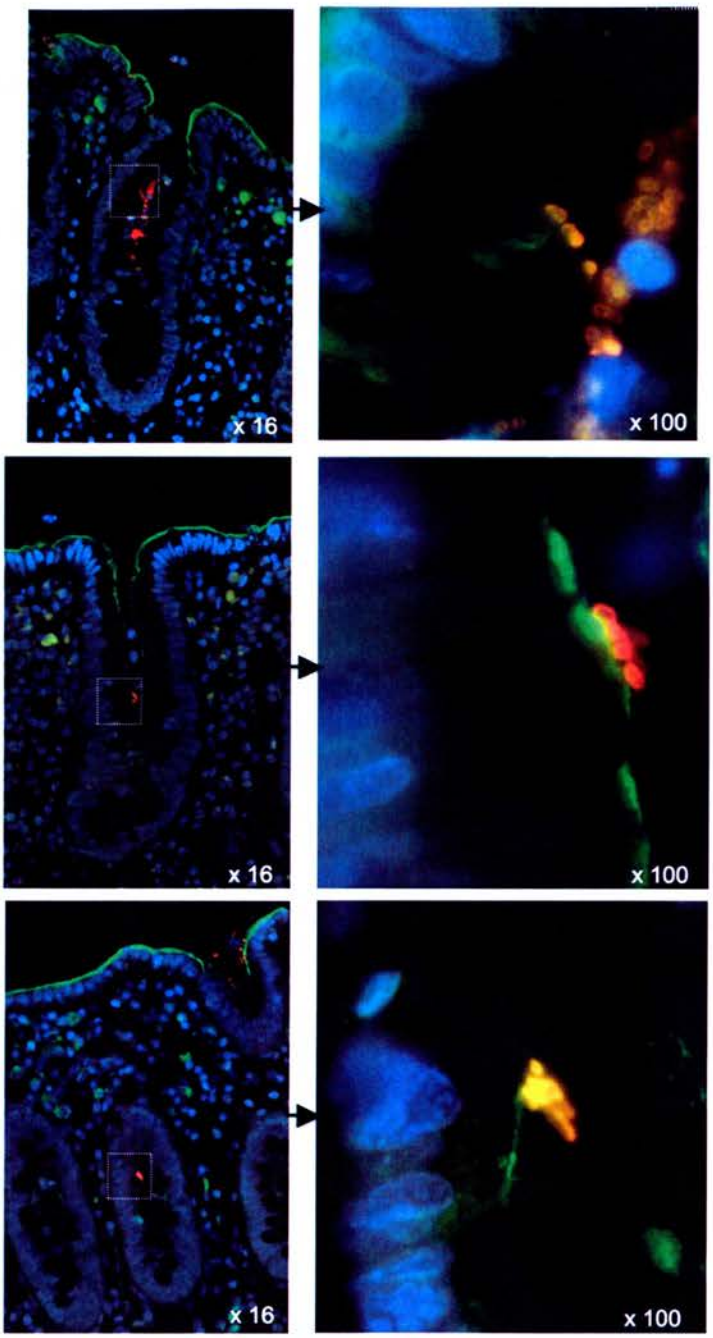


Figure 4.9. EPEC enters colonic crypts of *ex vivo* human colonic mucosa. Colon mucosa was separated from full thickness colon tissue removed from surgical patients. Mucosa was co-cultured with wild-type (E2348/69) EPEC for 12 h then washed and fixed in formalin. Immunosections were stained for *E. coli* (red) and ezrin (green), nuclei were counter stained with DAPI (blue). Broken white boxes delineate area of magnification in subsequent image.

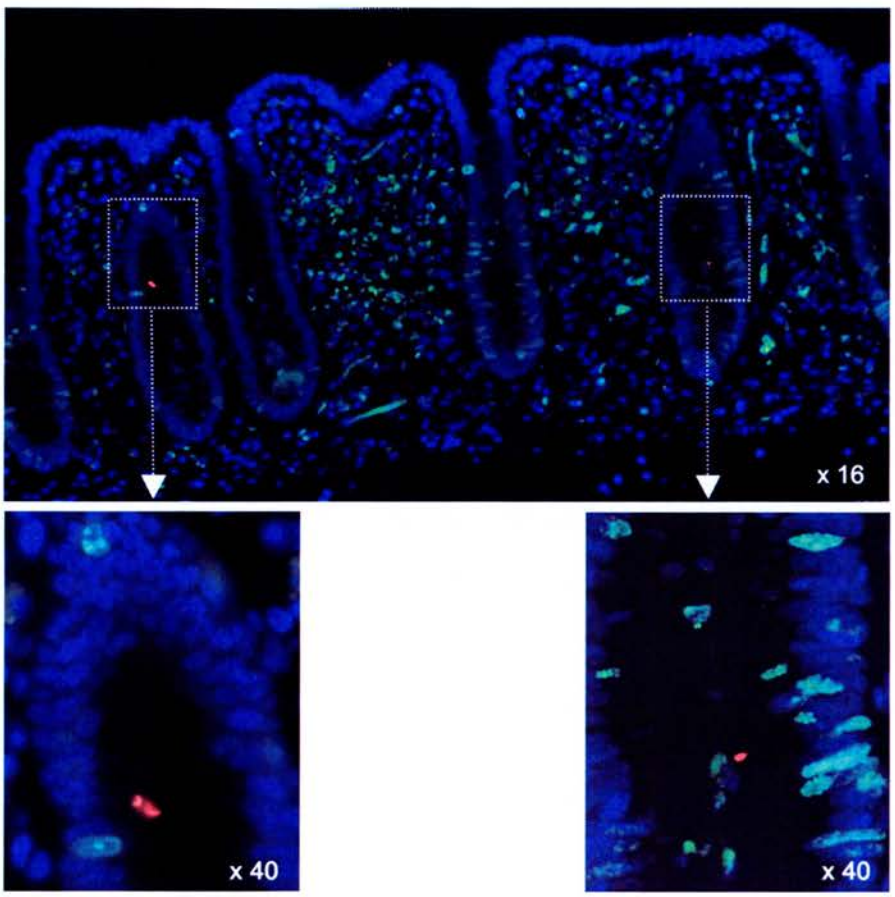


Figure 4.10. EPEC enters proliferative compartment of colonic crypts. Colon mucosa was separated from full thickness colon tissue removed from surgical patients. Mucosa was co-cultured with wild-type (E2348/69) EPEC for 12 h then washed and fixed in formalin. Immunosections were stained for *E. coli* (red) and ki67 (green), nuclei were counter stained with DAPI (blue). Broken white boxes delineate area of magnification in subsequent image.

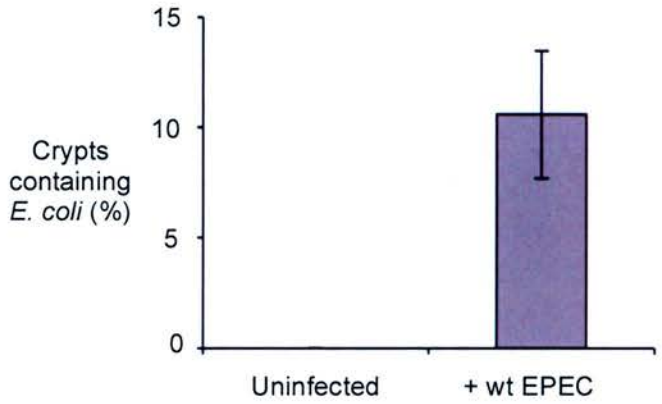


Figure 4.11. Frequency of crypt infection in *ex vivo* human colon tissue. Colon mucosa was separated from full thickness colon tissue removed from surgical patients (n = 3). Uninfected pieces of mucosa were washed and fixed in formalin. Matched pieces of mucosa were washed and then co-cultured with wild-type (E2348/69) EPEC for 12 hours, then washed and fixed in formalin. Immunosections were stained for *E. coli* and the number of crypts containing *E. coli* were counted and calculated as a percentage of total crypts (>300 crypts counted in each instance). Error bars represent standard error of mean.

4.2.3. Prevalence of AE *E. coli* infection and *eae* in colorectal cancer patients

It was hypothesised that EPEC infection is a risk factor in the development of colon cancer in man, and therefore, that tissue from colorectal cancer patients would harbour residual EPEC infection. Formalin fixed, paraffin embedded samples of colorectal adenocarcinoma and normal colon tissue from 20 randomly selected patients were obtained from archives of the Department of Histopathology, Edinburgh University. Due to the absence of a commercial antibody for EPEC it was not possible to unambiguously identify EPEC. However, by using a generic anti-*E.coli* antibody and PCR for intimin, it was possible to confirm the presence of *E. coli* with AE ability.

Immunofluorescent staining revealed the presence of mucosa associated *E. coli* in colonic crypts of normal tissue (Figure 4.12). Within adenocarcinoma tissue large numbers of *E. coli* were found. In a number of cases the *E. coli* were closely associated with the mucosa, often within (tubulo-villus) crypts and sometimes seemingly integrated into the adenocarcinoma tissue itself (Figure 4.13). PCR amplification identified the presence of *eae* in a number of normal and tumour tissue samples (Figure 4.14). One adenocarcinoma showed a particularly high level of *E. coli* infection, with bacteria penetrating deep into the crypts and hundreds of *E. coli* very closely associated with the surface epithelium (Figure 4.15b). A repeat PCR on this tissue confirmed the presence of the bacterial *eae* gene (Figure 4.15c).

Overall it was found that 95% of adenocarcinomas had mucus associated *E. coli*, and 50% had mucosa associated *E. coli*. Of the normal tissue samples, 60% held mucus associated *E. coli* and 15% had mucosa associated *E. coli*. The presence of the bacterial *eae* gene was identified in 25% of adenocarcinomas and 0% of normal tissue samples (Table 4.3). These results therefore show that a significant percentage of cancer patients harboured AE *E. coli* within their colon, and that AE *E. coli* were frequently associated with tumour rather than normal tissue in these patients.

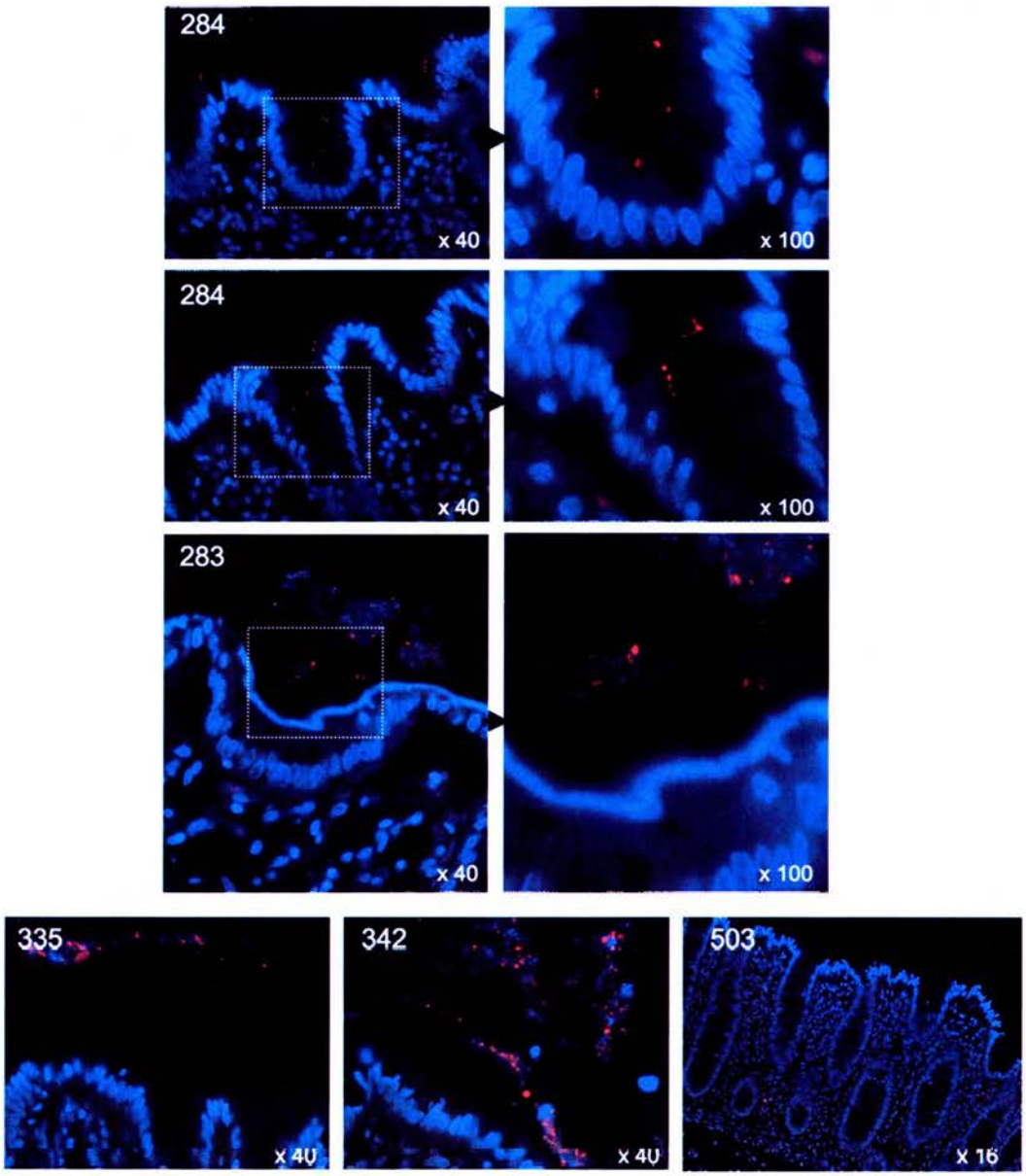


Figure 4.12. Identifying *E. coli* in normal human colon tissue. Normal tissue from 20 colorectal cancer patients was tested for the presence of *E. coli* (red) by immunofluorescence, nuclei were counterstained with DAPI (blue). Dashed boxes delineate magnified field to the right. Case numbers in top left corner of first image in sequence. *E. coli* can be seen as mucosa associated (284), mucus associated (283, 335 & 342) or absent (503). Original magnification shown in bottom right-hand corner of each image.

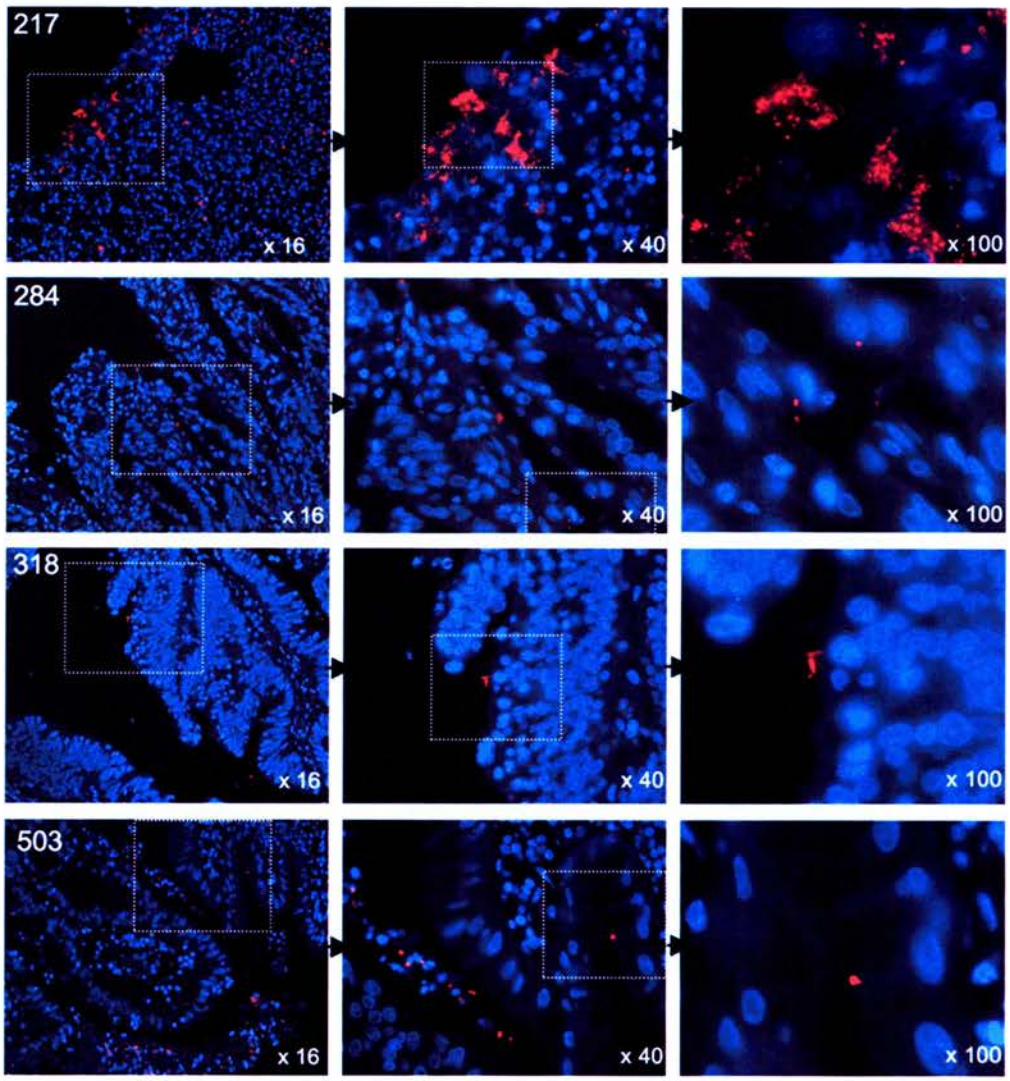


Figure 4.13. Identifying *E. coli* in human adenocarcinoma tissue.
Adenocarcinoma tissue from 20 patients was tested for the presence of *E. coli* (red) by immunofluorescence, nuclei were counterstained with DAPI (blue). Dashed boxes delineate magnified field to the right. Case numbers in top left corner of first image in sequence. Original magnification shown in bottom right-hand corner of each image.

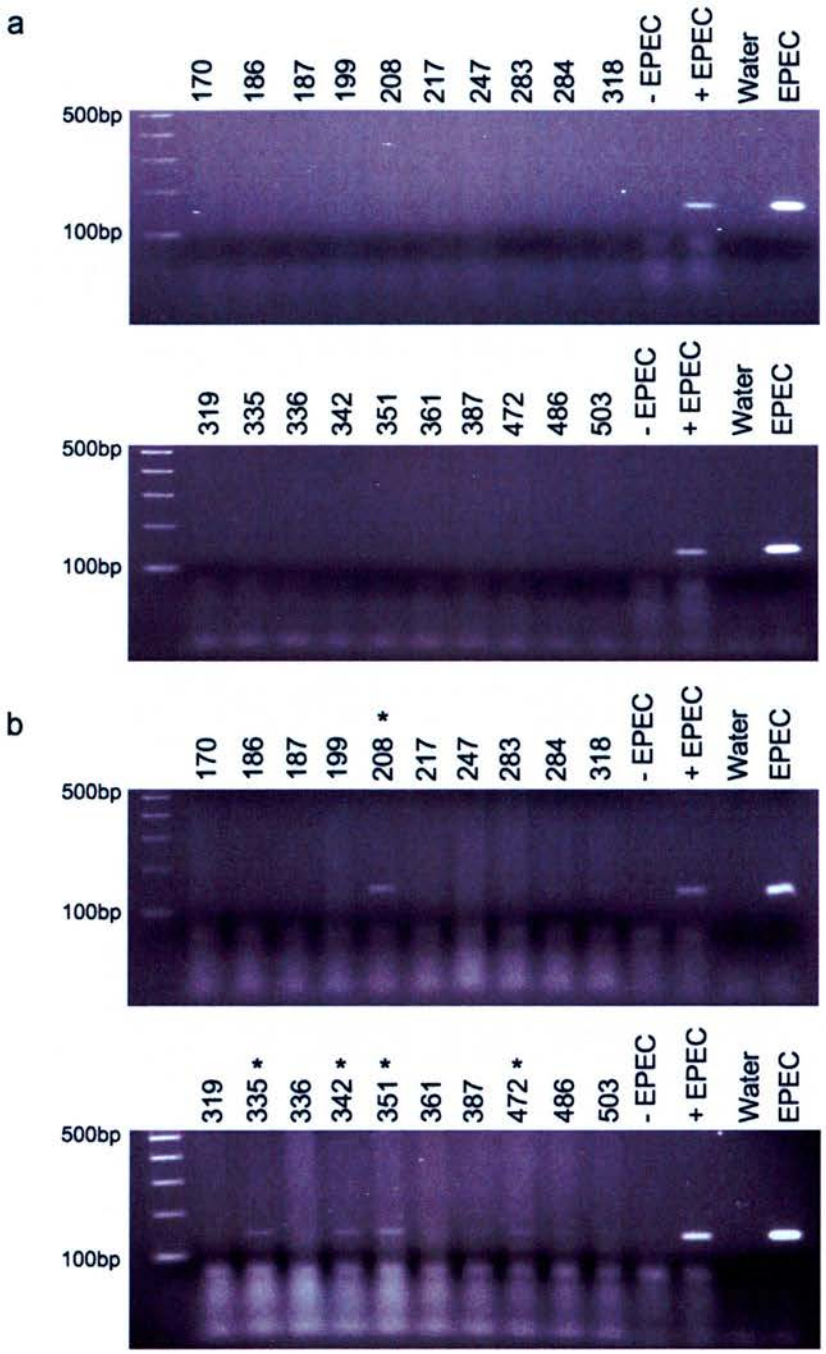


Figure 4.14. Detection of *eae* in human normal and adenocarcinoma tissue. Paraffin embedded sections (20μm) of normal mucosa (**a**) and adenocarcinoma (**b**) from 20 randomly selected patients were deparaffinised and subjected to proteinase K digestion. DNA was obtained by phenol chloroform extraction and PCR for the AE bacteria gene *eae* was performed. Uninfected (- EPEC) and EPEC infected (+ EPEC) human *ex vivo* tissue sections were used as negative and positive controls. DNA extracted directly from overnight culture of EPEC was also used as a positive control (EPEC). *Cases judged positive. PCR reactions performed by Scott Bader.

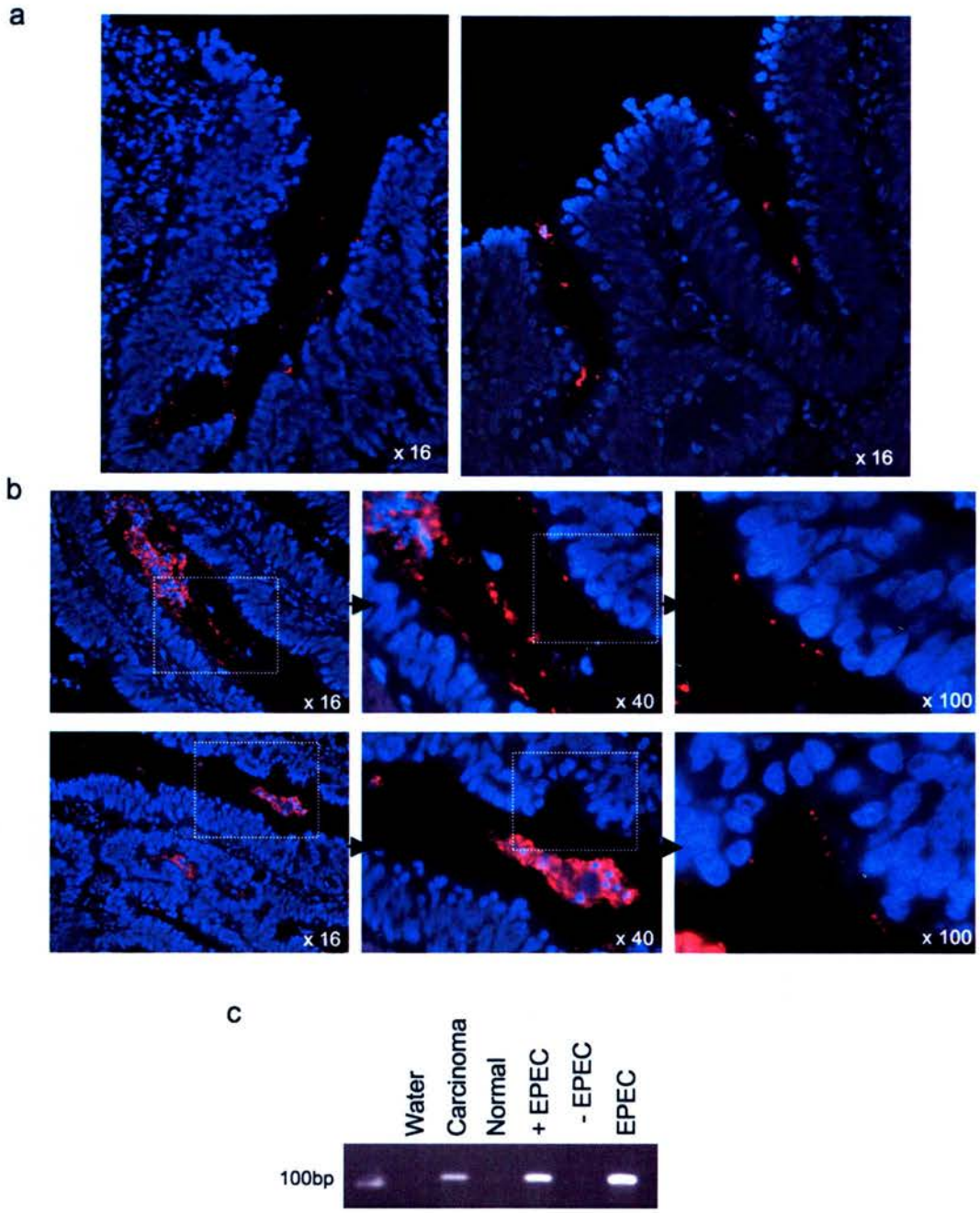


Figure 4.15. Example of an adenocarcinoma highly infected with adherent AE *E. coli.* Adenocarcinoma case 342 immunostained for *E. coli* (red), nuclei were counterstained with DAPI (blue). *E. coli* were identified in tubulovillus crypts on the surface of the tissue (**a**) and deeper within the adenocarcinoma (**b**). A repeat PCR for *eae* was performed on tissue sections using a different reverse primer (**c**). Human *ex vivo* colon tissue sections infected with wild-type EPEC (+ EPEC) or uninfected (- EPEC) were used as additional positive and negative controls for PCR. Broken white boxes delineate areas of magnification shown in subsequent images.

Case No.	Normal			Adenocarcinoma		
	Mucus *E. coli*	Mucosal *E. coli*	Intimin	Mucus *E. coli*	Mucosal *E. coli*	Intimin
170	+	-		+	-	
186	-	-		-	-	
187	-	-		+	-	
199	-	-		+	-	
208	++	-		++	+	♦
217	+	-		+++	+++	
247	-	-		+	+	
283	+	-		+++	-	
284	++	+		+++	+	
318	+	-		++	+	
319	++	-		+	-	
335	+	-		+	+	♦
336	++	-		++	-	
342	++	+		+++	+++	♦
351	+++	+		++	+	♦
361	-	-		+	-	
387	-	-		+	-	
472	-	-		++	+	♦
486	-	-		+	-	
503	+	-		+++	+	
Total	**12/20 60%**	**3/20 15%**	**0/20 0%**	**19/20 95%**	**10/20 50%**	**5/20 25%**

Table 4.3. *E. coli* infection and *eae* identification in human colon tissue. Paraffin embedded adenocarcinoma and matched normal tissue from 20 patients was tested for the presence of *E. coli* by immunofluorescence and for expression of the *eae* gene (encoding intimin) by PCR (♦). The level of *E. coli* infection was scored as zero (-), tens (+), hundreds (++) or thousands (+++) of bacteria. Both mucus associated and mucosa associated *E. coli* were evaluated.

4.3. Discussion

Citrobacter rodentium produced characteristic hyperplasia in C57Bl/6 mice, whereas mice infected with EPEC did not display any signs of hyperplasia. In man, EPEC infection is not known to cause hyperplasia, this initial result therefore suggests that infection of mice with EPEC rather than *C. rodentium* might represent a more pathologically relevant model. However, counts of adherent and non-adherent EPEC in the lower gut of infected animals revealed low levels of EPEC and a short time-course of infection. After 3 days, no adherent EPEC were detected in the cecum or colon. Savkovic *et. al.* (2005) report that after 3 days infection of C57Bl/6 mice with EPEC, cecum (~16,300) CFU) and colon (~6,500 CFU) tissue held relatively high levels of adherent EPEC. In an attempt to improve the model (i.e. to increase gut colonisation by EPEC) C3H/HeN mice were infected, this strain shows greater susceptibility to *C. rodentium* infection (Vallance *et. al.,* 2003). Higher levels of adherent and non-adherent EPEC were found in these animals compared to C57Bl/6 mice, however, the levels were still low in comparison the published data. Furthermore, infection did not persist in these animals in the medium term.

The inability to recreate this model may be attributable to a number of factors, including differences in the host animals and differences in technique. Tissue was homogenised using curved scissors and syringing, whereas the paper of Savkovic *et. al.* (2005) is unclear as to the methods of homogenisation. Agar supplemented with nalidixic acid rather than ampicillin was used to count bacteria, a difference in selectivity / potency of these antibiotics may account for greater counts in the published report. Host gut microflora would have a direct effect on colonisation by foreign bacteria. Mice used in this study were obtained from a different supplier than those in the published report; hence differences in the commensal organism populations within the mice would be expected to differ. However, despite any differences in mice, it was notable that it was possible to recreate the *C. rodentium* infection model with ease. Overall, results suggest that establishing EPEC infection model in mice is not straightforward.

In light of *in vivo* mouse results, co-culture studies were performed to evaluate the species-specific properties of EPEC and *C. rodentium in vitro*. Infection of a mouse colon cancer cell line with EPEC did not produce the same characteristic changes in protein expression seen in human cells. Furthermore, infection of mouse cells with *C. rodentium* also failed to produce the characteristic protein changes caused by EPEC in human cells. It was also noted that adherence of both EPEC and *C. rodentium* to CMT-93 cells was considerably lower than for EPEC with HT29 cells, supporting the results of Tobe and Sasakawa (2002). It is possible therefore that CMT-93 cells do not have surface residues conducive to bacterial attachment. However, these results do suggest that EPEC pathology (and potentially adherence) are species specific, and conflict with the conclusions of Savkovic *et. al.* (2005) that mice provide a viable animal model for EPEC infection.

In summary, the pathological effects of *C. rodentium* infection in mice are unlike those of EPEC in man, and are in fact analogous to inflammatory bowel disease in humans (Newman *et. al.,* 2001). Hence, although *C. rodentium* may provide a convenient model for the AE process *per se*, it does not necessarily represent a viable model for the biological consequences of EPEC infection in man. The *C. rodentium* model could however be potentially used to study how inflammatory diseases such as Crohn's disease and Ulcerative Colitis predispose to cancer in man. Due to the inability to induce persistent EPEC infection in mice, and the species specific effects observed in cell lines, it was concluded that infection of C57Bl/6 and C3H/HeN mice with EPEC did not represent viable models for EPEC infection in man either. After observing the *in vitro* specificity of EPEC, further enhancement of the mouse model was not attempted. However, as one possible modification, pre-treatment of mice (with antibiotics) to reduce numbers of commensal bacteria would have been likely to increase infection. Another more complex approach would be to engineer EPEC strains with enhanced affinity for mouse tissue. In studying intimin subtypes, Phillips & Frankel (2000) constructed EPEC expressing chimera intimin-α/γ. A similar approach, using *C. rodentium* derived adhesion factors could allow human virulent EPEC strains to attach more effectively to the mouse gut.

Evidently, at the same time as the experiments in this study were being performed, other workers were drawing the same conclusions about the validity of the EPEC mouse model. Mundy *et. al.* (2006) also studied EPEC infection in mice, using C57Bl/6 and C3H/HeJ strains, comparing their results to *C. rodentium* infection and also non-pathogenic (commensal) *E. coli*. The authors used homogenised tissue bacterial counts to assess adherent bacteria, and stool counts for non-adherent bacteria. Although Mundy *et. al.* (2006) report higher levels of adherent bacteria, and longer time-course of infection than this study, the authors conclude that EPEC: were unable to effectively colonise the colonic epithelium; were unable to expand *in vivo*; demonstrate a commensal rather than pathogenic organism in the mouse; and that infection of mice with EPEC has very limited usefulness. The authors note that their results are supported by aspects of a recent study that showed EPEC did not colonise mice (Klapproth *et. al.* 2005) and conflict with Savkovic *et. al.* (2005).

By adapting published organ culture protocols a system to co-culture viable colonic mucosa with EPEC for 12 hours was established. Co-culture resulted in the migration of EPEC into the colonic crypts, hence after 12 hours just over 1 in 10 crypts held EPEC. Additionally, EPEC were found in the mid-crypt region, often half way down the crypt. Although staining of ezrin did not conclusively demonstrate AE lesion formation, ezrin accumulation below EPEC was noted in some cases. It was clear that EPEC associated very closely with the surface of the crypt epithelial cells. Immunofluorescence also confirmed that EPEC penetrated far enough into the crypts to reach the proliferative progenitor cell compartment. It was also notable that EPEC rarely associated with cells of the surface epithelium. Importantly, these results demonstrate – potentially for the first time – how EPEC might behave within the healthy adult colon.

Undifferentiated proliferative progenitor cells lie in the lower two-thirds of colonic crypts (Sancho *et. al.*, 2004), and are thought to have the potential to initiate tumour formation (van der Wettering *et. al.*, 2002). Loss of control over differentiation / apoptosis or cell cycle by these cells (e.g. due to mutation induced by dietary carcinogens) would almost certainly facilitate tumourigenesis. If EPEC intimately

attach to crypt progenitor cells *in vivo,* it is quite possible that EPEC induced changes in host cell signalling pathways could influence the susceptibility of these cells to cancer development.

Using enhanced immunostaining and microscopy techniques in conjunction with this model would make it possible to clarify whether EPEC is able to form AE lesions and attach intimately within crypts. Scanning electron microscopy (SEM) and particularly transmission electronic microscopy (TEM) would be capable of this. *In situ* staining of proteins such as Nck, N-WASP and Tir (as recently demonstrated by Schuller *et. al.,* 2007) would help to identify actin pedestals. Directly staining actin with fitc-phalloidin (an adapted FAS test) was attempted in this study, however this method failed to stain actin consistently in fixed tissue (not shown). Using frozen rather than formalin fixed tissue would probably have improved results. Similar methods could also be used to analyse EPEC induced protein changes in host cells. The ability of EPEC to enter crypts is potentially achieved via propulsion by flagella. This could be tested by comparing the behaviour of EPEC cultured in DMEM (which inhibits flagella expression: Zhou *et. al.,* 2003) versus EPEC grown in other media.

In fixed normal and adenocarcinoma tissue from colorectal cancer patients EPEC was most often seen in the mucus layer, mucosa associated bacteria were found on half of the adenocarcinomas but only on a few normal tissue samples. The bacterial gene for intimin *eae* was also more prevalent in the tumour compared to normal tissue, and was identified in a quarter of tumours. Within the sample of 20 cases, *E. coli* and AE *E. coli* were more commonly found in association with tumour tissue than normal tissue in the human colon. These results support the findings of Swidsinski *et. al.* (1998) and Martin *et. al.* (2004) who also found *E. coli* more commonly associated with tumour rather than normal tissue in colorectal cancer patients. This is also potentially the first study to find an association between AE *E. coli* and colorectal adenocarcinoma. Martin *et. al.* (2004) attribute the apparent preferential binding to the expression of bacterial adhesins with specificity for

"oncofetal" carbohydrate antigens over-expressed by mucosal glycoconjugates in cancer.

It is well established that cancer cells display altered surface characteristics compared to normal cells; these include the appearance of new antigens, proteoglycans, glycolipids and mucins (Kufe *et. al.,* 2003). Aberrant glycosylation is thought to be a key factor in these changes, and may result from three processes: attenuated synthesis of normally expressed carbohydrate chains; activation of glycosyltranferase enzymes; or reorganisation of tumour cell membrane glycolipids (Kufe *et. al.,* 2003). These changes represent a realistic basis for altered adhesion by AE *E. coli.* At present the exact residue(s) to which EPEC binds on host cells remains unclear. By screening a range of glyco- and phospholipids, Barnett-Foster *et. al.* (1999) found that EPEC and EHEC bound in a specific and dose dependent manner to phosphatidylethanolamine and correlated this to bfp expression. Other candidate receptors include gangliotriaosylceramide, and gangliotetraosylceramide, 32-33 kDa glycoproteins and lactosamine sequences lacking sialic acid (reviewed by Tobe and Sasakawa, 2002). Carbohydrates, such as D-mannose also influence EPEC binding to epithelial cells, with the ability to enhance or inhibit adherence depending on concentration (Schaeffer *et. al.,* 1980).

Although *eae* was not identified in any normal tissue samples by PCR, three samples had mucosa associated *E. coli,* and in at least one case these seemed to be adherent to crypt cells. This supports the findings from the *ex vivo* model where EPEC apparently bound preferentially to colonic crypt epithelial cells within adult colon tissue. Higher numbers of *E. coli* were clearly found on tumour samples compared to normal tissue. Aside from differences in surface residues, the difference in detection of *E. coli* and *eae* between normal and tumour tissue may be partially explained by the architecture of tumour versus normal tissue. The vastly increased surface area of adenocarcinoma provides more opportunities for binding. Also, the bacteria enclosed within folds of the tumour would be less likely to be shed *in vivo* or washed off while tissue was prepared and fixed. Hence AE *E. coli* numbers may have been too low in normal tissue (and some tumour samples) to detect *eae* by standard PCR.

A potential explanation for the apparent affinity of EPEC for crypt cells is the change in expression of mucosal surface glycoproteins during migration of epithelial cells from crypt to surface epithelium (Kim *et. al.*, 1971; Etzler & Branstrator, 1974). Within the crypts, mitotically active, undifferentiated crypt cells (in similarity to fetal cells) express active glycosyltransferase enzymes and acceptor sites that are glycoproteins with incomplete polysaccharide chains (Weiser, 1973). A selective ability to bind to these residues could also provide an explanation for the affinity of AE *E. coli* for tumour tissue, and possibly for the more severe pathology of EPEC infection in the infant intestine. The ability of EPEC to enter crypts of the adult human colon and to adhere to cells in the proliferative region are novel findings, and illustrate a potential niche environment for EPEC in the (asymptomatic) adult colon.

Further study of tissue from cancer patients would help to confirm an association between AE *E. coli* and cancer. One of the limitations of this study was that fixed tissue was used. Hence, PCR was performed on small numbers of bacteria that had been embedded in paraffin for a number of years, probably leading to nucleic acid degradation and cross-linking. Fresh tissue would be preferable to fixed tissue as bacteria could be sub-cultured and identified more readily (e.g. by PCR and phenotypic assays such as FAS test). Due to time limitations, only PCR for *eae* was performed, although this confirmed the presence of AE *E. coli*, it did not confirm that these were EPEC. Further PCR for *stx*, the gene for shiga toxin, expressed only by EHEC would have enabled us to differentiate between EHEC and EPEC. It would also be very interesting to find out what the levels of EPEC carriage are amongst the healthy adult population; analysis of stool samples or biopsy tissue could help to achieve this. This data could then be compared to carriage levels in cancer patients. Furthermore, long-term follow-up of EPEC carriers would fundamentally determine any link between EPEC and colon cancer.

Chapter 5.

Effect of EPEC infection on DNA mismatch repair protein expression and apoptosis in human colorectal cell lines.

5.1. Introduction

5.1.1. DNA mismatch repair

The hypothesis that EPEC increases susceptibility to colon cancer is founded on the contention that the molecular effects of infection are oncogenic. Microarray analysis of gene expression in cells co-cultured with EPEC provides evidence of molecular changes with the potential to increase the susceptibility of infected cells to mutation. Genes encoding DNA mismatch repair proteins showed a greater than two-fold reduction in expression after co-culture of HT29 cells with wild-type EPEC (Chapter 3). DNA mismatch repair disruption is a causative factor in the development of both familial and sporadic colorectal cancer. *Helicobacter pylori* infection has been shown to impair DNA mismatch repair (MMR) via reduction in MSH2 and MLH1 protein levels in gastric epithelial cells (Kim *et al.,* 2002; Park *et al.,* 2005). Patients with MSI-positive tumours are also more likely to have active *H. pylori* infection than patients with MSI-negative tumours (Leung *et. al.,* 2000). An aim of this study was therefore to investigate the effects of EPEC infection on the expression of DNA mismatch repair proteins using the extended time-course co-culture model.

The presence of mechanisms for the correction of DNA base pair mismatches were first identified using *E. coli,* in which the MutS and MutL proteins are key components of the MMR pathway (Modrich *et al.,* 1991). Human 'MutS homologues' (Msh2, Msh3, Msh6) and 'MutL homologues' (Mlh1, Mlh3, Pms1, Pms2) are the principal proteins of MMR pathways in man (Peltomaki, 2001). The MMR genes can be described as indirect tumour suppressor genes. Their role is to correct post-replication DNA base pair mismatches and insertion-deletion loops created by DNA polymerases (Jiricny, 2000[a]). Failure of the DNA MMR system confers significant genomic instability, promoting rapid accumulation of point and frame-shift mutations in repeated sequence motifs (Jiricny, 2000[b]), a scenario termed microsatellite instability (Thibodeau *et. al.,* 1993). Mutation of MMR genes, resulting in microsatellite instability (MSI) is the defining genetic characteristic of the most common form of inherited colorectal cancer, Hereditary Non-Polyposis Colorectal Cancer (HNPCC) (Lynch & de la Chapelle 1999). MMR gene mutation

and MSI are also found in approximately 15% of sporadic cases of colorectal cancer (Liu *et al.,* 1995). The genomic instability resulting from MMR disruption is implicated in cancer development by facilitating and accelerating the accumulation of mutations in tumour suppressor and oncogenes such as *APC*, *TP53* and *K-ras*. Hence, cancers with disrupted MMR function are said to display a 'mutator' phenotype.

5.1.2. DNA mismatch repair protein apoptosis signalling

In addition to their role in DNA repair, MMR proteins also have a vital role in signalling DNA damage induced apoptosis. Cytotoxic and carcinogenic chemical agents (such as 5-fluorouracil, cisplatin, alkylating agents and methylating agents) create DNA damage that is recognised by the MMR system (Jascur & Boland, 2006). In detecting severe DNA damage, MMR proteins can either direct cell cycle arrest (in order to allow more time for repair), or if the damage is too great, apoptosis (Bernstein *et. al.,* 2002). Hence the absence of functional MMR proteins significantly reduces the ability of cytotoxic agents to cause apoptosis. MMR protein pro-apoptotic signalling can occur via stabilisation of the anti-apoptotic mediators p53 (Luo *et. al.,* 2004), or p73 (Shimodaira *et. al.,* 2003). Furthermore, these studies showed that p53 and p73 mediated apoptosis are dependent on MMR protein expression. When overexpression of MSH2 or MLH1 protein is induced in repair proficient or deficient cells, apoptosis is triggered, and in cells isolated from MSH2 null mice, the ability to undergo mutagen induced apoptosis is lost (Zhang *et. al.,* 1999).

In the colon epithelium (and elsewhere) induction of apoptosis is an essential mechanism for maintaining normal tissue homeostasis and also preventing the clonal expansion of cells with tumourigenic mutations. The role of MMR proteins in pro-apoptotic signalling therefore provides an additional mechanism by which the disruption of MMR can increase susceptibility of cells to transformation. MSH2 null mice (*Msh2*$^{-/-}$) are predisposed to malignancy (Toft *et. al.,* 1999). Although, mice carrying a specific homozygous mutation in *Msh2* (that attenuates mismatch repair but not apoptosis signalling) are still highly prone to tumours, tumourigenesis occurs

more slowly in comparison to MSH2 null animals (Lin *et. al.,* 2004). This experiment therefore proves that disruption of MMR does indeed promote tumourigenesis by (at least) two mechanisms, i.e. attenuation of DNA repair *and* DNA damage induced apoptosis signalling.

5.1.3. DNA mismatch repair protein regulation

Although much research has been carried out into the mechanisms by which MMR proteins undertake DNA repair (discussed in Chapter 1), less information exists on how the expression of these proteins is regulated. A confounding factor in defining MMR protein regulation mechanisms is that a wide range of processes trigger changes in MMR protein expression. At the transcriptional level, MMR protein expression can be suppressed by hypoxia (Koshiji *et. al.,* 2005), and in colon cancer both by DNA methylation (Fang *et, al.,* 2006) and Bcl-2 over expression (Youn *et. al.,* 2005). Whereas, increased transcription of MMR genes has been observed in response to stimulated cell growth (Iwanaga *et. al.,* 2004) and PKC activation (Humbert *et. al.,* 2003). Aspirin treatment also causes a marked upregulation of MMR protein *in vitro* (Goel *et. al.,* 2003).

At the post-translational level, phosphorylation of MMR proteins by PKCζ induces activation and nuclear translocation (Hernandez-Pigeon *et. al.,* 2005). Ubiquitination is an established mechanism for DNA repair protein regulation (Huang & Andrea, 2006), and MMR protein degradation can occur via ubiquitination initiated proteosomal degradation (Hernandez-Pigeon *et. al.,* 2004). However, this process is blocked via PKCζ catalysed MMR protein phosphorylation (Hernandez-Pigeon *et. al.,* 2005). Additionally, MMR proteins achieve a degree of self-regulation, whereby the expression of MSH2 and MLH1 dictate the level of MSH3, MSH6 and PMS2 protein (Chang *et. al.,* 2000). Hence, in the absence of MSH2 protein, MSH3 and MSH6 are not expressed, and without MLH1 protein, the expression of PMS2 protein is suppressed, despite the presence of PMS2 mRNA. Chang *et. al.* (2000) also demonstrated that MSH2 (followed by MLH1) was the most abundant MMR

protein in MMR proficient cells, further qualifying the importance of these two proteins.

The half-life of MSH2 in human epithelial (HeLa) cells can be estimated by extrapolating the results of Hernandez-Pigeon *et. al.* (2004) who studied the turnover of MMR proteins by inhibiting *de novo* protein synthesis using cyclohexamine. It is notable that MSH2 and MSH6 protein half-lives varied considerably between cell lines. In HeLa cells MSH2 half-life can be estimated as approximately 80 hours and the half-life of MSH6 approximately 30 hours. HeLa cells were the only epithelial cell line tested, and expressed relatively high baseline levels of MMR protein. In U937 (monocytic) cells and HL-60 (myelocytic) cells MSH2 half-life was 14 hours and 8 hours respectively (Hernandez-Pigeon *et. al.*, 2004).

5.1.4. Experimental evidence for the consequences of DNA MMR disruption

The rate of endogenous DNA damage is estimated to be from $10^4 - 10^6$ damages per cell, per day (Ames *et. al.,* 1991; Holmquist, 1998), and reactive oxygen species are thought to be a major causative factor in this process. Cells exposed to exogenous genotoxic agents have much higher rates of damage (Bernstein *et. al.,* 2002). Mouse models using gene knockout techniques have proven that MMR disruption prevents cells from dealing appropriately with DNA damage caused by endogenous and exogenous stimuli, directly resulting in increased mutation rates and tumour formation. Hence, mice heterozygous or homozygous for *Mlh1* gene knockout are predisposed to gastrointestinal tumour formation, and generate tumours deficient in Apc protein (Edelmann *et. al.,* 1999). Tumour growth in response to X-ray radiation is also enhanced by loss of Mlh1 (Tokairin *et. al.,* 2006). In Msh2 null mice, spontaneous mutation frequency is 8 – 9 fold higher in all parts of the colon compared to Msh2 competent mice (Zhang *et. al.,* 2002). Consequently, the food borne mutagen PhIP (2-amino-1-methyl-1-6-phenolimidazo[4,5-b]pyridine) induces a significantly greater increase in mutation frequency in Msh2 null verses normal mice (Zhang *et. al.,* 2001). The methylating agent and mutagen DMH (1,2-dimethylhydrazine) induces apoptosis of colonic epithelial cells dependant on Msh2

expression, and accelerates the death of Msh2 null mice (versus Msh2 competent mice) due to colorectal tumours and lymphomas (Colussi *et. al.,* 2001).

Significantly, Msh2 knockout also enhances somatic mutation of *Apc* and *p53* in $Apc^{+/-}Mlh1^{-/-}$ mice. Sohn *et. al.* (2003) report that $Apc^{+/-}Mlh1^{+/+}$ mice generate tumours via *Apc* LOH, but in $Apc^{+/-}Mlh1^{-/-}$ mice tumours originate via somatic mutation of the remaining *Apc* allele (i.e. without LOH). Furthermore, 45% of adenomas from $Apc^{+/-}Mlh1^{-/-}$ mice carried *p53* mutations, whereas $Apc^{+/-}Mlh1^{+/+}$ tumours did not carry this mutation. The authors conclude that Msh2 deficiency causes a state of hypermutabilty. These results also suggest that somatic mutations occur more rapidly than LOH as the cause of *Apc* gene silencing in Msh2 deficiency.

5.1.5. Mutation frequency analysis

Based on the link between MMR protein expression and mutation, the effect of EPEC infection on mutation frequency was analysed. Mutation frequency can be analysed *in vitro* by exposing cells to toxins that depend on the expression of specific genes to trigger cell death. Mutation of these genes allows cells to survive toxicity; hence the number of surviving cells indicates the number of cells that have acquired mutations. In our initial mutation frequency experiments we used the toxin ouabain, which causes cell death via blockade of Na^+,K^+-ATPase (Capella *et. al.,* 2001). As an alternative method for mutation frequency analysis we used inter-Alu PCR. Repeated Alu sequences are scattered throughout the human genome and are preferential targets of replication errors. When inter-Alu PCR products are resolved through polyacrylamide gels 40-60 bands are detected. Inter-Alu PCR has been used to detect changes in these repeated sequences in cancer patients; Krajinovic *et. al.* (1996) compared DNA from normal tissue to DNA extracted from microsatellite unstable tumours and found differences in 20% of bands. Changes in band signal intensities were common, and loss or gain of bands also occurred, hence inter-Alu PCR was able to detect the mutator phenotype in cancer cells.

5.2. Results

5.2.1. Effect of EPEC infection on MSH2 and MLH1 protein expression

Western blots revealed that the expression of MSH2 and MLH1 was reduced considerably in HT29 cells (relative to actin and PCNA expression) after 9-12 hours co-culture with wild-type EPEC (Figure 5.1a). In HT29 cells co-cultured with non-AE mutant EPEC, MSH2 and MLH1 expression remained relatively strong after 9-12 hours (Figure 5.1b). Removal of wild-type EPEC (by treatment with antibiotics) subsequent to co-culture allowed MSH2 and MLH1 expression to return to pre-infection levels after 36-39 hours (Figure 5.1c). As cell division does not occur until after 48-60 hours of recovery (Section 3.2, Figure 3.1) it can be concluded that the recovery of MHS2 and MLH1 expression cannot be accounted for by newly formed daughter cells. Therefore, MSH2 and MLH1 protein downregulation is a reversible consequence of intimate attachment of EPEC to HT29 cells.

Western blots of SW480 cells showed that MSH2 and MLH1 expression was also downregulated by wild-type EPEC infection (Figure 5.2a). The effects of EPEC on LS513 cells were less pronounced: MSH2 expression remained strong after 9 hours co-culture with wild-type EPEC, after 12 hours expression was reduced but detectable. MLH1 expression was reduced but still detectable after 9 hours and below detection after 12 hours (Figure 5.2b).

Immunofluorescent staining of HT29 cells allowed analysis of changes in protein expression *in situ*. In untreated HT29 cells, MSH2 and MLH1 staining was localised to cell nuclei. Most nuclei showed intermediate staining intensity, some cells showed very strong staining intensity and a small number of cells had no visible nuclear stain. In mutant EPEC infected controls nuclear MSH2 and MLH1 staining showed a similar pattern to uninfected controls, with most cells demonstrating intermediate nuclear staining intensity. In cells infected with wild-type EPEC for 9 hours a shift in staining pattern was observed; although most cells showed intermediate nuclear staining, very few showed strong nuclear staining, with many more cells having no

nuclear MSH2 or MLH1 signal compared to controls. After 12 hours co-culture with wild-type EPEC no cells showed strong nuclear staining, a small number of cells showed intermediate staining with almost all cells having no nuclear stain. Nuclear expression of PCNA (western blot loading control) was retained by cells co-cultured with wild-type EPEC, showing a small reduction after 12 hours (Figure 5.3). Similar trends in MSH2, MLH1 and PCNA protein expression respectively were observed in SW480 cells (Figure 5.4).

In order to more accurately quantify the observed changes in nuclear MSH2 and MLH1 staining digital images of immunofluorescent stained HT29 cells were analysed with image analysis software. In uninfected and 9 hours mutant infected controls approximately 90% of cells displayed intermediate or strong nuclear MSH2 and MLH1 staining. However, after 9 hours co-culture with wild-type EPEC only 60-70% of cells had intermediate or strong staining. After 12 hours co-culture 75-80% of mutant infected cells retained intermediate or strong nuclear staining for MSH2 and MLH1, whereas in wild-type infected cells only 5% displayed intermediate staining with all other cells showing no nuclear staining (Figure 5.5a,b).

As a positive control, nuclear PCNA signal was also analysed. In uninfected and mutant infected controls PCNA staining showed a similar pattern to MSH2 and MLH1 staining, with the vast majority of cells showing intermediate or strong staining. Although a small reduction in positive cells was observed after 9 and 12 hours co-culture with wild-type EPEC, the reduction was far less than that seen for MLH1 and MSH2. After 9 and 12 hours co-culture with wild-type EPEC, 72% and 64% of cells showed intermediate or strong PCNA staining respectively (Figure 5.5c). Immunofluorescent staining of MLH1, MSH2 and PCNA was also performed with SW480 cells co-cultured with mutant and wild-type EPEC. Image analysis revealed the same respective trends in nuclear staining of these proteins. However, in SW480 cells the downregulation of MSH2 and MLH1 protein expression was more rapid compared to HT29 cells; i.e. approximately 95% of SW480 cells had no nuclear MHS2 or MLH1 stain after only 9 h co-culture with wild-type EPEC (Figure 5.6a-c).

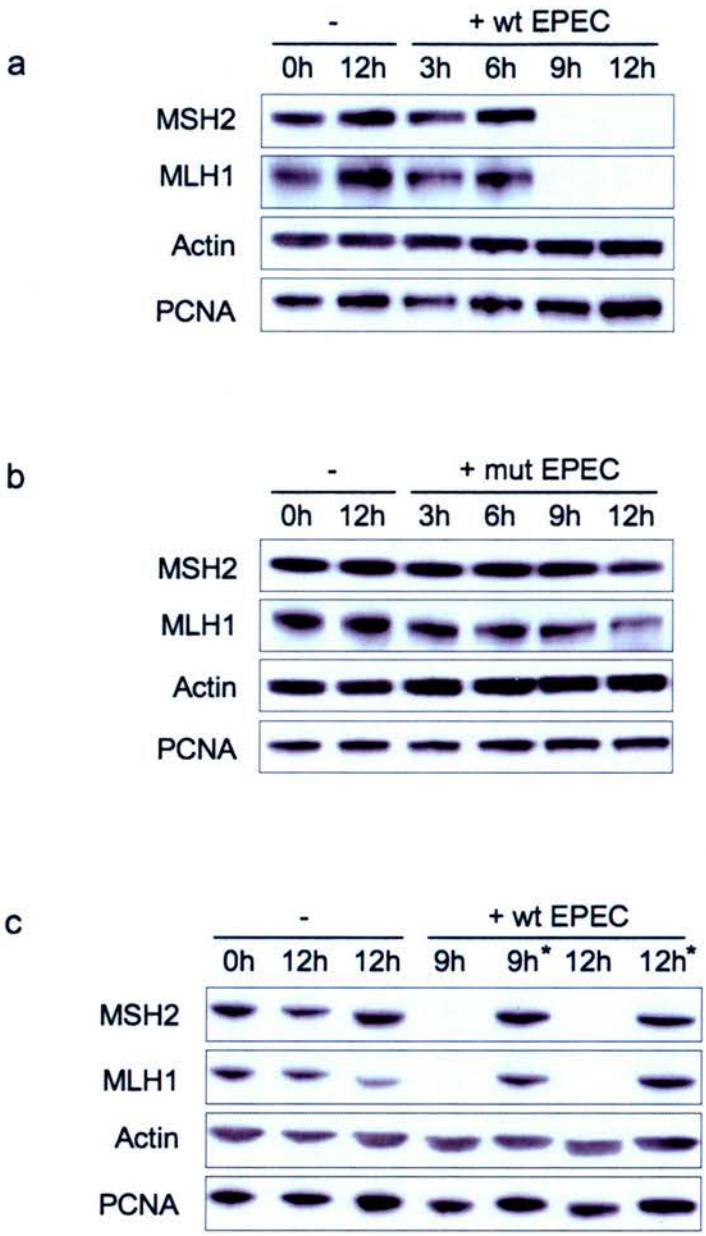


Figure 5.1. Western blots of HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC **(a & b).** Removal of EPEC by treatment with antibiotics for 39 -36 hours (9h* & 12h* respectively) allowed cells to recover from infection (**c**). Whole cell protein extracts were resolved through polyacrylamide gels and transferred to PVDF membranes which were probed with primary and secondary antibodies.

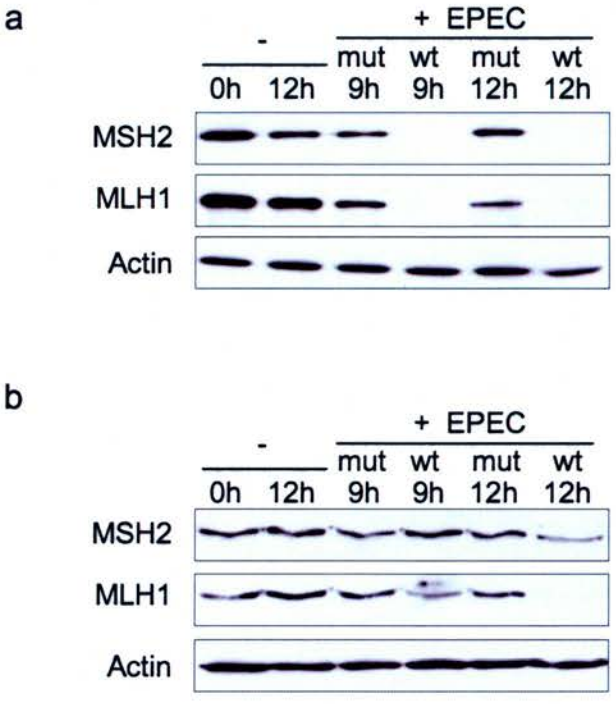


Figure 5.2. Western blots of other colorectal cell lines co-cultured with EPEC. SW480 (**a**) or LS513 (**b**) cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 - 12h. Whole cell protein extracts were resolved through polyacrylamide gels and transferred to PVDF membranes which were probed with primary and secondary antibodies. Cell culture & western blot in (**b**) performed by Scott Bader.

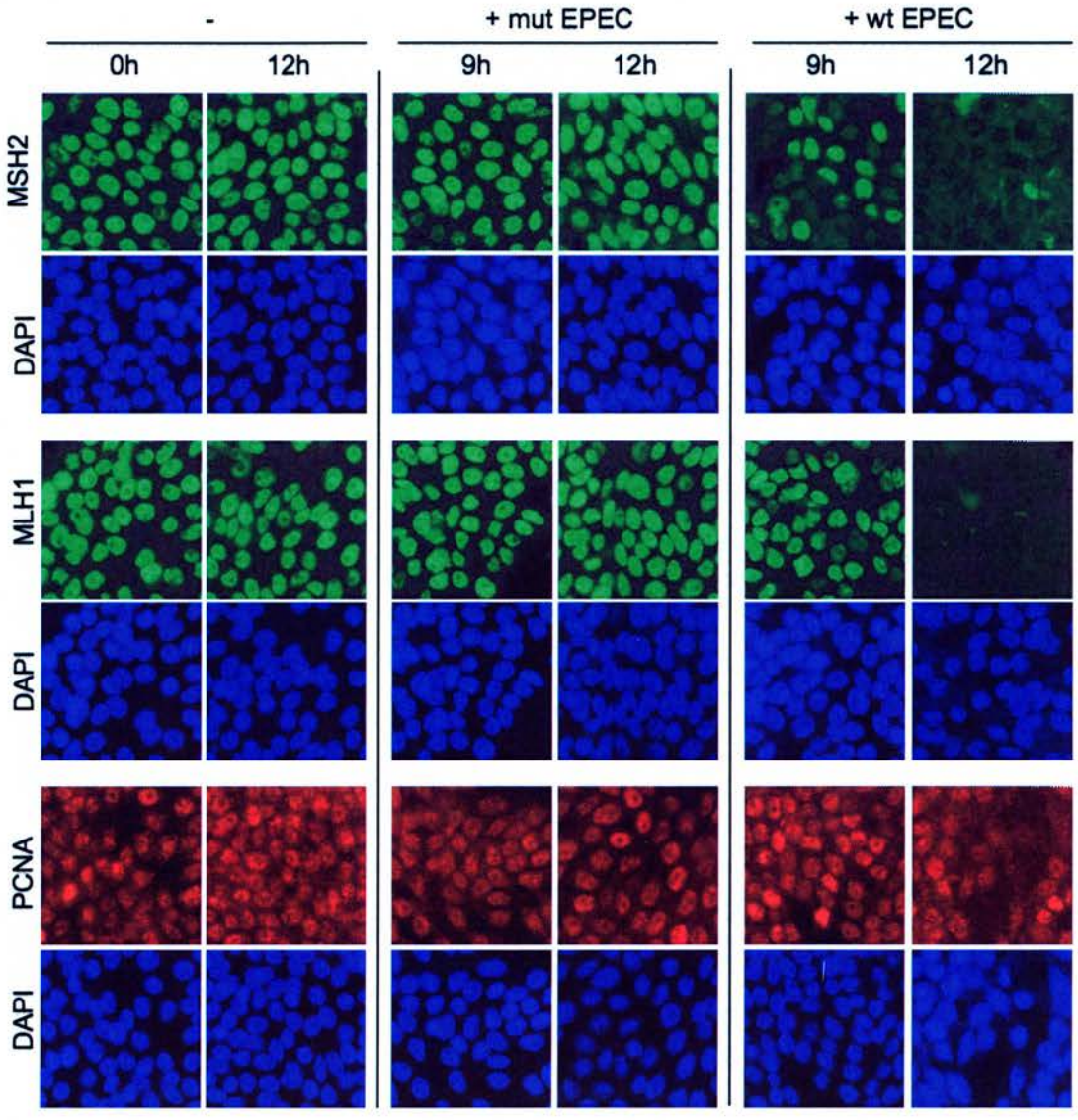


Figure 5.3. Immunofluorescence staining of mismatch repair proteins in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9-12 hours. Cells were stained for MSH2, MLH1 (green) and PCNA (red), nuclei were counterstained with DAPI (blue). Images used are representative of images used for quantitative image analysis. Original magnification, x 40.

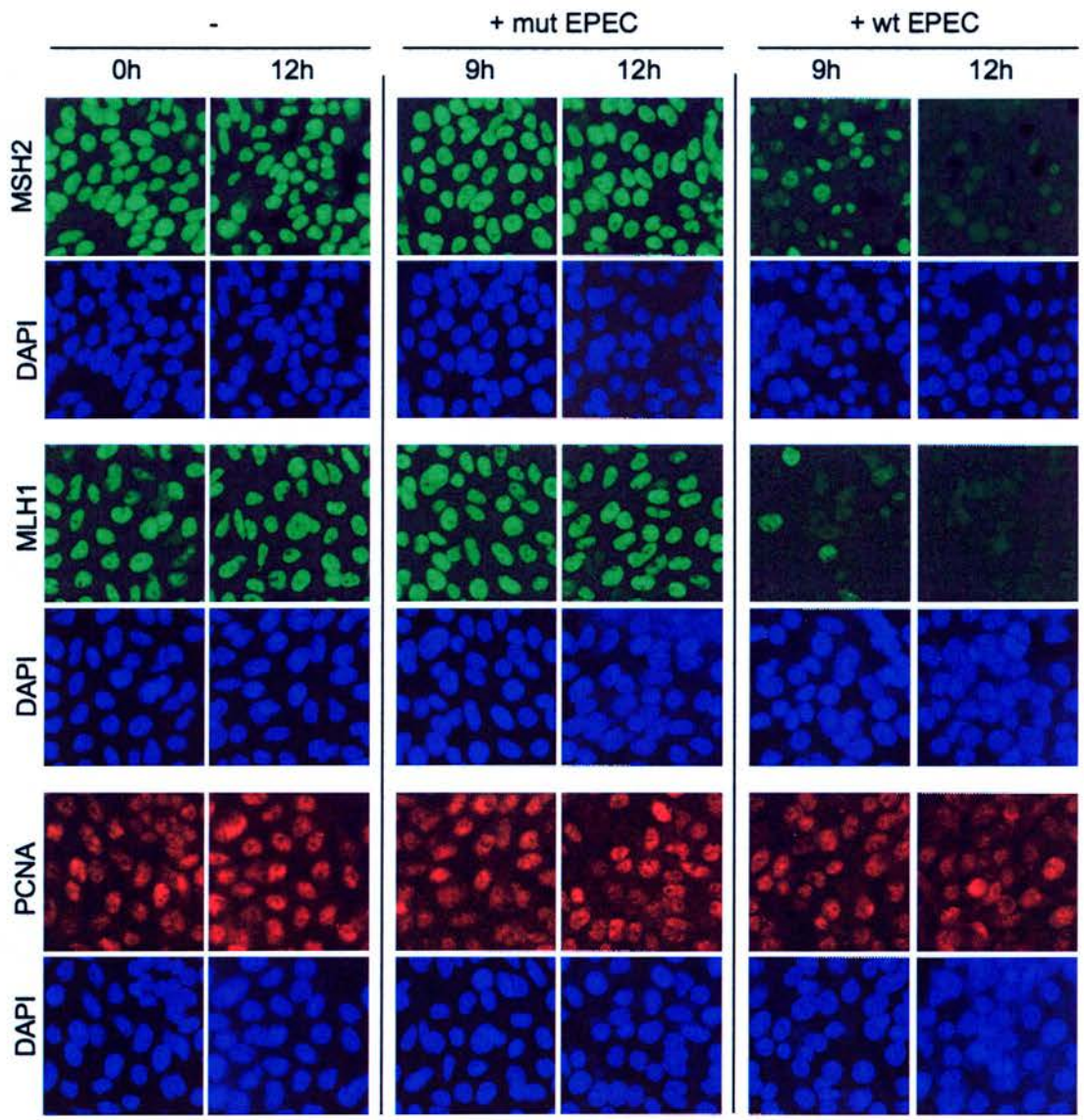


Figure 5.4. Immunofluorescence staining of mismatch repair proteins in SW480 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9-12 hours. Cells were stained for MSH2 , MLH1 (green) and PCNA (red), nuclei were counterstained with DAPI (blue). Images used are representative of images used for quantitative image analysis. Original magnification, x 40.

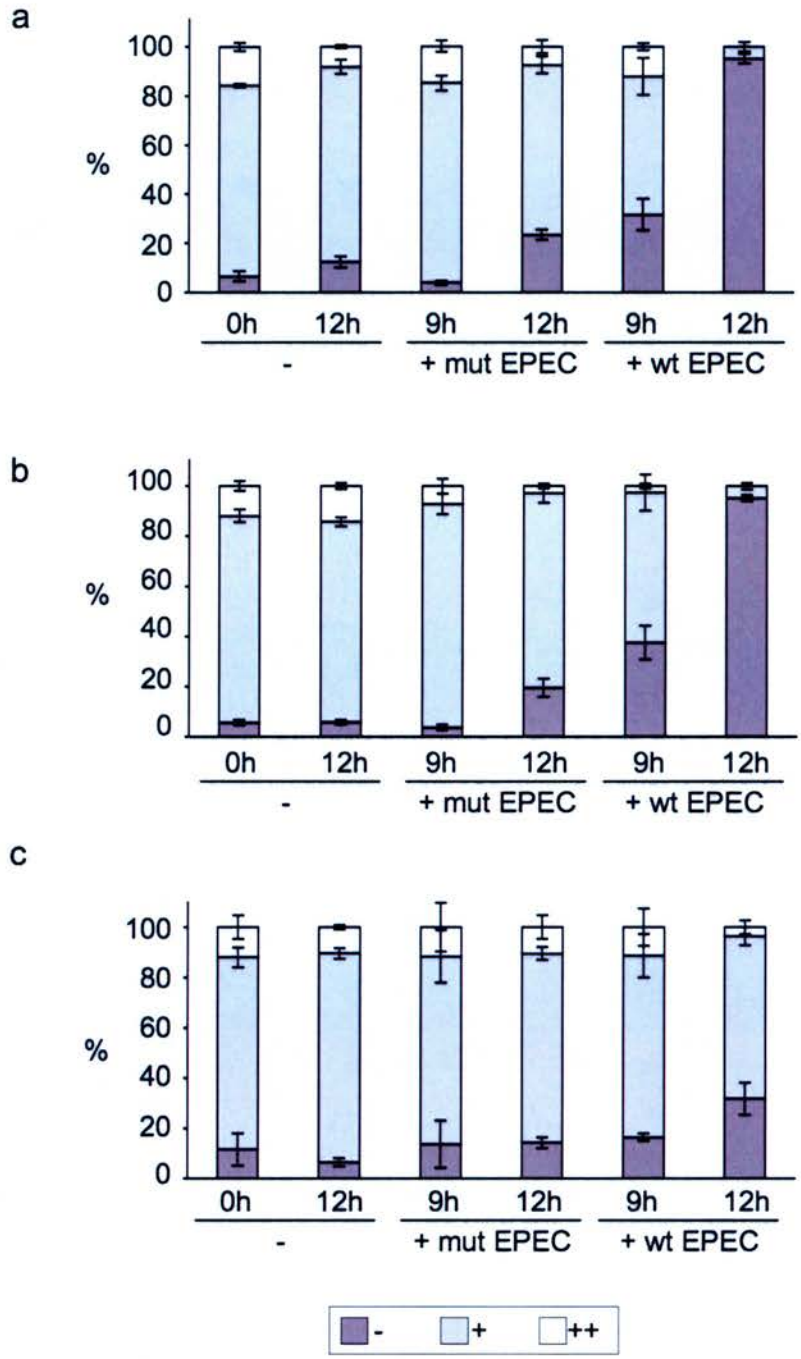


Figure 5.5. Quantitative image analysis of immunostained HT29 cells. Digital images of MSH2 (**a**), MLH1 (**b**) and PCNA (**c**) protein immunofluorescence staining in HT29 cells co-cultured with EPEC were quantitatively analysed with IPlab® software. Based on nuclear staining intensity values, cells were categorized as having either very low (-), intermediate (+) or very strong (++) signal. Error bars represent standard error of mean (n = 3).

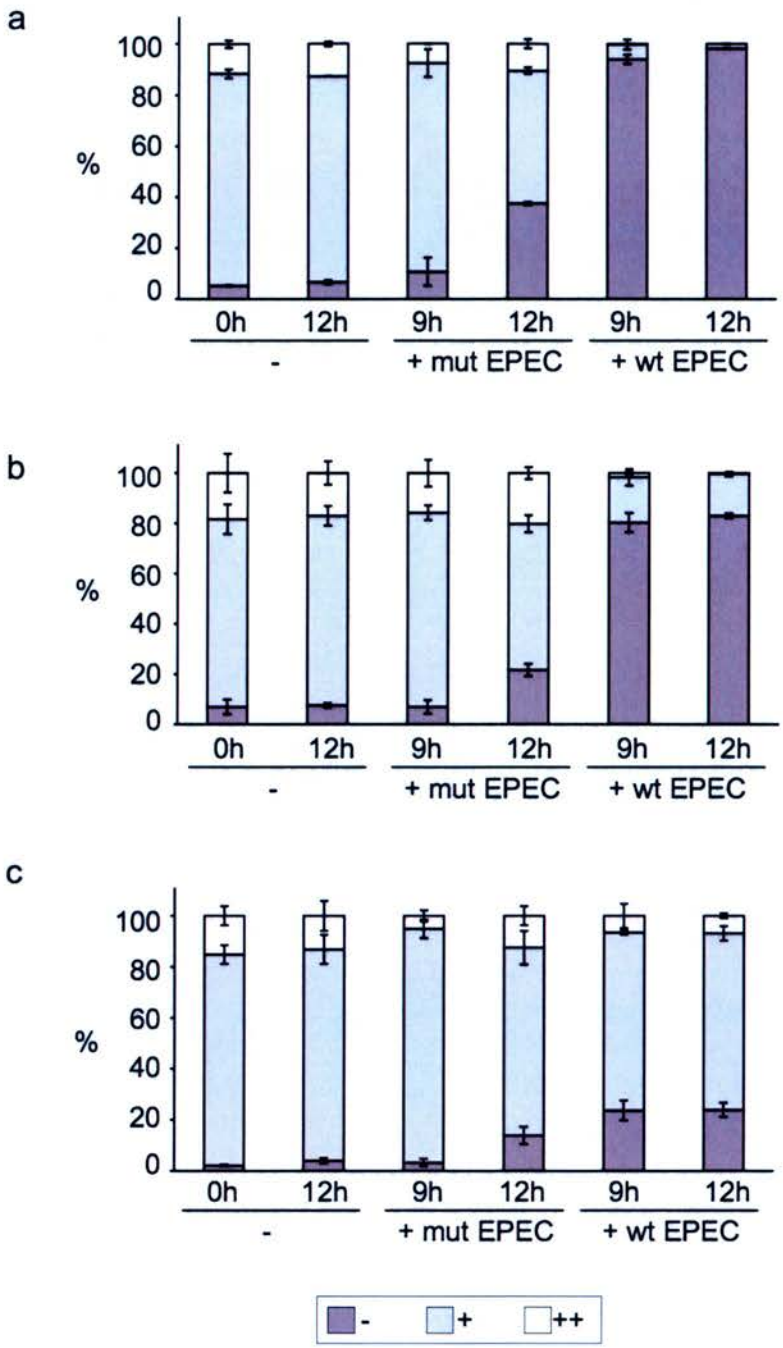


Figure 5.6. Quantitative image analysis of immunostained SW480 cells. Digital images of MSH2 (**a**), MLH1 (**b**) and PCNA (**c**) protein immunofluorescence staining in HT29 cells co-cultured with EPEC were quantitatively analysed with IPlab® software. Based on nuclear staining intensity values, cells were categorized as having either very low (-), intermediate (+) or very strong (++) signal. Error bars represent standard error of mean (n = 3).

5.2.2. Effect of EPEC infection on cell death and relation to MLH1 expression

It was notable from DAPI staining that a change in nuclear morphology of some HT29 cells occurred after infection with wild-type EPEC. This study therefore investigated whether the effects of EPEC infection on MMR protein expression in HT29 cells were associated with induction of apoptotic or necrotic mechanisms.

An *in situ* (TUNEL) cell death detection kit was used to identify cells undergoing necrosis / late stage apoptosis. Images show that the majority of cells co-cultured with EPEC for 9 or 12 hours were not apoptotic or necrotic (Figure 5.7). Counts revealed that less than 1% of uninfected and mutant infected HT29 cells were TUNEL positive. In cells co-cultured with wild-type EPEC for 9 and 12 hours, 3% and 7% of cells were TUNEL positive respectively (Figure 5.8). This sub-population of apoptotic cells was therefore far too small to co-segregate with cells showing down-regulation of MMR proteins. Furthermore, images reveal that many cells showing low or no nuclear MLH1 stain subsequent to EPEC infection were not identified as apoptotic / necrotic (Figure 5.9).

Cells undergoing the early stages of apoptosis are not identified by TUNEL staining. Cytochrome *c* staining was performed to identify cells committed to apoptosis but not at a stage advanced enough for TUNEL detection. Immunofluorescent staining of cytochrome *c* in uninfected control cells revealed strong punctate staining localised to mitochondria (confirmed by MTCO2 staining). Cells infected for 9 or 12 hours with wild-type EPEC also showed discrete cytochrome *c* distribution localised to mitochondria. Cell treated with Etoposide for 2 hours acted as positive controls for apoptosis and displayed diffuse cytochrome *c* staining, indicating the release of mitochondrial contents, an early marker of commitment to apoptosis (Figure 5.10). Simultaneous staining of MLH1 protein confirmed that MLH1 expression was downregulated in non-apoptotic cells subsequent to EPEC infection, and that MLH1 expression remained strong in cells committed to apoptosis subsequent to Etoposide treatment (Figure 5.11).

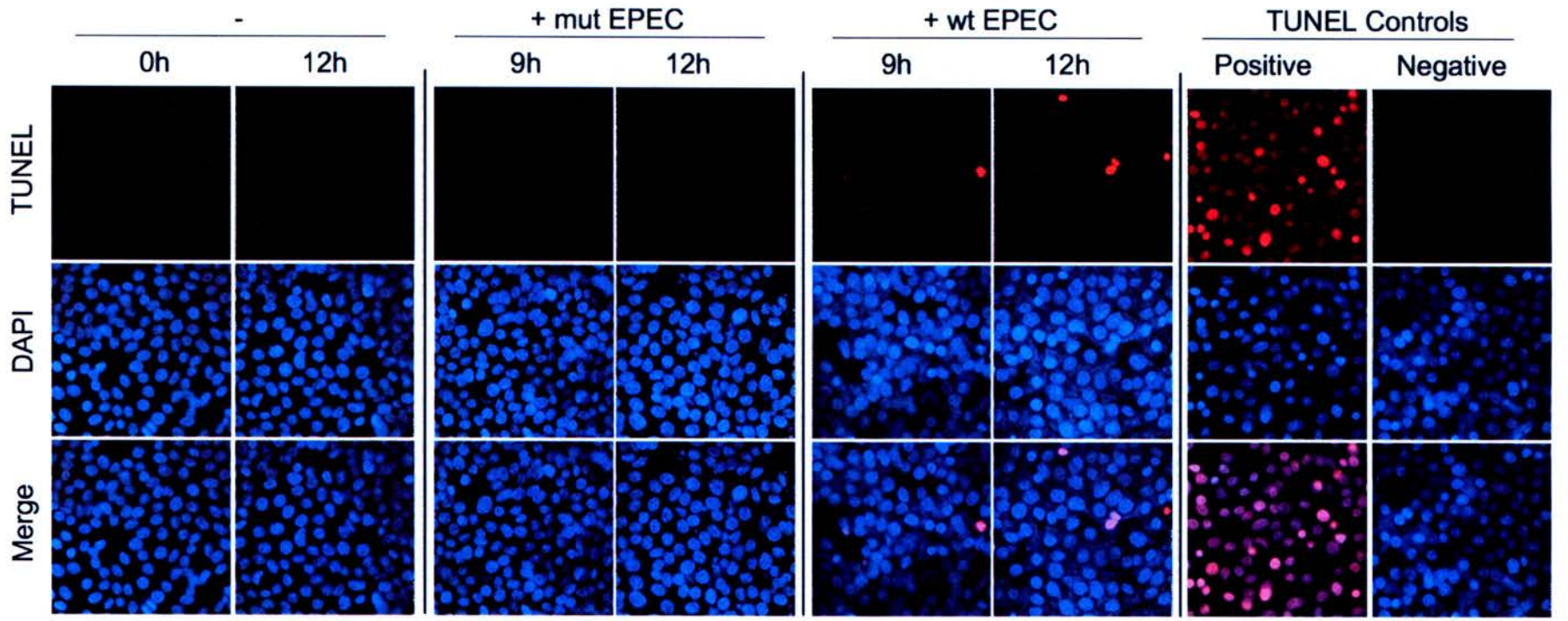


Figure 5.7. *In situ* cell death detection in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9-12h. HT29 cells treated with DNAse (to create DNA strand breaks) were used as positive controls, negative controls did not receive the deoxynucleotidyle transferase enzyme (used to bind DNA stand breaks). Original magnification, x 40.

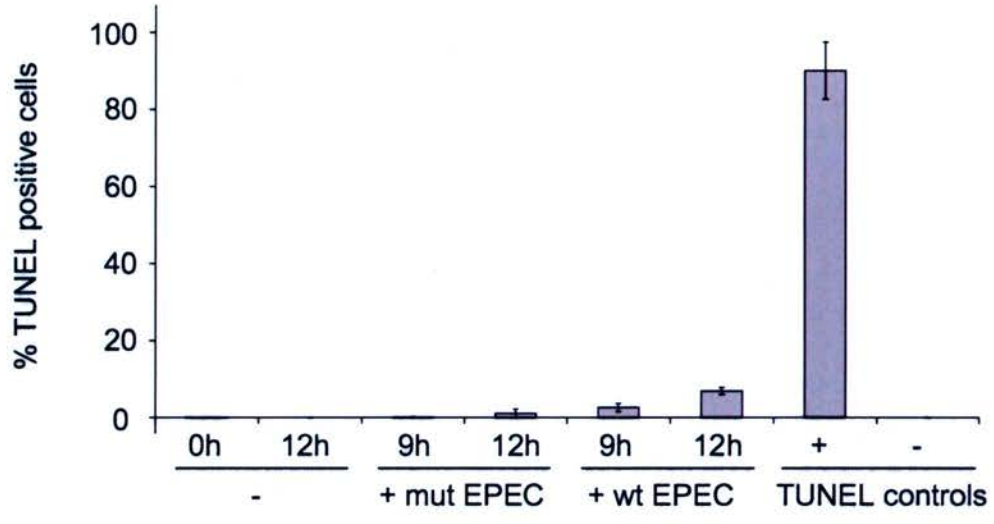


Figure 5.8. Quantification of *in situ* cell death in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC. Cells undergoing late stage apoptosis / necrosis were identified using an *in situ* cell death (TUNEL) detection kit and counted as a percentage of total cells (at least 300 cells counted in each instance). Error bars represent standard error of mean (n = 3).

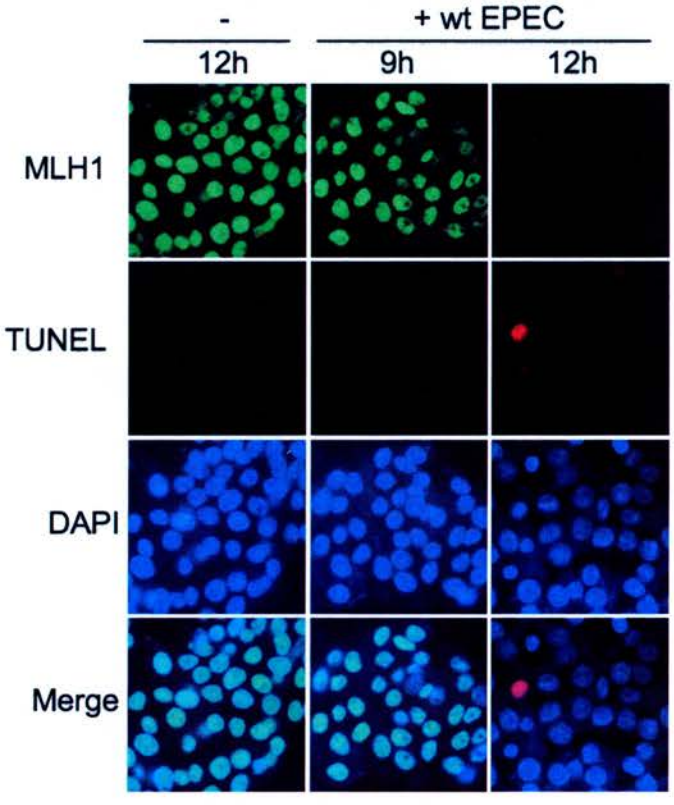


Figure 5.9. *In situ* cell death detection and MLH1 expression in HT29 cells. Cells were either uninfected (-) or co-cultured with wild-type (E2348/69) EPEC for 9-12h. Apoptotic / necrotic cells (red) were identified with an *in situ* cell death (TUNEL) detection kit and stained for MLH1 (green). The immunostaining and image capture used in (**b**) were performed under my supervision by Abigail Short. Original magnification x 40.

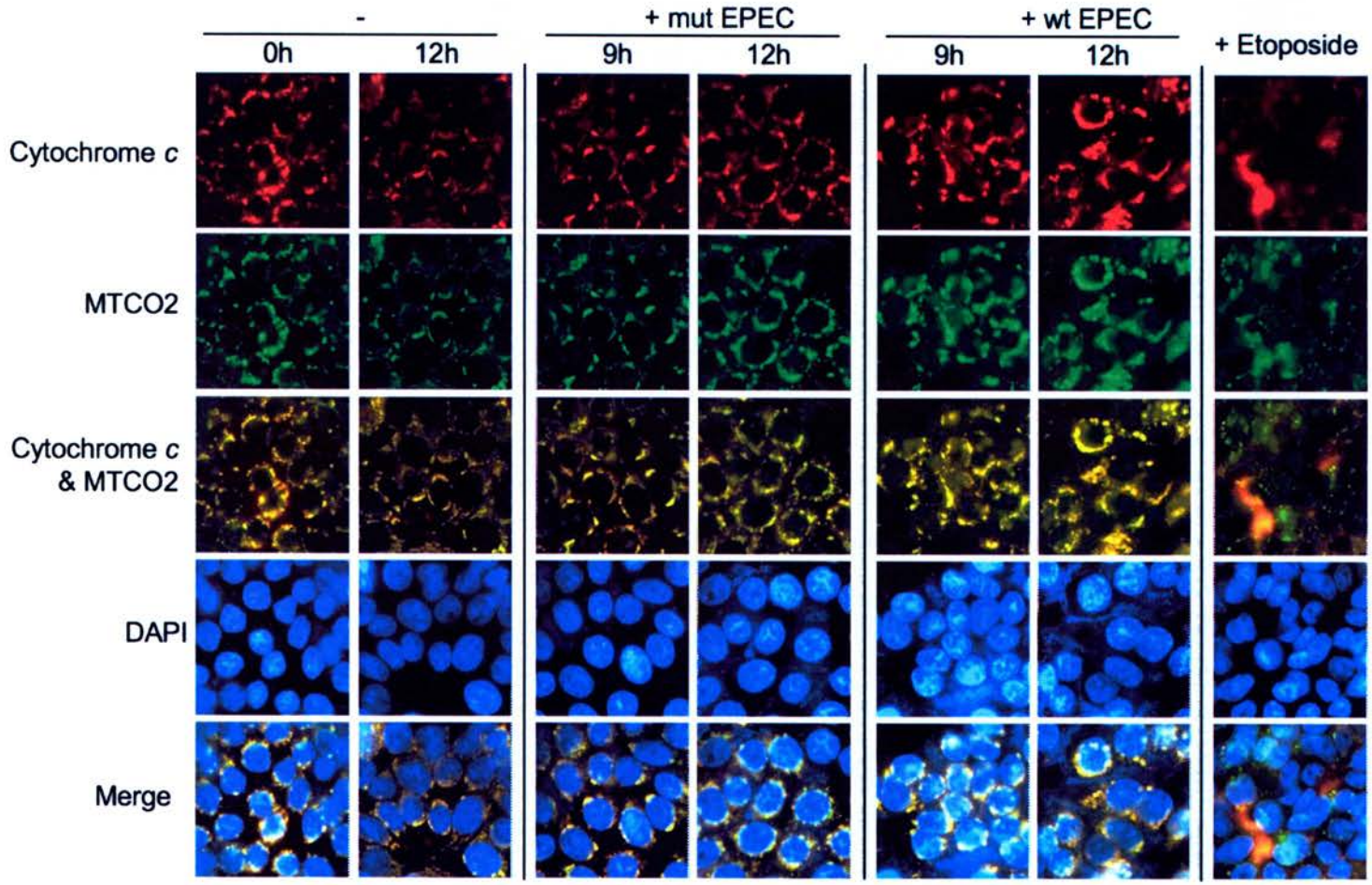


Figure 5.10. Co-immunofluorescence staining of caspase co-activator cytochrome *c* and mitochondrial marker MTCO2 in HT29 cells. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9-12h. HT29 cells treated with etoposide 1uM for 2h (then 6h in normal media) were used as positive controls for apoptosis . Cells were immunostained for cytochrome c (red) and MTCO2 (green), nuclei were counterstained with DAPI (blue). Original magnification, x 100.

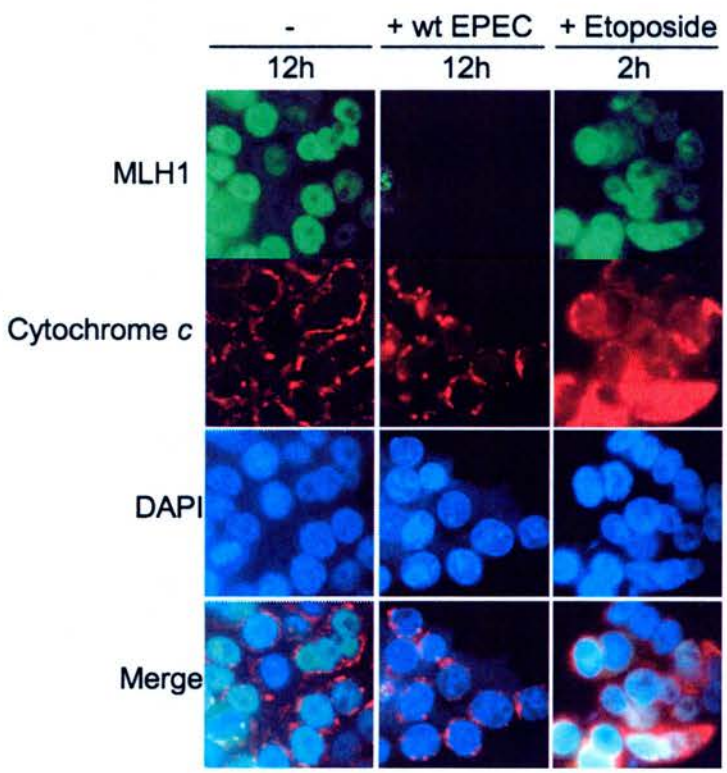


Figure 5.11. Co-immunofluorescence staining of caspase co-activator cytochrome *c* and MLH1 in HT29 cells. Cells were either uninfected (-) or co-cultured with wild-type (E2348/69) EPEC or treated with etoposide. Cells were immunostained for cytochrome *c* (red) and MLH1 (green), nuclei were counterstained with DAPI (blue). The immunostaining and image capture used in this figure were performed under my supervision by Abigail Short. Original magnification x 100.

5.2.3. Effect of EPEC infection on mutation frequency *in vitro*

The consequences of MMR protein downregulation on the mutation frequency of cells co-cultured with EPEC were investigated. It was hypothesised that cells infected with wild-type EPEC would accumulate mutations (caused by endogenous factors) at a greater rate than uninfected or mutant EPEC infected cells. In initial mutation frequency experiments the toxin ouabain and HT29 cells were used. Firstly a baseline mutation frequency rate was established (Table 5.1). Unfortunately, repeated experiments (not shown) showed a wide variation in baseline mutation frequency values. The observed variability was most likely due to the relatively low mutation frequency of HT29 cells as detected by ouabain resistance, hence many more flasks of cells than was practical to use would be required to produce statistically reliable data.

Treatment	Cells per dish	Average surviving colonies	Plating efficiency	Mutation frequency / 10^6 cells
-	250	127 (n = 3)	0.51	-
Ouabain 1uM	1×10^6	1.7 (n = 10)	-	3.3
Ouabain 1uM	2×10^6	2.5 (n = 10)	-	2.5

$$\text{Mutation frequency / } 10^6 \text{ cells} = \left[\frac{10^6}{\text{Plating efficiency} \times \text{Cells per dish}} \right] \times \text{Average No. surviving colonies}$$

Table 5.1. Calculating baseline mutation frequency in HT29 cells. Cells were seeded at various densities and grown in the presence of ouabain for 12 days, surviving colonies were stained with crystal violet and counted. Cells grown the absence (-) of ouabain (3 dishes) were used to calculate the plating efficiency. Mutation frequency (mutants / million) was calculated with the equation above.

Due to this baseline variability, inter-Alu PCR was employed as an alternative method for mutation frequency analysis. For these experiments SW480 cells were used, as MMR protein downregulation was seen to be more rapid in these cells compared to HT29 cells. To test the reproducibility of this method repeat (n = 10) inter-Alu PCR reactions on DNA isolated from three separate populations of cells

were performed. Over 30 bands were detected, and band patterns were consistent across all samples, indicating good reproducibility (Figure 5.12). Comparison of PCR product bands from cells that were untreated or co-cultured with wild-type or mutant EPEC did not reveal any overt differences in band pattern (Figure 5.13). Hence there was no observable difference in mutation frequency between the samples tested.

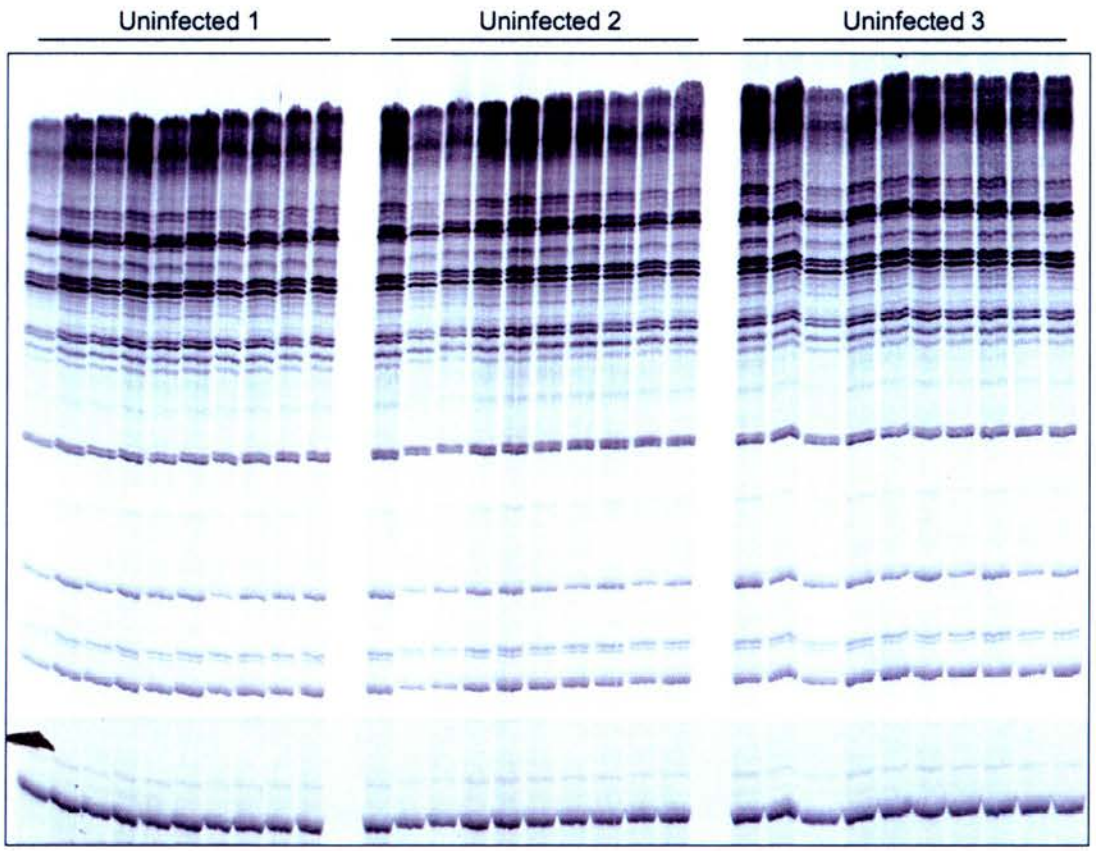


Figure 5.12. Inter-Alu PCR: method reproducibility. DNA was isolated from three flasks of uninfected SW480 cells. Inter-Alu PCR (10 repeat reactions / flask) was performed. Radiolabelled (P^{33}) products were resolved through denaturing 6% polyacrylamide gels and visualised by exposure of dried gels to radiographic film.

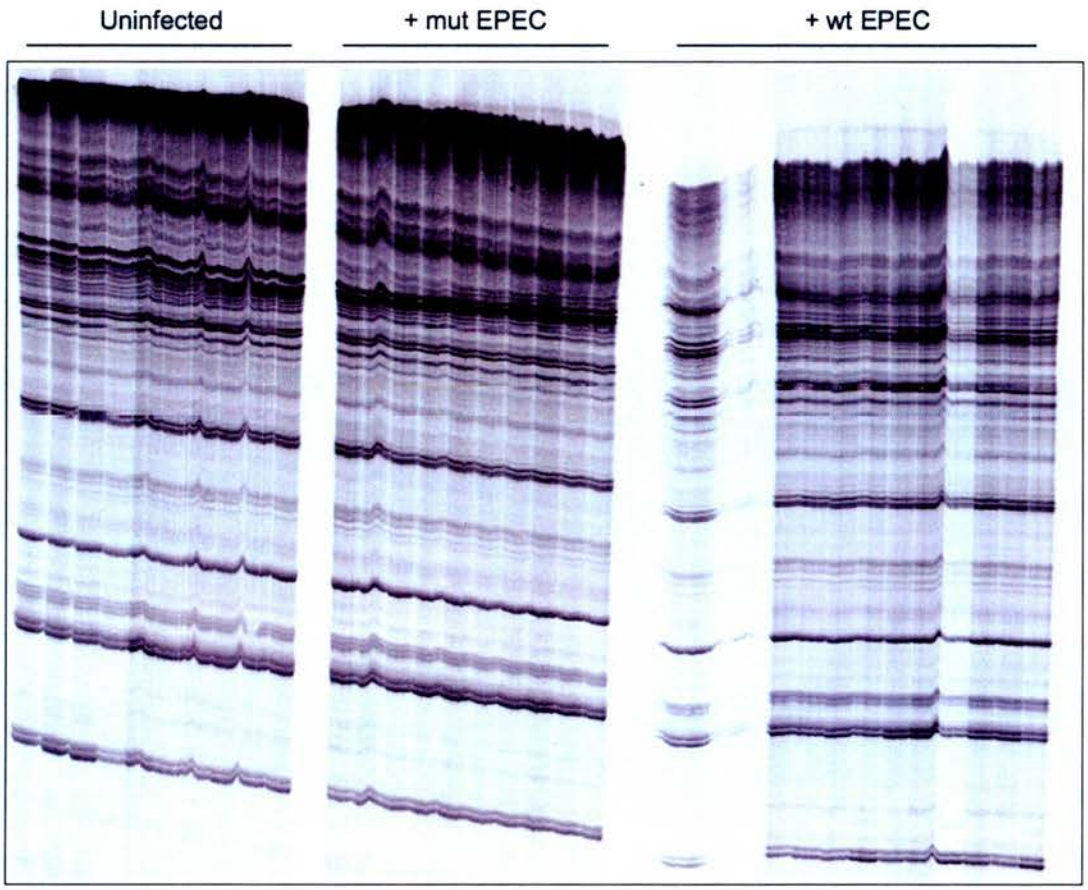


Figure 5.13. Inter-Alu PCR mutation frequency analysis. Single flasks of SW480 cells were either uninfected or co-cultured with mutant (+ mut EPEC) or wild-type EPEC (+wt EPEC) for 12 hours. Cells were treated with antibiotics and allowed to recover from infection, after reaching confluency, flasks were split into 10 new flasks and grown to confluency. DNA was isolated from each flask and Inter-Alu PCR was performed. Radiolabelled (P^{33}) products were resolved through denaturing 6% polyacrylamide gels and visualised by exposure of dried gels to radiographic film.

5.3. Discussion

EPEC (E2348/69) was clearly able to cause a dramatic reduction in MSH2 and MLH1 protein levels in colorectal cancer cell lines. The inability of mutant EPEC (UMD864) to induce the same effects suggests that MMR downregulation is mediated specifically via EPEC effector protein translocation and / or intimin signalling. EPEC induced MMR protein downregulation was only detected (in HT29 cells) after 9 hours infection, demonstrating the value of an extended time-course co-culture model. The ability of cells to recover MMR protein expression post-infection further demonstrates that this was an EPEC specific effect, and that infection was not overtly toxic. It was notable that in SW480 cells MMR protein downregulation was more complete than in HT29 cells after 9 hours, implying that that this effect is more rapid in SW480 cells. Conversely, in LS513 cells the effects of EPEC on MMR proteins were less marked than in HT29 cells. The differences between cell types could be due to differences in cell signalling / protein metabolism pathways; hence further investigation could provide an insight into the mechanism of EPEC's effects. In addition, SW480 cells show less features of differentiation (e.g. brush border) than HT29 cells (Chantret *et. al.*, 1988). The preferential binding of EPEC to crypt cells in *ex vivo* tissue (Chapter 4) suggests that EPEC has higher affinity for the surface of undifferentiated cells. In addition, it is possible that HT29 and LS513 cells are partially protected form EPEC adhesion by increased mucus secretion. LS513 cells were derived from a mucus-secreting tumour, and in culture both HT29 and LS513 cells were observed to produce more secretions than SW480 cells.

There is overwhelming evidence that attenuated MSH2 and MLH1 protein expression results in increased mutation frequency and reduced DNA damage induced apoptosis (leading to enhanced colorectal tumourigenesis). Inter-Alu PCR would be expected to detect mutations caused by disruption of MMR as Alu sequences are microsatellite repeats susceptible to DNA replication errors normally corrected by MMR. Despite establishing a reproducible inter-Alu PCR assay, no change in mutation frequency due to EPEC infection was detected. This was either because no increase in mutation frequency occurred, or because the method used was

not sensitive enough. Mutations caused by endogenous factors (e.g. DNA polymerase slippage) occur most frequently during DNA replication (prior to cell division). As very little cell division occurred during the period in which MMR protein was downregulated (i.e. between 9-12 hours of infection and during some of the recovery period) it is unlikely that many mutations would have occurred. *In vivo*, EPEC induced MMR downregulation would have greater potential to increase mutation frequency due to the presence of exogenous (e.g. food borne) mutagens. Also, infection of individual cells by EPEC *in vivo* could occur over a chronic period (i.e. days), extending the time period over which MMR protein is downregulated. Therefore, future mutation frequency analysis experiments could include the addition of mutagens (such as PhIP or DMH), or use repeated infections (on the same population of cells) to more closely replicate *in vivo* conditions.

Inter-Alu PCR provided highly reproducible results, a key requirement in mutation frequency analysis experiments. Hence further use of this method (in conjunction with the modifications stated above) in detecting mutations due to MMR disruption could be made. Alternative (and potentially more sensitive) mutation frequency analysis techniques could also be employed. One such method involves transfecting a DNA plasmid (containing microsatellite repeats) into cells and then after several days, extracting the plasmid to check for mutations (Diem & Runger, 1998). Insertion of plasmids containing mismatched bases and a fluorescent reporter has been used to measure DNA mismatch repair capability in live cells (Lei *et. al.*, 2004). It would also be interesting to evaluate EPEC induced mutation in cells deficient in DNA repair (e.g. HCT116 cells lacking MLH1). This could replicate the effect of EPEC infection on an individual carrying MMR gene mutation(s).

It was notable that the nuclei of cells infected with wild-type EPEC often showed altered nuclear DNA staining compared to uninfected or mutant EPEC infected cells. Cells undergoing apoptosis display changes in nuclear morphology and DNA organisation. These changes result from the activation of DNAse enzymes that cleave DNA during apoptosis. Activation of protease enzymes (such as caspases) also occurs during apoptosis. These enzymes degrade proteins (e.g. DNA repair

proteins) that, by their normal action, oppose apoptotic changes (i.e. DNA damage) (Bernstein *et. al.,* 2002). In cancer cells treated with etoposide to induce apoptosis, MLH1 is proteolysed by caspase-3 (Chen *et. al.,* 2004). The literature reports pro- and anti-apoptotic responses to EPEC infection *in vitro*, however, this study uses a longer time-course co-culture model than those used previously. Therefore it was possible that EPEC induced MMR protein degradation in this model was caused by induction of pro-apoptotic mechanisms leading to DNA cleavage and proteolysis of MMR proteins.

To identify cells undergoing late stage apoptosis or necrosis, a cell death detection kit designed to label DNA strand breaks was used. Results show that although cells infected with wild-type EPEC displayed higher levels of cell death than control cells, only a small fraction (3-7%) of the infected cells tested positive for cell death. These results therefore demonstrate that EPEC induced MMR protein downregulation was not a non-specific consequence of apoptosis or necrosis. Nuclear and DNA alterations caused by EPEC infection have previously been reported, and were attributed to disruption of the cell cycle rather than apoptosis (Marches *et. al.,* 2003). This study (i.e. the results of this chapter and chapter 3) therefore support these findings. It has been claimed that these nuclear 'cytopathic' effects of EPEC are non-reversible (Nougayrede *et. al.,* 2001), however this study shows that cells are not irreversibly damaged by EPEC infection. The relationship between EPEC infection, cell cycle arrest, DNA changes and apoptosis therefore requires further investigation.

As a marker of early apoptosis cytochrome *c* distribution was analysed. Early in the execution of apoptosis, a multimeric complex of dATP, Apaf-1 and cytochrome *c* (termed the apoptosome) is formed. This complex activates caspase-9, which causes the downstream activation of caspase-3, leading to proteolysis (reviewed by Bernstein *et. al.,* 2002). In order to join the apoptosome, cytochrome *c* must first be released from the mitochondria. In cells co-cultured with or without EPEC, cytochrome *c* distribution was limited to mitochondria; indicating cells had not entered early apoptosis. Furthermore, treatment of cells with etoposide (to induce apoptosis) did not cause MLH1 degradation but did cause cytochrome *c* release,

demonstrating that apoptotic MLH1 cleavage takes time to occur even when apoptosis has been initiated.

As it has been established that EPEC did not cause MSH2 and MLH1 downregulation via non-specific apoptotic effects, it is important to consider the mechanism by which this change occurred. As previously mentioned, MMR protein regulation can occur by a variety of transcriptional and post-translational pathways. Microarray analysis has revealed that EPEC infection suppressed MSH2 and MLH1 expression (chapter 3). Suppression of MSH2 has been observed in cancer cells via inhibition of the transcription factor E2F by Bcl-2 (Youn *et. al.,* 2005). In order to become active, Bcl-2 requires phosphorylation (Ito *et. al.,* 1997), a task carried out by mitochondrial PKCα (Ruvolo *et. al.,* 1998). EPEC increases membrane bound PKCα activity and the PKCα content of cancer cell membranes (Crane & Oh, 1997). It is therefore possible that EPEC induced activation of PKCα leads to Bcl-2 activation, followed by suppression of MMR protein transcription.

In support of this proposed mechanism, microarray analysis revealed a 2.6 fold increase in PKCα expression, and an 11.0 fold increase in the expression of the PKCα activator endothelin-2 in response to EPEC infection. The rapidity and extent of EPEC's effects on MSH2 and MLH1 strongly suggest that degradation of protein, as well as transcriptional silencing has a role in MMR protein downregulation in this model. This is certainly true if the half-life of MSH2 in HT29 and SW480 cells is similar to that in HeLa cells. The homology between mammalian and bacterial MMR proteins is well established, and it has been demonstrated that human MLH1 interacts with bacterial MutS and MutL proteins when expressed in *E. coli,* subverting their function (Quaresima *et. al.,* 2003). This raises the possibility that *E. coli* secreted proteins are able to directly interact with MMR proteins to influence their expression.

Bcl-2 can mediate anti-apoptotic effects by preventing mitochondrial cytochrome *c* release (Yang *et. al.,* 1997; Kluck *et. al.,* 1997), and is overexpressed in many cancers. Although it has been previously reported that EPEC infection induces

cytochrome *c* release from mitochondria via EspF signalling (Nougayrede & Donnenberg, 2004), this did not occur in the present study. Therefore, EPEC induced activation of Bcl-2 also provides a mechanism to support the failure of EPEC to induce cytochrome *c* release in our experiments. The lack of widespread apoptosis in response to EPEC could also be a feature of MMR protein downregulation itself, as induction of apoptosis can be dependent on MMR protein expression (Luo *et. al.*, 2004; Shimodaira *et. al.*, 2003; Zhang *et. al.*, 1999). Prevention of MMR protein pro-apoptotic signalling could in fact be main advantage of MMR protein downregulation to EPEC.

The ability of EPEC to cause a reduction in MSH2 and MLH1 protein is a potentially significant finding, as it provides a direct link between EPEC infection and molecular aberrations known to be a causative factors in the development of colorectal cancer. If this *in vitro* effect is also able to manifest itself during *in vivo* infection, EPEC has the potential to increase mutation frequency and suppress DNA damage induced apoptosis in the colonic epithelium. These changes would strongly predispose infected epithelial cells to the effects of exogenous mutagens and therefore tumourigenesis.

The key aim of further work would therefore be to establish whether EPEC induces MMR protein downregulation in human tissue. Use of the *ex vivo* model established in this study could be used for this purpose. The mechanism of EPEC induced MMR protein downregulation may be complex due to the variety of pathways potentially involved. Establishing the normal half-life of MSH2 and MLH1 in HT29 and SW480 cells would help to reveal whether transcriptional or post-translational mechanisms dominate the regulation of these proteins. Also, using proteosome inhibitors during EPEC infection could reveal whether EPEC induces active proteolysis of MSH2 and MLH1. Quantitative real-time PCR could be used to confirm the MMR gene expression results generated by microarray analysis. It is important to consider that elucidating the mechanism of EPEC induced MMR protein downregulation is less important than establishing whether these effects occur *in vivo* (though the former may assist in the latter); as EPEC's effects can be prevented without any knowledge

of the mechanism, simply by eradicating the bacteria. The finding that Aspirin induces MMR protein upregulation could partly explain its protective effect from colorectal cancer. Hence it would be interesting to know whether Aspirin can inhibit EPEC induced MMR protein downregulation, providing another avenue for further investigation.

Chapter 6.

Effect of EPEC infection on the expression of β-catenin, cell-cell adhesion and cytoskeletal proteins *in vitro*.

6.1 Introduction

6.1.1. β-catenin signalling and distribution

The majority of colorectal cancers, at some point in their development, gain mutations of the *APC* gene. Inactivating *APC* mutations prevent phosphorylation and ubiquitination of β-catenin, resulting in cytoplasmic β-catenin accumulation and activation of oncogenic Wnt target genes. *In vitro* EPEC infection has been shown to alter β-catenin distribution (from the plasma membrane to the cytoplasm) after 3 hours (Malladi *et. al.,* 2004). It was hypothesised that intimate attachement of EPEC to human colonic epithelial cells could increase susceptibility to cancer by promoting β-catenin signalling in the *in vitro* model. Hence, an aim of this study was to analyse the effect of EPEC on the expression β-catenin and its associated adhesion proteins in HT29 and SW480 cells after 9-12 hours infection.

Bacterial activation of β-catenin has been previously reported in HeLa and T84 cells infected with *Salmonella typhimurium*. Sun *et. al.* (2004) found that cells infected with a non-pathogenic *S. typhimurium* displayed nuclear accumulation of β-catenin and activation of β-catenin/TCF signalling, leading to enhanced cell proliferation. This effect was shown to occur via inhibition of β-catenin ubiquitination, and be dependent on the *S. typhimurium* secreted effector protein AvrA. Hence a link between bacterial attachment and insertion of effector molecules with a pathway central to tumourigenesis within the human intestine has been established. Investigation of EPEC induced disruption of cell-cell adherens junctions in Caco-2 cells has revealed that wild-type EPEC caused dissociation of E-cadherin from β-catenin (Malladi *et. al.,* 2004). This dissociation was a result of PKCα activation and contributes to EPEC induced epithelial barrier disruption. A potentially more important consequence of EPEC induced adherens junction breakdown is the subsequent release of β-catenin into the cytoplasm, with potential to initiate oncogenic Wnt signalling pathways. Indeed, after 3 hours EPEC infection β-catenin was shown to translocate from the plasma membrane to the cytoplasm, however, downstream Wnt signalling was not investigated (Malladi *et. al.,* 2004).

6.1.2. β-catenin associated proteins

In addition to the effects caused by β-catenin signalling, disruption of adherens junctions has the potential to induce other tumourigenic effects. Within adherens junctions, catenins (including α- catenin, β-catenin and γ-catenin) anchor E-cadherin to actin filaments of the cytoskeleton (Alberts *et. al.,* 2002). Downregulation of the E-cadherin/catenin/cytoskeleton complex is a common finding in cancer, and is thought to be a key step in the transition to an invasive i.e. malignant phenotype (Mareel *et. al.,* 1997). Suppression of E-cadherin expression is observed in gastric and other cancers, it has been proposed that loss of E-cadherin mediated cell-cell adhesion is essential for tumour cell invasion and metastasis formation (reviewed by Chan, 2006). γ-catenin (also known as plakoglobin) is structurally related to, and has similar functions in signalling and cell-cell adhesion as β-catenin (Peifer *et. al.,* 1992). γ-catenin is found in association with cadherins in both cell-cell desmosomes and adherens junctions (Alberts *et. al.,* 2002). Functional loss of γ-catenin has been implicated in the development of prostate, lung and bladder cancer (Shiina *et. al.,* 2005; Winn *et. al.,* 2002; Syrigos *et. al.,* 1998). Unlike adherens junctions, desmosomes connect intracellularly to intermediate filaments (Alberts *et. al.,* 2002), a cytoskeletal network also modulated by EPEC infection (reviewed by Caron *et. al.,* 2006).

Another molecule tasked with anchoring the cytoskeleton to the plasma membrane is ezrin. Ezrin interacts with both E-cadherin and β-catenin, and has been found to regulate cell-cell and cell-matrix adhesion (Hiscox & Jiang, 1999). Suppression of ezrin expression in colorectal cancer cells causes reduced cell-cell adhesion and gain in motile and invasive behaviour (Hiscox & Jiang, 1999). *In vitro* EPEC infection causes activation (via phosphorylation) of ezrin, increasing ezrin association with the cytoskeleton, resulting in tight junction disruption after 3 hours (Simonovic *et. al.,* 2001). Goosney *et. al.* (2001) report that ezrin is recruited to EPEC induced pedestals, hence it is probable that ezrin has a function in the anchorage of cytoskeletal components to the plasma membrane within these structures. Despite

causing re-distribution and phosphorylation of ezrin, the total cellular concentration of ezrin is unchanged after (5 hours) co-culture with EPEC (Simonovic *et. al.,* 2001).

The effects of EPEC attachment on actin are well known; high concentrations of cytoskeletal actin are a prominent feature of AE lesions (Knutton *et. al.,* 1989; Finlay *et. al.,* 1992). It is possible that some of the actin accumulated in AE pedestals is remodelled from microvilli that have been effaced. Also, actin that has dissociated from adherens junctions (via EPEC induced effects on catenins and ezrin) could contribute to pedestals. The actin cytoskeleton plays a key role in the regulation of many cellular processes, including migration, locomotion, cytokinesis and cell polarity (Caron *et. al.,* 2006). It has been proposed that disruption of the actin cytoskeleton within intestinal epithelial cells could prevent normal cell migration within the crypt, and that this could be a uniquely important mechanism of tumour formation in gut tissue (Nathke, 2006).

The carcinogenic bacterium *Helicobacter pylori* has recently been found to influence cell-cell adhesion pathways in gastric cells. *H. pylori* induces scattering of cultured gastric epithelial cells, an effect related to disruption of E-cadherin / catenin containing adherens junctions, and dependent on insertion of the effector molecule CagA (Suzuki *et. al.,* 2005). In rat gastric epithelium oncogenic *H. pylori* caused CagA dependent β-catenin activation, and nuclear accumulation of β-catenin was observed in gastric epithelium from humans infected with Cag^+ but not Cag^- *H. pylori* (Franco *et. al.,* 2005). *In vitro* co-culture experiments have demonstrated disruption of epithelial junctional β-catenin expression (without nuclear accumulation) in response to pathogenic *H. pylori*, but independent of Cag (Bebb *et. al.,* 2006). CagA has also been shown to physically interact with E-cadherin, thus impairing β-catenin / E-cadherin complexation, resulting in nuclear and cytoplasmic β-catenin accumulation (Murata-Kamiya *et. al.,* 2007).

6.2. Results

6.2.1. Effect of EPEC on β-catenin expression *in vitro*

Western blots of HT29 cells show that wild-type EPEC infection clearly caused a downregulation in the expression of β-catenin after 9 or 12 hours co-culture (Figure 6.1a). It was notable that β-catenin expression was also reduced (but by a lesser degree) in uninfected washed control cells (-, 12h) and mutant EPEC infected cells versus uninfected, untreated control cells (-, 0h). This suggests that the experimental protocol may have influenced β-catenin expression in this instance.

Immunofluorescent staining of HT29 cells also demonstrated that wild-type EPEC infection caused β-catenin downregulation (Figure 6.1b). In uninfected and mutant-EPEC infected cells, β-catenin was located at the periphery of cells in the plasma membrane. Co-culture with wild-type EPEC for 9 hours resulted in reduced peripheral β-catenin expression, with some staining seen in the cytoplasm, and after 12 hours co-culture, no peripheral β-catenin expression was detected, although some low level nuclear staining was observed, but may have been due to background. Using an antibody for non-phosphorylated β-catenin (targeted to the N-terminal) produced similar results to the C-terminal antibody (described above). Thus demonstrating the absence of significant levels of phosphorylated β-catenin in either infected or uninfected cells (Figure 6.2a,b).

Western blots of SW480 cells co-cultured with EPEC revealed the same trends in β-catenin expression: while cells co-cultured with mutant EPEC retained β-catenin expression, wild-type EPEC infection caused a clear reduction in expression (Figure 6.3a). There is also little difference in β-catenin detection between C-terminal and N-terminal antibodies, indicating a lack of phosphorylated β-catenin (Figure 6.3b).

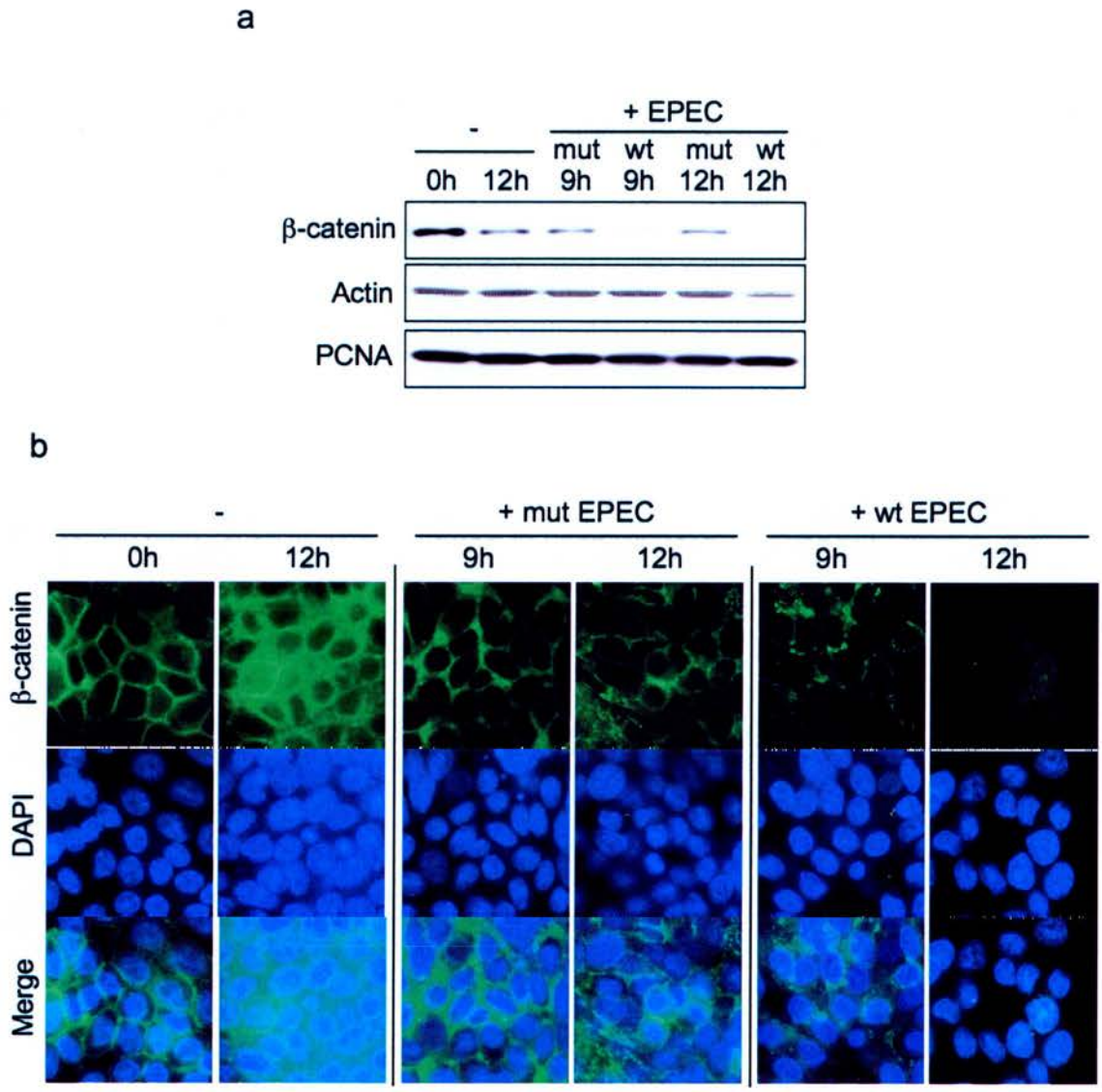


Figure 6.1. Total β-catenin expression in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 hours. Western blots were performed on whole cell protein extracts and probed with an anti-β-catenin C-terminal antibody (**a**). Fixed cells were immunostained with the same antibody, nuclei were counter stained with DAPI (blue) (**b**). Original magnification x 100.

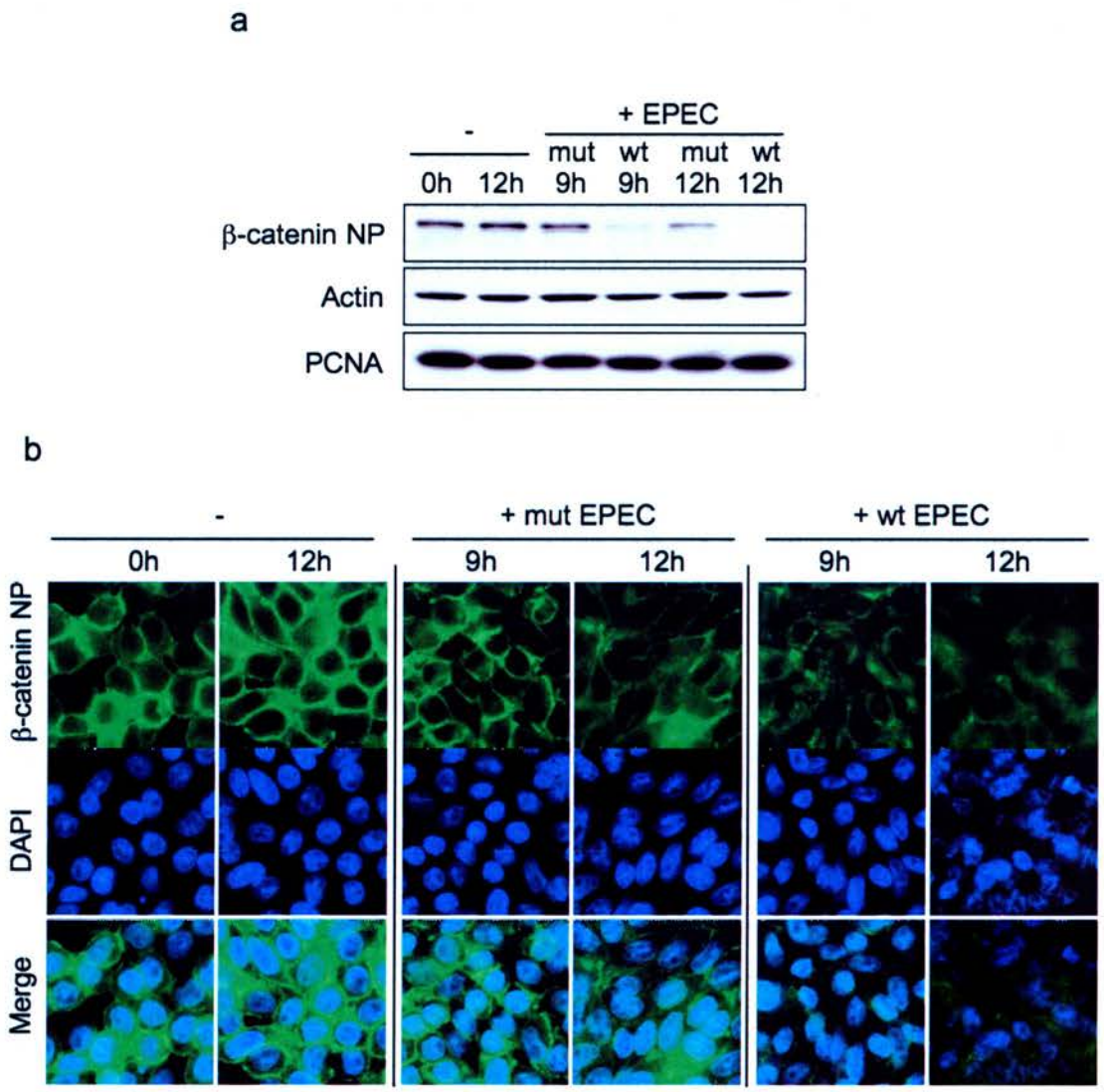


Figure 6.2. Non-phosphorylated β-catenin expression in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Western blots were performed on whole cell protein extracts and probed with a anti-non-phosphorylated β-catenin (NP) antibody (**a**). Fixed cells were immunostained with the same antibody (green), nuclei were counter stained with DAPI (blue) (**b**). Original magnification x 100.

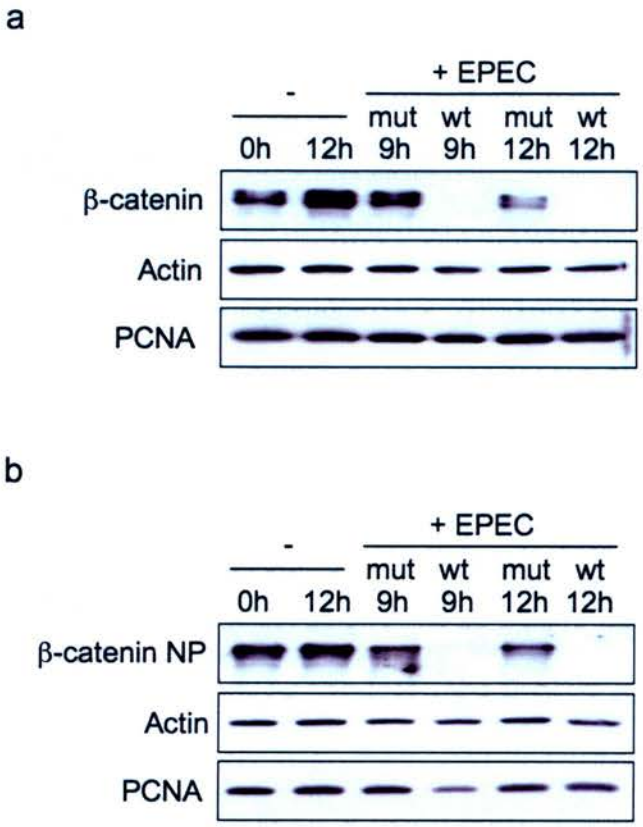


Figure 6.3. β-catenin expression in SW480 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Western blots were performed on whole cell protein extracts and probed with anti-β-catenin (c-terminal) (**a**) or with a anti-non-phosphorylated β-catenin (NP) antibody (**b**).

6.2.2 Effect of EPEC on γ-catenin expression *in vitro*

Western blots show that γ-catenin expression was also reduced after co-culture of HT29 cells with wild-type EPEC. In mutant EPEC infected cells, γ-catenin expression was strong after 9 hours (possibly showing increased expression) and remained comparable to uninfected cells after 12 hours (Figure 6.4a). Like β-catenin, γ-catenin is also expressed in the periphery of epithelial cells. As with β-catenin, immunofluorescent staining of γ-catenin revealed a loss of peripheral

expression after 12 hours co-culture with wild-type EPEC, with possible residual nuclear expression (Figure 6.4b). Images show that cells infected with mutant EPEC retain strong γ-catenin expression after 9 -12 hours, supporting western blot results.

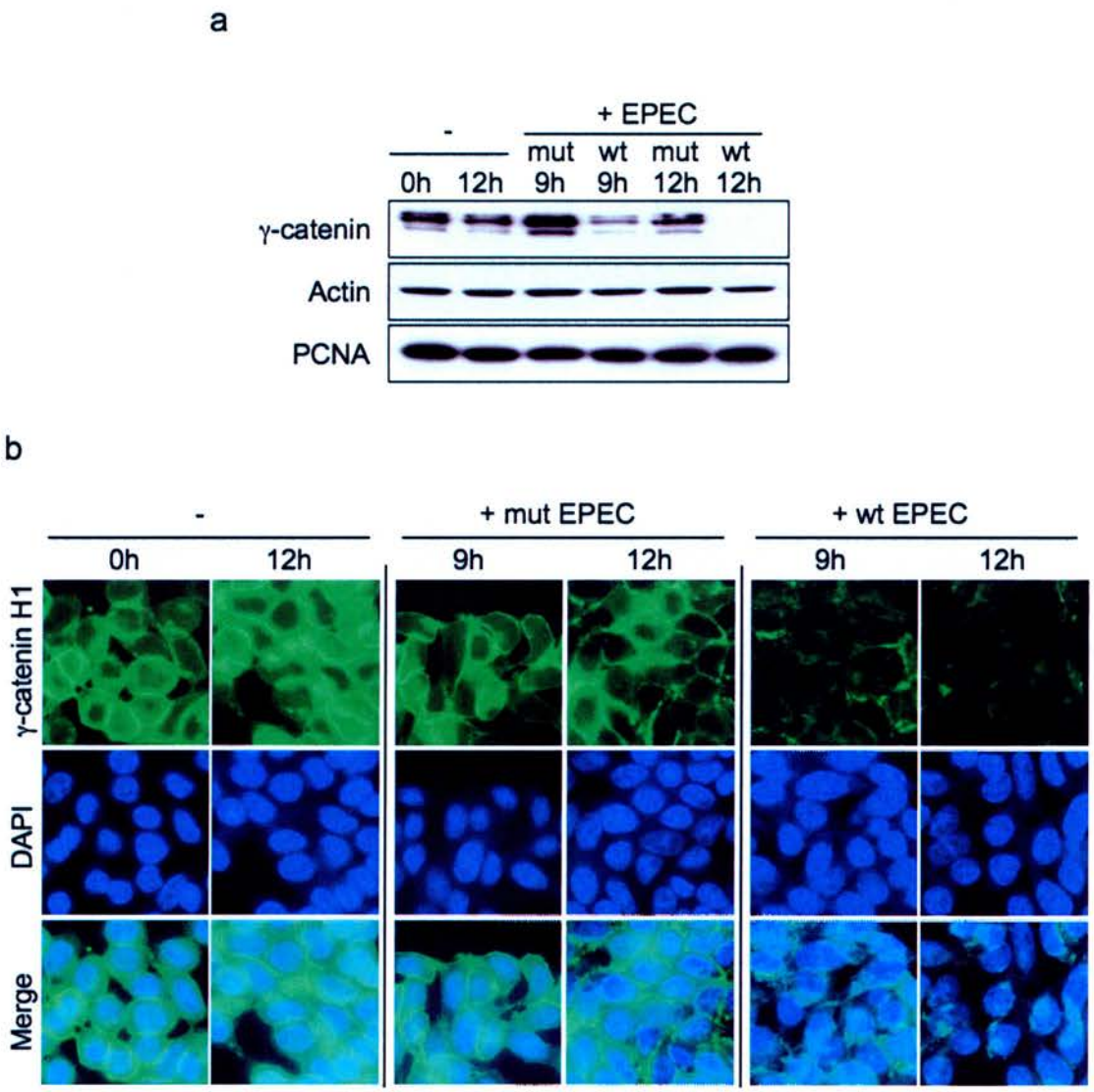


Figure 6.4. γ-catenin expression in HT29 cells co-cultured with EPEC. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Western blots were performed on whole cell protein extracts and probed with anti-γ-catenin (**a**). Fixed cells were immunostained with anti-γ-catenin (green), nuclei were counter stained with DAPI (blue) (**b**). Original magnification x 100.

6.2.3 Effect of EPEC on E-cadherin expression *in vitro*

Based on expression of the loading controls actin and PCNA, western blots of HT29 cells co-cultured with EPEC show that unlike γ-catenin and β-catenin, expression of E-cadherin remains relatively constant under all experimental conditions, except perhaps after 9 hours co-culture with wild-type EPEC (Figure 6.5a). E-cadherin expression is potentially increased in HT29 cells. In cells co-cultured with mutant EPEC, E-cadherin expression levels are similar to the levels seen in uninfected controls. E-cadherin expression in SW480 cells was too low to detect via western blot.

Immunofluorescent staining of HT29 cells gave a more accurate picture of E-cadherin expression and allowed comparison of E-cadherin distribution with the location of adherent EPEC. Images of E-cadherin expression and EPEC location were taken in both the nuclear plane (middle – base of cell) and the apical plane (surface of cell) and at high (x 100), medium (x 40) and low (x 16) magnification.

High magnification images in the nuclear focal plane show that E-cadherin is strongly expressed in the periphery (i.e. plasma membrane) of uninfected cells where adjacent cells meet (Figure 6.5b). Co-culture with mutant EPEC for 9 hours had little effect on E-cadherin expression. However, after 12 hours with mutant EPEC, the E-cadherin distribution was disrupted so that instead of continuous, uniform peripheral expression, E-cadherin is present in small discrete spots within the membrane. In HT29 cells co-cultured with wild-type EPEC for 9 hours, E-cadherin expression was similar to that seen in uninfected cells, however, in some areas, strong E-cadherin expression potentially co-localised with EPEC. After 12 hours co-culture with wild-type EPEC, peripheral expression of E-cadherin was seen in most cells. However, peripheral expression is lower than in uninfected cells and expression was also detected in the cytoplasm and in discrete nuclear regions of several cells (marked by arrow heads).

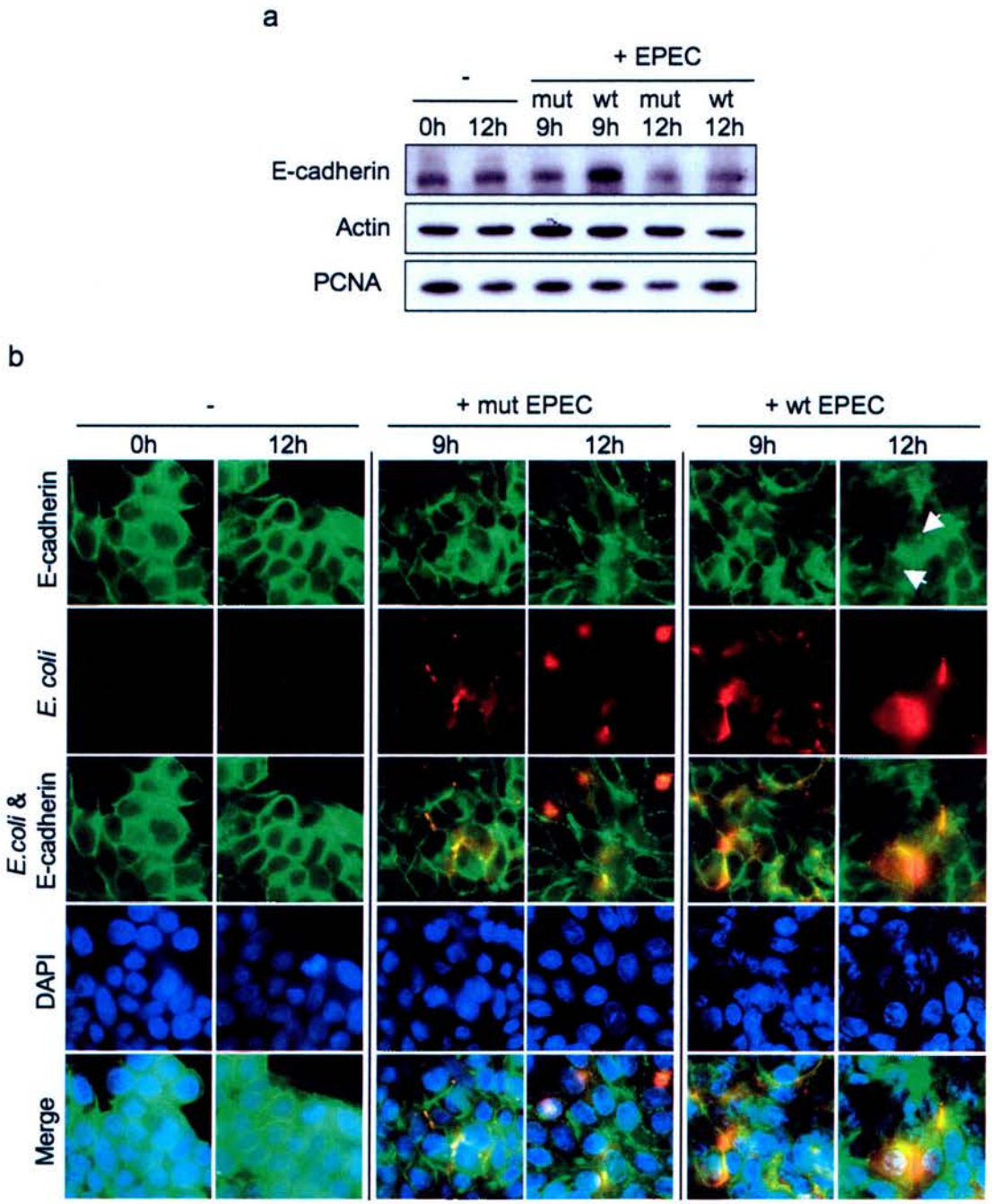


Figure 6.5. Co-localisation of E-cadherin and *E .coli* in HT29 cells co-cultured with EPEC: Nuclear x 100. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Western blots were performed on whole cell protein extracts (**a**). Fixed cells were immunostained for E-cadherin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue) (**b**). Arrow heads indicate areas of nuclear E-cadherin accumulation. Nuclear focal plane, original magnification x 100.

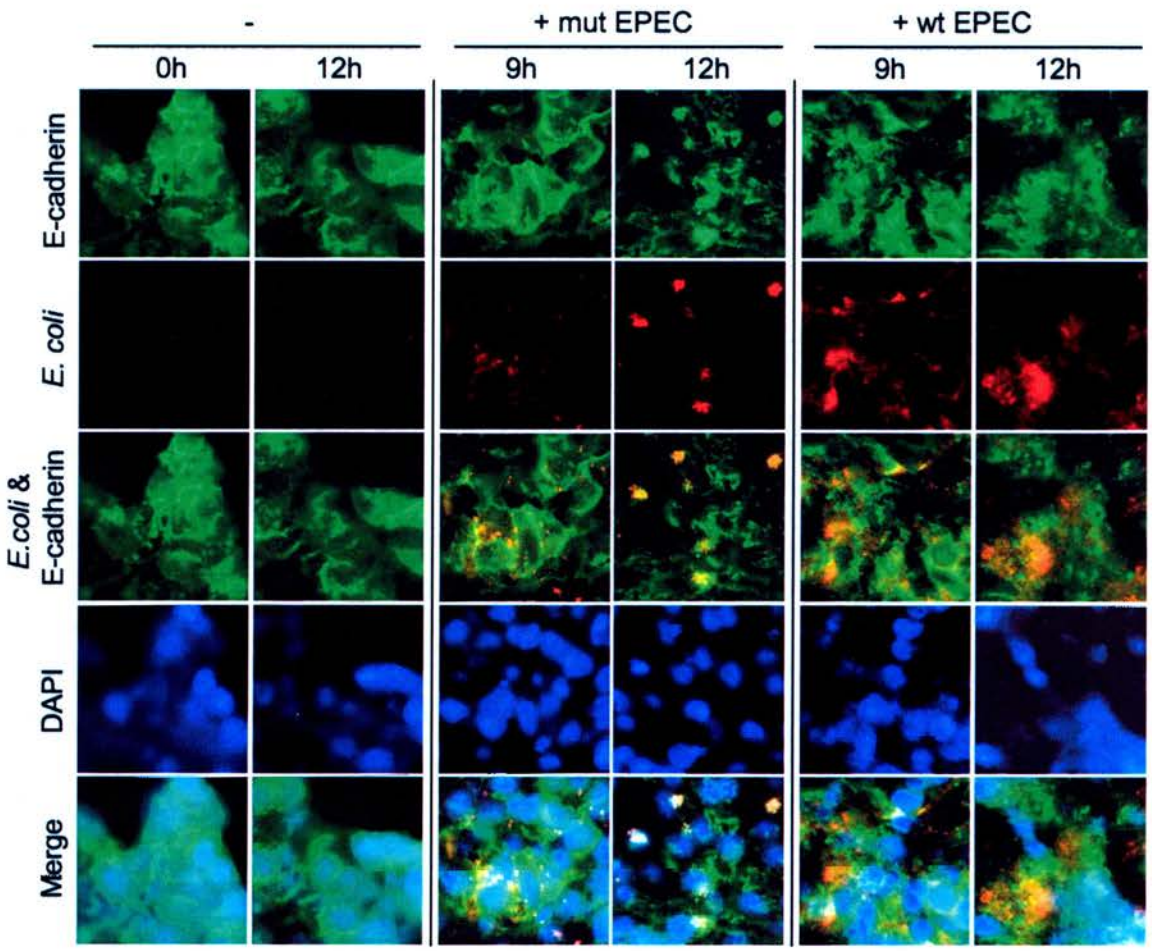


Figure 6.6. Co-localisation of E-cadherin and *E .coli* in HT29 cells co-cultured with EPEC: Apical x 100. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for E-cadherin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue) (**b**). Apical focal plane, original magnification x 100.

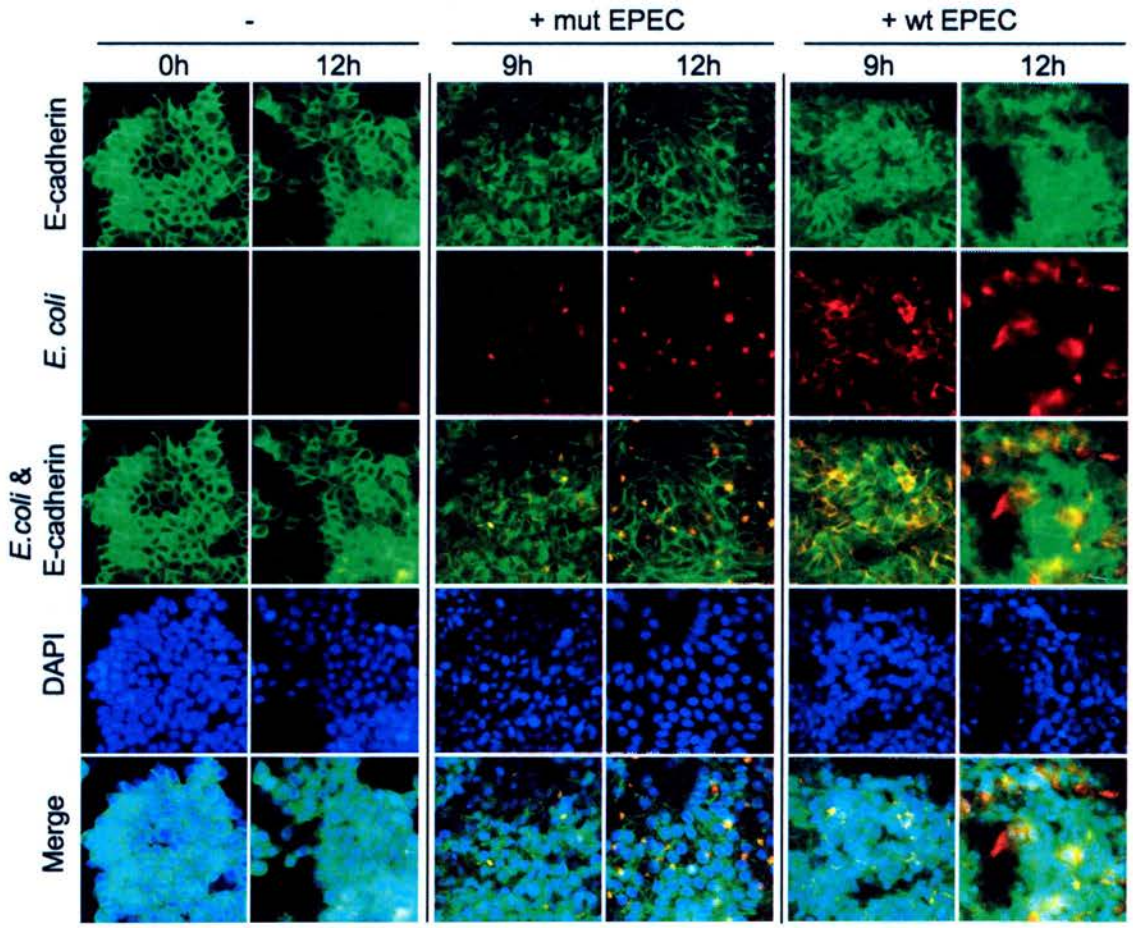


Figure 6.7. Co-localisation of E-cadherin and *E .coli* in HT29 cells co-cultured with EPEC: Nuclear x 40. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for E-cadherin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Nuclear focal plane, original magnification x 40.

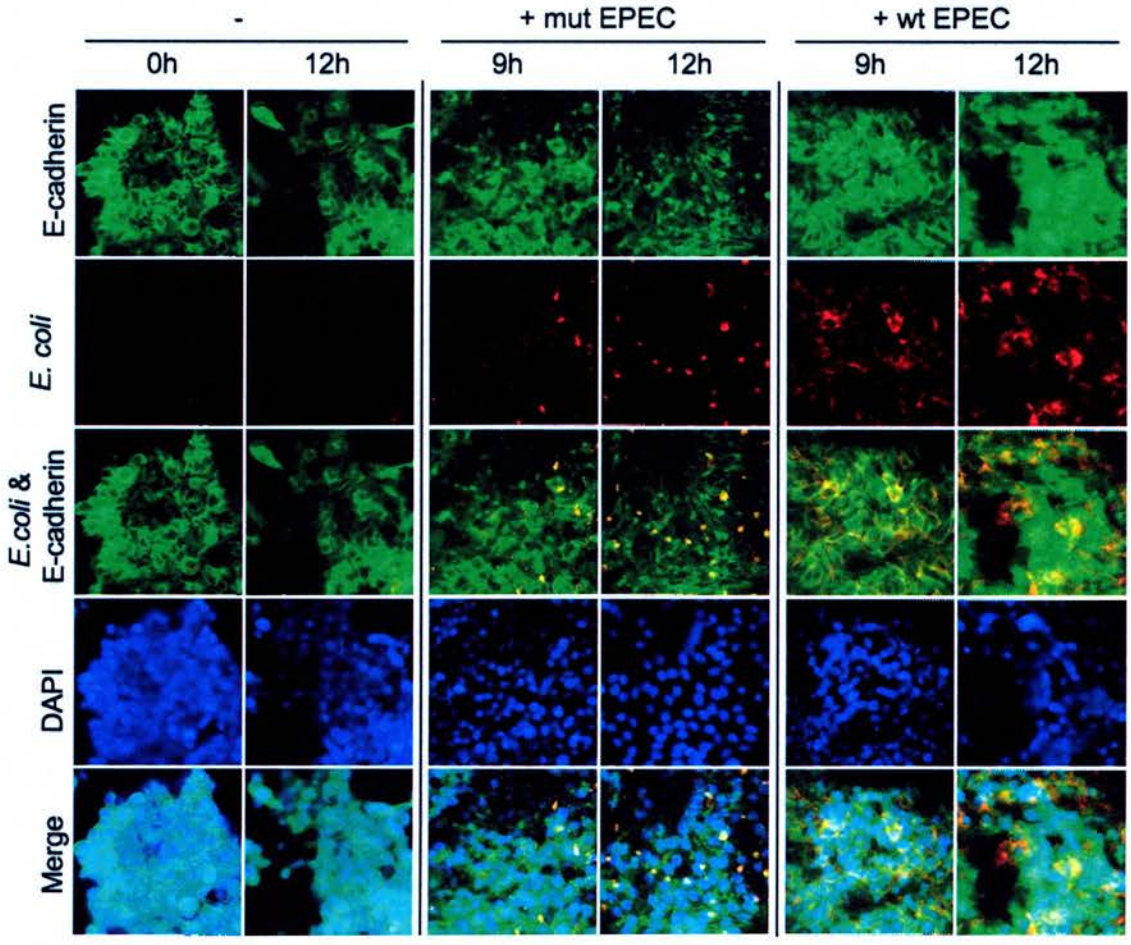


Figure 6.8. Co-localisation of E-cadherin and *E .coli* in HT29 cells co-cultured with EPEC: Apical x 40. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for E-cadherin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Apical focal plane, original magnification x 40.

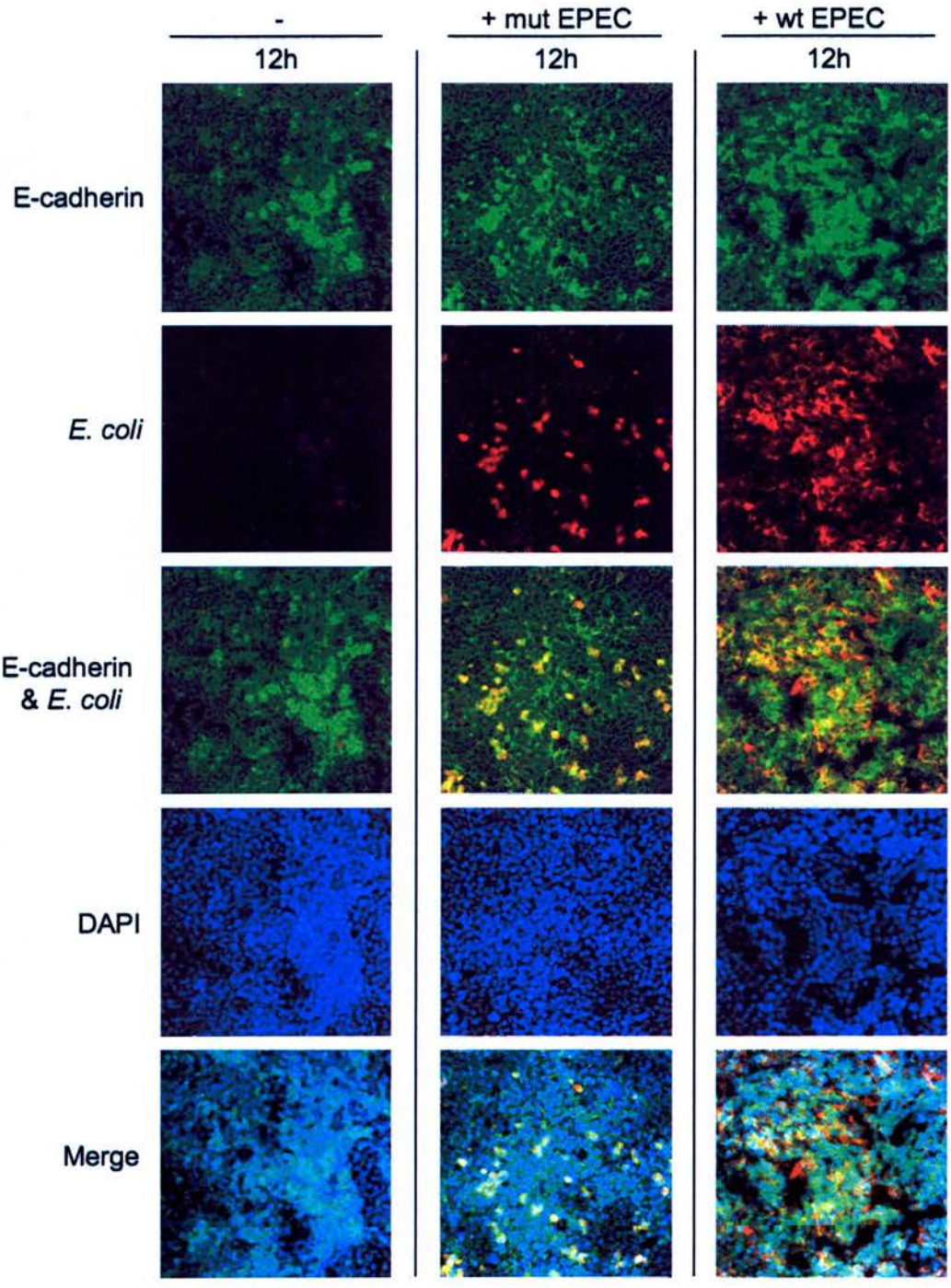


Figure 6.9. Co-localisation of E-cadherin and *E .coli* in HT29 cells co-cultured with EPEC: x 16. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Original magnification x 16.

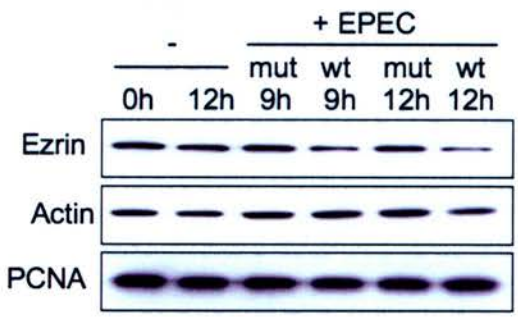
High magnification images in the apical focal plane show E-cadherin localised to sites of EPEC adherence (Figure 6.6). In uninfected cells E-cadherin is expressed diffusely at the apical surface of cells, with stronger staining at cell boundaries. Co-culture with mutant EPEC for 9 hours had little effect on E-cadherin distribution, however, after 12 hours patches of mutant EPEC (attached by "localised adherence") co-localise with strong E-cadherin expression. Evidence of E-cadherin-EPEC co-localisation was also seen after co-culture with wild-type EPEC, particularly after 9 hours.

Images taken at intermediate magnification also show EPEC induced disruption of E-cadherin expression in nuclear focal plane (Figure 6.7), and accumulation of E-cadherin where adherent EPEC were detected in the apical focal plane (Figure 6.8). When viewed at low magnification, the accumulation of E-cadherin at sites of EPEC adherence is clear, particularly in mutant infected cells where discrete colonies of EPEC correspond to the sites of highest E-cadherin expression (Figure 6.9). In cells co-cultured with wild-type EPEC for 12 hours it is more difficult to discern co-localisation due to widespread EPEC staining, however, overall E-cadherin expression is seen to be strong.

6.2.4. Effect of EPEC on Ezrin expression *in vitro*

Western blots show that in HT29 cells, ezrin expression was reduced after co-culture with wild-type but not mutant EPEC (Figure 6.10a). However, unlike β-catenin and γ-catenin expression, the reduction was relatively small, and ezrin was still expressed in HT29 cells (at approximately equal levels) after 9 and 12 hours co-culture. Western blots demonstrated that co-culture of wild-type EPEC with SW480 cells also resulted in reduction of ezrin expression (Figure 6.10b). However, in SW480 cells the reduction in ezrin appeared to be more severe as expression was below levels of detection. This difference may be accounted for by the relatively low expression of ezrin in SW480 cells compared to HT29 cells. Immunostaining of HT29 cells for ezrin was unsuccessful due an apparent lack of affinity of the antibody for ezrin in fixed cells.

a



b

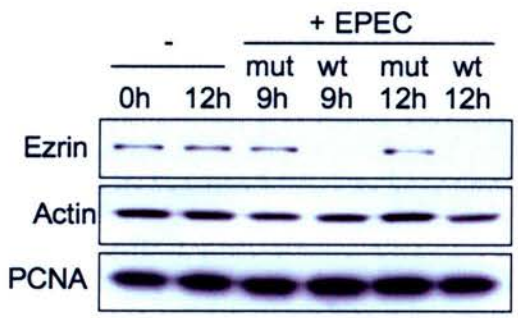


Figure 6.10. Ezrin expression in human colorectal cells co-cultured with EPEC. HT29 (**a**) or SW480 (**b**) cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Western blots were performed on whole cell protein extracts and probed for ezrin.

6.2.5. Effect of EPEC on actin expression *in vitro*

Actin, in the form of filamentous actin (F-actin) is known to accumulate at the apical membrane where EPEC adheres to cells. Having used actin as a loading control for western blots we wished to confirm the effects of EPEC infection on actin expression *in situ*. In the nuclear focal plane of uninfected HT29 cells, actin was expressed in the cytoplasm and at the periphery of cells. Although, actin expression was quite evenly distributed, some small spots of high expression were visible. After infection with mutant EPEC for 9 and 12 hours, actin continues to be expressed within the cytoplasm; however, its distribution was more granular than seen in uninfected cells. In cells infected with wild-type EPEC, actin expression was more diffuse than in control cells, with several relatively large, very strong spots of expression (Figure 6.11). In the apical focal plane, accumulation of actin at sites of mutant and wild-type EPEC adherence was evident. However, wild-type EPEC induced much larger and more concentrated spots of actin than mutant bacteria (Figure 6.12).

Intermediate magnification images of HT29 cells clearly show that in wild-type EPEC infected cells actin expression was reduced in the nuclear focal plane, particularly after 12 hours co-culture (Figure 6.13). However, in the apical focal plane actin is highly expressed in wild-type infected cells and is distributed in highly concentrated spots, which are likely to correspond with adherent EPEC (Figure 6.14). Low magnification images of co-cultured cells support the ability of EPEC infection to cause actin remodelling but also show that overall expression levels are similar in uninfected, mutant EPEC infected and wild-type EPEC infected cells (Figure 6.15). Therefore the use of actin as a loading control for western blots is justified.

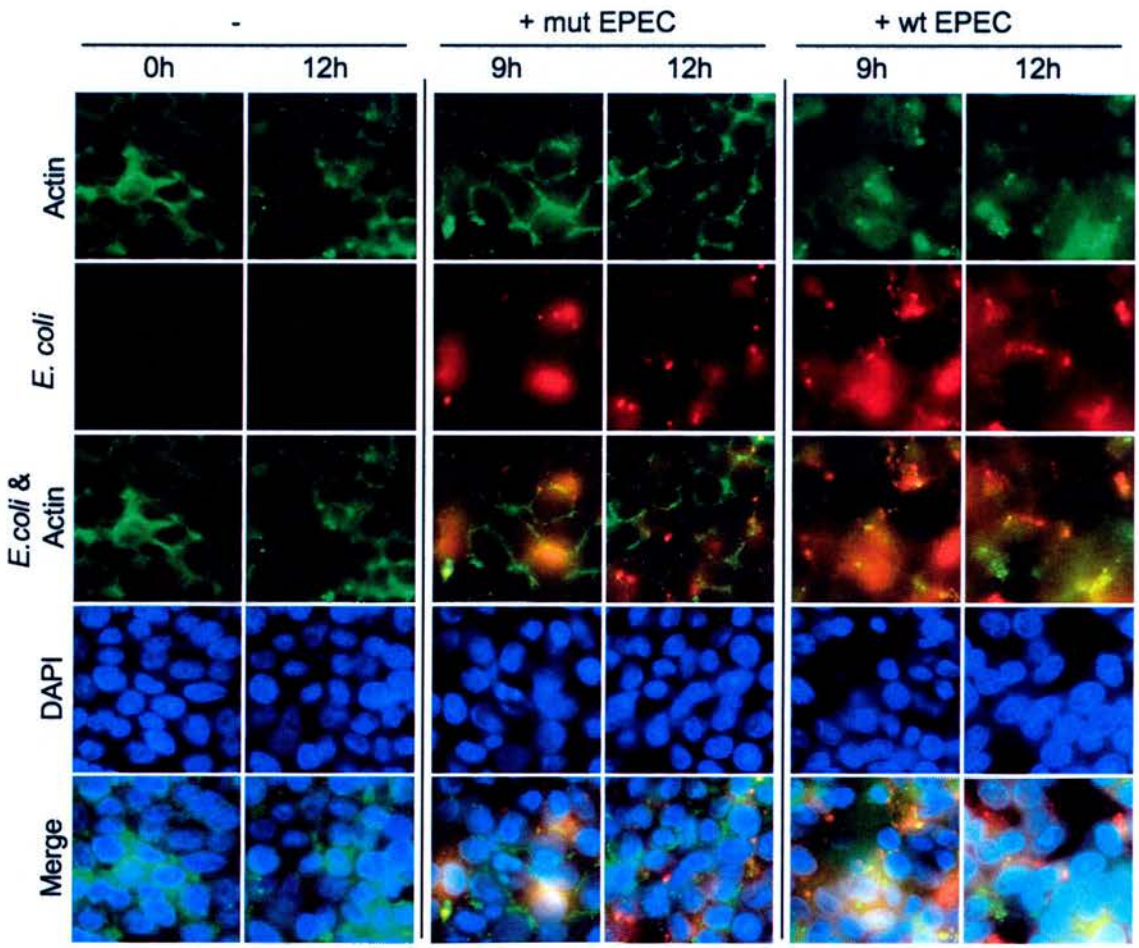


Figure 6.11. Co-localisation of actin and *E .coli* in HT29 cells co-cultured with EPEC: Nuclear x 100. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Nuclear focal plane, original magnification x 100.

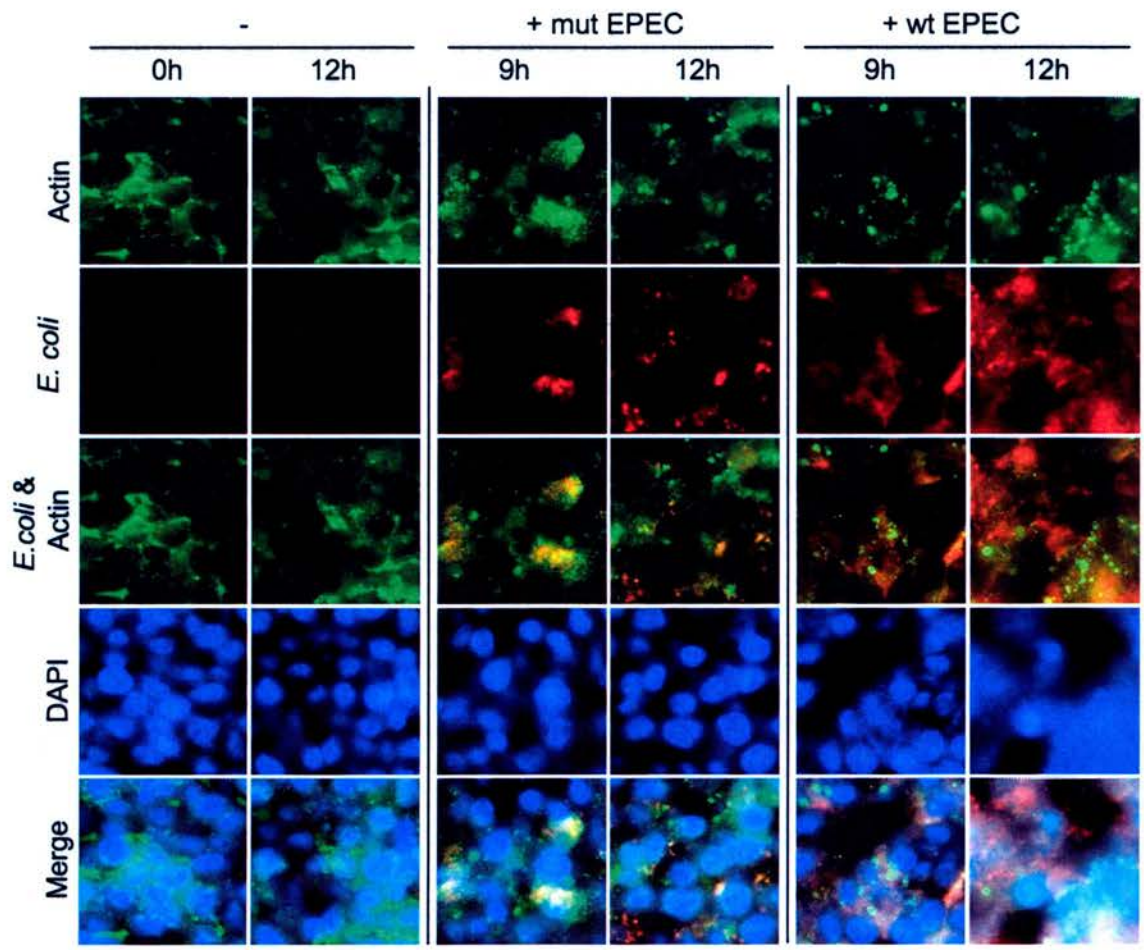


Figure 6.12. Co-localisation of actin and *E .coli* in HT29 cells co-cultured with EPEC: Apical x 100. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Apical focal plane, original magnification x 100.

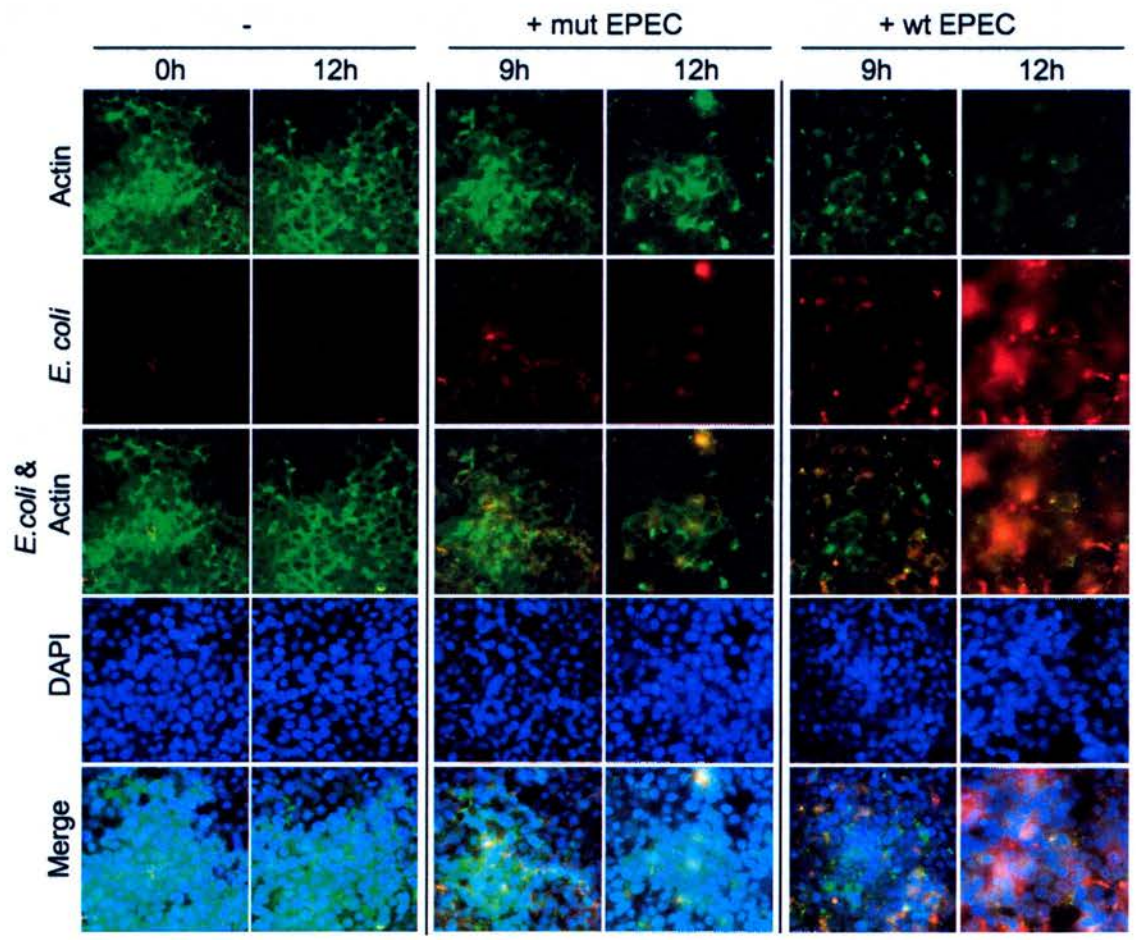


Figure 6.13. Co-localisation of actin and *E .coli* in HT29 cells co-cultured with EPEC: Nuclear x 40. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Nuclear focal plane, original magnification x 40.

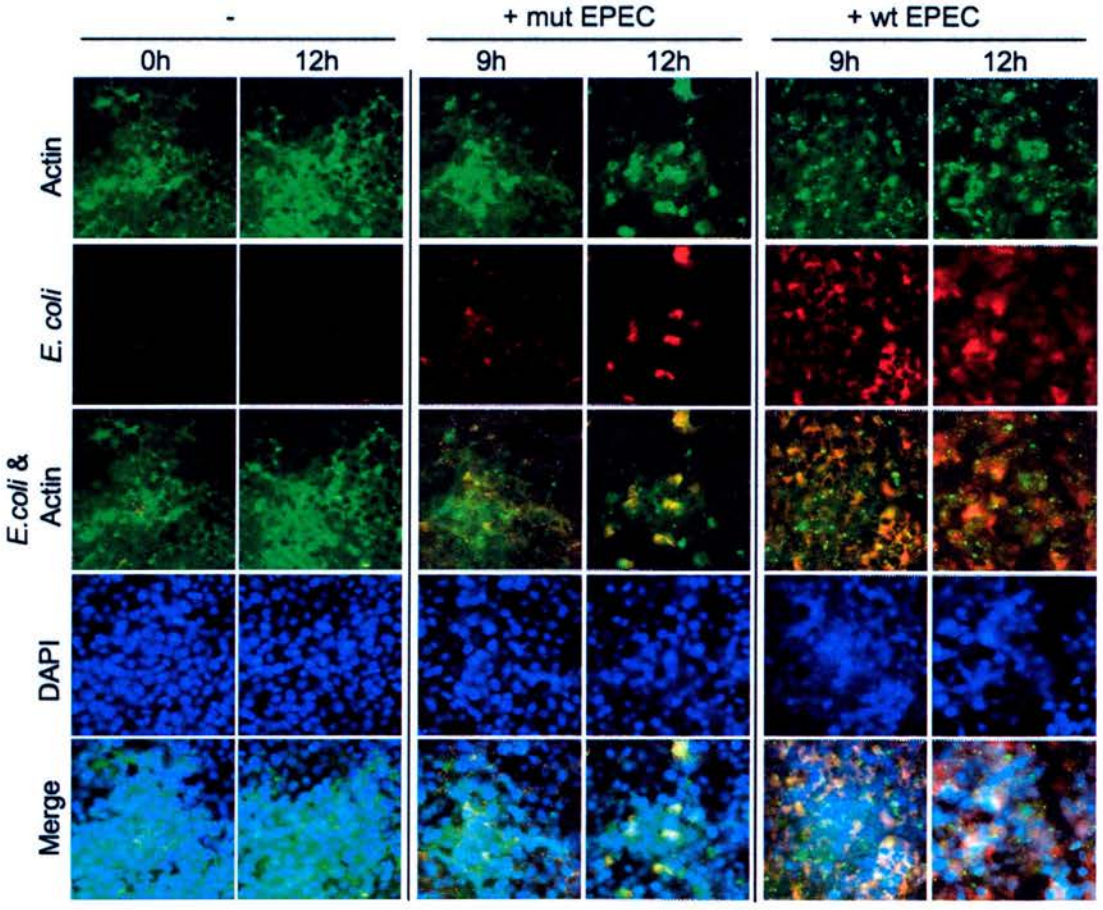


Figure 6.14. Co-localisation of actin and *E .coli* in HT29 cells co-cultured with EPEC: Apical x 40. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 9 -12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Apical focal plane, original magnification x 40.

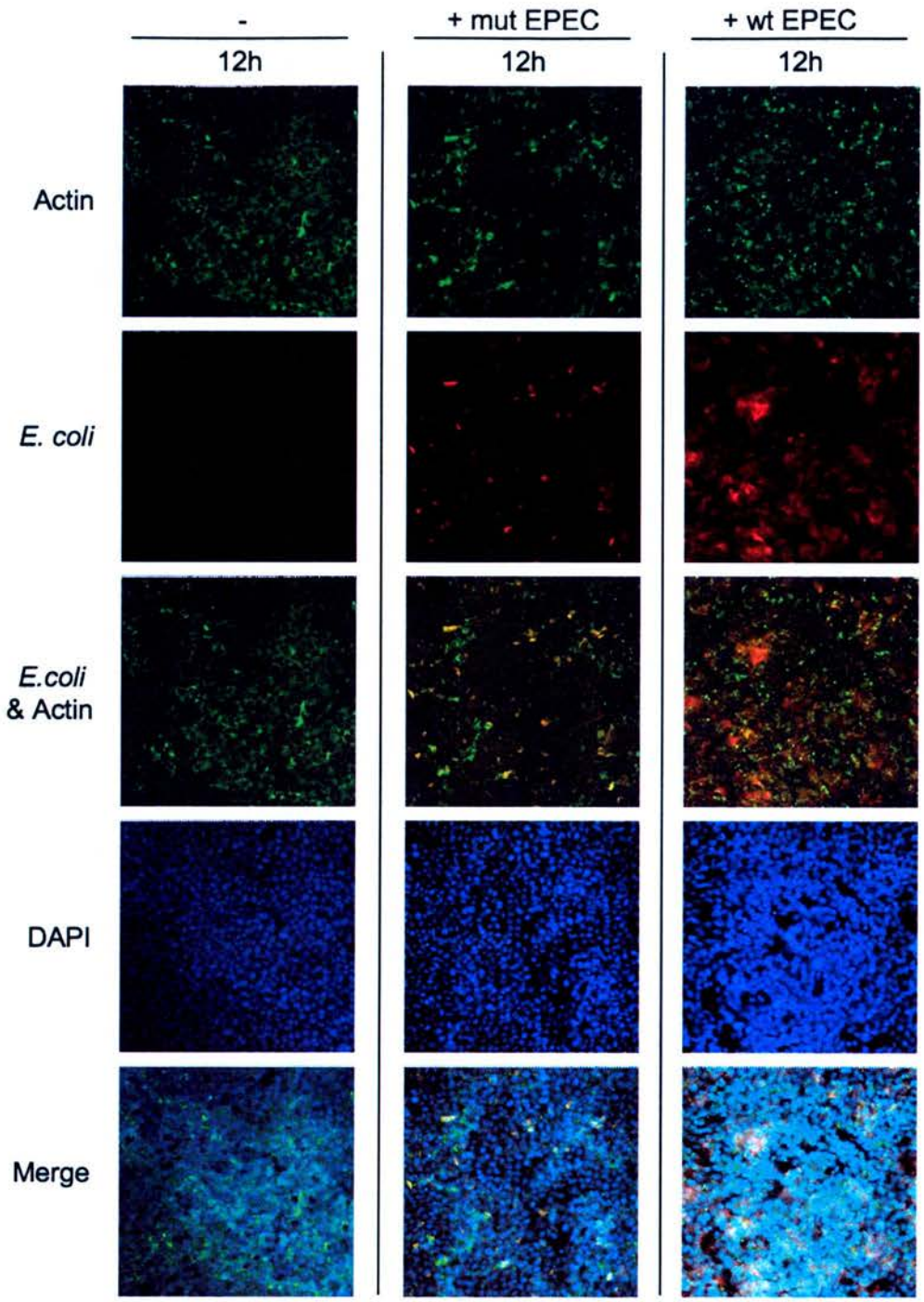


Figure 6.15. Co-localisation of actin and *E .coli* in HT29 cells co-cultured with EPEC: x 16. Cells were either uninfected (-) or co-cultured with mutant (UMD864) or wild-type (E2348/69) EPEC for 12 h. Fixed cells were immunostained for actin (green) and *E. coli* (red), nuclei were counter stained with DAPI (blue). Original magnification x 16.

	Protein				
	β-catenin	γ-catenin	E-cadherin	Ezrin	Actin
HT29 cells	↓	↓	↑/ ↔	↔/↓	↔
SW480 cells	↓	ND	ND	↓	↔

Table 6.1. EPEC induced changes in protein expression: results summary. Changes in protein expression in response to wild-type EPEC infection in HT29 and SW480 cells. γ-catenin and E-cadherin were not detected (ND) in SW480 cells.

6.3. Discussion

EPEC E2348/69 clearly downregulated total β-catenin expression in HT29 and SW480 cells after 9 – 12 hours co-culture. Images also suggest that redistribution of β-catenin (from the periphery to the cytoplasm) may have occurred, particularly after 9 hours. EPEC UMD864 also influenced β-catenin expression, but to a lesser degree than the wild-type bacteria, causing a reduction in expression most notable after 12 hours co-culture. Canonical β-catenin degradation involves serine/threonine phosphorylation at its amino terminal (catalysed by glycogen synthase kinase-3β complexed with APC and axin), followed by ubiquitination and proteosomal degradation (reviewed by Kolligs *et. al.,* 2002). Using different antibodies we determined that the vast majority of β-catenin (in uninfected and infected HT29 cells) was in a non-phosphorylated form, this is a logical finding as phosphorylated β-catenin is rapidly degraded and is hardly detectable (1-2% of total β-catenin) in normal cultured cells (Sadot *et. al.,* 2002).

These results therefore fit the canonical model of β-catenin degradation as the mechanism for EPEC induced β-catenin downregulation. However, like most colon cancer cells, both HT29 and SW480 cells carry mutations in their *APC* genes. Consequently both cell lines express truncated APC protein and have reduced β-catenin degradation ability compared to normal cells (Sadot *et. al.,* 2002). Hence it is possible that EPEC induced β-catenin degradation in these cells is supplemented by additional mechanisms. EPEC induced PKCα activation (Crane & Oh, 1997) has

the potential to fulfil this role. Breast cancer cells over-expressing of PKCα have reduced β-catenin stability (Williams & Noti, 2001). Furthermore, chemical activation of PKCα results in amino-terminal serine phosphorylation and degradation of β-catenin *in vitro* (Gwak *et. al.,* 2006), although this is presumably via the APC-Axin-GSK-3β pathway. In HT29 cells, γ-catenin expression changed in response to EPEC infection in a similar way to β-catenin expression. After 9 hours infection with wild-type EPEC, γ-catenin expression was reduced to roughly half the control levels, and by 12 hours, expression was virtually undetectable. Whereas, cells infected with mutant EPEC retained strong γ-catenin expression. This is unsurprising considering the structural and functional similarities between γ-catenin and β-catenin. γ-catenin contains a glycogen synthase kinase-3β consensus motif phosphorylation site (Shiina *et. al.,* 2005), suggesting its degradation occurs via the same pathways as β-catenin. Hence EPEC induced degradation of γ-catenin may well occur via the same pathways as β-catenin.

In contrast to catenins, E-cadherin expression was unchanged or even upregulated after 9 hours co-culture with EPEC. This response was unexpected as EPEC is observed to cause a reduction in cell-cell adhesion, usually a feature of E-cadherin downregulation. That elevated levels of E-cadherin were observed in cells showing reduced cell-cell adhesion can be explained by the inability of E-cadherin to function properly in the absence of β-catenin (and possibly γ-catenin). That is, without β-catenin, the cytoplasmic domain of E-cadherin is unstructured and does not link to the cytoskeleton (Huber *et. al.,* 2001). It is possible that increased E-cadherin expression was triggered in infected cells as an attempt to overcome loss of cell-cell adhesion. It was notable that E-cadherin accumulated in the apical plane of cells at sites of wild-type and mutant EPEC adherence, this suggests E-cadherin could play a role in non-intimate attachment of EPEC. *Listeria monocytogenes* is a human enteric pathogen that adheres to the intestinal epithelium via E-cadherin binding. *L. monocytogenes* uses E-cadherin as a receptor for the bacterial protein internalin, which is required for entry of the bacteria into epithelial cells (Mengaud *et. al.,* 1996). Though EPEC is generally accepted not to enter cells, it is possible that E-cadherin could be involved in surface adherence of EPEC.

Although wild-type EPEC induced a reduction in ezrin expression in HT29 cells and SW480 cells, this reduction was not as marked in HT29 cells as with catenin expression. Hence, ezrin expression was retained in HT29 cells after 9-12 hours co-culture with EPEC, but was below detection in SW480 cells. EPEC infection has been shown to activate ezrin via phosphorylation in human intestinal epithelial (T84) cells, without reducing overall expression (Simonovic *et. al.,* 2001). It is possible that in our extended time-course model degradation of ezrin occurs subsequent to phosphorylation. The difference between the two cell lines may be a consequence of divergent cell signalling pathways in these cell lines. Alternatively the apparent difference in mucus secretion by these cell lines (HT29 cells were observed to release more mucus than SW480 cells) may account for a protective effect in HT29 cells, as with MMR protein changes (Chapter 5).

EPEC infection undoubtedly had marked effects on actin distribution within HT29 cells, causing actin condensation at sites of EPEC attachment, without changing its overall expression. These results correlate with the fluorescent staining test results, which utilised phalloidin to stain actin in EPEC induced pedestals (Chapter 3). This was expected, as actin rearrangement is a fundamental feature of EPEC attachment (Knutton *et. al.,* 1989; Finlay *et. al.,* 1992). Preservation of actin expression can therefore be viewed as an important strategy for EPEC pathogenesis. These finding help to further characterise the pathways involved in EPEC induced loss of cell-cell and cell-lamina adhesion, contributing to epithelial barrier disruption.

Although actin rearrangement and disruption of cell-cell adhesion are beneficial to EPEC, the consequences to the host cells should also be considered. The literature suggests that EPEC infection could promote carcinogenesis by causing cytoplasmic accumulation of β-catenin, leading to the activation of Wnt target genes. However, this study shows that after 9 – 12 hours infection, EPEC in fact caused a marked downregulation of β-catenin. Therefore, in terms of the canonical Wnt/APC pathway of colonic tumourigenesis, this change should prevent rather than promote cancer. However, as β-catenin is a multi-functional molecule its downregulation may

promote tumourigenesis via another pathway, i.e. cell-cell adhesion. Although downregulation of adhesion proteins is usually associated with advanced tumours, changes in these proteins within the normal colon could also be related to tumourigenesis.

A recent review draws attention to the role of the actin cytoskeleton in cells migrating within the intestinal epithelium, suggesting that disturbance of cell architecture could prevent normal migration (Nathke, 2006). The rapid turnover of cells within the intestinal epithelium protects from tumourigenesis by ejecting cells before they have a chance to accumulate sufficient mutations to become cancerous. Retardation of cellular migration within the crypt would have the potential to extend the life of cells within the relatively toxic environment of the gut, providing a greater chance for mutations to accumulate, and also potentially expose cells to inappropriate cues. Hence, it is "mandatory that any event that can induce tumours has to include a 'brake' to allow cells to remain in the tissue" (Nathke, 2006). Aberrant expression of adhesion proteins is also likely to perturb migration, as cell-cell signalling via direct contact would be interrupted. The ability of cells to organise their distribution based on surface protein expression is illustrated by cadherin dependent cell sorting. Cells are able to arrange themselves either based on expression of different forms of cadherin (e.g. E-cadherin versus N-cadherin), or based on expression levels of the same cadherin (e.g. low versus high E-cadherin expression) (Alberts *et. al.,* 2002). This study shows that EPEC has profound effects on cytoskeletal and cell adhesion pathways *in vitro*. Translation of these effects into colonic proliferative progenitor cells *in vivo* would be highly likely to disturb normal migration and hence provide a braking effect, hence increasing susceptibility to tumourigenesis in the healthy colon.

These results constitute a preliminary investigation into the effects of EPEC on cell-cell adhesion and cytoskeletal pathways in an extended time-course *in vitro* model. Confirmation of the exact changes in protein distribution would help to clarify EPEC's effects. Separating nuclear, cytoplasmic and cell membrane fractions prior to probing for protein expression could help to achieve this. The mechanisms

underlying the observed changes in protein expression also warrant further investigation. A more thorough investigation into protein phosphorylation (particularly of β-catenin) would help to determine degradation mechanisms. Using additional phosphorylation specific antibodies could aid this process. Comparing the response to EPEC of cell lines expressing wild-type APC and β-catenin (such as HCA7) with HT29 and SW480 cells would also be of value. Also, use of enzyme inhibitors (such as sphingosine and bisindolylmaleimide to inhibit PKC) would help to establish the involvement of specific enzymes in protein degradation.

The association of proteins of interest with potential degradation enzymes could be analysed, immunoprecipitation could be utilised for this purpose. The consequences of EPEC induced changes on cell migration and expression of Wnt target genes (such as *c-myc* and *cyclin D1*) could also be investigated. The ability of E-cadherin to act as an adhesion protein for EPEC could be assessed by blocking free E-cadherin with antibodies prior to incubation with EPEC. Most importantly, the translation of *in vitro* effects to the *in vivo* scenario (potentially via analysis using *ex vivo* tissue) could be investigated.

Chapter 7.

Final discussion

The aim of this study was to test the hypothesis that infection of colonic epithelial cells by EPEC can increase the susceptibility of these cells to neoplastic transformation in man. It was not possible to establish an *in vivo* mouse model of EPEC infection, largely due to the species specificity of EPEC E2348/69. Biologically relevant and repeatable *in vitro* and *ex vivo* models for EPEC infection were established and provided a means to test the hypothesis. Consequently this study has provided novel insights into the behaviour of EPEC in the adult colon and the effects of EPEC on the classic molecular pathways of colorectal carcinogenesis. That is, the ability of EPEC to downregulate MSH2, MLH1 and β-catenin protein expression. Additionally, this study has established that EPEC enter the crypts of normal human colonic mucosa, and associate with proliferative epithelial cells. This illustrates a potential niche environment for chronic EPEC infection in man, and more importantly, places EPEC in a position to exert its effects on cells capable of initiating tumour formation.

Translation of the *in vitro* effects of EPEC to the human colon *in vivo* would have potentially serious implications for infected crypt cells. Prolonged reduction of MSH2 and MLH1 protein expression is likely to confer significant genomic instability, and reduce DNA damage associated apoptosis signalling. This study shows that cell division is slowed by EPEC infection and that alterations in host cell DNA occur subsequent to infection; this supports a previous study, which found that EPEC induces cell cycle arrest without stopping DNA replication (Marches *et. al.,* 2003). Continued replication of DNA in the absence of functional DNA mismatch repair or DNA damage associated apoptotic signalling would present an environment highly conducive to the accumulation of mutations. MMR disruption is proven to facilitate mutation of tumour suppressor and oncogenes within the colon, leading to enhanced tumour formation (Sohn *et. al.,* 2003). Hence EPEC could induce a paradoxical situation whereby cell cycle arrest promotes rather than inhibits accumulation of mutations. This study also supports the ability of EPEC to disrupt the host cytoskeleton and cell-cell adhesion mechanisms. Induction of these changes *in vivo* would have the potential to retard epithelial cell migration, thus extending cell life and delaying differentiation. Infected cells would consequently be exposed

to food borne carcinogens for longer, and sustain increased exposure to proliferative signals (supplied to cells while in the lower two thirds of the crypt). The combined effect of these molecular changes therefore represents a significant increase in the risk of cell transformation.

A reduction in host cell apoptotic signalling and cell migration (both resulting in extended cell life) would be potentially beneficial for an attaching and effacing bacteria such as EPEC. The AE process requires a significant investment of bacterial cell resources, and results in a highly specialised binding site, potentially providing access to nutrients and a degree of protection from immune cells. By extending the life of the host cell, EPEC would preserve this favourable situation. EPEC has the ability to move across the apical surface of epithelial cells (Narato & Kaper, 1998). Therefore, once anchored within the colonic crypt it is possible that EPEC could move (downwards) from cell to cell, perpetuating its residence within the crypt. Bacterial migration would also allow cells at one time infected, to become free of infection. This study has shown that even cells exposed to high levels of EPEC infection for an extended time-course can recover from infection and undergo division. Daughter cells produced from a progenitor exposed to chronic EPEC infection would, for the reasons already discussed, have an increased chance of inheriting mutations. Further cell division and clonal expansion could therefore result in tumour formation.

In addition to the *in vitro* and *ex vivo* findings, analysis of human adenocarcinomas for AE *E. coli* has revealed an association between these bacteria and tumour tissue. This is also a novel finding and although it does not necessarily imply a causative role for adherent *E. coli* in tumourigenesis, it does perhaps provide an explanation for bacteria induced carcinogenesis. Initially it seems counter intuitive that a bacteria could derive benefit from inducing a lethal disease in its host organism, and that this would be unlikely to confer an evolutionary advantage to the bacteria. Firstly, it must be remembered that colorectal cancer takes decades to develop; more than enough time for EPEC to propagate and infect (via the faecal oral route) further hosts. Secondly, as this study has demonstrated, adenocarcinomas provide an excellent

environment for EPEC. Tumours have vastly increased surface area compared to normal mucosa, and evidently surface antigens that are preferable to normal tissue for AE *E. coli* adhesion. These results support previous studies that have found that adherent *E. coli* associate with tumour tissue in preference to normal mucosa (Martin *et. al.,* 2004; Swidsinski *et al.,* 1998). Therefore, carcinogenesis of the colon could actually be an advantage to EPEC, providing an opportunity for population expansion, leading to increased bacterial shedding and hence increased propensity for the host to pass on infection to others.

The precise surface antigens to which EPEC initially binds are unknown. The results of this study demonstrate that within the adult human colon, EPEC binds selectively to crypt proliferative progenitor cells and to tumour tissue. The severe pathology of EPEC infection in infants suggests that the infant intestinal epithelium also represents an excellent binding surface. Surface residues shared by these tissues are therefore candidate targets for EPEC adherence. A similarity between these cell types is that they are more mitotically active and 'less differentiated' than cells at the surface epithelium of the adult colon, where EPEC did not adhere. In normal tissue, mitotically active, undifferentiated cells express active glycosyltransferase enzymes, resulting in the expression of surface glycoproteins with incomplete polysaccharide chains (Weiser, 1973). Cancer cells also express active glycosyltransferase enzymes and have different surface antigens compared to normal, differentiated cells.

It has already been acknowledged that the surface antigens of fetal and tumour cells display some homology, leading to the suggestion by Martin *et. al.* (2004) that adherent *E. coli* bind to 'oncofetal' surface antigens. The present study suggests that mitotically active, undifferentiated crypt cells also express 'oncofetal' surface antigens. It is logical that EPEC would target more immature cells when invading a host. Firstly, these cells are likely to live for longer than differentiated cells, providing a longer lasting binding site. Secondly, it is possible that EPEC's molecular and cellular effects are more easily translated in undifferentiated cells, where cell fate is yet to be decided. The observation that MMR protein downregulation was more rapid in SW480 cells versus HT29 cells supports this

theory. Although the surface antigens expressed in infants are not necessarily the same as those in the fetal intestine, it is logical to assume that the infant intestine contains a higher percentage of immature cells than the adult, as organ growth is still occurring. In conclusion, it seems likely that EPEC binds preferentially to undifferentiated cells, and that this binding affinity can explain; (a) the severe pathology of EPEC infection in children, (b) the ability of adults to carry EPEC without symptoms, potentially in the niche of the colonic crypts, and (c) the association of AE *E. coli* with adenocarcinoma.

There are striking similarities between the behaviour and effects of EPEC and those of *Helicobacter pylori*, a bacterium responsible for carcinogenesis in the stomach. In similarity to EPEC, *H. pylori* downregulates DNA mismatch repair proteins in gastric epithelial cancer cells *in vitro* (Kim *et. al.,* 2002); an effect found to translate to the gastric epithelium *in vivo* (Park *et. al.,* 2005). *H. pylori* also reduces cell-cell adhesion by disrupting E-cadherin / catenin adherens junctions (Suzuki *et. al.,* 2005). Like EPEC, *H. pylori* associates closely with the host epithelium and inserts effector molecules via a bacterial secretion system. Based on the observation that not all *H. pylori* strains are carcinogenic, recent work on *H. pylori* induced carcinogenesis has focussed on strains carrying the *cag* pathogenicity island. The EPEC LEE pathogenicity island and *cag* are analogous in that they both encode multiple genes for secretion systems and secreted effector molecules. *H. pylori* strains that harbour *cag* have increased ability to cause cancer versus *cag* negative strains (Blaser *et. al.,* 1995). It is also notable that despite the ability to cause cancer in its host, *H. pylori* is able to thrive among the human population. As with *H. pylori*, it is true that many more people harbour *E. coli* than develop colorectal cancer. It is therefore an appealing hypothesis that the difference between carcinogenic and non-carcinogenic bacteria is the possession of virulence determinants that allow bacterial attachment and insertion of effector molecules into the host epithelium.

If EPEC is indeed able to increase the risk of colorectal cancer, this raises a paradox, in that EPEC infection is more prevalent in developing countries where colorectal cancer rates are lower than in the developed world. This could be explained by a

number of factors. Firstly, modern epidemiological information on EPEC in developed countries is severely lacking. The reduction in infant mortality due to EPEC in developed countries means routine screening is no longer performed in hospitals; hence EPEC carriage by adults in developed countries is virtually unknown and may be higher than expected. Secondly, it is probable that the diet in developed countries contains a greater variety, and higher concentrations of carcinogens (e.g. in processed food) compared to the more natural diet of developing countries. Therefore, if EPEC increases the susceptibility of colonic cells to mutation, it would have a greater chance of initiating cancer in developed countries.

A more accurate evaluation of EPEC carriage amongst adults in developed countries would be extremely useful in evaluating a relationship between EPEC and cancer. Presently, studies on the prevalence of EPEC infection focus on children, and show that up to 11% of asymptomatic (i.e. healthy) children carry EPEC in developed countries. It is quite possible that a similar proportion of adults also carry EPEC. In this study AE *E. coli* (i.e. EPEC / EHEC) were identified in 25% of patients with adenocarcinoma, results suggest that this proportion may have been higher with more sensitive detection techniques or if fresh samples had been used. Even so, a 25% detection rate is approximately double the combined prevalence EPEC and EHEC in studies on children in developed countries. Therefore, not only does this study support the carriage of EPEC by adults in the UK, it also suggests that patients with colorectal cancer may have a higher prevalence of EPEC carriage than individuals without cancer (assuming adult EPEC carriage is ~10%). This crucial result is potentially of genuine significance and establishes the need for further epidemiological investigation of EPEC, particularly in comparing EPEC carriage in adults with and without colorectal cancer.

In addition to the specific changes in protein expression analysed in this study, the wider proteomic and transcriptomic effects observed demonstrate the complexity and variety of EPEC's effects. Though this study has not focussed on the mechanisms of these changes there are a number of clues to suggest how EPEC causes its effects. The inability of the mutant EPEC strain to induce the same changes as wild-type

strain indicates that secreted effector proteins and / or intimin signalling are key to the transduction of molecular effects in the host. Reference to the literature has provided putative mechanisms for EPEC induced MMR and adhesion protein downregulation. Protein kinase enzymes are activated by EPEC (Crane & Oh, 1997) and represent prime candidates to mediate many of the effects identified in this study; PKCα is particularly conspicuous in this respect. The diversity of transcriptomic and proteomic effects demonstrated by this and other studies (e.g. Hardwidge *et. al.,* 2004) in response to EPEC infection should encourage the research of EPEC to extend beyond simply AE lesion formation and diarrhoea. If adult populations do indeed carry EPEC, further exploration of the role of this pathogen in more complex adult diseases such as inflammatory bowel disease and intestinal cancers is warranted.

In conclusion, this study has met its initial aims, and adds support to the hypothesis that infection of colonic epithelial cells by enteropathogenic *Escherichia coli* can increase the susceptibility of these cells to neoplastic transformation in man. In addition, the novel findings presented in this thesis establish many opportunities for further research.

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Appendix 2. Microarray functional annotation clustering.

Gene ontology: Pathways with altered gene expression in HT29 cells infected with EPEC, using VSN normalisation and SAM analysis. Genes with statistically significant fold-change (false discovery rate 0.1, $P<0.05$) were subjected to functional annotation clustering searches via DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/tools.jsp). The enrichment score for the group is based on the P-value of each term member; the higher the score the more enriched. The number of differentially expressed genes found in the pathway is displayed under 'Count'. P_value = modified Fisher Exact P-value for gene enrichment analysis (or EASE score); the smaller the value the more enriched the pathway, 0 represents perfect enrichment.

-0h / -12h		
Cluster 1. Enrichment Score: 0.6	Count	P_Value
lipid metabolism	3	1.60E-02
primary metabolism	4	5.30E-01
cellular metabolism	4	5.70E-01
intracellular	3	8.20E-01
Cluster 2. Enrichment Score: 0.39	Count	P_Value
cellular physiological process	6	2.20E-01
metabolism	5	3.00E-01
physiological process	6	4.70E-01
cellular process	6	5.30E-01
cell	5	6.70E-01
+mut 9h / -12h		
Cluster 1. Enrichment Score: 2.68	Count	P_Value
apoptosis	10	5.50E-05
programmed cell death	10	5.70E-05
cell death	10	7.40E-05
death	10	7.80E-05
negative regulation of apoptosis	6	8.50E-05
negative regulation of programmed cell death	6	8.80E-05
regulation of apoptosis	7	8.40E-04
negative regulation of cellular physiological process	9	8.50E-04
regulation of programmed cell death	7	8.70E-04
negative regulation of physiological process	9	1.10E-03
negative regulation of cellular process	9	1.60E-03
apoptosis	5	2.00E-03
negative regulation of biological process	9	2.60E-03
anti-apoptosis	4	7.10E-03
regulation of biological process	19	3.50E-02
positive regulation of biological process	5	1.40E-01
positive regulation of physiological process	4	1.90E-01
positive regulation of cellular physiological process	3	4.20E-01
positive regulation of cellular process	3	5.10E-01
Cluster 2. Enrichment Score: 2.06	Count	P_Value
angiogenesis	4	1.30E-03
vasculature development	4	1.50E-03
blood vessel development	4	1.50E-03
blood vessel morphogenesis	4	1.50E-03
angiogenesis	3	2.80E-03
cell differentiation	7	5.10E-03
differentiation	4	1.50E-02
organ morphogenesis	4	3.10E-02
morphogenesis	6	4.00E-02
developmental protein	4	9.00E-02
organ development	4	2.40E-01
Cluster 3. Enrichment Score: 1.91	Count	P_Value
cytokine	7	3.00E-06
cytokine activity	8	3.80E-06
receptor binding	11	1.20E-05
extracellular space	9	6.40E-05
response to stress	13	1.20E-04
Small chemokine, C-X-C/Interleukin 8	3	6.60E-04
signal	15	8.70E-04
response to external stimulus	8	1.10E-03
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	7	1.70E-03
extracellular region	11	5.30E-03
Small chemokine, interleukin-8-like	3	5.50E-03
SCY	3	5.50E-03
chemotaxis	4	7.20E-03
taxis	4	7.20E-03
chemokine activity	3	7.30E-03
chemokine receptor binding	3	7.30E-03
inflammatory response	3	7.50E-03
locomotory behavior	4	8.00E-03
response to wounding	6	8.20E-03
chemotaxis	3	9.30E-03
Cytokines and Inflammatory Response	3	9.90E-03
cell fraction	8	1.00E-02
G-protein-coupled receptor binding	3	1.00E-02

response to stimulus	16	1.60E-02
soluble fraction	4	2.40E-02
behavior	4	2.40E-02
disulfide bond	14	2.70E-02
cell-cell signaling	6	2.70E-02
response to pest, pathogen or parasite	6	3.30E-02
response to chemical stimulus	5	3.50E-02
cell communication	18	3.50E-02
growth factor	3	3.90E-02
response to other organism	6	4.10E-02
signal transducer activity	15	4.20E-02
signal peptide	14	5.20E-02
response to abiotic stimulus	5	5.40E-02
immune response	8	6.70E-02
signal transduction	16	6.80E-02
growth factor activity	3	9.20E-02
defense response	8	1.00E-01
response to biotic stimulus	8	1.20E-01
inflammatory response	3	1.50E-01
glycoprotein	11	2.10E-01
cell surface receptor linked signal transduction	8	2.60E-01
sensory transduction	3	3.40E-01
organismal physiological process	10	3.80E-01
sensory perception	3	6.70E-01
neurophysiological process	3	8.10E-01
glycosylation site:N-linked (GlcNAc...)	9	8.20E-01
Cluster 4. Enrichment Score: 1.77	**Count**	**P_Value**
epidermis development	4	2.30E-03
ectoderm development	4	3.20E-03
tissue development	4	2.20E-02
structural molecule activity	4	5.10E-01
Cluster 5. Enrichment Score: 1.76	**Count**	**P_Value**
negative regulation of cellular physiological process	9	8.50E-04
negative regulation of physiological process	9	1.10E-03
negative regulation of cellular process	9	1.60E-03
negative regulation of biological process	9	2.60E-03
cell cycle arrest	3	1.90E-02
regulation of progression through cell cycle	5	6.00E-02
regulation of cell cycle	5	6.10E-02
negative regulation of progression through cell cycle	3	8.90E-02
cell cycle	5	1.90E-01
cell proliferation	3	5.10E-01
Cluster 6. Enrichment Score: 1.63	**Count**	**P_Value**
cell differentiation	7	5.10E-03
repeat:1	4	2.40E-02
repeat:2	4	2.40E-02
repeat:3	3	1.00E-01
Cluster 7. Enrichment Score: 1.58	**Count**	**P_Value**
Cells and Molecules involved in local acute inflammatory response	3	4.60E-03
Adhesion and Diapedesis of Granulocytes	3	4.60E-03
58.(CD40L)_immnosurveillance	3	1.50E-02
18.Cytokine_astocytes	3	1.50E-02
INFECTION	3	3.60E-02
IMMUNE	5	1.40E-01
cell adhesion	4	3.70E-01
Cluster 8. Enrichment Score: 1.02	**Count**	**P_Value**
METABOLIC	6	9.60E-03
CANCER	4	1.20E-01
IMMUNE	5	1.40E-01
NEURODEG	3	1.80E-01
CARDIOVASCULAR	3	3.00E-01
Cluster 9. Enrichment Score: 0.96	**Count**	**P_Value**
domain:Leucine-zipper	4	3.10E-03
transcription cofactor activity	5	5.20E-03
nuclear protein	15	7.00E-03
Basic-leucine zipper (bZIP) transcription factor	3	8.80E-03
BRLZ	3	9.20E-03
transcription factor binding	5	9.70E-03
protein dimerization activity	4	1.10E-02
dna-binding	9	1.90E-02

transcription corepressor activity	3	2.50E-02
regulation of cellular physiological process	18	2.50E-02
regulation of physiological process	18	3.30E-02
regulation of biological process	19	3.50E-02
regulation of cellular process	18	3.90E-02
transcription regulation	8	5.00E-02
transcription factor activity	7	5.20E-02
DNA-binding region:Basic motif	3	5.30E-02
DNA binding	4	7.50E-02
repressor	3	9.10E-02
transcription	7	1.10E-01
sequence-specific DNA binding	4	1.20E-01
DNA binding	10	1.50E-01
transcription regulator activity	7	1.70E-01
transcription from RNA polymerase II promoter	4	2.00E-01
regulation of transcription, DNA-dependent	10	2.40E-01
transcription, DNA-dependent	10	2.70E-01
regulation of metabolism	11	2.80E-01
regulation of transcription	10	3.10E-01
regulation of nucleobase, nucleoside, nucleotide & nucleic acid metabolism	10	3.20E-01
transcription	10	3.60E-01
nucleus	15	3.70E-01
regulation of cellular metabolism	10	3.90E-01
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	12	6.00E-01
nucleic acid binding	10	6.40E-01
intracellular organelle	21	7.40E-01
organelle	21	7.40E-01
intracellular membrane-bound organelle	17	7.90E-01
membrane-bound organelle	17	7.90E-01
intracellular	24	8.50E-01
primary metabolism	20	9.60E-01
metabolism	22	9.70E-01
cellular metabolism	20	9.80E-01
Cluster 10. Enrichment Score: 0.31	**Count**	**P_Value**
cellular process	45	1.80E-01
cellular physiological process	34	7.70E-01
physiological process	39	8.90E-01
Cluster 11. Enrichment Score: 0.26	**Count**	**P_Value**
immunoglobulin domain	3	4.20E-01
Immunoglobulin subtype	3	5.20E-01
IG	3	5.50E-01
Immunoglobulin-like	3	6.20E-01
plasma membrane	6	6.50E-01
Cluster 12. Enrichment Score: 0.16	**Count**	**P_Value**
transmembrane protein	4	2.40E-01
membrane	11	3.40E-01
plasma membrane	6	6.50E-01
integral to plasma membrane	4	7.50E-01
intrinsic to plasma membrane	4	7.50E-01
transmembrane	7	8.60E-01
transmembrane region	7	9.70E-01
membrane	13	9.80E-01
integral to membrane	9	9.80E-01
intrinsic to membrane	9	9.80E-01
Cluster 13. Enrichment Score: 0.16	**Count**	**P_Value**
structural molecule activity	4	5.10E-01
cytoskeleton	4	5.50E-01
non-membrane-bound organelle	5	7.90E-01
intracellular non-membrane-bound organelle	5	7.90E-01
cell organization and biogenesis	4	8.80E-01
Cluster 14. Enrichment Score: 0.08	**Count**	**P_Value**
plasma membrane	6	6.50E-01
transmembrane receptor activity	3	9.20E-01
receptor activity	4	9.50E-01
Cluster 15. Enrichment Score: 0.05	**Count**	**P_Value**
metal-binding	6	5.90E-01
zinc-finger	3	8.60E-01
transition metal ion binding	5	9.20E-01
zinc	3	9.20E-01
ion binding	7	9.60E-01

metal ion binding	7	9.60E-01
cation binding	6	9.70E-01
zinc ion binding	3	9.80E-01
Cluster 16. Enrichment Score: 0.04	**Count**	**P_Value**
establishment of localization	8	8.80E-01
localization	8	8.80E-01
transport	6	9.60E-01
Cluster 17. Enrichment Score: 0.03	**Count**	**P_Value**
nucleotide-binding	3	8.30E-01
purine nucleotide binding	3	9.70E-01
nucleotide binding	3	9.80E-01
Cluster 18. Enrichment Score: 0.03	**Count**	**P_Value**
phosphorus metabolism	3	8.10E-01
phosphate metabolism	3	8.10E-01
protein modification	4	9.20E-01
biopolymer modification	4	9.30E-01
biopolymer metabolism	5	9.90E-01
protein metabolism	6	9.90E-01
cellular protein metabolism	4	1
cellular macromolecule metabolism	4	1
macromolecule metabolism	7	1

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Cluster 1. Enrichment Score: 3.53	**Count**	**P_Value**
negative regulation of cellular process	17	1.10E-08
negative regulation of cellular physiological process	16	2.10E-08
negative regulation of biological process	17	3.10E-08
negative regulation of physiological process	16	3.40E-08
apoptosis	13	1.70E-06
programmed cell death	13	1.70E-06
cell death	13	2.50E-06
death	13	2.60E-06
apoptosis	7	7.80E-05
regulation of progression through cell cycle	10	8.90E-05
regulation of cell cycle	10	9.00E-05
cell cycle arrest	5	1.50E-04
negative regulation of apoptosis	6	2.50E-04
negative regulation of programmed cell death	6	2.60E-04
negative regulation of progression through cell cycle	6	4.00E-04
cell cycle	11	4.80E-04
regulation of apoptosis	8	5.00E-04
regulation of programmed cell death	8	5.10E-04
anti-apoptosis	4	1.30E-02
positive regulation of biological process	6	1.10E-01
induction of programmed cell death	3	1.10E-01
induction of apoptosis	3	1.10E-01
positive regulation of physiological process	5	1.20E-01
positive regulation of apoptosis	3	1.40E-01
positive regulation of programmed cell death	3	1.40E-01
mutagenesis site	7	2.60E-01
positive regulation of cellular physiological process	4	2.70E-01
positive regulation of cellular process	4	3.60E-01
Cluster 2. Enrichment Score: 3.05	**Count**	**P_Value**
Basic leucine zipper	4	2.20E-05
Basic-leucine zipper (bZIP) transcription factor	5	3.10E-05
BRLZ	5	6.90E-05
protein dimerization activity	6	2.30E-04
Fos transforming protein	3	3.70E-04
SF001719:fos transforming protein	3	6.00E-04
domain:Leucine-zipper	5	7.10E-04
DNA-binding region:Basic motif	5	1.70E-03
transcription factor binding	6	3.20E-03
transcription from RNA polymerase II promoter	7	1.30E-02
sequence-specific DNA binding	6	1.40E-02
repressor	3	1.50E-01
Cluster 3. Enrichment Score: 2.71	**Count**	**P_Value**
response to stress	20	2.50E-08
cytokine	9	6.20E-08
cytokine activity	10	7.70E-08
receptor binding	13	1.50E-06
response to external stimulus	12	5.20E-06

extracellular space	11	8.20E-06
Small chemokine, C-X-C/Interleukin 8	4	1.50E-05
response to wounding	10	2.50E-05
response to stimulus	25	4.30E-05
response to chemical stimulus	9	1.50E-04
signal	19	2.60E-04
response to pest, pathogen or parasite	10	3.70E-04
Small chemokine, interleukin-8-like	4	3.70E-04
response to abiotic stimulus	9	4.00E-04
chemokine activity	4	4.80E-04
chemokine receptor binding	4	4.80E-04
response to other organism	10	5.70E-04
SCY	4	6.60E-04
inflammatory response	4	6.90E-04
G-protein-coupled receptor binding	4	8.30E-04
behavior	6	1.10E-03
extracellular region	14	1.10E-03
Small chemokine, C-X-C	3	1.20E-03
chemotaxis	5	1.50E-03
taxis	5	1.50E-03
inflammatory response	6	1.50E-03
SF002522:beta-thromboglobulin	3	1.60E-03
locomotory behavior	5	1.80E-03
immune response	12	4.90E-03
response to biotic stimulus	13	5.00E-03
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	7	6.30E-03
soluble fraction	5	7.70E-03
growth factor	4	8.10E-03
defense response	12	1.00E-02
chemotaxis	3	1.60E-02
cell-cell signaling	7	2.00E-02
growth factor activity	4	2.30E-02
signal peptide	18	4.80E-02
organismal physiological process	15	1.00E-01
regulation of cell proliferation	4	1.20E-01
negative regulation of cell proliferation	3	1.20E-01
signal transducer activity	15	1.40E-01
disulfide bond	15	1.40E-01
cell proliferation	5	1.70E-01
glycoprotein	13	2.90E-01
cell surface receptor linked signal transduction	9	3.20E-01
sensory transduction	3	4.80E-01
sensory perception	3	7.90E-01
neurophysiological process	3	9.00E-01
G-protein coupled receptor protein signaling pathway	3	9.40E-01
glycosylation site:N-linked (GlcNAc...)	10	9.60E-01
Cluster 4. Enrichment Score: 1.92	**Count**	**P_Value**
TOLL-LIKE RECEPTOR SIGNALING PATHWAY	5	3.60E-03
T CELL RECEPTOR SIGNALING PATHWAY	5	3.80E-03
Cadmium induces DNA synthesis and proliferation in macrophages	3	1.00E-02
ADIPOCYTOKINE SIGNALING PATHWAY	4	1.20E-02
APOPTOSIS	4	2.40E-02
B CELL RECEPTOR SIGNALING PATHWAY	3	7.90E-02
Cluster 5. Enrichment Score: 1.73	**Count**	**P_Value**
angiogenesis	4	2.50E-03
blood vessel morphogenesis	4	2.90E-03
blood vessel development	4	2.90E-03
vasculature development	4	2.90E-03
angiogenesis	3	4.80E-03
cell differentiation	7	1.50E-02
differentiation	4	3.10E-02
organ morphogenesis	4	5.50E-02
developmental protein	4	1.70E-01
morphogenesis	5	2.20E-01
organ development	4	3.60E-01
Cluster 6. Enrichment Score: 1.69	**Count**	**P_Value**
cell communication	23	1.30E-02
signal transduction	21	2.10E-02
intracellular signaling cascade	10	3.20E-02
Cluster 7. Enrichment Score: 1.32	**Count**	**P_Value**

RESTENOSIS	3	6.80E-03
Adhesion and Diapedesis of Granulocytes	3	1.30E-02
Cells and Molecules involved in local acute inflammatory response	3	1.30E-02
INFECTION	4	1.40E-02
TYPE 2 DIABETES	6	2.50E-02
18.Cytokine_astocytes	3	4.00E-02
58.(CD40L)_immnosurveillance	3	4.00E-02
METABOLIC	7	4.00E-02
IMMUNE	8	4.30E-02
CANCER	6	4.90E-02
SYSTEMIC LUPUS ERYTHEMATOSUS	4	8.00E-02
BREAST CANCER	3	1.10E-01
cell adhesion	5	2.80E-01
CARDIOVASCULAR	4	2.90E-01
NEURODEG	3	4.00E-01
Cluster 8. Enrichment Score: 1.19	**Count**	**P_Value**
dna-binding	14	4.60E-04
nuclear protein	21	5.00E-04
regulation of cellular process	25	3.00E-03
transcription factor binding	6	3.20E-03
regulation of biological process	26	3.30E-03
regulation of cellular physiological process	24	3.60E-03
regulation of physiological process	24	5.30E-03
DNA binding	6	9.70E-03
transcription from RNA polymerase II promoter	7	1.30E-02
sequence-specific DNA binding	6	1.40E-02
DNA binding	14	2.40E-02
transcription regulation	10	3.40E-02
transcription cofactor activity	4	5.10E-02
transcription	9	7.20E-02
nucleus	22	8.10E-02
regulation of transcription, DNA-dependent	14	8.20E-02
transcription, DNA-dependent	14	9.80E-02
transcription factor activity	7	1.00E-01
regulation of transcription	14	1.20E-01
regulation of metabolism	15	1.30E-01
regulation of nucleobase, nucleoside, nucleotide & nucleic acid metabolism	14	1.30E-01
transcription	14	1.60E-01
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	19	1.60E-01
transcription regulator activity	8	1.60E-01
regulation of cellular metabolism	14	1.80E-01
nucleic acid binding	14	3.50E-01
intracellular membrane-bound organelle	25	4.10E-01
membrane-bound organelle	25	4.20E-01
intracellular organelle	29	4.60E-01
organelle	29	4.60E-01
cellular physiological process	45	5.10E-01
primary metabolism	30	7.10E-01
intracellular	32	7.10E-01
cellular metabolism	30	7.90E-01
metabolism	32	8.20E-01
cell	46	9.70E-01
Cluster 9. Enrichment Score: 1.17	**Count**	**P_Value**
ank repeat	4	3.30E-02
repeat:ANK 5	3	4.00E-02
repeat:ANK 4	3	5.70E-02
Ankyrin	4	7.10E-02
ANK	4	7.50E-02
repeat:ANK 3	3	8.70E-02
repeat:ANK 1	3	1.10E-01
repeat:ANK 2	3	1.10E-01
Cluster 10. Enrichment Score: 1.13	**Count**	**P_Value**
epidermis development	3	4.30E-02
ectoderm development	3	5.30E-02
tissue development	3	1.70E-01
Cluster 11. Enrichment Score: 1.02	**Count**	**P_Value**
cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)	3	3.10E-03
DNA metabolism	4	5.10E-01
cell organization and biogenesis	7	5.80E-01
Cluster 12. Enrichment Score: 0.94	**Count**	**P_Value**

IMMUNE	8	4.30E-02
MULTIPLE SCLEROSIS	3	9.40E-02
TYPE 1 DIABETES	3	3.70E-01
Cluster 13. Enrichment Score: 0.75	**Count**	**P_Value**
Ras small GTPase, Rho type	3	5.90E-02
Ras small GTPase, Ras type	3	6.60E-02
Ras small GTPase, Rab type	3	8.50E-02
Small GTP-binding protein domain	3	1.00E-01
Ras GTPase	3	1.10E-01
nucleotide-binding	8	1.20E-01
gtp-binding	3	1.90E-01
nucleotide phosphate-binding region:GTP	3	2.50E-01
small GTPase mediated signal transduction	3	3.10E-01
GTP binding	3	3.80E-01
purine nucleotide binding	8	3.80E-01
guanyl nucleotide binding	3	3.80E-01
nucleotide binding	8	5.40E-01
Cluster 14. Enrichment Score: 0.57	**Count**	**P_Value**
cellular process	57	6.90E-02
cellular physiological process	45	5.10E-01
physiological process	52	5.60E-01
Cluster 15. Enrichment Score: 0.37	**Count**	**P_Value**
actin cytoskeleton organization and biogenesis	3	1.30E-01
actin filament-based process	3	1.50E-01
organelle organization and biogenesis	5	4.40E-01
cytoskeleton organization and biogenesis	3	4.80E-01
cell organization and biogenesis	7	5.80E-01
cytoskeleton	4	7.10E-01
intracellular non-membrane-bound organelle	6	8.00E-01
non-membrane-bound organelle	6	8.00E-01
Cluster 16. Enrichment Score: 0.35	**Count**	**P_Value**
phosphorylation	13	9.30E-03
nucleotide-binding	8	1.20E-01
phosphoric monoester hydrolase activity	3	2.00E-01
kinase	5	2.30E-01
phosphotransferase activity, alcohol group as acceptor	5	2.50E-01
phosphoric ester hydrolase activity	3	3.00E-01
serine/threonine-protein kinase	3	3.00E-01
Serine/threonine protein kinase, active site	3	3.40E-01
protein kinase activity	4	3.70E-01
purine nucleotide binding	8	3.80E-01
active site:Proton acceptor	4	3.90E-01
hydrolase activity, acting on ester bonds	4	3.90E-01
atp-binding	5	4.10E-01
kinase activity	5	4.10E-01
protein serine/threonine kinase activity	3	4.20E-01
Tyrosine protein kinase	3	4.70E-01
Serine/threonine protein kinase	3	4.90E-01
domain:Protein kinase	3	5.20E-01
phosphorus metabolism	5	5.30E-01
phosphate metabolism	5	5.30E-01
transferase activity, transferring phosphorus-containing groups	5	5.30E-01
nucleotide binding	8	5.40E-01
Protein kinase	3	5.40E-01
biopolymer modification	8	5.50E-01
binding site:ATP	3	5.60E-01
hydrolase	5	5.80E-01
biopolymer metabolism	12	6.20E-01
hydrolase activity	8	6.20E-01
nucleotide phosphate-binding region:ATP	4	6.40E-01
ATP binding	5	6.70E-01
protein modification	7	6.80E-01
adenyl nucleotide binding	5	7.00E-01
protein amino acid phosphorylation	3	7.30E-01
transferase	4	7.60E-01
phosphorylation	3	8.30E-01
transferase activity	5	8.90E-01
Shatner's Bassoon macromolecule metabolism	16	8.90E-01
protein metabolism	11	9.20E-01
cellular protein metabolism	8	9.90E-01

cellular macromolecule metabolism	8	9.90E-01
Cluster 17. Enrichment Score: 0.18	Count	P_Value
electron transport	3	5.40E-01
generation of precursor metabolites and energy	4	5.50E-01
transport	7	9.80E-01
Cluster 18. Enrichment Score: 0.11	Count	P_Value
metal-binding	9	3.70E-01
zinc-finger	5	6.50E-01
zinc	5	8.00E-01
transition metal ion binding	7	8.40E-01
zinc ion binding	5	9.20E-01
ion binding	9	9.40E-01
metal ion binding	9	9.40E-01
cation binding	7	9.80E-01
Cluster 19. Enrichment Score: 0.08	Count	P_Value
membrane	14	3.30E-01
plasma membrane	6	8.30E-01
integral to plasma membrane	4	8.70E-01
intrinsic to plasma membrane	4	8.70E-01
transmembrane	8	9.40E-01
membrane	15	9.90E-01
transmembrane region	8	1
integral to membrane	10	1
intrinsic to membrane	10	1
Cluster 20. Enrichment Score: 0.02	Count	P_Value
establishment of localization	9	9.40E-01
localization	9	9.40E-01
transport	7	9.80E-01
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Cluster 1. Enrichment Score: 21.91	Count	P_Value
nuclear protein	302	2.40E-45
intracellular membrane-bound organelle	469	8.50E-27
membrane-bound organelle	469	9.20E-27
nucleus	348	5.70E-22
intracellular	587	7.90E-22
intracellular organelle	514	2.30E-21
organelle	514	2.50E-21
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	293	1.60E-15
cell	719	7.70E-03
Cluster 2. Enrichment Score: 11.47	Count	P_Value
primary metabolism	544	8.80E-21
metabolism	579	3.00E-18
cellular metabolism	549	3.70E-18
cellular physiological process	687	9.10E-16
physiological process	730	5.00E-05
cellular process	742	1.40E-04
cell	719	7.70E-03
Cluster 3. Enrichment Score: 11.25	Count	P_Value
dna replication	24	1.00E-15
DNA replication	41	8.50E-13
DNA-dependent DNA replication	26	1.20E-11
DNA replication initiation	12	9.40E-08
Cluster 4. Enrichment Score: 8.2	Count	P_Value
transit peptide	44	3.10E-11
mitochondrion	61	2.10E-10
transit peptide:Mitochondrion	44	1.00E-08
mitochondrion	66	2.40E-05
Cluster 5. Enrichment Score: 8.05	Count	P_Value
membrane-enclosed lumen	66	2.20E-11
organelle lumen	66	2.20E-11
nuclear lumen	50	1.70E-08
nucleoplasm	29	7.80E-04
Cluster 6. Enrichment Score: 7.07	Count	P_Value
DNA metabolism	89	8.60E-15
DNA repair	41	7.40E-11
response to DNA damage stimulus	42	4.90E-10
response to endogenous stimulus	43	1.00E-09
dna damage	21	5.50E-07
dna repair	19	1.60E-06
response to stress	79	9.50E-03

response to stimulus	118	1
Cluster 7. Enrichment Score: 5.45	**Count**	**P_Value**
cell cycle	85	1.30E-11
regulation of progression through cell cycle	60	1.10E-09
regulation of cell cycle	60	1.20E-09
cell cycle	39	1.70E-08
negative regulation of progression through cell cycle	21	3.50E-04
negative regulation of cellular process	57	3.30E-03
negative regulation of cellular physiological process	52	4.30E-03
negative regulation of biological process	59	6.30E-03
negative regulation of physiological process	52	8.60E-03
Cluster 8. Enrichment Score: 5.34	**Count**	**P_Value**
RNA processing	53	1.80E-10
RNA metabolism	58	4.50E-09
mrna processing	24	1.70E-07
mrna splicing	20	8.70E-07
RNA binding	54	1.60E-05
mRNA processing	28	2.30E-05
mRNA metabolism	30	3.30E-05
RNA splicing	23	3.50E-05
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	20	5.90E-05
nuclear mRNA splicing, via spliceosome	20	5.90E-05
RNA splicing, via transesterification reactions	20	5.90E-05
spliceosome complex	10	9.00E-03
Cluster 9. Enrichment Score: 5.07	**Count**	**P_Value**
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	293	1.60E-15
nucleic acid binding	276	3.00E-14
transcription	118	1.50E-10
dna-binding	115	6.60E-09
transcription regulation	110	9.00E-08
DNA binding	168	3.40E-07
regulation of cellular physiological process	230	5.70E-05
regulation of physiological process	235	8.00E-05
regulation of cellular process	238	9.30E-05
regulation of biological process	251	1.30E-04
transcription	163	2.40E-03
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	157	3.50E-03
regulation of cellular metabolism	163	5.80E-03
regulation of metabolism	167	6.10E-03
regulation of transcription	152	8.10E-03
transcription, DNA-dependent	145	1.40E-02
regulation of transcription, DNA-dependent	138	2.70E-02
transcription regulator activity	69	7.00E-01
Cluster 10. Enrichment Score: 5.07	**Count**	**P_Value**
DNA replication initiation	12	9.40E-08
domain:MCM	6	7.20E-06
MCM	6	3.10E-05
MCM	6	4.50E-05
DNA-dependent ATPase activity	11	4.80E-05
Cluster 11. Enrichment Score: 4.69	**Count**	**P_Value**
macromolecule metabolism	363	1.80E-15
biopolymer metabolism	245	2.60E-14
protein metabolism	215	3.40E-02
cellular protein metabolism	192	1.30E-01
biopolymer modification	106	1.30E-01
cellular macromolecule metabolism	193	1.60E-01
protein modification	96	3.60E-01
Cluster 12. Enrichment Score: 3.43	**Count**	**P_Value**
PYRIMIDINE METABOLISM	23	1.10E-07
nucleotidyltransferase	18	8.30E-07
nucleotidyltransferase activity	25	2.10E-06
dna-directed rna polymerase	10	6.00E-06
DNA-directed RNA polymerase I activity	8	1.60E-04
DNA-directed RNA polymerase II activity	8	1.60E-04
DNA-directed RNA polymerase III activity	8	1.60E-04
DNA-directed RNA polymerase activity	11	2.50E-04
RNA POLYMERASE	8	1.50E-03
PURINE METABOLISM	21	2.90E-03
DNA-directed RNA polymerase III complex	4	7.60E-03
transcription from RNA polymerase III promoter	6	1.10E-02

RNA polymerase complex	6	1.70E-02
obsolete molecular function	20	1.90E-01
DNA-directed RNA polymerase II, core complex	3	2.60E-01
Cluster 13. Enrichment Score: 3.37	**Count**	**P_Value**
replication fork	9	8.30E-08
replisome	6	2.50E-05
replication fork (sensu Eukaryota)	6	2.50E-05
replisome (sensu Eukaryota)	6	2.50E-05
DNA replication factor A complex	4	6.40E-04
Nucleic acid-binding, OB-fold, subgroup	8	2.00E-02
nucleic acid binding, OB-fold, tRNA/helicase-type	4	2.30E-02
single-stranded DNA binding	6	3.20E-02
structure-specific DNA binding	7	4.40E-02
Cluster 14. Enrichment Score: 3.26	**Count**	**P_Value**
AAA	19	7.40E-05
AAA ATPase, central region	11	3.10E-04
AAA ATPase	16	7.30E-03
Cluster 15. Enrichment Score: 3.02	**Count**	**P_Value**
nuclease activity	24	7.10E-06
exonuclease activity, active with ribo/deoxyribonucleic acids & producing 5'-phosphomonoesters	8	1.50E-05
rRNA metabolism	12	8.10E-05
ribosome biogenesis	13	1.30E-04
ribosome biogenesis and assembly	14	2.20E-04
exonuclease	8	4.40E-04
cytoplasm organization and biogenesis	15	5.40E-04
3'-5' exonuclease activity	8	5.60E-04
rRNA processing	10	7.80E-04
nuclease	11	2.40E-03
3'-5'-exoribonuclease activity	5	3.10E-03
exonuclease activity	10	3.10E-03
rrna processing	7	3.50E-03
exosome	4	5.10E-03
exoribonuclease activity, producing 5'-phosphomonoesters	5	5.40E-03
exoribonuclease activity	5	5.40E-03
exosome (RNase complex)	4	1.10E-02
Exoribonuclease	3	1.10E-01
Cluster 16. Enrichment Score: 2.93	**Count**	**P_Value**
zinc-finger	104	1.40E-06
metal-binding	144	3.40E-06
zinc	120	3.80E-06
zinc ion binding	138	1.60E-03
transition metal ion binding	160	2.90E-03
Zinc finger, C2H2-type	64	5.80E-03
metal ion binding	206	1.50E-01
ion binding	206	1.50E-01
cation binding	185	3.70E-01
Cluster 17. Enrichment Score: 2.88	**Count**	**P_Value**
ribosomal protein	33	1.70E-09
ribonucleoprotein	34	7.60E-09
ribonucleoprotein complex	56	9.70E-05
cellular biosynthesis	95	5.30E-03
biosynthesis	101	1.40E-02
structural constituent of ribosome	29	8.40E-02
protein biosynthesis	53	1.20E-01
macromolecule biosynthesis	57	1.50E-01
ribosome	26	3.00E-01
structural molecule activity	55	3.60E-01
Cluster 18. Enrichment Score: 2.83	**Count**	**P_Value**
ribonuclease activity	13	1.60E-04
exonuclease activity	10	3.10E-03
endonuclease activity	12	6.50E-03
Cluster 19. Enrichment Score: 2.59	**Count**	**P_Value**
hereditary nonpolyposis colorectal cancer	5	9.70E-05
maintenance of fidelity during DNA-dependent DNA replication	6	1.30E-02
mismatch repair	6	1.30E-02
Cluster 20. Enrichment Score: 2.48	**Count**	**P_Value**
exonuclease activity, active with ribo/deoxyribonucleic acids & producing 5'-phosphomonoesters	8	1.50E-05
exodeoxyribonuclease activity	3	1.40E-02
exodeoxyribonuclease activity, producing 5'-phosphomonoesters	3	1.40E-02
deoxyribonuclease activity	5	4.30E-02

Cluster 21. Enrichment Score: 2.46	Count	P_Value
nucleotide-binding	107	1.30E-08
nucleoside-triphosphatase activity	45	6.10E-03
AAA ATPase	16	7.30E-03
hydrolase activity, acting on acid anhydrides	47	7.90E-03
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	46	9.70E-03
pyrophosphatase activity	45	1.30E-02
ATPase activity, coupled	21	1.50E-01
ATPase activity	23	2.20E-01
Cluster 22. Enrichment Score: 2.45	**Count**	**P_Value**
rna-binding	41	3.40E-06
RRM	21	6.80E-03
domain:RRM	10	1.60E-02
RNA-binding region RNP-1 (RNA recognition motif)	21	2.50E-02
Nucleotide-binding, alpha-beta plait	21	6.30E-02
Cluster 23. Enrichment Score: 2.42	**Count**	**P_Value**
delta-DNA polymerase cofactor complex	4	6.40E-04
SF004274:phage T4 DNA polymerase accessory protein 44	4	1.70E-03
obsolete cellular component	6	6.00E-03
Replication factor C conserved region	4	6.90E-03
DNA replication factor C complex	3	1.70E-02
Cluster 24. Enrichment Score: 2.21	**Count**	**P_Value**
transferase	114	2.90E-12
nucleotide-binding	107	1.30E-08
atp-binding	83	8.20E-07
transferase activity	147	2.00E-06
nucleotide phosphate-binding region:ATP	65	5.20E-05
nucleotide binding	149	7.60E-05
transferase activity, transferring phosphorus-containing groups	88	3.60E-04
purine nucleotide binding	126	6.10E-04
adenyl nucleotide binding	103	7.20E-04
ATP binding	100	7.30E-04
active site:Proton acceptor	43	2.20E-03
kinase	53	3.70E-03
phosphotransferase activity, alcohol group as acceptor	56	1.60E-02
serine/threonine-protein kinase	24	5.30E-02
protein kinase activity	44	8.10E-02
protein kinase CK2 activity	16	8.60E-02
kinase activity	63	8.70E-02
cyclic nucleotide-dependent protein kinase activity	16	9.80E-02
cAMP-dependent protein kinase activity	16	9.80E-02
binding site:ATP	29	1.10E-01
protein serine/threonine kinase activity	30	1.10E-01
biopolymer modification	106	1.30E-01
domain:Protein kinase	27	1.30E-01
protein amino acid phosphorylation	38	3.40E-01
S_TKc	16	3.50E-01
protein modification	96	3.60E-01
phosphorus metabolism	54	4.40E-01
phosphate metabolism	54	4.40E-01
Serine/threonine protein kinase, active site	21	4.90E-01
Serine/threonine protein kinase	28	5.10E-01
Protein kinase	30	5.70E-01
phosphorylation	43	5.70E-01
Tyrosine protein kinase	25	6.70E-01
Cluster 25. Enrichment Score: 2.19	**Count**	**P_Value**
regulation of cyclin dependent protein kinase activity	10	3.20E-04
regulation of kinase activity	17	2.60E-03
regulation of protein kinase activity	17	2.60E-03
regulation of transferase activity	17	2.80E-03
regulation of enzyme activity	20	8.70E-02
MAPKKK cascade	9	1.40E-01

+wt 12h / -12h

Cluster 1. Enrichment Score: 42.08	Count	P_Value
nuclear protein	580	5.10E-64
intracellular membrane-bound organelle	1000	4.80E-56
membrane-bound organelle	1000	5.80E-56
intracellular	1260	1.50E-46
intracellular organelle	1097	9.40E-44
organelle	1097	1.10E-43

nucleus	669	4.50E-25
cell	1560	2.40E-07
Cluster 2. Enrichment Score: 27.24	**Count**	**P_Value**
mitochondrion	161	2.60E-37
transit peptide	102	1.00E-28
mitochondrion	178	3.60E-24
transit peptide:Mitochondrion	101	1.20E-21
Cluster 3. Enrichment Score: 18.64	**Count**	**P_Value**
cellular physiological process	1473	2.00E-30
cellular metabolism	1128	5.80E-24
metabolism	1191	2.30E-23
primary metabolism	1093	4.60E-23
cellular process	1600	7.10E-08
physiological process	1567	1.70E-07
Cluster 4. Enrichment Score: 17.25	**Count**	**P_Value**
membrane-enclosed lumen	139	5.40E-24
organelle lumen	139	5.40E-24
nuclear lumen	101	1.90E-15
nucleoplasm	65	1.80E-08
Cluster 5. Enrichment Score: 16.8	**Count**	**P_Value**
cell cycle	167	5.50E-19
cell cycle	84	9.20E-18
regulation of cell cycle	120	5.30E-17
regulation of progression through cell cycle	119	2.30E-16
Cluster 6. Enrichment Score: 12.21	**Count**	**P_Value**
RNA processing	107	1.40E-19
RNA binding	137	4.30E-19
RNA metabolism	121	4.80E-18
mrna processing	53	6.70E-17
mrna splicing	44	4.30E-15
mRNA processing	61	1.70E-11
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	44	5.70E-11
nuclear mRNA splicing, via spliceosome	44	5.70E-11
RNA splicing, via transesterification reactions	44	5.70E-11
RNA splicing	49	1.40E-10
mRNA metabolism	64	1.80E-10
spliceosome	19	4.70E-07
spliceosome complex	24	7.00E-07
Cluster 7. Enrichment Score: 10.97	**Count**	**P_Value**
DNA metabolism	152	8.40E-16
response to DNA damage stimulus	76	2.80E-14
DNA repair	71	3.60E-14
response to endogenous stimulus	77	3.30E-13
dna damage	40	2.40E-11
dna repair	36	1.70E-10
response to stress	154	1.50E-02
Cluster 8. Enrichment Score: 10.68	**Count**	**P_Value**
dna replication	32	2.00E-15
DNA replication	58	1.70E-10
DNA-dependent DNA replication	32	2.70E-08
Cluster 9. Enrichment Score: 7.49	**Count**	**P_Value**
rna-binding	103	6.60E-19
RRM	49	5.40E-07
RNA-binding region RNP-1 (RNA recognition motif)	50	2.00E-05
Nucleotide-binding, alpha-beta plait	54	2.10E-05
domain:RRM	21	2.50E-04
Cluster 10. Enrichment Score: 6.68	**Count**	**P_Value**
nucleotide-binding	225	4.80E-15
atp-binding	168	5.90E-10
nucleotide binding	324	1.30E-09
purine nucleotide binding	266	2.90E-06
adenyl nucleotide binding	214	1.10E-05
nucleotide phosphate-binding region:ATP	126	1.10E-05
ATP binding	207	1.30E-05
kinase activity	121	2.30E-01
Cluster 11. Enrichment Score: 6.49	**Count**	**P_Value**
methyltransferase	31	8.40E-09
methyltransferase activity	40	6.00E-08
transferase activity, transferring one-carbon groups	40	8.90E-08
S-adenosylmethionine-dependent methyltransferase activity	27	5.70E-06

SAM (and some other nucleotide) binding motif	19	1.40E-05
Cluster 12. Enrichment Score: 5.68	Count	P_Value
macromolecule metabolism	743	1.10E-23
protein metabolism	473	4.10E-04
cellular protein metabolism	429	3.80E-03
cellular macromolecule metabolism	433	5.60E-03
biopolymer modification	233	1.60E-02
protein modification	220	5.50E-02
Cluster 13. Enrichment Score: 5.26	Count	P_Value
negative regulation of progression through cell cycle	43	2.10E-07
negative regulation of biological process	131	7.80E-06
negative regulation of cellular process	123	9.10E-06
negative regulation of cellular physiological process	113	1.00E-05
negative regulation of physiological process	114	3.30E-05
Cluster 14. Enrichment Score: 5.12	Count	P_Value
ribonucleoprotein	74	2.90E-19
ribosomal protein	62	9.70E-15
ribonucleoprotein complex	118	1.10E-08
biosynthesis	212	1.40E-03
cellular biosynthesis	192	1.80E-03
structural constituent of ribosome	62	1.30E-02
macromolecule biosynthesis	124	3.70E-02
protein biosynthesis	111	5.90E-02
ribosome	52	3.30E-01
structural molecule activity	106	7.60E-01
Cluster 15. Enrichment Score: 4.86	Count	P_Value
unfolded protein binding	43	4.70E-06
chaperone	31	6.80E-06
protein folding	56	8.40E-05
Cluster 16. Enrichment Score: 4.72	Count	P_Value
AAA ATPase, central region	20	1.70E-06
AAA	32	8.20E-06
AAA ATPase	31	4.80E-04
Cluster 17. Enrichment Score: 4.48	Count	P_Value
ribosome biogenesis and assembly	25	3.80E-06
ribosome biogenesis	22	6.10E-06
cytoplasm organization and biogenesis	27	2.10E-05
rRNA metabolism	18	4.90E-05
rRNA processing	16	1.80E-04
rrna processing	12	2.90E-04
Cluster 18. Enrichment Score: 4.45	Count	P_Value
mitotic cell cycle	51	5.00E-07
M phase	45	1.40E-05
mitosis	24	2.00E-05
cell division	33	2.40E-05
M phase of mitotic cell cycle	37	2.50E-05
mitosis	36	4.50E-05
regulation of mitosis	15	2.30E-04
cell division	35	2.60E-03
Cluster 19. Enrichment Score: 4.45	Count	P_Value
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	106	3.50E-06
nucleoside-triphosphatase activity	101	4.80E-06
hydrolase activity, acting on acid anhydrides	106	6.60E-06
pyrophosphatase activity	104	7.90E-06
ATPase activity	60	1.30E-03
ATPase activity, coupled	52	1.90E-03
Cluster 20. Enrichment Score: 4.43	Count	P_Value
cell organization and biogenesis	241	6.10E-07
protein transport	59	3.60E-06
intracellular transport	106	2.20E-05
establishment of cellular localization	107	2.30E-05
cellular localization	107	3.30E-05
protein targeting	38	5.90E-05
protein transport	96	1.30E-04
protein localization	101	1.40E-04
establishment of protein localization	97	2.80E-04
intracellular protein transport	60	4.20E-04
Cluster 21. Enrichment Score: 3.92	Count	P_Value
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	560	5.40E-16
nucleic acid binding	518	7.00E-13

transcription	209	8.10E-09
dna-binding	203	1.10E-06
transcription regulation	195	1.30E-05
regulation of cellular process	481	6.30E-05
regulation of biological process	509	9.20E-05
regulation of cellular physiological process	459	9.60E-05
DNA binding	302	2.20E-04
regulation of physiological process	466	3.50E-04
transcription	307	8.90E-02
transcription, DNA-dependent	277	1.70E-01
regulation of nucleobase, nucleoside, nucleotide & nucleic acid metabolism	289	2.10E-01
regulation of cellular metabolism	304	2.20E-01
regulation of metabolism	311	2.80E-01
regulation of transcription	280	3.30E-01
regulation of transcription, DNA-dependent	260	3.80E-01
transcription regulator activity	150	7.30E-01
Cluster 22. Enrichment Score: 3.74	**Count**	**P_Value**
organelle membrane	90	1.10E-05
organelle envelope	61	3.70E-05
envelope	61	7.40E-05
mitochondrial envelope	43	1.10E-04
inner membrane	20	2.40E-04
mitochondrial membrane	38	5.60E-04
mitochondrial inner membrane	32	1.20E-03
organelle inner membrane	34	2.10E-03
Cluster 23. Enrichment Score: 3.74	**Count**	**P_Value**
nucleotidyltransferase	28	2.60E-07
dna-directed rna polymerase	15	5.00E-07
PYRIMIDINE METABOLISM	33	1.50E-06
nucleotidyltransferase activity	38	1.00E-05
DNA-directed RNA polymerase III activity	12	2.40E-05
DNA-directed RNA polymerase I activity	12	2.40E-05
DNA-directed RNA polymerase II activity	12	2.40E-05
DNA-directed RNA polymerase activity	17	9.80E-05
RNA POLYMERASE	12	8.90E-04
PURINE METABOLISM	36	4.90E-03
DNA-directed RNA polymerase III complex	5	9.30E-03
RNA polymerase complex	9	1.40E-02
transcription from RNA polymerase III promoter	7	7.40E-02
DNA-directed RNA polymerase II, core complex	4	3.60E-01
Cluster 24. Enrichment Score: 3.57	**Count**	**P_Value**
small nucleolar ribonucleoprotein complex	12	2.00E-04
Sm	9	2.20E-04
Like-Sm ribonucleoprotein, eukaryotic and archaea-type, core	10	2.80E-04
Like-Sm ribonucleoprotein, core	10	4.30E-04
Cluster 25. Enrichment Score: 3.51	**Count**	**P_Value**
DNA replication initiation	13	3.00E-05
DNA-dependent ATPase activity	15	1.40E-04
domain:MCM	6	3.60E-04
MCM	6	1.10E-03
MCM	6	1.70E-03
+mut 9h / +wt 9h		
Cluster 1. Enrichment Score: 0.98	**Count**	**P_Value**
intracellular signaling cascade	3	3.10E-02
protein binding	4	6.00E-02
organismal physiological process	3	1.30E-01
signal transduction	3	2.10E-01
cell communication	3	2.40E-01
Cluster 2. Enrichment Score: 0.96	**Count**	**P_Value**
regulation of physiological process	5	3.00E-03
regulation of biological process	5	4.20E-03
regulation of cellular process	4	4.40E-02
protein binding	4	6.00E-02
binding	5	2.10E-01
regulation of cellular physiological process	3	2.20E-01
physiological process	5	5.50E-01
cellular process	5	6.00E-01
cell	5	6.70E-01
cellular physiological process	4	7.20E-01
Cluster 3. Enrichment Score: 0.38	**Count**	**P_Value**

regulation of cellular process	4	4.40E-02
intracellular organelle	3	6.90E-01
organelle	3	6.90E-01
cellular physiological process	4	7.20E-01
intracellular	3	8.20E-01
+mut 12h / +wt 12h		
Cluster 1. Enrichment Score: 17.73	Count	P_Value
mitochondrion	115	1.90E-24
transit peptide	72	1.80E-18
mitochondrion	130	5.10E-17
transit peptide:Mitochondrion	73	6.90E-14
Cluster 2. Enrichment Score: 16.12	Count	P_Value
intracellular membrane-bound organelle	695	2.20E-27
membrane-bound organelle	695	2.50E-27
intracellular	907	5.50E-27
nuclear protein	362	6.80E-23
intracellular organelle	776	1.10E-22
organelle	776	1.30E-22
nucleus	428	4.10E-06
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	363	1.30E-04
cell	1142	3.00E-04
nucleic acid binding	334	1.40E-03
Cluster 3. Enrichment Score: 12.55	Count	P_Value
cellular physiological process	1077	4.40E-22
metabolism	863	5.10E-15
cellular metabolism	813	1.20E-14
primary metabolism	785	1.40E-13
cellular process	1180	3.20E-08
physiological process	1148	4.10E-06
Cluster 4. Enrichment Score: 9.52	Count	P_Value
protein transport	66	3.70E-14
protein localization	104	3.60E-12
protein transport	99	5.40E-12
establishment of protein localization	101	7.10E-12
intracellular transport	101	9.40E-11
cellular localization	102	1.40E-10
establishment of cellular localization	101	2.00E-10
transport	147	1.50E-09
intracellular protein transport	59	6.10E-08
cell organization and biogenesis	186	4.90E-07
protein targeting	34	2.90E-06
Cluster 5. Enrichment Score: 9.44	Count	P_Value
organelle lumen	96	3.30E-14
membrane-enclosed lumen	96	3.30E-14
nuclear lumen	66	6.60E-08
nucleoplasm	42	2.50E-04
Cluster 6. Enrichment Score: 8.23	Count	P_Value
regulation of progression through cell cycle	83	2.70E-10
regulation of cell cycle	83	3.00E-10
cell cycle	55	2.30E-09
cell cycle	108	7.30E-09
negative regulation of progression through cell cycle	38	1.20E-08
anti-oncogene	20	2.80E-06
Cluster 7. Enrichment Score: 7.51	Count	P_Value
macromolecule metabolism	545	1.10E-17
primary metabolism	785	1.40E-13
biopolymer metabolism	345	4.80E-12
protein metabolism	371	4.10E-06
cellular macromolecule metabolism	344	3.50E-05
cellular protein metabolism	338	5.20E-05
biopolymer modification	181	3.60E-03
protein modification	176	4.00E-03
Cluster 8. Enrichment Score: 7.1	Count	P_Value
negative regulation of progression through cell cycle	38	1.20E-08
negative regulation of cellular physiological process	97	4.90E-08
negative regulation of physiological process	99	7.80E-08
negative regulation of biological process	108	2.60E-07
negative regulation of cellular process	102	2.70E-07
Cluster 9. Enrichment Score: 6.9	Count	P_Value
RNA binding	96	5.50E-12

RNA processing	69	8.30E-10
mrna processing	35	1.80E-09
RNA metabolism	79	3.80E-09
mrna splicing	27	2.30E-07
RNA splicing	35	2.90E-07
mRNA processing	39	6.60E-06
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	28	9.40E-06
nuclear mRNA splicing, via spliceosome	28	9.40E-06
RNA splicing, via transesterification reactions	28	9.40E-06
mRNA metabolism	42	1.00E-05
Cluster 10. Enrichment Score: 5.95	**Count**	**P_Value**
golgi stack	58	5.30E-09
Golgi stack	59	7.70E-06
Golgi apparatus	69	3.40E-05
Cluster 11. Enrichment Score: 5.38	**Count**	**P_Value**
rna-binding	80	2.00E-15
RRM	34	2.40E-04
domain:RRM	17	7.30E-04
RNA-binding region RNP-1 (RNA recognition motif)	35	1.40E-03
Nucleotide-binding, alpha-beta plait	37	2.40E-03
Cluster 12. Enrichment Score: 4.79	**Count**	**P_Value**
unfolded protein binding	34	1.40E-05
protein folding	47	1.40E-05
chaperone	25	2.10E-05
Cluster 13. Enrichment Score: 4.73	**Count**	**P_Value**
nucleotide-binding	169	1.00E-11
nucleotide binding	240	9.00E-08
atp-binding	118	5.60E-06
purine nucleotide binding	200	1.10E-05
adenyl nucleotide binding	156	2.40E-04
ATP binding	147	1.00E-03
nucleotide phosphate-binding region:ATP	87	6.20E-03
phosphotransferase activity, alcohol group as acceptor	74	1.60E-01
Cluster 14. Enrichment Score: 4.56	**Count**	**P_Value**
cell death	88	1.80E-07
death	88	2.50E-07
apoptosis	85	2.60E-07
programmed cell death	85	3.10E-07
apoptosis	40	1.10E-06
regulation of apoptosis	56	1.20E-05
regulation of programmed cell death	56	1.40E-05
positive regulation of cellular process	76	1.90E-05
positive regulation of apoptosis	30	1.30E-04
positive regulation of programmed cell death	30	1.50E-04
positive regulation of biological process	81	2.40E-04
induction of programmed cell death	26	5.70E-04
induction of apoptosis	26	5.70E-04
positive regulation of cellular physiological process	60	8.30E-04
positive regulation of physiological process	61	1.30E-03
induction of apoptosis by extracellular signals	9	4.40E-03
Cluster 15. Enrichment Score: 4.32	**Count**	**P_Value**
electron transporter activity	51	4.90E-07
electron transport	63	1.50E-04
generation of precursor metabolites and energy	86	1.40E-03
Cluster 16. Enrichment Score: 4.26	**Count**	**P_Value**
transport	147	1.50E-09
localization	316	1.30E-04
establishment of localization	313	2.00E-04
transport	290	2.80E-04
transporter activity	158	4.70E-02
Cluster 17. Enrichment Score: 4.17	**Count**	**P_Value**
ribonucleoprotein	53	8.40E-13
ribosomal protein	44	1.00E-09
ribonucleoprotein complex	86	3.00E-06
protein biosynthesis	29	6.40E-05
ribosome	15	3.00E-04
structural constituent of ribosome	47	1.90E-02
ribosome	44	9.90E-02
structural molecule activity	87	3.10E-01
RIBOSOME	18	9.70E-01

Cluster 18. Enrichment Score: 4.02	Count	P_Value
er-golgi transport	16	2.30E-06
ER to Golgi vesicle-mediated transport	19	5.00E-06
Golgi vesicle transport	23	1.60E-05
secretory pathway	33	1.60E-04
secretion	34	4.40E-03
vesicle-mediated transport	47	5.40E-03
Cluster 19. Enrichment Score: 4	**Count**	**P_Value**
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	80	2.50E-05
pyrophosphatase activity	79	3.50E-05
nucleoside-triphosphatase activity	76	3.80E-05
hydrolase activity, acting on acid anhydrides	80	4.20E-05
ATPase activity	49	4.10E-04
ATPase activity, coupled	41	1.80E-03
Cluster 20. Enrichment Score: 3.81	**Count**	**P_Value**
DNA-dependent DNA replication	21	8.80E-05
regulation of DNA replication	8	1.10E-04
regulation of DNA metabolism	11	3.80E-04
Cluster 21. Enrichment Score: 3.56	**Count**	**P_Value**
mrna splicing	27	2.30E-07
spliceosome complex	14	3.70E-03
spliceosome	9	2.50E-02
Cluster 22. Enrichment Score: 3.45	**Count**	**P_Value**
I-kappaB kinase/NF-kappaB cascade	26	1.40E-05
protein kinase cascade	45	7.80E-05
regulation of I-kappaB kinase/NF-kappaB cascade	19	2.80E-04
positive regulation of I-kappaB kinase/NF-kappaB cascade	18	3.40E-04
positive regulation of signal transduction	18	3.50E-03
regulation of signal transduction	32	5.80E-03
Cluster 23. Enrichment Score: 3.4	**Count**	**P_Value**
DNA-dependent DNA replication	21	8.80E-05
dna replication	15	1.30E-04
DNA replication	34	2.70E-04
DNA replication initiation	8	7.60E-03
Cluster 24. Enrichment Score: 3.4	**Count**	**P_Value**
magnesium ion binding	43	1.50E-04
magnesium	39	2.80E-04
metal ion-binding site:Magnesium	17	1.50E-03
Cluster 25. Enrichment Score: 3.32	**Count**	**P_Value**
organelle membrane	77	2.50E-07
organelle envelope	49	3.40E-05
mitochondrial envelope	30	3.70E-03
mitochondrial membrane	27	7.50E-03
organelle inner membrane	24	1.90E-02
mitochondrial inner membrane	22	2.00E-02