A new model for studying the effects of NSAIDs on cell migration in the canine gastric epithelium

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Abstract

NSAIDs, which act as COX antagonists, are widely used in veterinary practice to provide long-term pain relief, however a major limiting complication of their use in dogs is gastric ulceration. Gastric ulceration may result from either direct toxic effects on gastric epithelial cells or systemic action, including the inhibition of gastric epithelial cell migration. The role of cell migration in this effect and the specific signalling pathways involved are not known. COX-derived prostaglandins are known to have an important role in gastric defence and cytoprotection through the promotion of gastric mucosal blood flow and mucus secretion and the inhibition of gastric acid secretion. Given the importance of gastric epithelial cell migration in re-establishing gastric mucosal integrity following injury, the aim of this investigation was to test the hypothesis that paracrine PGE_2 signalling modulates gastric epithelial cell migration.

In order to address this hypothesis, a primary cell culture model which incorporates intact canine gastric glands that spread to form monolayer cell islands, was first characterised and then used. The effects of non-selective and COX-2 selective antagonism on cell spreading in this model and wound healing in immortalised cell monolayers was assessed. Furthermore, the involvement of subtype-specific EP receptor signalling in PGE_2-mediated modulation of epithelial cell migration was investigated through treatment with specific agonists and antagonists.

Both non-selective and COX-2 selective antagonism inhibited PGE_2 production and epithelial cell migration, thus providing evidence that COX-2-derived PGE_2 is important for the modulation of epithelial cell migration. Furthermore, non-selective and COX-2 selective antagonism inhibited cellular protrusive activity. The effects of PGE_2 on epithelial cell migration were shown to be mediated through EP3 and EP4 receptor signalling. Expression of COX-2, EP3 and EP4 was found to be readily induced in response to stressors. Interestingly, COX-2 expression was up-regulated in patients infected with spiral bacteria.

These findings provide evidence that COX-2-derived PGE_2 stimulates epithelial cell migration and the formation of cellular protrusions. Thus, reduced PGE_2 production in the gastric mucosa may inhibit gastric epithelial migration and contribute to the ulcerogenic effects associated with COX antagonist therapy.

List of Abbreviations

ACTH: adrenocorticotropic hormone

ADAM: A disintegrin and a metalloprotease domain

AP: Activator protein

ARE: AU-rich element

Arp: Actin related proteins

BCA: Bicinchoninic acid

bFGF: Basic fibroblast growth factor

bp: Base pair

BSA: Bovine serum albumin

C/EBP-α: CCAAT/enhancer binding protein α

Ca: Calcium

cagA: Cytotoxin-associated gene A

CagA: Cytotoxin-associated gene A protein

cAMP: Cyclic adenosine monophosphate

CCK: Cholecystokinin

CD3: Cluster of differentiation 3

CD8: Cluster of differentiation 8

Cdc42: Cell division control protein 42 homolog

cDNA: Complementary deoxyribonucleic acid

CHO: Chinese hamster ovary cell

CLASS: Celecoxib Long-term Arthritis Safety Study

c-met: Mesenchymal-epithelial transition factor

COX: Cyclooxygenase

CRE: Cyclic AMP response element

CRH: Corticotrophin releasing hormone

D cell: Delta cell

DAB: 3,3′-diaminobenzidine tetrahydrochloride

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DP: D-prostanoid receptor

DTT: Dithiothreitol

E-cadherin: Epithelial cadherin

ECL cell: Enterochromaffin-like cell

ECL solution: Enhanced chemiluminescence solution

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EMEM: Eagle's minimum essential medium

EMT: Epithelial-mesenchymal transition

EP: E-prostanoid receptor

ERK: Extracellular Receptor Kinase

FAK: Focal adhesion kinase

FBS: Foetal bovine serum

FITC: Fluorescein isothiocyanate

FP: F-prostanoid receptor

G cell: Gastrin-secreting cell

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC: Glucocorticoid

GER – Gastric epithelial restitution

GFP: Green fluorescent protein

GI: Gastrointestinal

G_i: Inhibiting G-protein

G-protein: Guanine nucleotide-binding protein

GR: Glucocorticoid receptor

GRE: Glucocorticoid responsive elements

G_s: Stimulatory G-protein

H^+K^+ATP: Hydrogen potassium adenosine triphosphate

H2: histamine type 2

HBSS: Hank's balanced salt solution

HCl: Hydrochloric acid

HGF: Hepatocyte growth factor

HPA: Hypothalamic pituitary adrenal

HRP: Horseradish peroxidase

Hsp: Heat shock proteins

Hz: Hertz

IBD: Inflammatory bowel disease

ICC: Immunocytochemistry

Ig: Immunoglobulin

IL: Interleukin

ILE: Isoleucine

INF: Interferon

IP: I-prostanoid receptor

ISMF: Intestinal sub-epithelial myofibroblasts

K_d: Dissociation constant

Km: Michaelis constant

LPS: Lipopolysaccharide

LT: Leukotriene

MAPK: Mitogen-activated protein kinase

MDCK: Madin Darby Canine Kidney

MMP: Matrix metalloproteinase

mRNA: Messenger RNA

NaHCO3: Sodium bicarbonate

NF-IL-6: Nuclear factor interleukin-6

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

NS-398: N-(2, cyclohexyloxy-4-nitrophenyl) methane sulphonamide

NSAID: Non-steroidal anti-inflammatory drug

PAGE: Polyacrylamide gel electrophoresis

PAR1: Polarity-regulating kinase

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PG: Prostaglandin

PGI: Prostacyclin

PI: Phosphoinositide

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLA2: Phospholipase A2

PLC: Phospholipase C

RGM: Rat gastric mucosal

RNA: Ribonucleic acid

rpm: Revolutions per minute

RSPCA: Royal Society for the Prevention of Cruelty to Animals

RT-PCR: Reverse transcription polymerase chain reaction

RU-38486: Mifepristone

SCAR: suppressor of cAMP receptor

SDS: Sodium dodecyl sulphate

SEM: Standard error of the mean

SHP2: Src Homology Phosphatase 2

siRNA: Small interfering RNA

Spp.: Species

SPSS: Statistical Package for the Social Sciences

TAE: Tris-acetate-EDTA

TFF: Trefoil factor

TGF: Transforming growth factor

TIA-1: T-cell Intracellular Antigen-1

TLR: Toll like receptor

TNF: Tumour necrosis factor

TP: Thromboxane receptor

TPA: 12-O-tetradecanoylphorbol-13-acetate

UTR: Untranslated region

UV: Ultraviolet

vacA: Vacuolating cytotoxin A gene

VacA: Vacuolating cytotoxin A protein

Val: Valine

VASP: Vasodilator-stimulated phosphoprotein

VEGF: Vascular endothelial derived growth factor

VIGOR: Vioxx Gastrointestinal Outcomes Research

WASP: N-Wiskott-Aldrich syndrome protein

WAVE: WASP-family verprolin-homologous protein

WSAVA: World Small Animal Veterinary Association

Chapter 1 - Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in both veterinary and human practice to treat pain and inflammation. Gastric ulceration is a limiting complication of their use and the major mechanism through which NSAIDs appear to cause gastric injury is via the inhibition of COX-1 and -2, leading to decreased prostaglandin production. Prostaglandins have an important role in gastric defence and cytoprotection, via the promotion of blood flow and mucus secretion and the inhibition of gastric acid secretion. Small areas of damage in the gastric epithelium are usually rapidly repaired via a process known as 'gastric epithelial restitution'. During restitution, underlying epithelial cells migrate from the gastric pits to cover the denuded areas. The role of restitution in NSAID-induced gastric ulceration has not been widely studied.

1.1 Canine stomach anatomy

1.1.1. Structure of the canine stomach

The canine stomach is located between the oesophagus and the small intestine and plays a vital role in the second phase of digestion through the secretion of acid and gastric enzymes. The functional stomach is divided into two distinct parts, the proximal stomach that consists of the cardia, fundus and a proportion of the body and the distal stomach, consisting of the antrum and the pylorus. The proximal stomach is involved in food storage and is capable of expanding considerably, while the distal section is responsible for the peristaltic contractions that mix food and acid together, thus aiding digestion. These contractions also encourage the movement of chyme, a semi-fluid material produced during the mechanical mixing of food in the stomach, into the duodenum.

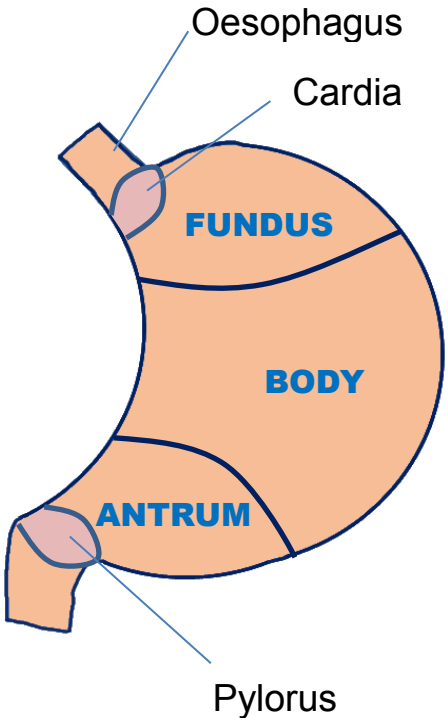

Figure 1.1- Diagram illustrating the basic stomach anatomy

The wall of the stomach is composed of four layers, the serosa, the muscularis layer, the submucosa and the mucosa. The serosa is the outermost layer that serves to reduce friction on the stomach. The inner lining of the stomach, the mucosa, consists of the lamina propria, the muscularis mucosae and densely packed gastric pits leading to the gastric glands. The lamina propria comprises many different cell types, including T and B lymphocytes, macrophages, plasma cells and myofibroblasts (Wu et al., 1999). Intraepithelial lymphocytes are also present in the normal gastric mucosa, with the majority being $CD3^+$ T lymphocytes and approximately 70% expressing CD8 on the cell surface (Oberhuber et al., 1998). The submucosa is a layer of collagen-rich connective tissue that provides most of the gastric wall strength and supports the mucosa. The mucosa and submucosa are separated by the muscularis mucosae, a smooth muscle sheet consisting of an inner circular and an outer longitudinal layer. Separating the submucosa and the serosa is the muscularis propria, which also consists of longitudinal and circular fibres and serves to impede gastric ulcer progression (Clayburgh et al. 2004). When the stomach is empty, elastic fibres in the submucosa and the muscularis mucosae push the mucosa to create

characteristic folds, known as rugae. The anatomy of the canine stomach is very similar to that of the human stomach, both are glandular and consist of cardiac, fundic and pyloric mucosal areas. These areas occupy similar proportions of both the canine and human stomachs (Kararli, 1995).

1.1.2. Structure and function of the canine gastric mucosa

There are two types of gastric gland found in the stomach, the oxyntic or fundic gland and the pyloric gland, named for their location. The pyloric glands consist of predominately mucus-secreting epithelial cells and gastrin-secreting G-cells, while the fundic glands are primarily composed of mucus-secreting epithelial cells and acid-secreting parietal cells (Schubert & Peura, 2008). Other cells present in the fundic glands include somatastatin-secreting D-cells, ECL cells and pepsinogen-secreting chief cells, while the pyloric glands also contain D cells. Functionally, the parietal cells are the most important cells in the fundic glands. They express the transmembrane enzyme H^+/K^+ ATPase, a proton pump that maintains the acidification of the stomach by catalysing the exchange of one hydrogen ion out of the cytoplasm for one potassium ion from the gastric lumen (Law et al., 2008). G-cell derived gastrin primarily stimulates acid secretion in an endocrine manner, via activation of the CCK_2 receptor on ECL cells, causing release of histamine. Histamine then binds to H2 receptors on the parietal cell and activates adenylate cyclase, causing generation of cAMP. Gastrin can also signal directly to parietal cells via CCK_2 receptors, activating PLC which causes a release of cytosolic Ca^{++} and promotes acid secretion (Schubert & Peura, 2008). Chief cells, located in the fundic glands, also work synergistically with parietal cells. They release the inactive precursor enzyme pepsinogen, which is converted into its active form, pepsin, by secreted acid. Pepsin is one of the primary proteolytic enzymes present in the digestive system (Raufman, 2004).

Gastric acid is an important secretion that prevents bacterial overgrowth, thus helping to protect against the development of enteric infection, and also provides a negative feedback loop for gastrin secretion (Spencer & Metz, 2010). However, abnormally high levels of gastric acid have been linked to gastric ulcer formation in the mucosa (Schubert & Peura, 2008), thus, regulatory mechanisms to tightly control gastric acid secretion are important. Somatostatin, a peptide hormone

secreted by D cells, is involved in regulating the endocrine system via G-protein coupled receptors; it acts as an inhibitor of gastrin-stimulated acid secretion (Shamburek & Schubert, 1993). Furthermore, the surface mucous cells, located primarily within the top half of the gland (Figure 1.2), secrete thick, acid-resistant mucus that lubricates and protects the mucosal lining from any acid-induced damage (Laine et al., 2008). The surface mucus also contains bactericidal components, such as, macrophages, lysozyme and IgA (Nakagawa et al., 2000).

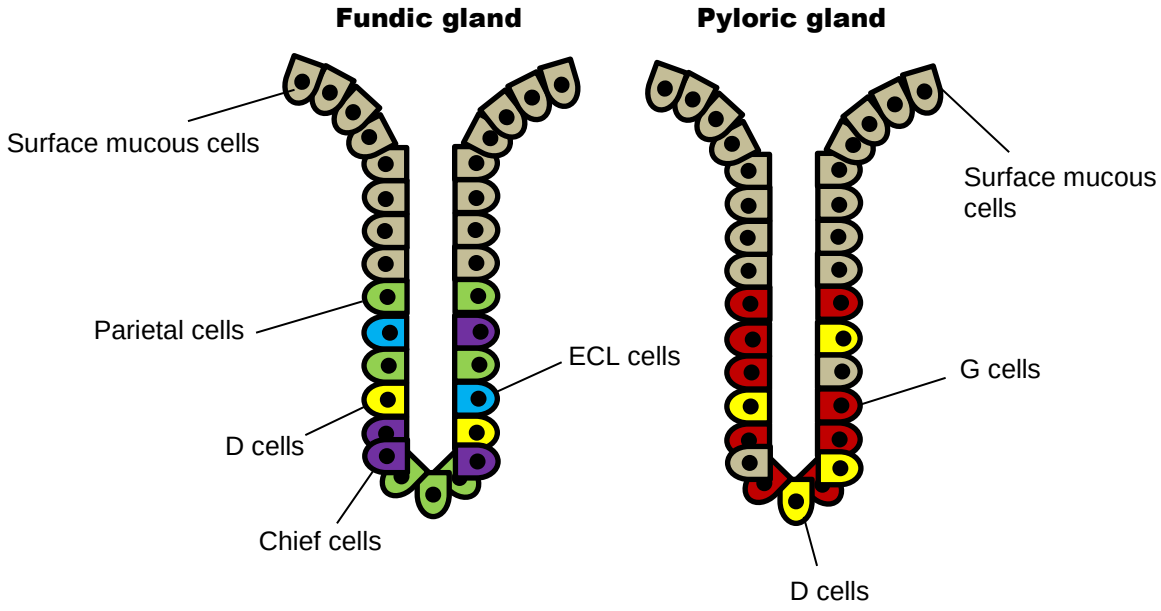

Figure 1.2- Diagram illustrating the structure of the fundic and pyloric glands

1.1.3. Paracrine signalling in the gastric epithelium

Paracrine signalling between the cells of the gastric epithelium may have an important regulatory role. Activation of the gastrin-CCK_B receptor via paracrine gastrin release has been shown to induce MMP-9 expression, leading to increased cell invasion in a gastric epithelial cell line (Wroblewski et al., 2002) and to induce gastric epithelial cell migration via activation of the EGF receptor, the erbB-2 receptor tyrosine kinase and the MAP kinase pathway (Noble et al., 2003). Gastrin release also induces IL-8 production in gastric epithelial cells via activation of the transcription factors, NF-κB and AP-1 (Hiraoka et al., 2001). In addition, HGF produced by gastric fibroblasts, acts on neighbouring epithelial cells to promote gastric epithelial proliferation and migration (Takahashi et al., 1995). Prostaglandins are known to strongly induce HGF release, and HGF may

modulate the protective effects of prostaglandins in the stomach (Takahashi et al., 1996). The HGF receptor gene, *c-met* is up-regulated in rat gastric mucosa during the healing process, indicating a role for HGF and its receptor in gastric mucosal healing (Tsuji et al., 1995). It is believed that HGF mediates interactions between mesenchymal and epithelial cells predominately during the remodelling stage of mucosal healing (Schmassman et al., 1997).TGF–α, released by parietal cells also appears to act in a paracrine manner to regulate the growth of canine oxyntic mucosal cells (Chen et al., 1993).

1.1.4. Mechanisms of gastric protection

The gastrointestinal tract is in constant contact with potentially damaging substances, including gastric acid and pepsin, both of which are capable of digesting tissue (Laine et al., 2008), thus several defence mechanisms exist to protect the gastric tissue from injury. The gastric mucosal barrier consists of a lining of columnar epithelial cells which secrete bicarbonate and mucus to neutralise gastric acid and also generate prostaglandins, which can act in a paracrine manner to inhibit acid secretion and stimulate mucus and bicarbonate production (Laine et al., 2008). The surface epithelial cells also exhibit tight junctions between them in order to maintain a physical barrier and prevent the back diffusion of acid (Shorrock & Rees, 1988). Microcirculation around the mucosa enables the delivery of cytoprotective agents, such as prostaglandins and EGF, and promotes acidic neutralisation via bicarbonate delivery (Abdel-Salam, et al., 2001). The TFF family have also been shown to play an essential role in gastric cytoprotection (Farrell et al., 2002; Babyatsky et al., 1996; Marchbank et al., 1998) via the stimulation of cellular proliferation and the inhibition of gastric acid secretion (Farrell et al., 2002). All three TFF's are expressed in the tissue of the stomach (Madsen et al., 2007) and exhibit gastric cytoprotective and healing effects (Taupin & Podolsky, 2003). Furthermore, mucosal NO protects the gastric mucosa against NSAID-induced damage through the maintenance of blood flow and the promotion of prostaglandin production (Khattab, 2001).

1.2.Cell migration

1.2.1.Importance in embryogenesis

Cell migration is a critical process in embryogenesis, for instance in giving rise to key embryogenic structures such as the ectoderm, the mesoderm and the endoderm (Strachan & Read, 2004). Perturbation of some of the important components involved in embryonic cell migration can lead to severe developmental defects (Kurosaka & Kashina, 2008), as evidenced using knockout mouse models, for example, knocking out E-cadherin caused a loss of trophectoderm epithelium formation, resulting in lethality at the time of implantation (Larue et al., 1994).

1.2.2.Importance in wound healing

Cell migration plays an important role in the repair of superficial or partial-thickness wounds in the adult. These wounds occur when the epidermis and superficial layers of the dermis are injured but the basal layer of cells remains intact and cells migrate into the gap in order to close the wound (Enoch & Leaper, 2005). Cell migration is an important homeostatic process involved in maintaining the integrity of skin, corneal, intestinal and gastric tissue. Migration appears to follow a similar process in all cell types and environments that have been investigated so far. Repair of damage within the cornea is a complex process as, due to the function of the eye, it is essential that the transparent properties of the cornea remain unaffected. During the first four to six hours following injury, corneal epithelial cells polarise and the actin cytoskeleton is reorganised, then when actively migrating, the epithelial cells flatten and spread out to cover the wound area, projecting both lamellipodia and filopodia from the leading edge (Dua et al., 1994). Glycogen metabolism provides the energy required for this process (Dua et al., 1994; Kuwabara et al., 1976). Cell migration within the gastrointestinal tract is very similar to the process involved in corneal wound repair. IBD is a group of conditions which cause the intestinal mucosa to become chronically inflamed and this inflammation can lead to repeated injury of the intestinal wall and thus the requirement for constant tissue repair. In the case of IBD, mucosal epithelial cells adjacent to the wound edge polarise, undergo

cytoskeletal changes and spread out in order to fill the gap (Sturm & Dignass, 2008). Rapid repair of superficial GI mucosal injury is important to prevent the occurrence of more widespread damage. As this project focusses on cell migration in the context of gastric mucosal repair, this process will be discussed in more detail. Cells within the stomach are exposed to a highly acidic environment, constant friction from ingested food and potential contact with pathogens, thus the stomach is an active site for rapid cell migration into denuded areas (Wallace and Granger, 1996).

1.2.3. Rapid epithelial restitution in the gastric mucosa

Gastric epithelial cell migration in response to gastric damage is referred to as rapid epithelial restitution (Lacy & Ito, 1984). Lacy and Ito (1984) conducted research looking at the cellular mechanisms involved in the repair of epithelial cells in the rat gastric mucosa following injury with absolute ethanol. The process of restitution started within three minutes of injury and was virtually complete after one hour. Lacy and Ito (1984) characterised restitution into four distinct stages based on the microscopic appearance of the cells (Figure 1.3). At stage one, the necrotic epithelium was still attached to viable cells within the gastric pits and no cell migration had occurred. The necrotic tissue was detached at stage two, leaving the basal lamina denuded. Migrating squamous cells with extended lamellipodia covered the basal lamina at stage three and changed to columnar cells at stage four to complete the migration process. A reduced level of cell migration occurred in areas containing breaks in the basal lamina (Lacy & Ito, 1984) thus suggesting that an intact basal lamina is important.

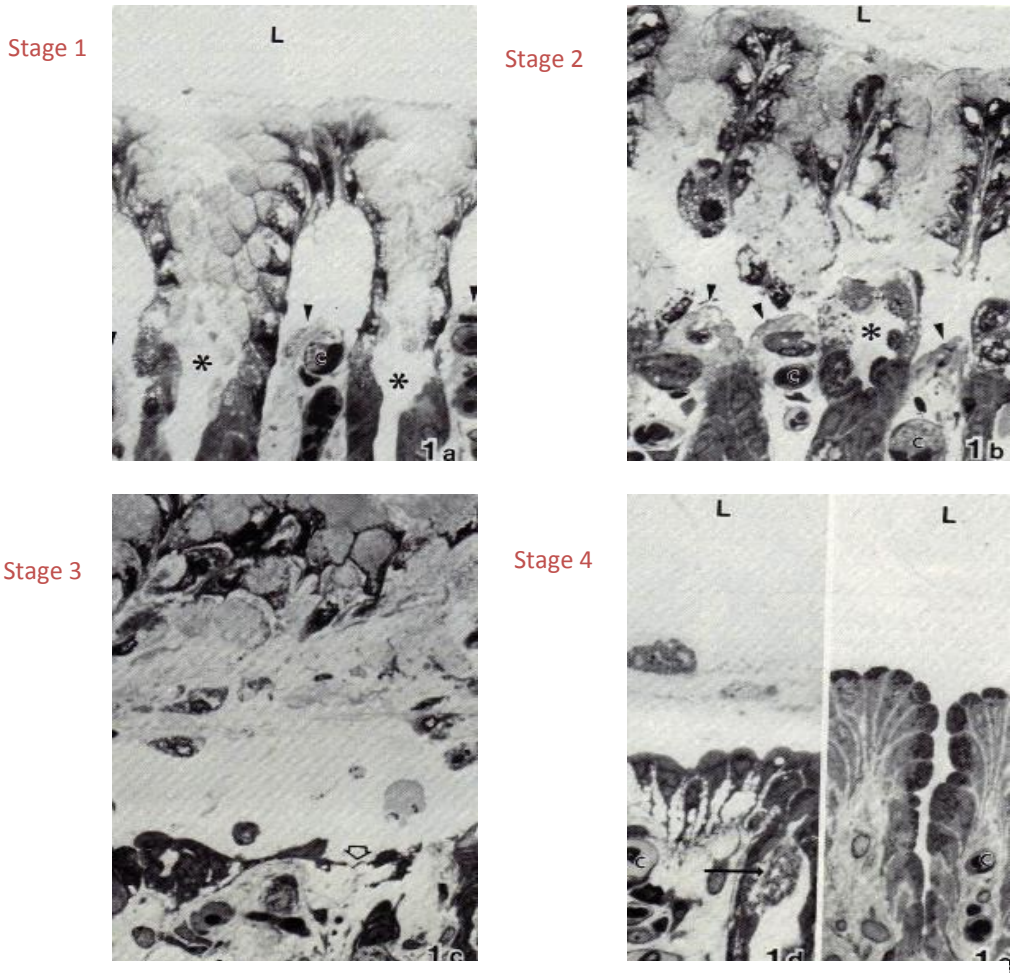

Figure 1.3- Photomicrographs of gastric mucosae illustrating the various stages involved in restitution; * = gastric pit, L = lumen, C = capillary, the arrowheads indicate the denuded basal lamina and the arrow indicates a parietal cell in the gastric pit.

(Figure reproduced from Lacy & Ito, 1984)

Prior to migration, cells undergo a series of characteristic changes in preparation for the process. Initially, migrating cells must polarise, with lamellipodia, microtubules and the Golgi complex locating towards the front of the cell and myosin II locating towards the rear (Ridley et al., 2003). Cell polarity is regulated by various signalling molecules, for instance, epithelial cell-cell contact, mediated by E-cadherin, has been shown to induce polarity (Desai et al., 2009). An intact actin cytoskeleton and Cdc42 signalling are also required (Desai et al., 2009) and Cdc42 is known to have a central role in cell polarisation (Etienne-Manneville, 2004).

Following polarisation, migrating cells extend protrusions from their plasma membrane at the leading edge. Actin polymerisation at the leading edge is stimulated by the activation of Rac or Cdc42 (Horwitz & Webb, 2003) and functions to push the membrane forward (Figure 1.4). There are four specific types of cellular protrusions involved in cell migration, namely lamellipodia, filopodia, blebs and invadopodia (Ridley, 2011). Lamellipodia are sheet-like, actin cytoplasmic projections that function to "pull" the migrating cell forwards and these were first identified by Abercrombie in 1980. The α6 and α3β1 integrins have a role in the formation of lamellae (Lotz et al., 2000). Filopodia were first described in 1961 by Gustafson and Wolpert, who referred to them as pseudopods and observed them in primary mesenchymal cells of the sea urchin embryo. Filopodia are finger-like projections that extend beyond the leading edge of the lamellipodia and function to explore the local environment. Blebs are spherical protrusions supported by the actomyosin cortex (Charras & Paluch, 2008); however, blebbing migration has not been widely studied. Invadopodia are protrusions typically extended by invasive tumoural or transformed cells. These protrusions are proteolytic in nature and their function is to degrade the extracellular matrix (Ayala et al., 2006), allowing further invasion. The pathways that activate the formation of these cellular protrusions differ. Lamellipodia formation is activated via Rac and the downstream WAVE2 complex, while filopodia formation is induced via WASP, which is activated downstream of Cdc42 (Takenawa & Miki, 2001). Bleb protrusions are Rho/ROCK dependant (Charras & Paluch, 2008) and invadopodia protrusions are RhoC dependant (Bravo-Cordero et al., 2011).

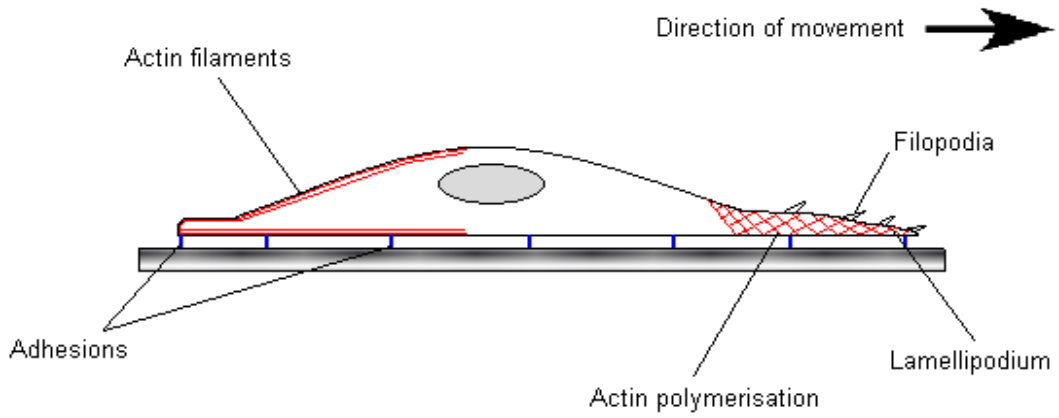

Figure 1.4- Illustration highlighting the processes involved in rapid epithelial restitution

In order for cellular protrusions to occur, areas of extension must overcome the resistive forces of cell membrane tension. A direct inverse relationship between lamellipodial extension rate and membrane tension has been described (Raucher & Sheetz, 2000). Once established, protrusions are stabilised via the binding of adhesions with the substratum (Horwitz & Webb, 2003). Adhesion formation is dependent on both Rac and Cdc42 (Ridley et al., 2003) and adhesions function as both traction sites and mechanosensors (Galbraith et al., 2002).

Finally, in order to complete its forward movement, a contractive force is exerted onto the rear of the cell in a myosin-dependant process (Horwitz & Webb, 2003). Adhesions at the rear of the cell are disassembled to allow movement, this disassembly can occur via two mechanisms, either the strong contractive force can physically 'tear' the adhesion bond or signals from regulatory pathways may activate the process (Lauffenburger & Horwitz, 1996).

1.2.4. Signalling molecules linked to migration

The cells within gastric glands may release many molecules, such as growth factors, cytokines and prostaglandins and several of these signalling molecules have been shown to be involved in the regulation of cell migration. These include EGF (Maldonado & Furcht, 1995), HGF (Netzer et al., 2003), TFF's (Taupin & Podolsky, 2003; Xue et al., 2010) and prostaglandins (Buchanan et al., 2003). Furthermore, FAK, a signalling molecule implicated in initiating many downstream processes, including tyrosine phosphorylation appears to have a role in leading edge formation during cell migration (Tilghman et al., 2005). Cell-cell junctions are also important in the regulation of cell migration, for instance, E-cadherin mediates cell polarity (Desai et al., 2009) and occludin regulates directional migration (Du et al., 2010). As well as functioning as adhesion sites, junctions can act as signal carriers to limit a cell's growth or to communicate a cell's position (Dejana, 2004).

1.2.5. Models used for studying cell migration

Due to its importance in a variety of biological processes, various experimental models have been developed to enable the study of cell migration. The most

common *in vitro* method used is the scratch wound healing assay, which is simple to perform, relatively cheap and mimics the migratory behaviour of cells seen *in vivo* (Liang, Park & Guan, 2007). Crude wounds are made in confluent cell monolayers and migration into the wound space is measured over a period of time. As wounded cells migrate as a sheet, normal cell-cell interactions are preserved (Fotheringham et al., 2012). This technique has been successfully used in a variety of cell types (Herren et al., 2001; Lu et al., 2004; Huang et al., 2008), however as a large quantity of cells are required, this is not usually the assay of choice for primary cell experiments. The Boyden chamber assay provides a means for studying directional cell migration in response to chemoattractants. Cells are seeded in serum-free medium over a porous insert and chemoattractants are placed in the well beneath, thus migration through the pores of the insert can be recorded (Chen, 2005). These assays are summarised in Figure 1.5. Both assays involve the use of a single cell type, which does not fully recapitulate the cell to cell interactions that occur *in vivo*. From a clinical perspective, analysis of the interactions between all cell types within the gastric gland would be more valuable.

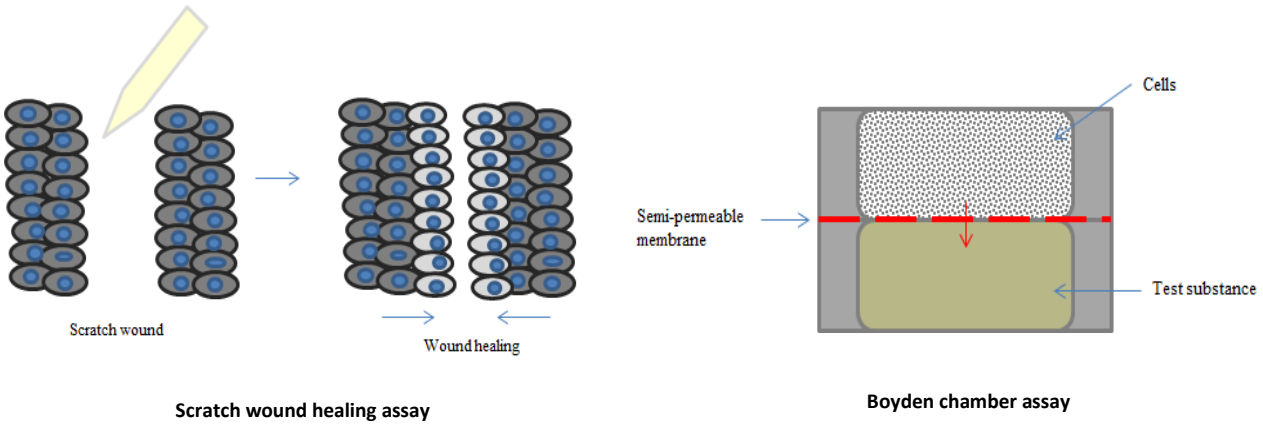

Figure 1.5- Diagram illustrating assays commonly used to study cell migration

1.3. Prostaglandins

1.3.1. Prostaglandin structure and synthesis

Prostaglandins are synthesised within the cell from the fatty acid precursor, arachidonic acid. The COX enzymes, COX-1 and COX-2, are involved in the metabolism of arachidonic acid, which is mainly stored in membrane phospholipids. PLA2 mediates the release of arachidonic acid from the cell's membrane, arachidonic acid is then oxidised by COX into PGG_2, and reduced to PGH_2. PGH_2 acts as a substrate for the COX enzymes, leading to production of the five prostaglandins (Hata & Breyer, 2004) (Figure 1.6). Once synthesised, the prostaglandins are released from the cell and have a short half-life *in vivo*, thus they act primarily on neighbouring cells (Narumiya et al., 1999). The prostaglandins are rapidly inactivated by the cytosolic enzymes, 15-ketoprostaglandin Δ^{13}-reductase and 15-hydroxyprostaglandin dehydrogenase (Legler et al., 2010). Prostaglandins are produced primarily when required; they do not appear to be stored in either tissues or cells in any appreciable quantity (Gibson, 1977).

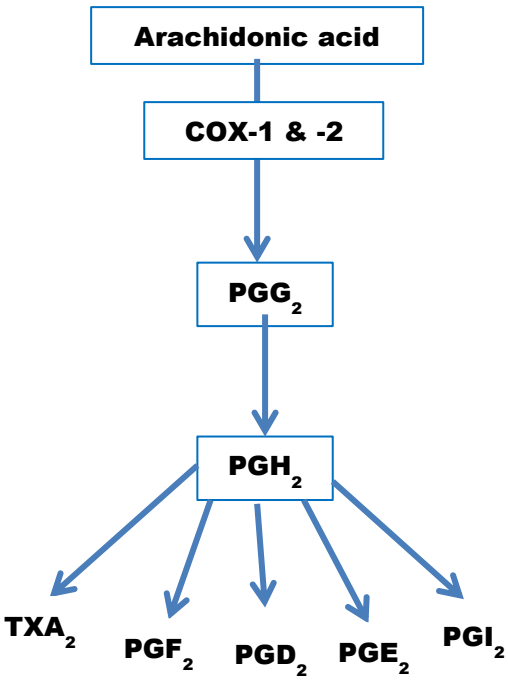

Figure 1.6- Schematic diagram illustrating the biosynthesis of prostaglandins

1.3.2. Functions of prostaglandins

1.3.2.1.General

Prostaglandins exert their effects via a diverse array of G-protein coupled receptors (Hata & Breyer, 2004), resulting in a variety of physiological effects within the body, including effects on reproduction and cardiovascular (Miller, 2006), renal (Schlondorff & Ardaillou, 1986), and gastrointestinal function (Robert, 1984). Prostaglandins are involved in various aspects of reproduction, including menstrual regulation, ovulation and stimulation of uterine contractions during labour (Embrey, 1981). Within the cardiovascular system, prostaglandins inhibit platelet aggregation (Miller, 2006), resulting in an anti-coagulant effect and also stimulate the vasodilation of endothelial cells. Prostacyclin has a vital role in regulation of renal blood flow, glomerular filtration rate and renin secretion and PGE_2 produced via the interstitial cells of the renal medulla regulates salt and water excretion (Miller, 2006). Furthermore, prostaglandins modify the inflammatory response through stimulation of vasodilation and vascular permeability (Goodwin & Webb, 1980). PGE_2 mediates arterial dilation, leading to redness and swelling and acts directly on peripheral sensory neurons, causing pain (Ricciotti & FitzGerald, 2011). Prostaglandins have also been shown to exhibit anti-inflammatory effects in allergic lung disease (Gavett et al., 1999) and inflammatory colitis (Morteau et al., 2000). Immune responses are initiated by T lymphocytes and prostaglandins may modulate the development and maturation of T cells and influence the function of mature lymphocytes. PGE_2 can inhibit many T and B cell functions including the inhibition of T lymphocyte activation and proliferation (Chouaib et al., 1985) and the inhibition of IgE production by B lymphocytes (Pène et al., 1988).

1.3.2.2.Gastrointestinal specific

Prostaglandins derived via COX activity, in particular PGE_2, are believed to have an important role in gastric defence and cytoprotection. Prostaglandins are synthesised throughout the gastrointestinal tract, with PGE_2 and $PGF_{2\alpha}$ being the most abundantly produced prostaglandins in the human gastric mucosa (Eberhart & Dubois, 1995). PGD_2 and PGI_2 are also produced in the gastric mucosa but at

significantly lower concentrations (Eberhart & Dubois, 1995). Prostaglandins contribute to gastric mucosal protection by increasing the production of bicarbonate and mucus (Laine et al., 2008), inhibiting gastric acid secretion (Whittle, 1980) and decreasing epithelial cell permeability to protect against acid back-diffusion (Takezono et al., 2004). Additionally, prostaglandins may protect the gastric mucosa by down-regulating the release of certain pro-inflammatory molecules, for example IL-1 (Kunkel et al., 1986) and LTB_4 (Ham et al., 1983) that can cause the development of gastric lesions. Prostaglandins may also protect epithelial cells from NSAID or ethanol-induced injury through a direct cellular effect (Tarnawski et al., 1988), although a mechanism for this was not clear. Furthermore, endogenous prostaglandins are potent vasodilators that can increase gastric mucosal blood flow. Gastric mucosal blood flow has a vital role in gastric cytoprotection and healing via the constant supply of oxygen and bicarbonate and the removal of excess acid and toxins. It is not known whether or not the cytoprotective effects of prostaglandins are a direct result of increased gastric mucosal blood flow or if prostaglandins increase blood flow to an appropriate level for restitution to occur (Abdel-Salam et al., 2001).

1.3.3.COX enzymes

1.3.3.1.Structural features of COX-1 and -2

Two isoforms of the COX enzymes (also known as prostaglandin endoperoxidase H_2 synthases) exist, namely COX-1 and COX-2. COX-1 was initially purified in 1976 from the vesicular glands of sheep (Hemler & Lands, 1976) and the COX-1 gene was sequenced in 1989 (Yokoyama & Tanabe, 1989), while the existence of a COX-2 isoform was first described in 1991 (Kujubu et al., 1991). The isoforms are each coded for by a different gene, although they have similar primary protein structures, sharing between 60 and 65% amino acid identity (Chandrasekharan & Simmons, 2004) and they both catalyse the conversion of arachidonic acid to PGG_2 and the reduction of PGG_2 to PGH_2. The isoforms also have similar three-dimensional structures, comprising of three structural domains, the N-terminal EGF domain, a membrane binding domain and a catalytic domain, containing a heme binding site and a COX site (Garavito & Mulichak, 2003). Despite having similar overall structures, the membrane binding domain sites of the isoforms are

only 38% identical (Spencer et al., 1999), with COX-2 having a much larger active site due to substitution of the COX-1 ILE523 residue with Val523. This increased binding site volume can be utilised by pharmaceutical companies in order to make drugs that specifically target the COX-2 enzyme, i.e. that are too large to bind to the COX-1 active site (Garavito et al., 2002).

1.3.3.2.Expression and regulation of COX-1 and -2

COX-1 mRNA and protein is expressed constitutively in most tissues and cells and is thought to be involved in normal homeostatic processes (Morita, 2002). The structure of the COX-1 gene is consistent with that of a housekeeping gene (Tanabe & Tohnai, 2002), i.e. it is GC-rich and lacks a canonical TATA or CAAT box in its promoter region (Martin Sanz et al., 2006). COX-2 expression is inducible upon cell activation and stimulation via proinflammatory stimuli (Hla et al., 1999). Proinflammatory factors that have been shown to induce COX-2 expression include IL-1, TNF-α, INF-γ and LPS (Tanabe & Tohnai, 2002). HGF has also been shown to induce COX-2 expression in rat gastric epithelial cells via phosphorylation of the c-Met/HGF receptor and activation of the ERK2 signalling pathway (Jones et al., 1999). The COX-2 gene has several regulatory elements, including NF-κB, NF-IL6 and CRE, which are involved in the transcriptional regulation of the gene (Tanabe & Tohnai, 2002). COX-2 expression is regulated at the transcriptional and post-transcriptional level (Hla et al., 1999) and activation of the MAPK cascade is involved in gene regulation at both levels (Tanabe & Tohnai, 2002).

1.3.3.3.Localisation of COX-1 and -2

The intracellular locations of COX-1 and -2 have been previously determined using quantitative confocal microscopy (Morita et al., 1995). COX-1 immunoreactivity was observed within the endoplasmic reticulum and the nuclear envelope and while COX-2 immunoreactivity was also seen in these areas, the intensity of staining in the nuclear envelope was 2-fold higher than that seen in the endoplasmic reticulum. Current evidence suggests that COX-1 and -2 function to produce prostaglandins for extracellular housekeeping and differentiative or replicative events respectively (Morita et al., 1995). Constitutive expression of

COX-1 makes it readily available to mediate prostaglandin synthesis for normal physiological functions, such as modulation of reproductive and cardiovascular processes (Miller, 2006), while COX-2 expression is known to be induced during differentiative or replicative events (Xie et al., 1991; Dewitt et al., 1993). Previous studies suggest that COX-1 and -2 utilise two distinct pools of arachidonic acid, with COX-2 responding to a stimulated pool of arachidonic acid, required for inflammatory responses, and COX-1 responding to a pool required for physiological functions (Reddy & Herschman, 1994). COX-2 expression is known to be required for prostaglandin production from endogenous arachidonic acid released in response to mitogen stimulation in murine fibroblasts and macrophages, while COX-1 is unable to utilise this store (Reddy & Herschman, 1994).

1.3.3.4.Functions of COX -1 and -2

Specific roles for COX-1 and -2 have been identified using gene disruption studies. While neither male nor female fertility appears to be affected in COX-1 deficient mice, complete lack of COX-1 through homozygous x homozygous mating, results in few live offspring (Langenbach et al., 1995), thus highlighting the importance of COX-1 in reproduction. Homozygous COX-1 mutant mice exhibit decreased platelet aggregation and arachidonic acid-induced inflammation (Langenbach et al., 1995), however, these mice survive well, do not exhibit any gastric pathology, show less susceptibility to indomethacin-induced gastric ulceration and exhibit minimal kidney abnormalities (Langenbach et al., 1995). These results are surprising, considering that COX-1 is believed to have a vital role in normal cellular physiology. COX-2 deficient mice show normal inflammatory responses to exogenous arachidonic acid and show no innate gastrointestinal pathology (Morham et al., 1995), they do, however, exhibit kidney abnormalities that progressively degenerate with age, and develop a susceptibility to peritonitis (Morham et al., 1995). In contrast, non-selective COX inhibition via NSAID treatments, has been shown to cause gastrointestinal damage (Wallace et al., 2000; Tanaka et al., 2001), thus suggesting that the COX enzymes do have an important role in gastrointestinal protection.

COX-2 has a role in tumorigenesis, through the stimulation of proliferation and angiogenesis and the suppression of apoptosis and the immune response (Howe et al., 2001). COX-2 has been shown to be up-regulated in pancreatic (Tucker et al., 1999), lung, breast and colon (Soslow et al., 2000) cancer. Furthermore, a reduced risk of colorectal cancer has been associated with the regular use of NSAIDs (Ruder et al., 2011) and the inhibition of COX-2 is believed to play a key role in this effect (Schrör, 2011). Aspirin treatment has been shown to reduce the risk of colorectal cancers overexpressing COX-2, but not those with weak or absent COX-2 expression (Chan et al., 2007). COX independent mechanisms which may play a role in this effect include modification of transcription factors, such as NF-κB, induction of apoptosis and DNA stabilisation (Schrör, 2011).

1.3.4. Prostaglandin receptors

1.3.4.1.Structure and localisation

Prostaglandin effects are mediated through the transmembrane, G-protein coupled prostanoid receptors, which were first classified in 1982 (Kennedy et al., 1982). There are five key members of the prostanoid receptor family, grouped by the ligand with which they bind to, these include DP, EP1-4, FP, IP and TP, which bind to PGD, PGE, PGF, PGI and thromboxane respectively (Ushikubi et al., 1995). Prostaglandins exhibit different biological functions, depending upon the receptor with which they bind, and effects may also differ depending on the cell type involved (Hata & Breyer, 2004). The prostanoid receptor gene structure has been shown to be similar between all receptors and across various species (Boie et al., 1995; Ogawa et al., 1995; Regan et al., 1994). The receptors consist of the seven hydrophobic, transmembrane domain, characteristic of G-protein coupled receptors and a putative extracellular-loop region (Breyer et al., 2001). As PGE_2 is considered to have the most important role in normal GI physiological processes, the focus of this review will be on the structure and function of the EP receptors to which PGE_2 binds.

Four sub-types of the EP receptor exist, namely EP1, EP2, EP3 and EP4, each with varying structures and functional roles. The diverse biological effects of PGE_2 may be attributed to the different signal transduction pathways that occur

upon activation of each receptor sub-type (Hata & Breyer, 2004). The EP receptors are encoded by different genes, but are well conserved throughout the mammalian species. All of the receptor sub-types are expressed on the plasma membrane, however EP3 and EP4 also localise at the nuclear envelope (Bhattacharya et al., 1999). EP3 is the only receptor that exhibits multiple alternatively spliced variants (Breyer et al., 1994), which can activate different second messenger signalling pathways (Pierce and Regan, 1998). Eight different EP3 isoforms have been recorded to date (Bilson et al., 2004).

EP receptor expression within the stomach varies greatly between species. For instance, in rat stomach tissue, EP1 mRNA was detected in the gastric muscle layers, while EP3 and EP4 mRNA was expressed primarily in the gastric mucosal layer (Ding et al., 1997). Within cultured gastric epithelial cells, EP3 and EP4 were expressed in parietal cells, while only EP4 was expressed in gastric mucous cells (Ding et al., 1997). In normal human gastric tissue, no EP1 protein was detected, EP2 was expressed on the luminal surface of the gastric epithelium, EP3 was expressed in the gastric epithelium only and was localised to the upper mucosal cells and intense EP4 expression was detected in the lamina propria mononuclear cells (Takafuji et al., 2002). Limited information is available with regards to the expression and localisation of the EP receptors in the normal canine gastric epithelium.

Out of the four EP receptor sub-types, EP1 has the lowest affinity for PGE_2 with a K_d of 16-25 nM (Dey et al., 2006). The affinity of EP2 for PGE_2 differs significantly between species, with the rat EP2 receptor having the highest affinity (K_d=5 nM) and the mouse receptor showing a much lower affinity (K_d=116 nM) (Dey et al., 2006). The EP3 and EP4 receptors both have a relatively high affinity for PGE_2, with K_d values of 0.33-2.9 nM and 0.59-1.27 nM respectively (Dey et al., 2006). These variations in affinity between the receptor sub-types may be due to the degree of G-protein subunit coupling. It has been reported that a receptor that is coupled exhibits a lower affinity for PGE_2 than an uncoupled receptor (Breyer et al., 2001).

1.3.4.2.Functions of the EP receptors

The roles of the specific EP receptors in gastric cytoprotection have been studied using both mouse 'knockout' models and experiments utilising sub-type specific EP receptor agonists and antagonists. Pre-treating rats with either PGE_2 or an EP1 specific agonist, dose-dependently prevented HCl/ethanol-induced gastric lesion development (Araki et al., 2000), thus it appears that PGE_2 provides gastric cytoprotection through activation of the EP1 receptor. This finding was confirmed using a mouse 'knockout' model, which demonstrated that the protective effect of PGE_2 disappeared in mice lacking the EP1 receptor (Araki et al., 2000). Furthermore, PGE_2 has been shown to dose-dependently protect the gastric mucosa against NSAID-induced damage, via activation of the EP1 receptor (Suzuki et al., 2001).

Prostaglandins have gastric cytoprotective effects via the regulation of acid, bicarbonate and mucus secretion through activation of the EP3, EP1 and EP4 receptors respectively (Takeuchi et al., 2010). PGE_2 has a biphasic effect on acid secretion via the activation of different receptors; for instance, EP3 and EP4 activation cause the inhibition and stimulation of acid secretion respectively (Dey et al., 2006). PGE_2-mediated inhibition of acid secretion via EP3 receptor activation, involves both a direct effect on parietal cells and an indirect effect via the inhibition of histamine secretion from ECL cells (Takeuchi et al., 2010). Additionally, gastric mucosal blood flow is increased via activation of the EP2, EP3 and EP4 receptors, but not the EP1 receptor (Araki et al., 2000). Activation of EP2 and EP4 may also be important for gastric cytoprotection, in particular, inhibition of ethanol-induced rat gastric mucosal damage has been shown to occur via activation of EP2 and EP4 leading to inhibition of LTC_4 production (Hattori et al., 2008). LTC_4 is considered to play an important role in the development of ethanol-induced gastric damage via the promotion of vascular disturbances (Higa et al., 1991). COX-2-derived PGE_2 has been shown to promote the healing of gastric ulcers via EP4 receptor activation and the up-regulation of VEGF (Hatazawa et al., 2007) and EP4 receptor activation is associated with the modulation of cell migration in a variety of cell types (Kim et al., 2010; Ma et al., 2006).

1.3.4.3.EP receptor signalling

Each EP receptor is coupled to a different intracellular signalling pathway (Figure 1.7). The EP1 receptor activates PLC, causing mobilisation of intracellular calcium, EP2 and EP4 activation results in an increase in intracellular cAMP levels and EP3 activation leads to a reduction in cAMP (Hull et al., 2004). The EP3 receptors are able to couple to multiple G-proteins, upon coupling they activate the G_i subunits, leading to inhibition of adenylyl cyclase. They may also activate G_s subunits causing an increase in cAMP production (Dey et al., 2006).

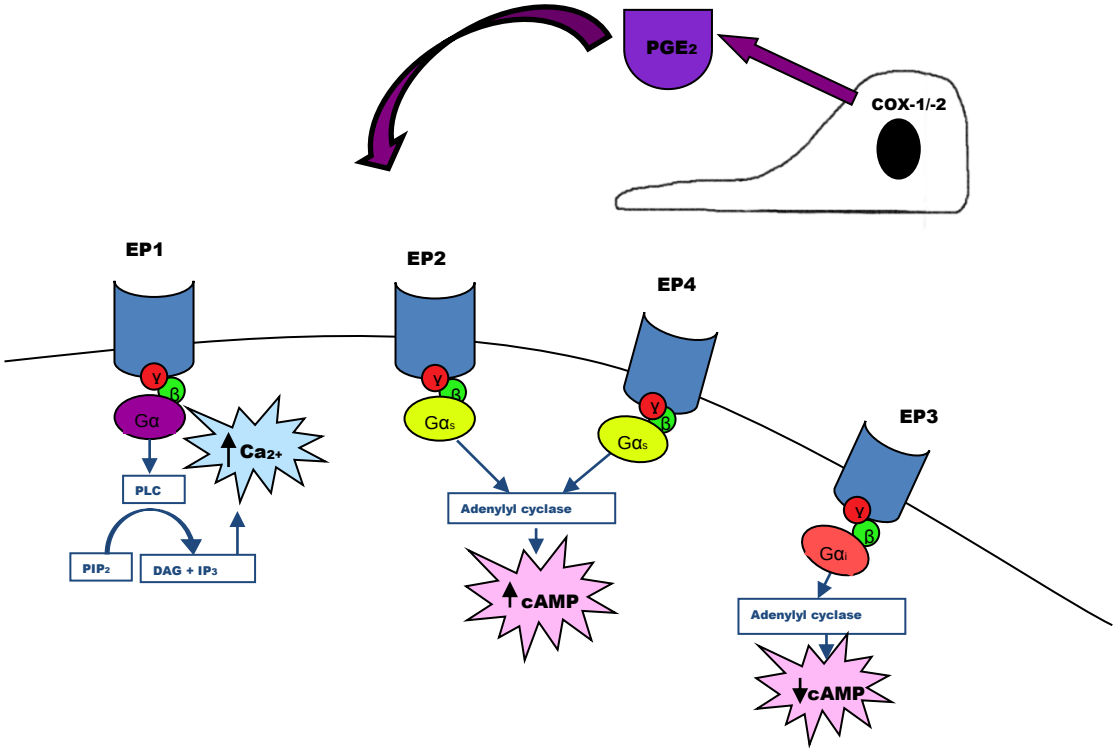

Figure 1.7- Diagram illustrating the signal transduction pathways induced via activation of the EP receptor sub-types

1.4. Non-steroidal anti-inflammatory drugs (NSAIDs)

1.4.1. Discovery and Clinical uses

NSAIDs are routinely used in both human and veterinary medicine to reduce inflammation and associated pain. NSAIDs exert their effects via the inhibition of prostaglandin production (Vane, 1971) and prostaglandin inhibition has been demonstrated in a wide variety of cell types (Vane & Botting, 1996). Prostaglandins cause increased vasodilation, which leads to an increase in plasma exudation induced by bradykinin and histamine (Williams & Peck, 1977). PGE_2 can also directly promote vasodilation through EP2 signalling (Tilley et al., 2001). These effects cause the typical symptoms present in cases of acute inflammation, i.e. erythema and oedema.

1.4.2. Adverse effects associated with NSAID use

NSAID use has been associated with adverse effects, including nephrotoxicity (Ejaz et al., 2004), adverse cardiovascular effects (Bennett et al., 2005) dyspepsia and gastric ulceration (Laine, 2002). In patients regularly taking NSAIDs, the point prevalence of gastric ulceration is 15-30% (Laine, 2001). NSAID-induced gastric damage may be caused either through topical or systemic action (Laine, 2002); however, a previous study found that there was no difference in the relative risk of developing upper-gastrointestinal bleeding between users of plain and enteric-coated aspirin (Kelly et al., 1996). As prostaglandins are involved in the regulation of a variety of gastric defence mechanisms, it seems likely that their inhibition will contribute to the gastrointestinal adverse effects associated with NSAID therapy, although NSAIDs may also induce apoptosis and necrosis of gastric mucosal cells *in vitro*, independently of COX inhibition (Tomisato et al., 2004). Furthermore, NSAIDs may prevent gastric repair through the reduction of mucosal blood flow, which is necessary for epithelial restitution, and by preventing EGF from initiating epithelial repair (Wallace, 2008).

As COX-1 is thought to be primarily involved in normal physiological processes, it was reasoned that the adverse effects associated with NSAID treatment could be avoided through selective COX-2 inhibition. Celecoxib, the first COX-2 selective

inhibitor (collectively known as coxibs) was introduced in December 1998 (DeWitt, 1999) and since then many more have been developed. COX-2 selective antagonists approved for use in dogs include acetamorphin, meclofenamic acid, carprofen, etodalac, vedaprofen, deracoxib, firocoxib and meloxicam (Thompson, 2011). Conflicting evidence exists, regarding the GI tolerability of coxibs in comparison with non-selective antagonists. Several endoscopic studies have been performed, comparing the incidence of gastric ulceration with coxib and non-selective NSAID therapy. These studies report a lower incidence of ulceration in patients prescribed coxibs, with incidence levels being similar to the placebo group (Laine et al., 1999; Simon et al., 1999; Hawkey et al., 2000). Two large, randomised controlled trials, the VIGOR and the CLASS trials, were also performed to analyse the occurrence of clinically relevant GI events during coxib and non-selective NSAID therapy (Bombardier et al., 2000; Silverstein et al., 2000). These trials also report decreases in clinically important GI events in patients taking coxibs compared to traditional non-selective NSAIDs. However, previous *in vivo* investigations have presented contrasting findings. Non-selective COX inhibition was found to significantly worsen IA-induced gastric damage in rats and selective inhibition of both COX-1 and COX-2 also exacerbated gastric lesions (Takeeda et al., 2004), thus suggesting that both enzymes are important for gastric protection. A similar study reported that selective COX-1 and -2 inhibition caused no gastric damage; however, non-selective inhibition caused gross damage (Tanaka et al., 2001). Thus, the role of COX-1 and -2 in gastric protection remains unclear.

COX-2 selective inhibitors have also been associated with increased cardiovascular risks, primarily an increased incidence of myocardial infarction (Bombardier et al., 2000). COX-2 selective inhibitors do not inhibit the prothrombotic prostaglandin thromboxane, however, they do inhibit the vasodilatory PGI_2 (Justice & Carruthers., 2005), thus this prostaglandin imbalance may lead to increased thrombosis and an increased cardiovascular risk. Due to these associated cardiovascular risks, one of the most widely used COX-2 selective inhibitors, rofecoxib, was withdrawn from the market in September 2004 (Jüni et al., 2004).

1.5. Glucocorticoids

1.5.1. Glucocorticoid structure and synthesis

The HPA axis is a complex system of endocrine interactions between the hypothalamus, pituitary and adrenal glands. The main role of the HPA axis is to maintain homeostasis under conditions of stress. CRH, released from the neurons of the paraventricular nucleus of the hypothalamus, regulates the HPA axis and stimulates ACTH secretion (Abel & Majzoub, 2005). Glucocorticoids are released from the adrenal cortex in response to ACTH stimulation (Chiras, 20011). Cortisol is the primary endogenous glucocorticoid in humans, while in rodents the main glucocorticoid is corticosterone (Kirschbaum et al., 1992). Glucocorticoids are made up of an initial four-ring cholesterol structure, as are all steroids (Feldman, 1992). The structures of the major endogenous (cortisol) and synthetic (dexamethasone) glucocorticoids are shown in Figure 1.8. Dexamethasone is a synthetic glucocorticoid, with potent anti-inflammatory and immunosuppressive effects, thus it has widespread clinical applications (Ong et al., 2006). Glucocorticoids play a role in critical processes, such as growth, reproduction, immune and inflammatory processes (Nicolaides et al., 2010) and the regulation of energy metabolism (Vegiopoulos & Herzig, 2007).

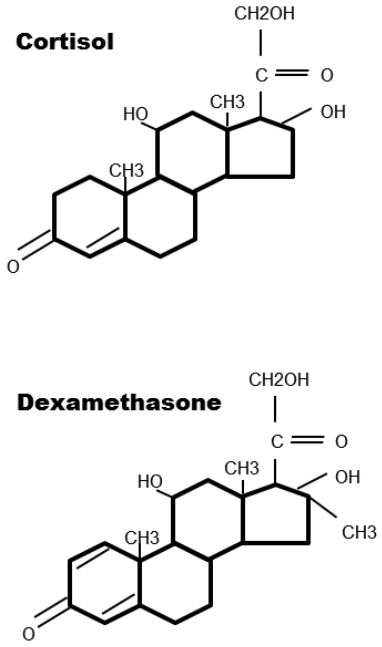

Figure 1.8- Diagram showing the chemical structure of two common glucocorticoids

1.5.2. Glucocorticoid receptor (GR)

The actions of glucocorticoids are mediated via the intracellular GR. The GR is expressed in most tissues; however tissue-specific variation in expression levels is evident (Kalinyak et al., 1987). The human GR gene sequence was isolated, via nucleotide sequence analysis of cDNA clones in 1985 (Hollenberg et al., 1985). The GR gene is located on chromosome 5 and consists of 9 exons (Rhen & Cidlowski, 2005), with exon 9 having two highly homologous isoforms, α and β (Nicolaides et al., 2010). The GR has a modular structure, comprising of four domains, the A/B region which is the N-terminal domain and the C, D and E regions, which correspond to the DNA-binding domains, the hinge region and the ligand binding domain respectively. The unbound GR exists as a cystolic multiprotein complexed with several Hsps, including Hsp90, Hsp70 and Hsp40 (Stahn et al., 2007). During activation, the glucocorticoid receptor forms a complex with a glucocorticoid molecule and translocates to the nucleus where it binds to the regulatory region of the gene. Once activated, the GR undergoes conformational changes and dissociates from the Hsp molecules (Almawi & Melemedjian, 2002). Activated GR can bind to specific GREs in the promoter region of the target gene, resulting in activation of gene transcription (Newton,

2000) and this process, commonly referred to as transactivation, is believed to be responsible for many of the effects of glucocorticoids. Activated GR can also interact, either directly or indirectly, with transcription factors involved in the regulation of pro-inflammatory gene expressions, such as NF-κB or AP-1.

1.5.3. Glucocorticoid functions

1.5.3.1.General

Glucocorticoids function primarily via the modulation of gene transcription and effects on translation and post-translational processes. They can affect the production of a wide variety of pro-inflammatory mediators, including cytokines, chemokines and adhesion molecules (Goulding & Flowers, 2001). The primary function of glucocorticoids involves the metabolism of glucose from non-carbohydrate sources, for example proteins within muscle tissue (Campbell & Reece, 2005); this is useful when the body requires more fuel than can be provided from normal glycogen stores. Glucocorticoids inhibit cellular degranulation, thus protecting against the release of pro-inflammatory cells, such as, granulocytes, mast cells and macrophages. Glucocorticoids also act to suppress components of the immune system, for example, they cause a decrease in normal B cell proliferation through decreased cytokine production and activate programmed cell death of T lymphocytes (Goodman, 2009). Glucocorticoids exert anti-inflammatory effects through inhibition of PLA2 production, a pre-cursor of arachidonic acid, leading to decreased prostaglandin production (Smith, 2009). Glucocorticoids are able to influence the expression of particular growth factors and growth hormones, thus allowing them to have a role in the formation of bone and cartilage (McMaster & Ray, 2008). Glucocorticoids have also been shown to have a regulatory role in vascular smooth muscle contractility (Kornel et al., 1993).

1.5.3.2.Glucocorticoids and the gastrointestinal tract

Controversy exists with regards to the effects of glucocorticoids on the gastrointestinal tract. High-dose steroid therapy has been associated with an increased risk of upper gastrointestinal complications in humans (Rodriguez &

Hernández-Diaz, 2001). Additionally, glucocorticoid administration to experimental rats' resulted in the formation of acute gastric erosions (Filaretova et al., 1998). The ulcerogenic effects of glucocorticoids are thought to occur due to increases in gastric acid and mucus production and gastrin and parietal cell hyperplasia (Schäcke et al., 2002). Glucocorticoid-induced gastric damage appears to be unrelated to inhibition of prostaglandin synthesis but may be related to inhibition of leukotriene synthesis (Wallace, 1987).

Contrastingly, endogenous glucocorticoids have been shown to provide protection to the gastric mucosa against injury. Acute increases in stress-induced plasma corticosterone levels appear to protect against the development of stress-induced ulcers (Filaretova et al., 1998). Glucocorticoids appear to exert gastroprotective effects via the modulation of mucosal blood flow (Filaretova et al., 1999) and mucus production and the inhibition of gastric motility and microvascular permeability (Filaretova et al., 2002). Endogenous glucocorticoids also provide protection against NSAID-induced gastric injury (Filaretova et al., 2001; Filaretova et al., 2002).

1.5.4. Clinical use

Due to their anti-inflammatory actions, glucocorticoids are routinely used to treat a variety of inflammatory conditions, such as IBD, bronchial asthma and glomerulonephritis (Waller et al., 2001) and replacement glucocorticoid therapy is standard practice for the treatment of adrenal insufficiency (Hahner & Allolio, 2009), during which inadequate amounts of steroid hormones are produced by the adrenal gland. Due to their suppressive effects on the immune system, glucocorticoids are often used as part of post-transplantation treatment to prevent allograft rejection and as a mainstay treatment for autoimmune disorders (Allison, 2000). Topical glucocorticoids are used in the treatment of a variety of skin disorders, such as psoriasis and dermatitis (Wiedersberg et al., 2008). Furthermore, glucocorticoids are often incorporated into cancer therapies, due to their ability to limit the growth of cancers associated with the lymphoid tissue and blood (Dawson et al., 2002).

1.5.5. Adverse effects associated with glucocorticoid use

Glucocorticoid therapy is associated with various adverse effects, including delayed wound healing (Schäcke et al., 2002), increases in gastric acid secretion during longer-term treatment (Cushman, 1970) and gastric ulceration (Smith, 2009). Children prescribed long-term glucocorticoid therapy may exhibit signs of retarded growth and delayed puberty (Schäcke et al., 2002). Continued use of glucocorticoid therapy can also lead to the development of glaucoma or cataracts (Smith, 2009) and an increased risk of developing osteoporosis (Schäcke et al., 2002). As glucocorticoids work to suppress the immune system, an increased susceptibility to infections is also associated with glucocorticoid therapy (Feldman, 1992).

1.6. Helicobacter spp.

1.6.1. Species characteristics

Helicobacter was first described in 1984, by Marshall & Warren, who demonstrated the presence of unidentified curved bacilli in the gastric epithelium of 58 patients with active chronic gastritis or ulceration. Initially, the bacterium was classified as a new species in the *Campylobacter* genus; however, in 1989 the new genus '*Helicobacter*' was established (Goodwin et al., 1989). Since classification, the *Helicobacter* genus now includes 18 different species (Table 1.1).

Helicobacter spp. are gram-negative, spiral-shaped, actively motile bacteria with flagella that colonize the gastrointestinal tract of both humans and animals (De Bock et al., 2006). *Helicobacter* spp. are urease positive, enabling them to survive the highly acidic environment in the stomach (Kusters et al., 2006). *H. pylori* has complex growth requirements and is therefore often hard to culture. It requires specialised growth medium, supplemented with blood or serum, and a microaerophilic environment, with optimal growth at 2-5% O_2/5-10% CO_2 and high humidity levels (Kusters et al., 2006). In older cultures, *H. pylori* tends to transform from its normal helical bacillary morphology into a coccoid form (Owen, 1998).

Species	Major Host
H. pylori	Human
H. canis	Dog, Human
H. felis	Cat, Dog, Human
H. heilmannii	Cat, Dog, Human
H. bizzozeronii	Dog
H. fennelliae	Human
H. pullorum	Poultry, Human
H. pametensis	Wild birds, Pig
H. cholecystus	Hamster
H. hepaticus	Mice
H. muridarum	Rat, Mice
H. trogontum	Rat
H. bilis	Mice, Dog
Flexispira rappini	Dog, Pig, Sheep
H. cinaedi	Human, Hamster
H. acinonyx	Cheetah
H. mustelae	Ferret
H. nemestrinae	Macaque monkey
H. suis	Pig

Table 1.1- The *Helicobacter* species and their major hosts

1.6.2. Clinical importance and pathogenicity

Nearly half of the world population are estimated to be infected with *Helicobacter pylori*, however many patients remain asymptomatic (Go, 2002). *Helicobacter* is thought to be transmitted via person-person contact, as clustering of infection has been found within families (Drumm et al., 1990) and institutionalised young people (Laporte et al., 2004). *H. pylori* organisms colonise the gastric epithelium and rarely penetrate into cells, tending to cluster around intracellular junctions (Peterson, 1991). More recently, intracellular localisation of *Helicobacter* spp. has been described in the parietal cells and macrophages of laboratory Beagle dogs (Lanzoni et al., 2011). Gastric colonisation with *H. pylori* stimulates cytokine secretion from gastric epithelial cells, leading to activation of immune and inflammatory cells and the development of chronic, active gastritis (Ernst & Gold, 2000), which can ultimately lead to gastric ulceration. In cases of acute gastritis,

H. pylori infection leads to the degeneration of surface epithelial cells, resulting in decreased mucin secretion and an infiltration of neutrophils. In chronic cases of gastritis, as well as epithelial degeneration and neutrophil infiltration, an influx of lymphocytes and plasma cells leads to chronic inflammation (Dixon, 1995), however, even when gastritis is present, only a minority of patients go on to develop clinical signs of disease (Kusters et al., 2006). Acute *Helicobacter* infections result in antral inflammation and hypochlorhydria, thought to be caused by inhibition of parietal cell function. *H. pylori* infection has been shown to inhibit H^+/K^+ATPase α-subunit gene expression in AGS cells (Göoz et al., 2000). *H. pylori* associated gastritis may also result in gastric acid hypersecretion, through the stimulation of gastrin release (McColl et al., 1998), thus patients colonised with *H. pylori* may present with either increased or decreased acid secretion (Schubert & Peura, 2008).

In 0.5-2% of *H. pylori* infections, patients go on to develop gastric cancer (Fritz & Van der Merwe, 2009) and both gastric lymphoma and adenocarcinomas are associated with *H. pylori* infection. Thus in 1994, *H. pylori* was classified as a type I carcinogen in humans by the International Agency for Research on Cancer (IARC, 1994). Clinical outcomes of *H. pylori* infection are mainly determined by the virulence factor of the bacterial strain and the most widely studied *H. pylori* virulence factor is CagA, encoded for by the *cagA* gene. Infection with strains of *H. pylori* carrying the *cagA* gene is associated with the development of gastric carcinoma (Hatakeyama, 2004). Pathogenic strains of *H. pylori* may also produce the vacuolating cytotoxin, VacA, encoded for by the *vacA* gene (Wada et al., 2004), which has been shown to cause cytoplasmic vacuolation in MKN-28 cells, (Ricci et al., 2002) and in primary human gastric epithelial cells (Smoot et al., 1996).

1.6.3. Helicobacter *spp. found in dogs*

Several species of *Helicobacter* are capable of infecting dogs, for instance, *H. felis, H. bizzozeronii, H. salmonis, H. bilis, H. heilmannii* and *Flexispira rappini* have all been cultivated from canine stomachs (Eaton et al., 1996; Jalava et al., 1998; Neiger et al., 1999). However, *H. pylori* has not been found in dogs (Neiger & Simpson, 2000), thus suggesting that dogs may not present a zoonotic risk for

this common strain of the bacteria. Previous studies have shown that living conditions can impact on the prevalence of *Helicobacter* infection, as dogs housed in animal shelters or bred in laboratory colonies were shown to have a higher prevalence of *Helicobacter* infection than pet dogs (Eaton et al., 1996). *Helicobacter* spp. are highly prevalent in both healthy and unwell dogs, suggesting that there is not a clear link between the infection and gastric disease in dogs (Simpson et al., 2000). *Helicobacter* infected dogs usually present with mononuclear gastritis of mild to moderate severity and no association between *Helicobacter* infection and gastric ulceration or neoplasia in dogs has been made to date (Simpson et al., 2000).

1.6.4. Helicobacter *infection and NSAID treatment*

As *Helicobacter* infected patients can remain asymptomatic, clinicians may unknowingly treat them for other conditions using NSAID therapy. Individually, both NSAIDs and *Helicobacter* infection are major causes of gastrointestinal disease, however, little is known about their potential interaction. In a study looking at long-term NSAID users, the risk of peptic-ulcer disease was significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients (Huang et al., 2002), suggesting a possible interaction between the two. Several studies have shown that *H. pylori* infection leads to increased COX-2 expression and PGE_2 synthesis (Fu et al., 1999; Romano et al., 1998; Chan et al., 2001) that is reduced following eradication (McCarthy et al., 1999). COX-2 expression was found to be localised at higher levels in the gastric antrum where *H. pylori* density is greatest (Fu et al., 1999). COX-1 expression also appears to be higher in biopsies from *H. pylori*-positive patients compared to non-infected patients (Franco et al., 1999). As *H. pylori* infection and NSAID therapy have opposing effects on PGE_2 production, an interaction between the two is likely and warrants further study.

*1.6.5.*Helicobacter *infection and cell migration*

In addition to causing direct gastric mucosal damage, colonisation with *H. pylori* may lead to changes in the modulation of gastric epithelial cell migration, and a number of different mechanisms for this effect have been studied. Focal adhesions

and the actin cytoskeleton both have a major role in cell migration, for instance, focal adhesion assembly and disassembly helps to coordinate cell movement across the ECM and actin polymerisation is essential for cellular protrusion and contraction (Ridley et al., 2003). *H. pylori*-infected gastric epithelial cells typically appear elongated, which may be caused by integrin/CagA signalling leading to stabilisation of focal adhesions at the rear of the cell (Wessler et al., 2011). *H. pylori* has also been shown to stimulate host cell motility via CagA interactions with SHP2, a protein-tyrosine phosphatase, and PAR1, a polarity-regulating kinase (Kikuchi et al., 2012). Furthermore, *H. pylori* VacA has been shown to inhibit re-epithelisation, possibly though the alteration of cytoskeleton associated proteins (Pai et al., 2000), while *H. pylori* adhesion to gastric epithelial cells has been associated with disruption of epithelial adhesion molecules, including a reduction in adherens junctions (Conlin et al., 2004) and induction of MMP-7 expression (Wroblewski et al., 2003).

1.7. Aims of the thesis

The overall aim of this thesis was to establish a suitable primary cell culture model to investigate the effects of COX antagonism on canine gastric epithelial cell migration and to highlight the specific signalling pathways that are involved.

The following key aspects were investigated:

- The effects of both non-selective and COX-2 selective antagonism on the modulation of epithelial cell migration.
- The effects of physiological and therapeutic glucocorticoid levels on the modulation of epithelial cell migration.
- The characterisation of COX-2 and EP receptor expression in the canine gastric epithelium.
- The characterisation of EP receptor subtypes involved in the modulation of epithelial cell migration.
- The relationship between COX-2, EP3 and EP4 protein expression and various clinical parameters, in order to determine their potential relevance as objective markers of GI disease.

Chapter 2 - Materials and Methods

2.1 Materials

2.1.1. General materials

All items were obtained from Sigma-Aldrich Company Ltd., The Old Brickyard, Dorset, SP8 4XT unless specified below:

Item	Supplier
17-Phenyl-trinor-PGE_2 (EP1 agonist)	Santa Cruz Biotechnology, Inc. Bergheimer Str. 89-2, 69115 Heidelberg, Germany.
AH 23848 (EP4 antagonist)	Santa Cruz Biotechnology, Inc. As above
Butaprost (EP2 agonist)	Santa Cruz Biotechnology, Inc. As above
Collagenase A	Roche Products Ltd. (Pharmaceuticals), Welwyn Garden City, AL7 1TW
Dexamethasone	Calbiochem-Novabiochem (UK) Ltd. c/o CN Biosciences (UK) Ltd, Beeston, NG9 2JR
DPX mounting medium	VWR International Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN
HBSS	Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF
ImProm II Reverse Transcription kit	Promega UK Branch Office, Southampton Science Park, SO16 7NS
Indomethacin	Calbiochem-Novabiochem (UK) Ltd. As above
Mastermix (2.5mM $MgCl_2$)	Abgene, Thermo Fisher Scientific UK Ltd, Loughborough, LE11 5RG
Novex® Sharp pre-stained protein standard	Life Technologies Ltd. As above
NS-398	Calbiochem-Novabiochem (UK) Ltd. As above
NuPage® 4-12% gradient gels	Life Technologies Ltd. As above
NuPage® MES SDS running	Life Technologies Ltd. As above

buffer (20X)	
NuPage® transfer buffer (20X)	Life Technologies Ltd. As above
ONO-AE1-329 (EP4 agonist)	A kind gift from ONO Pharmaceutical Co. Ltd. Osaka, Japan
Paraformaldehyde 16%	Thermo Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, LE11 5RG
Parameter™ PGE_2 kit	R&D systems, 19 Barton Lane, Abingdon Science Park, OX14 3NB
Perhydrol 30%	Thermo Fisher Scientific UK Ltd. As above
Pierce BCA protein assay kit	Thermo Fisher Scientific UK Ltd. As above
Propidium iodide	Calbiochem-Novabiochem (UK) Ltd. As above
QIAquick® PCR purification kit	Qiagen House, West Sussex, RH10 9NQ.
RNeasy® Plus Mini kit	Qiagen House. As above
siGENOME non-targeting siRNA controls	Thermo Fisher Scientific, Dharmacon Products, 2650 Crescent Drive, Suite 100 Lafayette, CO 80026, USA
SilenceMag™	OZ Biosciences, Parc scientifique de Luminy, Zone Luminy Entreprise, 163 avenue de Luminy - Case 922, 13288 Marseille cedex 9, France
siRNA buffer	Thermo Fisher Scientific, Dharmacon Products. As above
Sulprostone (EP3 agonist)	Santa Cruz Biotechnology, Inc. As above
TrackIt™ 100bp DNA ladder	Life Technologies Ltd. As above
VectaShield® mounting medium	Vector Laboratories Ltd. 3 Accent Park, Bakewell Road, Peterborough, PE2 6XS
Vectorstain® ABC kit	Vector Laboratories Ltd. As above
Western Lightning® Plus ECL solution	PerkinElmer, Chalfont Road, Buckinghamshire, Seer Green, HP9 2FX

Table 2.1- General materials and suppliers

2.1.2. Antibodies

Goat polyclonal anti-EP3, goat polyclonal anti-EP4 and goat polyclonal anti-COX-2 were all supplied by Santa Cruz Biotechnology, Inc. Bergheimer Str. 89-2, 69115 Heidelberg, Germany. An HRP-conjugated rabbit anti-goat IgG secondary and a goat polyclonal anti-beta actin were supplied by Abcam®, Cambridge, UK. A FITC-AffiniPure donkey anti-goat IgG secondary was supplied by Stratech Scientific, Oaks Drive, Suffolk CB8 7SY. A biotinylated horse anti-goat IgG secondary was supplied by Vector Laboratories Ltd. 3 Accent Park, Bakewell Road, Peterborough, PE2 6XS.

2.2. Media and buffers

Solution	Recipe
Amphotericin B	100 mg added to 50 ml ultra-pure water
Cell freezing medium	7 ml of DMEM/EMEM (without FBS added), 2 ml FBS, 1 ml DMSO
DMEM full medium	DMEM (as bought) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 0.1% amphotericin B and 0.1% gentamycin
EMEM full medium	100 ml 10X EMEM, 100 ml FBS, 10 ml non-essential amino acids, 10 ml NaHCO3, 10 ml penicillin-streptomycin, 10 ml L-glutamine, 1ml amphotericin B, made up to 1 L using ultra-pure water
Immunocytochemistry- blocking buffer	1X PBS, 10% donkey serum, 0.1% Triton™ X-100
Immunohistochemistry – Imidazole/HCl buffer	6.81 g imidazole made up to 1 L using distilled water, 500 ml 0.1M HCl added. pH adjusted to 7.1 using 0.1M HCl.
L-glutamine	2.92 g added to 100 ml ultra-pure water
4% Paraformaldehyde	10 ml Paraformaldehyde (16%) in 30 ml 1X PBS

PBS (10X)	100 tablets dissolved in 1 L ultra-pure water
PBS (1X)	88 ml 10X PBS added to 880 ml ultra-pure water
PBS/EDTA	0.18 g EDTA added to 900 ml 1X PBS
PBS/EDTA/Trypsin	1 ml of trypsin added to 50 ml PBS/EDTA
RNase buffer	1X PBS, 1% BSA, 10 µg/ml RNase solution
Stock TAE buffer (50X)	242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, made up to 1 L in double distilled water
TAE buffer (1X)	40 ml 50X TAE buffer and 1960 ml double distilled water
1X Tris buffered saline	0.8% saline prepared; 8 g NaCl made up to 1 L in distilled water. 100ml stock added to 850 ml saline and pH adjusted to 7.4 with 10M HCl. Volume adjusted to 1000 ml with 0.8% saline.
Western blot- cell lysis buffer	1.97 g Trizma HCl, 20 ml glycerol solution, 4 g (2%) SDS
Western blot- running buffer	25 ml NuPAGE® MES SDS buffer (20X) and 475 ml double distilled water
Western blot- sample buffer	62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue
Western blot- stripping buffer	15 g glycine, 1g SDS, 10 ml Tween® 20 made up to 1 L in double distilled water and pH adjusted to 2.2
Western blot- transfer buffer	50 ml NuPage™ transfer buffer (20X), 200 ml methanol, 750 ml double distilled water
Western blot- washing buffer	1X PBS, 0.1% Tween® 20
Western blot-blocking buffer	1X PBS, 5% milk powder, 0.1% Tween® 20

Table 2.2- Recipes for cell culture solutions and buffers

2.3. Canine endoscopy

Endoscopy samples were collected from patients presenting at the University of Liverpool Small Animal Teaching Hospital for vomiting, diarrhoea or weight loss. Following diagnostic evaluation, if an endoscopy was required, this was performed under general anaesthesia, using either an Olympus SIF O260 9mm enteroscope or an Olympus GIF-XQ240 9mm Gastroscope using 2.2mm disposable biopsy forceps. Biopsies were collected from the body of the stomach and were placed immediately in ice-cold HBSS solution. Owners of participating animals were provided with an information sheet prior to attending the clinic and animals were only included in the study where signed consent was provided. Procedures were scrutinised and approved by the University of Liverpool Ethics Committee.

2.4. Isolation of gastric glands

Tissue samples were obtained from either canine cadavers sourced from a local RSPCA centre or endoscopic biopsies sourced from patients undergoing routine upper GI endoscopy (Section 2.3). In the case of cadaver samples, a section of 4 cm^2 mucosal tissue was dissected from the stomach wall and placed in ice-cold HBSS prior to preparation. Biopsy samples were approximately 3 mm^2 and were treated in the same way. The samples were minced in a small volume of HBSS using a scalpel blade, to increase the exposed surface area. Samples were incubated with 1 mM DTT, for 15 min in a 37ºC water bath, shaking at 100 revs/min and provided with 95% O_2/5% CO_2. After washing, an HBSS solution containing 0.5 mg/ml collagenase A was added, and cells were incubated for a further 60 min under the same conditions, to create a suspension of disaggregated individual gastric glands. The preparation was triturated (aspirated and expressed through a Pasteur pipette) to enhance gland separation through shearing, and then left to stand for 45 sec, during which larger undigested tissue fragments sedimented. The supernatant was then removed and the isolated glands were sedimented out by incubation for 30 min on ice. The resultant supernatant, containing single cells and debris, was discarded and the isolated glands were re-suspended in full medium (Section 2.5.1).

2.5. Cell culture

2.5.1.Cell culture of isolated glands

Isolated glands were cultured in DMEM medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, 0.1% amphotericin B and 0.1% gentamycin. When plated into multi-well dishes, each well also contained the appropriate treatment (details of treatments used are described in the corresponding chapters). The cells were then incubated at 37ºC in a 5% CO_2 incubator for 48 h prior to analysis (glands derived from cadaver samples were incubated for 96 h prior to analysis).

2.5.2.Cell culture of immortalised cell lines

Primary cultures of AGS, MKN-45 and MDCK cells were maintained throughout the project. A stock of MDCK cells, at passage 6, was purchased from European Collection of Cell Cultures (ECACC, Porton Down, UK) and MDCK cells were used at passage number 6-22 throughout the project. AGS and MKN-45 cells, a kind gift from Andrea Varro (University of Liverpool), were used at passage number 40-65. Cells were grown to a 70% subconfluent monolayer in EMEM medium, supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% non-essential amino acids and 0.1% amphotericin B. Cells were maintained at 37ºC in a 5% CO_2 incubator, medium was changed twice weekly and the cells were sub-cultured when confluent at either 1:3 or 1:10 using a 1 ml trypsin/PBS/EDTA solution.

2.5.3.Cryopreservation of cells

Cell lines were cryopreserved for future use. Cell monolayers were trypsinised to obtain a cell suspension; the cells were then pelleted by centrifugation at 1400 rpm for 4 min and re-suspended in 1 ml of pre-warmed freezing medium (consisting of standard medium supplemented with 20% FBS and 10% DMSO) in a 1.5 ml cryovial. The cell suspension was frozen slowly in a polystyrene box at -80ºC before being transferred to liquid nitrogen for long-term storage.

2.6. Cell spreading assay

2.6.1.Treatments

Isolated glands were plated into 6-well plates and treated with either medium containing vehicle only (controls) or the relevant treatment. Treated glands were incubated at 37ºC in a 5% CO_2 incubator for either 48 h (biopsy-derived glands) or 96 h (cadaver-derived glands) prior to analysis, after which time they had adhered to the plate and spread out to form individual cell islands.

2.6.2.Cell fixation and propidium iodide Staining

Propidium iodide was used to stain individual cell nuclei to enable total cell counts to be performed. After 48 h in culture, the cell islands were fixed using a 4% paraformaldehyde solution and the cell membranes were permeabilised with a 0.2% solution of Triton™ X-100 for 30 min at room temperature. The cells were then incubated in PBS containing 1% BSA and 10 µg/ml RNase solution for 1 h at room temperature to eliminate background RNA staining. Finally the cells were incubated with a 2.5 µg/ml propidium iodide solution for 20 min at room temperature. The cells were washed three times using PBS in between all steps.

2.6.3.Analysis of epithelial cell island spreading

Cells migrate from individual glands to produce islands of cells and speed of cell migration is reflected in the rate of spreading of individual islands. Images were captured using a 20X objective on an Olympus CK40-SLP microscope (Olympus, Southend-on-Sea, UK), equipped with a cooled 3 megapixel CCD camera (Progres® C3, Jenoptik, Germany) and stored as 'TIFF' files for analysis using ImageJ software (Rasband, 1997-2012). The observer was blinded to the treatment used in individual wells. The total area of individual cell islands was measured and the total number of cells within each island was counted using fluorescence microscopy. The area encompassed by the cell nuclei was also measured, and subtracting this area from the total spread area provided a measurement of fringe area (Figure 2.1). The fringe area gives an approximation

of the protrusive activity of the cell island and can be corrected for by counting the cell nuclei surrounding the outside edge of the island, i.e. those cells which are contributing to the protrusive activity. Further details about the parameters used for analysis are provided in chapter 3 of this thesis.

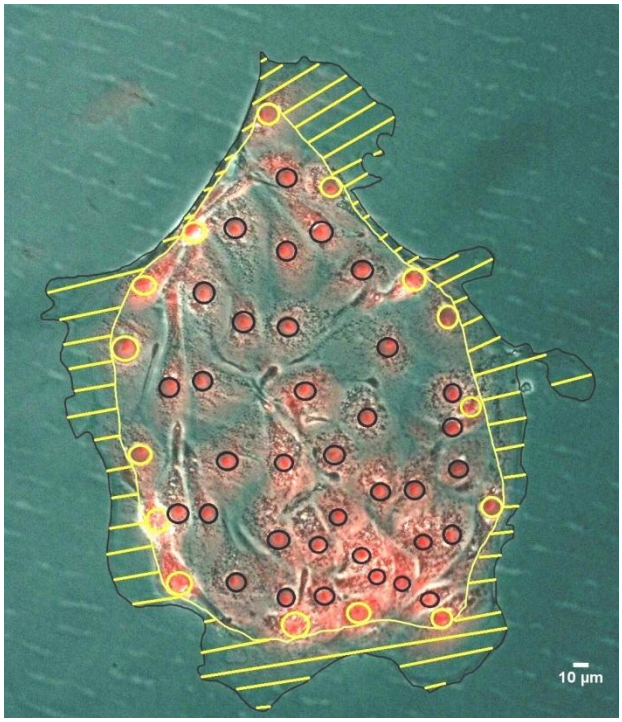

Figure 2.1- Analysis of cell island spreading using ImageJ. The black line delineates the total spread area, the yellow line delineates the area measurement around the nuclei, the yellow hatched area represents the fringe area and the yellow circles represent the edge nuclei. Scale bar: 10 µm

2.7. Scratch wound assay

Cells used for scratch wound experiments were grown to confluence, then serum-starved for 12 h prior to scratch wounding. Wounding was performed manually using a suitable-sized sterile filter pipette tip. Briefly, the pipette tip was scratched along the middle of each well to create a wound and dislodged cells were removed with two PBS washes. Wound sizes were found to be fairly consistent, with an approximate width of 1 mm. Following wounding, the cell medium was replaced, either with or without treatments, and the wound was imaged straight after wounding and at 24 h post-wounding. Two wounded areas were analysed for each well and the image position for a given region of wound was ensured by

positioning using a motorised stage. Total migration areas were calculated and normalised to control.

Specific time-course and dose-response experiments were performed using an Essen Bioscience Incucyte™ Live-Cell Imaging System (Essen BioScience, Inc., Welwyn Garden City, UK), allowing time-lapse image series to be gathered. A 96-pin scratch generator (WoundMaker®, Essen BioScience, Inc., Welwyn Garden City, UK) was used to create precise, reproducible wounds in a 96-well plate containing confluent monolayers of cells. Dislodged cells were removed through washing with PBS and the cell medium was replaced, as before. Wound healing was analysed via the IncuCyte™ software package, using three separate metrics. These metrics were wound width, wound confluence and Relative Wound Density.

2.8. Molecular methods

2.8.1.RNA isolation

RNA was extracted from either whole tissue or cultured cells using a Qiagen RNeasy® Plus Mini kit as per manufacturer's instructions. Approximately 30 mg of tissue or $1x10^7$ cells were used for the procedure. All steps of the protocol were performed in an RNase-free environment and all reagents were diluted in RNase-free water. Genomic DNA was removed using a gDNA Eliminator spin column, provided in the kit. The extracted RNA was eluted with 30 µl RNase-free water and quantified using a spectrophotometer. RNA samples were stored at -20ºC.

2.8.2.Reverse transcription

RNA was transcribed into cDNA using an IMProm II™ Reverse Transcription kit, as per manufacturer's instructions. The kit included a positive control of kanamycin RNA and specific up-stream and down-stream primers to ensure the reverse transcription stage was successful. As a further control, sham cDNA samples were produced, into which no reverse transcriptase was added, thus showing that the sample does not contain any genomic DNA. Oligo (dT) primer

was used to initiate transcription at the poly-A 3' tail of the RNA. The following thermocycler conditions were used for the reverse transcription reaction: anneal at 25º C for 3 min, extend at 42º C for 1 h, inactivate reverse transcriptase at 70º C for 15 min and hold at 4ºC. The cDNA samples were stored at 4ºC or -20ºC for long-term storage.

2.8.3.Polymerase chain reaction (PCR)

Reverse transcription PCR was used to study the expression of COX-2 and the prostaglandin receptors, EP3 and EP4. Primers for EP3, EP4, and COX-2, that span the exon-exon boundaries, were designed and supplied by Eurofins MWG Operon (Ebersberg, Germany). GAPDH primers were designed for use as an internal control and also supplied by Eurofins MWG Operon (Ebersberg, Germany). Sequences for the designed primers are provided in Table 2.3, and all primers were used at a concentration of 12.5 pmol/µl. Primers were designed using Primer Blast (http://www.ncbi.nlm. nih.gov/tools/primer-blast/index.cgi), based on the predicted cDNA sequence from the canine genome (Table 2.4).

A volume of 2 µl cDNA for each PCR assay was added to 25 µl of Mastermix (2.5 mM $MgCl_{2)}$ with 4 µl of each primer pair and 15 µl molecular grade water, to give a final reaction volume of 50 µl. The thermocycling parameters used were, an initial denaturation step at 94ºC for 5 min, then 40 amplification cycles of denaturation at 94 ºC for 20 sec, annealing at 50ºC for 20 sec and extension at 72ºC for 1 min. The final extension stage was at 72ºC for 5 min and the reaction was held at 4ºC.

Primer Name	Primer Sequence (5'-3')	Product length (bp)
GAPDH forward	TCCATCTTCCAGGAGCGAGA	781
GAPDH reverse	CCTTGGAGGCCATGTAGACC	
EP3 forward	AAAATGATCTTCAATCAGACA	167
EP3 reverse	CCTTCTTCGAAAGTTTTGTCA	
EP4 forward	CCGCTCGTGGTACGGGTGTTC	275
EP4 reverse	GCCATGTCCGGCCACTCTCG	
COX-2 forward	AGGATTGGGCCATGGGGTGGA	552
COX-2 reverse	GGGTTGCCGGTGGCAGGAAT	

Table 2.3 Primer sequences

cDNA sequence	Accession number
Canine GAPDH	NM_001003142
Canine EP3	NM_001002958
Canine EP4	NM_001003054
Canine COX-2	NM_001003354

Table 2.4 Accession numbers for the predicted cDNA sequences from the canine genome

2.8.4.Gel electrophoresis

The samples were run on a 1.5% agarose gel containing 5 µl ethidium bromide (500 µg/ml) in 50 ml of 1X TAE buffer. For each assay, 12 µl of sample was loaded per well, consisting of 10 µl of each product mixed with 2 µl of loading buffer. A ready-to-load TrackIt™ 100bp DNA ladder was used to determine band sizes. The gel was run at 120 volts for 30-40 min in 1X TAE buffer and visualised under UV light.

2.8.5.Sequencing of PCR products

Where possible, PCR products were sequenced commercially (Eurofins MWG Operon, Ebersberg, Germany), using the same primers that were used for the PCR amplification. A QIAquick® PCR purification kit was used to purify the amplified products prior to sequencing.

2.8.6.siRNA Transfection

siRNAs directed against EP3, EP4 and COX-2 (Table 2.5) were designed using the Dharmacon siDesign® centre (http://www.dharmacon.com/designcenter/designcenterpage.aspx), and two commercially available siGENOME non-targeting siRNA controls were purchased. These controls were compared against the canine genome by the manufacturers using BLAST (www.ncbi.nlm.nih.gov/BLAST), to ensure that they lacked homology with any known canine sequence. Both the control and custom-designed siRNAs were re-suspended in 5X siRNA buffer (diluted in RNase-free water) to create a 1 µM stock solution. Magnetofection was used to deliver the siRNAs into cultured cells, grown to confluence in 12-well plates. The siRNAs were diluted in 100 µl serum-free medium per well, to yield a final siRNA concentration of 10 nM (as recommended by the manufacturer). SilenceMag™, a transfection reagent, was vortexed prior to use and 2 µl was added to each 100 µl of diluted siRNA and thoroughly mixed through vigorous pipetting. The resulting siRNA/SilenceMag™ solution was incubated at room temperature for 20 min, after which the solution was added, drop by drop, onto cells cultured in 900 µl serum-free medium, to give a final cell culture volume of 1000 µl per well. The cell culture plate was then placed onto a magnetic plate for 15 min at room temperature. After transfection, the cells were cultured for 3 h in serum-free medium to improve gene silencing, as per manufacturer's instructions, after which they were cultured in medium containing 10% FBS for 24 h. Serum-free medium was then added to the cells for 12 h prior to analysis in order to induce the expression of EP3, EP4 and COX-2.

siRNA Name	siRNA sense sequence
EP3	UUUAUCUGCUGCUAAGAAAUU
EP4	GCAAAGCAAUAGAGAAGAUUU
COX-2	CAAAAGAGAUUGUGGAAAAUU

Table 2.5- siRNA sequences

2.8.7.Western Blotting

2.8.7.1.Protein extraction

MDCK cells were grown to confluence in either a 25cm^3 flask or a 12-well plate. Once confluent, cells were washed with ice-cold PBS, the PBS was drained and the cells were trypsinised, and then centrifuged twice in EMEM media for 4 min at 1400 rpm to obtain a cell pellet. The cells were lysed by adding 250 µl of lysis buffer to the cell pellet. Lysates were then sonicated (Sonicor, Sonicor Instrument Corporation, USA) for 3 x 10 sec bursts to shear the DNA and reduce sample viscosity. Protein was extracted from primary cell islands using the same method, however due to lower cell densities only 50 µl of lysis buffer was used. Protein was also extracted from mucosal tissue samples stored at -80ºC. A 5 mg sample of tissue was homogenised in 300 µl lysis buffer using a Qiagen TissueLyser II (Qiagen House, West Sussex) set at 11 Hz for 5 min. The tissue and lysis buffer solution was then agitated constantly for 2 h at 4ºC, after which the solution was centrifuged at 12,000 rpm for 20 min at 4ºC. The resulting supernatant, containing the extracted protein, was sonicated as described above.

2.8.7.2.Determination of protein concentration

To determine the protein concentration of the lysates, a Pierce BCA protein assay kit was used. A series of BSA standards were made up and 25 µl of all standards and unknown protein samples were pipetted into a 96-well plate in duplicate. Into each well, 200 µl of working reagent was added and the plate was incubated at 37ºC for 30 min. The absorbance was then measured at 570 nm using a Multiskan® FC microplate reader (Thermo Fisher Scientific UK, Loughborough). The average absorbance measurements for the blank standard replicates were

subtracted from the absorbance measurements of each individual standard and unknown sample replicates. A standard curve was constructed by plotting the average blank-corrected absorbance measurement for each standard against its concentration in µg/ml. Protein concentrations of the unknown samples were determined using the standard curve.

2.8.7.3.Western blots

Sample solutions were prepared in sample buffer to give a total solution volume of 10 µl. Samples were then heated at 80ºC for 10 min and centrifuged for 10 sec at 12,000 rpm. Ready-cast NuPage™ 4-12% gradient gels were used for electrophoresis in an Xcell *SureLock*™ mini-gel tank (Life Technologies Ltd, Paisley, UK). The tank was filled with running buffer and 15 µl of each sample and Novex® Sharp pre-stained protein standard were loaded. The gel was run at 200 volts for 35 min using a PowerPac™ power supply (BioRad House, Hemel Hempstead, UK). Separated proteins were transferred from the gel onto a nitrocellulose membrane at 30 volts for 90 min using transfer buffer. Non-specific binding sites were blocked on the blotted membrane by incubation with the blocking buffer at 4ºC overnight. The primary antibody, diluted in the blocking buffer, was added to the membrane and incubated at room temperature for 2 h. The EP3, EP4 and COX-2 primary antibodies were all diluted to 1:200. An HRP-conjugated secondary antibody, diluted to 1:10,000, was used to detect the bound antibodies using a Western Lightning® Plus ECL solution. Membranes were visualised using a UVP ChemiDoc-It® imaging system (Ultra-Violet Products Ltd, Cambridge, UK).

2.8.7.4.Stripping and re-probing membrane

Membranes were stripped and re-probed for beta-actin as a loading control. Membranes were incubated with stripping buffer in a 50ºC pre-heated roller blot for 30 min. The membrane was then washed 6 times in a 0.1% PBS/Tween® 20 solution, for 5 min per wash. Following this, the membrane was re-probed for beta-actin, diluted to 1:1000, using the method described previously.

2.8.7.5.Intra-assay variability of Western blot analysis

Intra-assay variability was evaluated by running replicate samples in a single assay and calculating a value for the coefficient of variation (Figure 2.2). Coefficient of variation was calculated as the ratio of the standard deviation to the mean, multiplied by 100, which was 10%.

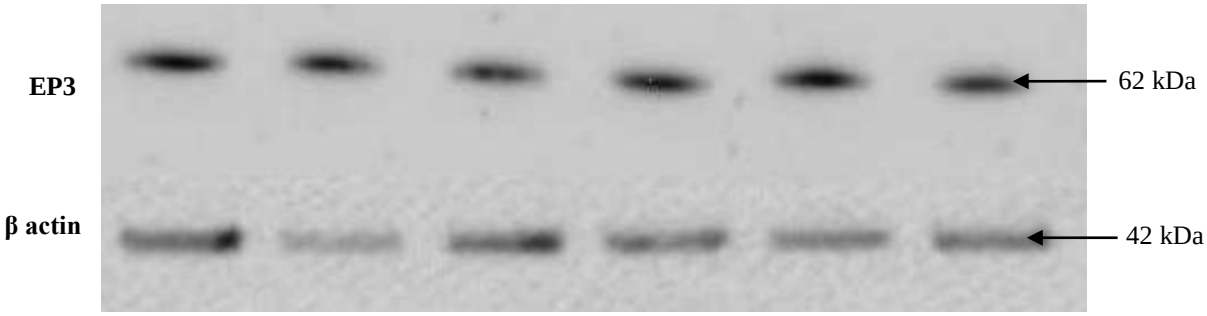

Figure 2.2- Western blot analysis of replicate samples; Six replicates containing equal amounts of MKN-45 protein were loaded and analysed for EP3 and beta actin expression. The EP3 band intensity was normalised to the corresponding beta actin intensity.

2.8.7.6.Dot blots

Protein extracts for dot blotting were prepared as described previously (Section 2.8.7.1) and the protein concentration of each sample was determined using the Pierce BCA protein assay (Section 2.8.7.2). 2 µl of each sample was dotted onto a nitrocellulose membrane, 1 cm apart. The membrane was allowed to dry for 30 min at room temperature, and then blocked by incubation with the blocking buffer at 4ºC overnight. The primary antibody, diluted in the blocking buffer, was added to the membrane and incubated at room temperature for 2 h. An HRP-conjugated secondary antibody was used to detect the bound antibodies using a Western Lightning® Plus ECL solution. Antibodies were diluted to the same concentrations as used for the western blot protocol (Section 2.8.7.3).

2.8.7.7.Densitometry

Densitometric quantification was performed using ImageJ software (Rasband, 1997-2012). The band intensity of the protein of interest was normalised to the corresponding beta actin band intensity.

2.9. Immunological Methods

2.9.1.Immunohistochemistry

Immunohistochemistry techniques were performed by Veterinary Pathology, University of Liverpool. Paraffin wax was dissolved in xylene and rehydrated in graded alcohols. Endogenous peroxidases were inactivated by 30 min incubation in freshly prepared 0.5% H_2O_2. Slides were washed with TBS (pH 7.4) and non-specific binding was blocked with horse serum for 10 min at room temperature. Slides were incubated with the primary antibodies (diluted in TBS to the appropriate concentration) overnight at 4°C. Slides were then washed with TBS and incubated with the secondary antibody (diluted in TBS) for 30 min at room temperature. Slides were washed again with TBS and incubated for 30 min with a Vectorstain® ABC solution (9 µl Reagent A, 9 µl reagent B in 100 µl TBS) (Vector Laboratories Ltd. Peterborough). Following a TBS wash, coverplates were removed and slides were incubated for 10 minutes with 3,3′-diaminobenzidine tetrahydrochloride (DAB) with 0.01% H_2O_2 in 0.1M imidazole buffer (pH 7.1) at room temperature. Slides were then washed three times with TBS and once with distilled water and counterstained using Papanicolaou's haematoxylin, dehydrated in ascending alcohols and cleared in xylene. Finally, the slides were mounted using DPX and a coverslip.

2.9.2. Immunocytochemistry

Cells were cultured on 4-well chamber slides (Lab-Tek II CC2 chamber slide system) and fixed in 4% paraformaldehyde. Cells were washed twice with PBS and incubated in blocking buffer for 30 min at room temperature. The PBS wash was repeated and the cells were incubated with the appropriate antibody for 2 h at room temperature. The primary antibodies were diluted to the appropriate concentration, as directed in the data sheets, using the blocking buffer as the antibody diluent. The cells were incubated with the conjugated secondary antibody at room temperature in a dark, moist atmosphere for 1 h, after which the chambers were removed and the slides were mounted using Vectashield® (to

minimise photobleaching) and coverslips. Stained cells were examined using the Olympus CK40-SLP microscope (Olympus, Southend-on-Sea, UK).

2.9.3.PGE_2 ELISA

PGE_2 ELISAs were performed using a Parameter™ PGE_2 kit. Cell culture supernatants were collected, aliquoted and stored at -20ºC prior to use. A set of PGE_2 standards were made, ranging in concentration from 2500 pg/ml to 39 pg/ml. The calibrator diluent was used as the zero standard (0 pg/ml). To each well, 150 µl of standard, control or sample was added followed by 50 µl of primary antibody solution. An additional set of wells, containing only 200 µl of calibrator diluent, were set up to detect non-specific binding. The plate was sealed and incubated for 1 h at room temperature on a microplate shaker (Grant Bio PMS-1000 microplate shaker, Thermo Fisher Scientific UK, Loughborough) set at 500 rpm. The PGE_2 conjugate was then added, the plate sealed and incubated for a further 2 h at room temperature on the shaker. Each well was aspirated and washed four times using the wash buffer provided in the kit. The plate was blotted dry against clean paper towels. Into each well, 200 µl of substrate solution was added and the plate was incubated for 30 min at room temperature in the dark. In order to terminate the reaction, 100 µl of the kits stop reagent was added to each well, causing a colour change from blue to yellow. The optical density of each well was read within 30 min, using a Multiskan® FC microplate reader (Thermo Fisher Scientific UK, Loughborough) set to 450 nm.

2.10. Statistics

Statistical analysis was performed as described in individual chapters, using Excel 2007 (Microsoft) and Stata Statistical Software: Release 11 (StataCorp. 2009) and the *xtmixed* command. Minitab® 16 Statistical Software (Minitab® Inc., 2010) was used to construct scatter plots and individual value plots for data analysis.

Chapter 3 - Characterisation of the model used for studying migration in primary gastric epithelial cell islands

3.1 Introduction

Gastric epithelial restitution (GER) has an important role in the maintenance of gastric epithelial integrity in response to injury (Lacy & Ito, 1984). GER is a complex process involving the coordination of various signalling cascades, for instance, signalling via EGF/TGF-α and activation of the EGF receptor and PI3K (Tétreault et al., 2008). Furthermore, dynamic changes to the actin cytoskeleton occur, including the initial formation of actin purse strings, followed by the formation of lamellae protrusions (Lotz et al., 2000). Local paracrine signalling pathways between multiple cell lineages present in gastric glands may also be important for the modulation of gastric epithelial migration. Several important paracrine mediators of gastric epithelial migration have been identified, in particular gastrin, a gastric hormone secreted by G cells. Gastrin has been shown to stimulate AGS cell migration *in vitro* via the paracrine release of EGF receptor ligands, leading to transactivation of the EGF receptor (Noble et al., 2003) and blockade of the EGF receptor using a monoclonal antibody leads to a reduction in cell migration (Kato et al., 1999).

Epithelial and mesenchymal cell interactions also play an important role in the paracrine regulation of gastric epithelial migration, for instance HGF, produced by gastric fibroblasts, has been shown to mediate prostaglandin effects on cell migration in a paracrine manner (Takahashi et al., 1996). HGF mRNA is not present in the gastric mucosa, thus suggesting that epithelial-mesenchymal interactions are essential for its effect (Tsuji et al., 1995). HGF has also been shown to induce activation of the COX-2 gene in gastric epithelial cells via phosphorylation of the *c-met*/HGF receptor and activation of the ERK2 signal transduction pathway (Jones et al., 1999).

Current models used for studying cell migration rely on the use of immortalised cell lines or suspensions of dissociated cells (Murakami et al., 1998; Pai et al.,

2001; Kim et al., 2012). To most accurately recapitulate the signalling mechanisms involved in *in vivo* gastric migratory processes, this investigation applied the use of a multicellular model. This model utilises individual isolated canine gastric glands consisting of a representative mixture of all cell types found *in vivo*, thus allowing the investigation of both local autocrine and paracrine signalling events. This type of model has previously been used to study *in vitro* gastric cell migration using glands isolated from rabbit (Berglindh & Öbrink, 1976), human (Wroblewski et al., 2003), mouse (Pagliocca et al., 2008) and rat (Azerkan et al., 2001) mucosal samples. Canine gastric mucosa has not been studied in this fashion, thus the aim of this chapter was to establish a reproducible model to enable the study of canine gastric epithelial cell migration.

3.2. Materials and methods

3.2.1.Sample collection

Samples were collected as described previously (Section 2.3 and Section 2.4). Canine gastric mucosal samples from both endoscopic biopsies and cadaver sources were used in this investigation.

3.2.2.Gastric gland isolation and culture

Intact gastric glands were isolated from mucosal tissue samples (Section 2.4) and cultured as described previously (Section 2.5.1).

3.2.3.Time-lapse experiments

Spreading of individual gastric glands was observed using the Essen Incucyte™ live-cell imaging system (Essen BioScience, Inc., Welwyn Garden City, UK), with images captured at 1 h intervals over a period of 54 h.

3.2.4.Cell spreading assays

Preliminary experiments were performed in order to better understand the process of cell spreading in our model and to determine the most appropriate parameters to use for measuring spreading. Cell spreading assays were performed as described previously (Section 2.6).

3.3. Results

3.3.1.Isolated gastric glands

The gastric glands isolated from mucosal tissue samples were intact and varied in length between approximately 0.2 mm and 0.9 mm (Figure 3.1), depending on the conditions of the protocol. However, once the protocol had been established, the majority of the isolated glands were nearer to 0.9 mm long.

In previous studies, collagenase digestion has been successfully used to isolate gastric glands from mucosal tissue samples (Wroblewski et al., 2003; Pagliocca et al., 2008; Berglindh & Öbrink, 1976). As described previously (Berglindh & Öbrink, 1976), tissue incubated with collagenase for a short period of time provided a low yield of intact gastric glands, due to incomplete tissue separation. However, longer incubation times resulted in suspensions of shorter gastric glands and areas of visible damage where single cells had dissociated from the gland (Figure 3.2), thus it was considered important to adhere to fairly stringent incubation times.

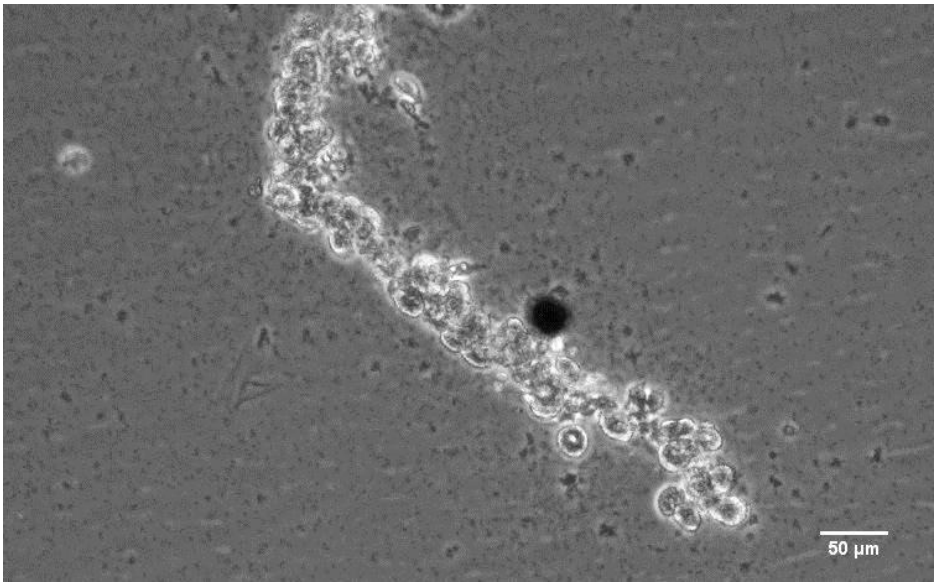

Figure 3.1- Photomicrograph of an intact viable gastric gland in culture.

Magnification of 20X; scale bar: 50µm

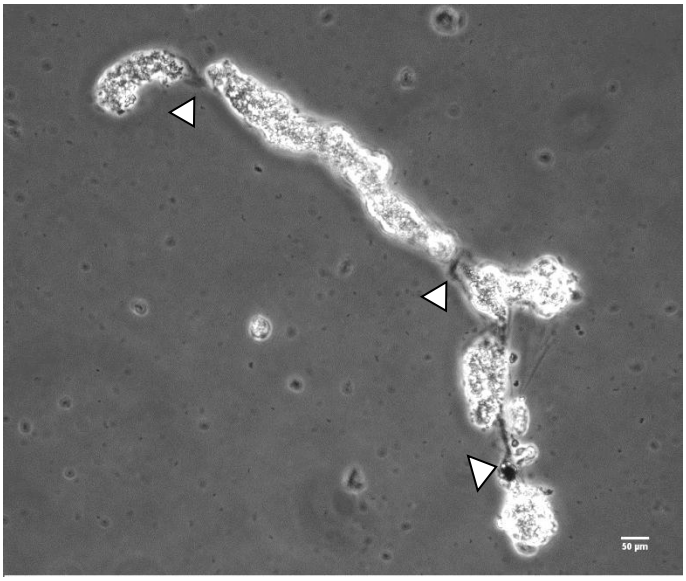

Figure 3.2- Photomicrograph of a damaged gastric gland in culture.

Damaged areas highlighted with arrowhead. Magnification of 20X; scale bar: 50µm

3.3.2.Time-lapse analysis characterising canine gastric gland cell spreading

Using time-lapse microscopy we were able to observe the sequence of events involved in the spreading of individual canine gastric glands. Isolated glands cultured on plasticware adhered to the substrate within 2 h and cells migrated out of the glands to form islands (Figure 3.3b). Once adhered, cells appear to flatten out and, after 12 h in culture, the cells located at the periphery of the island were seen to project active membrane protrusions (Figure 3.3d and Figure 3.4). Cell-cell contacts were maintained throughout the spreading process and cells fully spread to form monolayer cell islands after 48 h in culture (Figure 3.3g and Figure 3.5). A fragment of gastric gland tissue often remained at the centre of spreading islands (Figure 3.6), this has been described previously and confirms that the epithelial cells are migrating out of intact gastric glands (Smoot et al., 2000). The cell islands survived for up to 7 days in culture, after which they were seen to break apart and epithelial cells transformed into mesenchymal-like cells (Figure 3.7).

Frequent contaminations were initially observed in cell cultures isolated from cadaver-derived mucosal tissue (Figure 3.8). Through microbiological culture, the major contaminant was identified as a *Staphylococcus* species, although fungal organisms were also frequently identified. It was determined that this contamination was occurring during tissue collection, thus more stringent aseptic isolation practices were put in place, including the thorough disinfection of all dissection tools and surfaces in between sample collections. In addition, all cell cultures were supplemented with gentamycin, which is described as being more effective for the control of bacterial growth in culture than penicillin-streptomycin (Fischer, 1975). These control measures were effective at eliminating the contamination in subsequent cell cultures.

Differences in the spreading behaviour between gastric glands isolated from endoscopy- and cadaver-derived tissue were observed. Glands isolated from cadaver-derived tissue exhibited slower rates of spreading; with fully spread monolayer cell islands being achieved after 96 h in culture, compared with 48 h

for those derived from endoscopies. As a substantially greater number of glands were isolated from the larger volumes of cadaver-derived tissue, the effect of cell density on spreading rate was investigated. Cultures containing increasing numbers of glands were set up and observed, to identify differences in spreading speed. Although cultures containing lower numbers of cadaver-derived gastric glands exhibited quicker spreading speeds (data not presented), monolayer islands still took 96 h to fully form, and thus the differences observed are not explained by a cell culture ‘over-crowding’ effect.

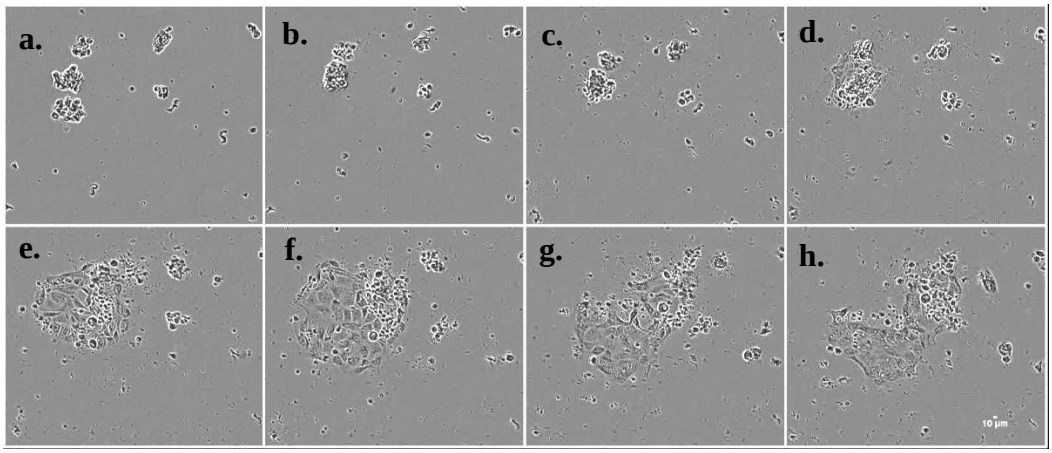

Figure 3.3- Time-lapse microscopy phase contrast images of an isolated canine gastric gland (derived from an endoscopic biopsy) spreading to form a cell island. (a.) 0 h,(b.) 2 h, (c.) 6 h, (d.) 12 h, (e.) 24 h, (f.) 36 h, (g.) 48 h, (h.) 54 h; magnification of 10X; scale bar: 10µm

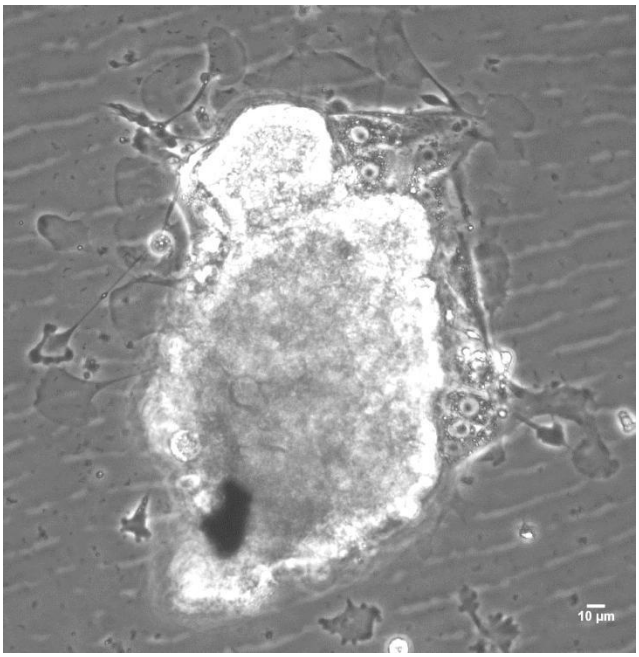

Figure 3.4- Photomicrograph of a cultured spreading gastric gland after 12 h in culture. Magnification of 40X; scale bar: 10µm

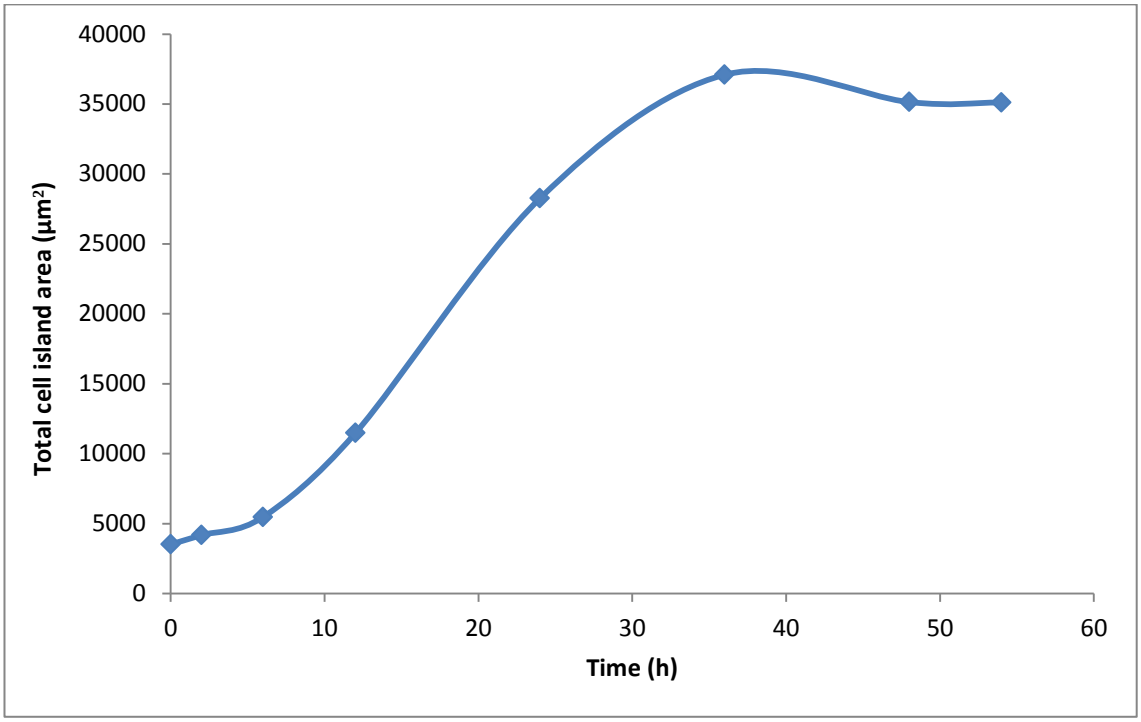

Figure 3.5- Quantification of biopsy-derived gastric gland spreading over 54 h. Data was derived from the time-lapse images presented in Figure 3.3.

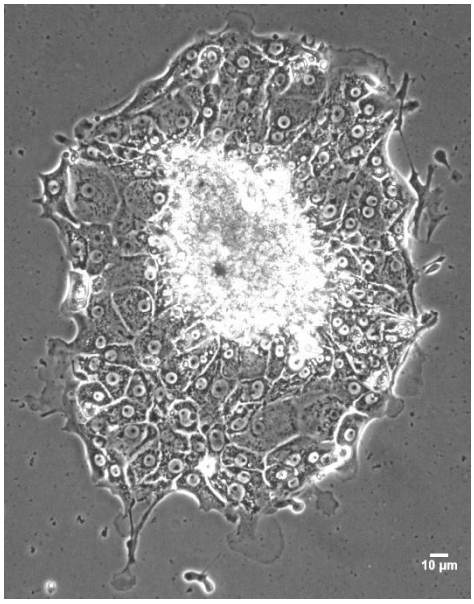

Figure 3.6- Photomicrograph showing a gland fragment present at the centre of a cultured spreading epithelial cell island. Magnification of 20X; scale bar: 10µm

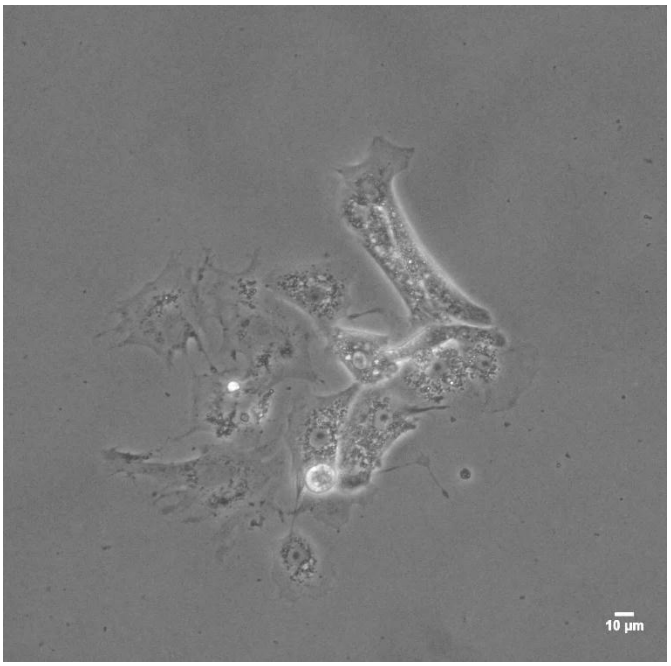

Figure 3.7- Photomicrograph showing cultured epithelial cells transforming into mesenchymal-like cells. Magnification of 20X; scale bar: 10µm

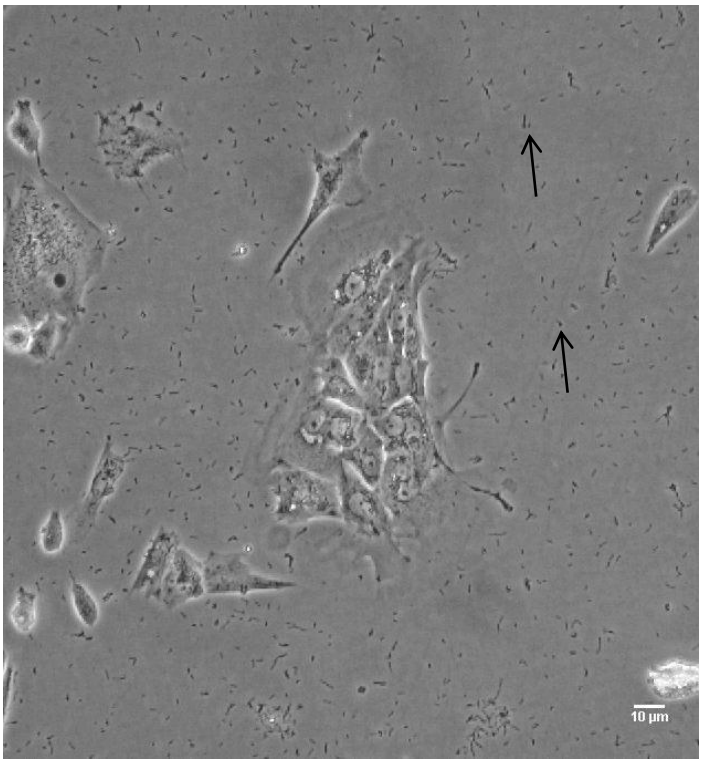

Figure 3.8- Photomicrograph showing bacterial contamination (seen as small black lines, highlighted by arrows) of cell culture. Magnification of 20X; scale bar: 10µm

Looking more closely at the cellular protrusions projected by cells at the periphery of the islands, there appears to be two distinct types of protrusion. The majority of cells project broad, thin, sheet-like protrusions which are characteristic of lamellipodia (Lauffenburger & Horwitz, 1996) (Figure 3.9a) and time-lapse images reveal that these protrusions are dynamic, constantly changing shape as the cells spread out. Certain cells also exhibit thin, needle-like protrusions, characteristic of filopodia (Lauffenburger & Horwitz, 1996) (Figure 3.9b).

Certain cells present within the epithelial islands exhibited a mottled appearance (Figure 3.10) and this is characteristic of parietal cells due to the presence of many mitochondria in the cytoplasm (Schultheis et al., 1998). There appeared to be many cells of this type within individual cell islands and they were present in the majority of islands. This is not suprising, given that parietal cells have been shown to constitute around 50% of the total cell volume of an isolated gastric gland (Berglindh & Öbrink, 1976). Cells adjacent to these appear to have a much smoother appearance (Figure 3.10). Spindle-shaped cells characteristic of fibroblasts (Murray et al., 2009) were also identified in culture (Figure 3.11).

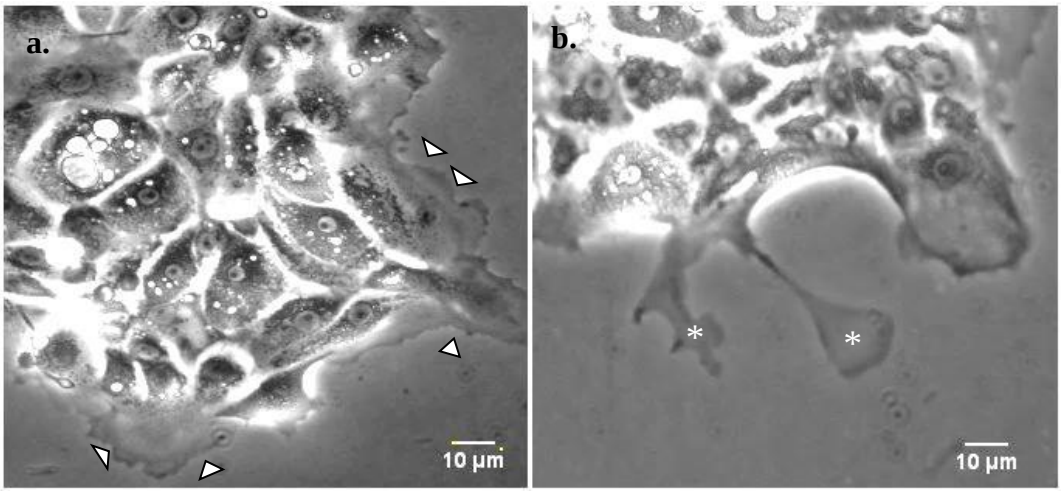

Figure 3.9- Cultured cell islands showing (a.) broad, sheet-like protrusions characteristic of lamellipodia (arrowhead) and (b.) finger-like protrusions characteristic of filopodia (*). Magnification of 40X; scale bar: 10µm

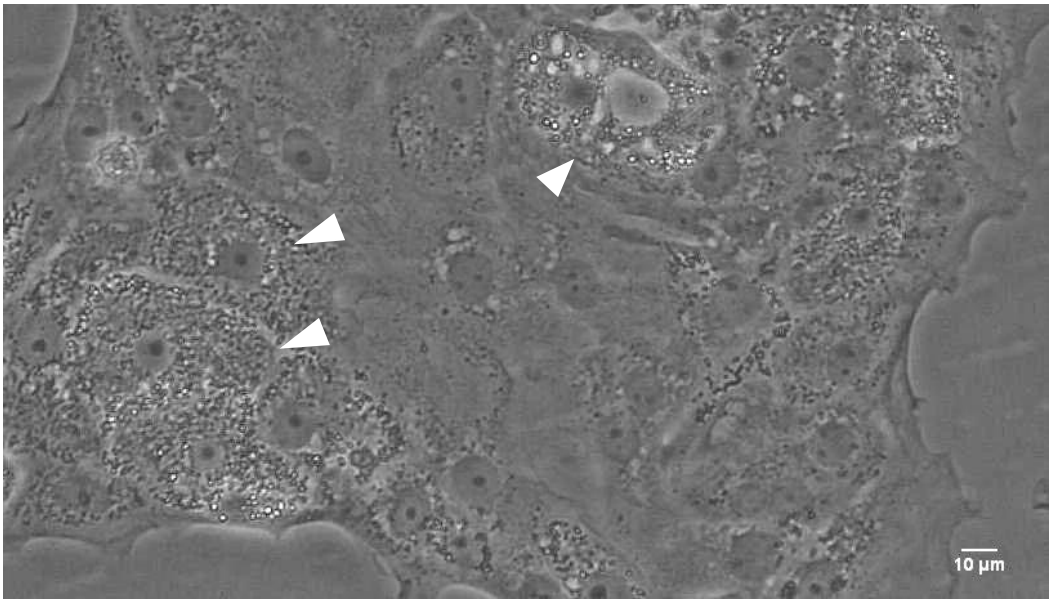

Figure 3.10- Representative photomicrograph highlighting the morphology of suspected parietal cells (arrowheads). Magnification of 40X; scale bar: 10µm

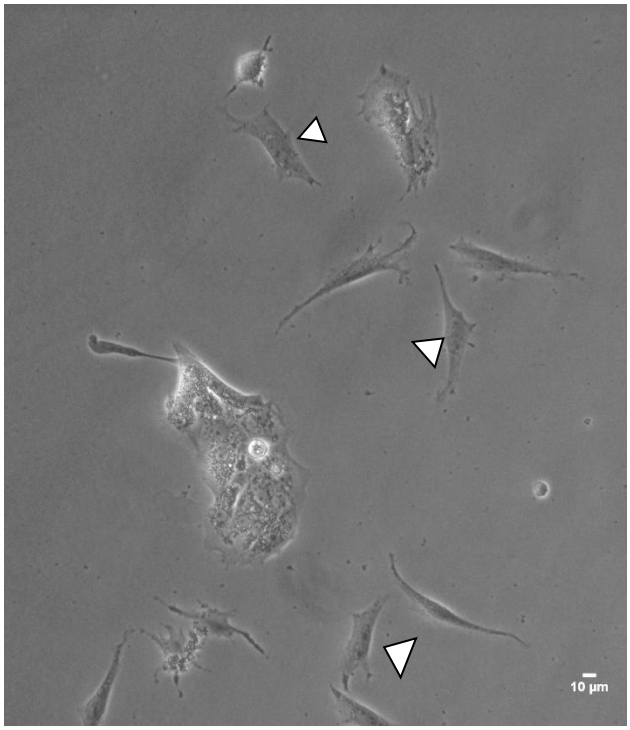

Figure 3.11- Representative photomicrograph highlighting the presence of fibroblasts in culture (arrowheads). Magnification of 20X; scale bar: 10µm

3.3.3.Parameters used to analyse cell spreading

In order to determine the most appropriate parameters for analysis of cell spreading in this model, preliminary spreading experiments were carried out using gastric epithelial cell islands cultured from canine endoscopic biopsy samples. In previous cell spreading studies, measurement of the area of individual cells has typically been used to represent migration speed (Arthur & Burridge, 2001; Flevaris et al., 2007; Sanders et al., 2009). In a study using a similar model to that described here, cell spreading was calculated as the mean cell island area, normalised for cell number (Wroblewski et al., 2003). When comparisons are made between the total spread area of individual cell islands, this value provides some indication of the speed of migration. Thus, to calculate cell spreading in this model, measurements were made of the total area encompassing each island after 48 h in culture. As individual cell islands are made up of varying cell numbers (ranging from approximately 2-90 cells per island), the relationship between total area and cell count was analysed. A positive linear association was found to exist between these parameters (Figure 3.12), thus the spreading behaviour of an individual cell can be represented by normalising the total island area for cell number.

The total spread area values for islands with cell counts > 60 were found to be smaller than expected, based on the linear relationship of the data (Figure 3.12– blue dots). Cell crowding in islands with larger cell counts may affect the spreading behaviour of cells and could explain this decrease in spreading activity. As shown visually in Figure 3.13, cells occupying islands containing > 60 cells remain more tightly packed than those in islands containing fewer cells. Based on these findings, a cut-off value of 60 cells was implemented in subsequent experiments. Cell islands consisting of less than 10 cells were also discounted, in order to ensure that a representative mixture of cell lineages was present in each cell island. Thus, only islands with cell counts of between 10 and 60 were analysed in subsequent experiments.

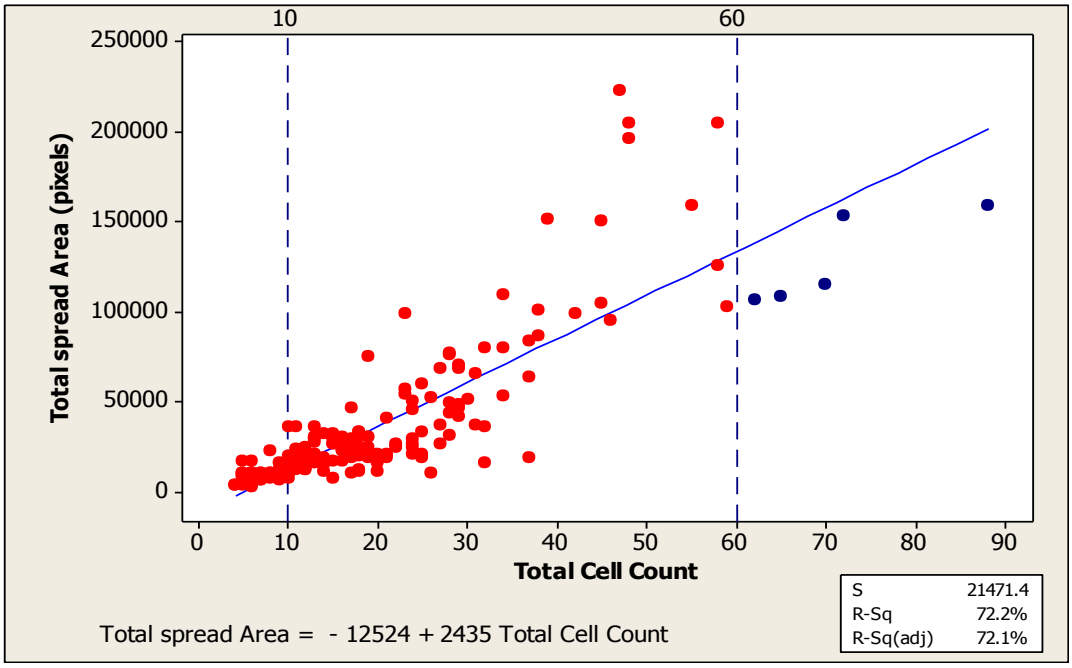

Figure 3.12- Scatterplot diagram to identify the relationship between total spread area and total cell count. Dashed lines represent the cut-off values used in subsequent investigations. The line on the graph indicates a linear regression line.

The projection of cellular protrusions, such as lamellae, is a crucial part of cell migration (Ridley et al., 2003), thus protrusive activity of cells within our model was also characterised. A characteristic fringe, representing cellular protrusions, is present around the periphery of cell islands. These protrusions are extended beyond the location of the cells, thus the protrusive area can be calculated by subtracting the area encompassed by the cell nuclei (using propidium iodide staining) from the total spread area (Figure 2.1). This novel calculated area is referred to as the fringe area and is larger for cells that are protruding more actively. As only cells around the periphery of islands are thought to contribute to the projection of cell protrusions, it was considered appropriate to normalise the fringe area to the edge cell count and the linear relationship between these two parameters is shown in Figure 3.14.

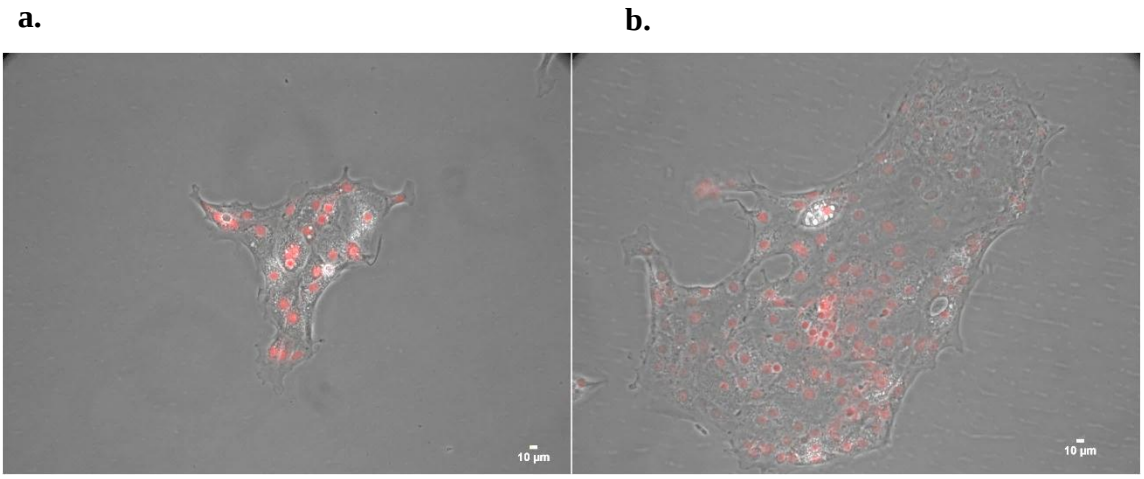

Figure 3.13- Photomicrographs highlighting the differences in spreading behaviour of islands with (a.) smaller and (b.) larger (> 60) cell counts. Magnification of 20X; scale bar: 10µm

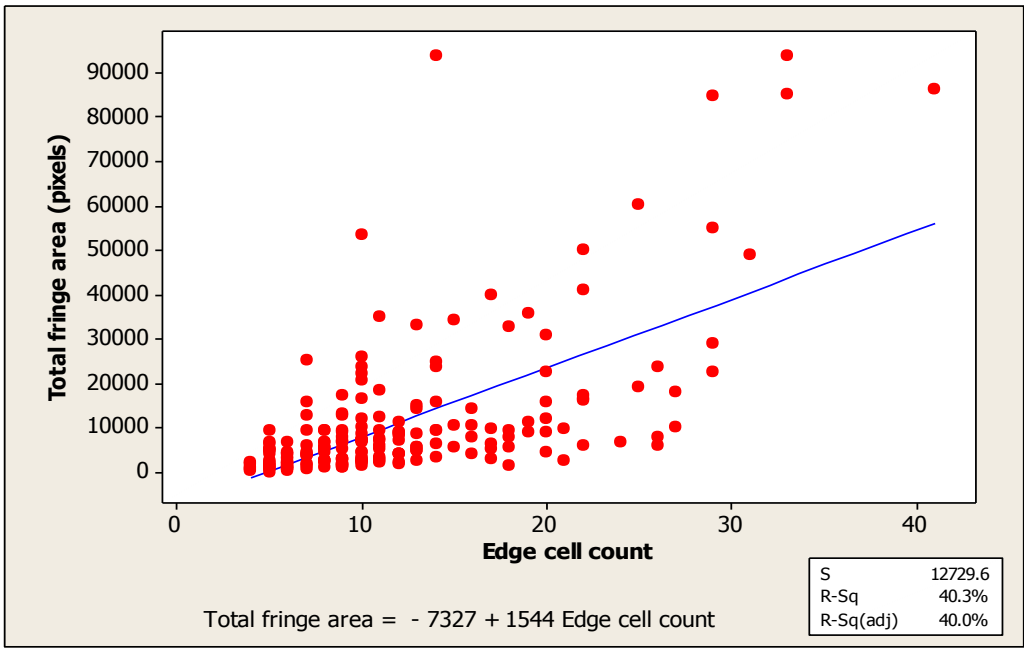

Figure 3.14- Scatterplot diagram to identify the relationship between total fringe area and edge cell count. The line on the graph indicates a linear regression line.

3.4. Discussion

This chapter describes the development of an appropriate model for studying the spreading of primary canine epithelial cells from isolated gastric glands. Much of the previous work on epithelial cell spreading and migration has relied upon the use of disaggregated cell suspensions or immortalised cell lines (Kato et al., 1999; Buchanan et al., 2003; Sanders et al., 2009; Kim et al., 2012). *In vitro* models representative of an intact gastric epithelium have been successfully used to study human (Wroblewski et al., 2003), mouse (Pagliocca et al., 2008) and rabbit (Berglindh & Öbrink, 1976) cells, however, to our knowledge, such a model has not previously been used to isolate glands from canine tissue. Thus, the model presented here uses previously published techniques (Wroblewski et al., 2003; Pagliocca et al., 2008; Berglindh & Öbrink, 1976), to isolate intact glands from the canine gastric epithelium. In published studies, cell islands prepared in this way contain representative mixtures of cell types present *in vivo* including chief cells, parietal cells, surface mucous cells, and additionally mesenchymal cells. Based on morphology we were able to identify cells characteristic of parietal cells within islands and mesenchymal-like cells in culture.

The protocol used in previous studies (Wroblewski et al., 2003; Pagliocca et al., 2008; Berglindh & Öbrink, 1976) was adapted to enable the reproducible isolation of viable canine gastric glands. A collagenase concentration of 0.5 mg/ml, as used previously (Wroblewski et al., 2003), was found to provide a suitable gland yield, thus this concentration was used in all subsequent experiments. Various collagenase digestion times were trialled in order to determine the optimal time for maximal gland isolation. Shorter digestion times were found to give a low yield of glands and left large fragments of tissue undigested, whereas enzymatic cell damage occurred when tissue was digested for longer time periods. The optimal digestion conditions were determined to be two 30 min collagenase incubations and washing the tissue samples with HBSS in between the digestion steps was found to limit the presence of excess tissue fragments in the final cell cultures.

Due to the limited availability of gastric mucosal samples from routine endoscopies, samples were also obtained from canine cadavers sourced from a local animal shelter. For endoscopy-derived samples, clinical data including *Helicobacter* status, the presence of certain inflammatory indicators and the final clinical diagnosis, including the presence of gastric adenocarcinoma or lymphoma, were recorded. Furthermore, the breed, sex, neutering status and age of the dog were recorded. The limitation of using cadaver-derived material is the absence of clinical data; only breed, neutering status and an estimation of age could be recorded for these samples. Despite this limitation, larger volumes of mucosa could be taken from the cadavers, allowing a high yield of gastric glands and a greater throughput of experiments.

Time-lapse microscopy enabled us to observe the spreading behaviour of epithelial cells from isolated gastric glands. Time-lapse analysis was performed using a heated incubation chamber, allowing cells to be observed in real-time. Cell-cell contacts were maintained throughout the spreading process and cells did not detach from the islands. The extension of dynamic cellular protrusions was observed after 12 h in culture and these protrusions were characteristic of both lamellipodia and filopodia. Lamellipodia and filopodia are formed at the leading edge of the cell via actin polymerisation and are essential for cell motility (Small et al., 2002). It has been suggested that lamellipodia function to promote cell movement over a surface, whereas filopodia perform an exploratory role in order to direct migration (Mejillano et al., 2004). The Arp 2/3 protein complex has a key role in the formation of lamellipodia, via promotion of actin assembly (Machesky, 2008) and this complex is regulated by the SCAR/WAVE complex (Ibarra et al., 2005). Filopodia formation is known to be controlled by proteins, such as fascin and Mena/VASP (Machesky, 2008). In order to stabilise these cellular protrusions, adhesions are made between the actin cytoskeleton and the extracellular matrix (Horwitz & Webb, 2003).

After 48 h in culture, biopsy-derived cells had fully spread from the isolated gastric glands to form monolayer cell islands, thus analysis of cell spreading in subsequent experiments was performed at this time point. In contrast, cells isolated from cadaver-derived tissue took substantially longer to spread, with

fully spread monolayer islands being observed after 96 h in culture. This difference in spreading behaviour was found to be independent of cell plating density. When cultured for longer time periods, epithelial cell islands survived for up to 7 days, adhered to the culture vessel with no biological substratum, as found previously (Basque et al., 1999). After 7 days in culture, cell islands were seen to break apart and epithelial cells transformed into mesenchymal-like cells. This process is referred to as epithelial-mesenchymal transition (EMT), and involves increases in MMP expression, basement membrane degradation and cell detachment, changes in the cytoskeletal arrangement (Murray, Knight & Laurent, 2009) and altered cell-cell interactions (Radisky, 2005). EMT plays an important role in embryonic development (Shook & Keller, 2003) and the metastatic spread of cancer cells (Iwatsuki et al., 2010).

These preliminary experiments were undertaken in order to characterise cell spreading in this model and to assess the suitability of certain parameters for measuring spreading. Previous studies have analysed cell spreading via the measurement of the relative area of individual cells (Arthur & Burridge; Sanders et al., 2009). Within this model, as in previous similar models (Wroblewski et al., 2003), cell spreading was calculated as the mean cell island area, normalised for cell number. Previous studies have found no evidence of proliferation in similar cell island models (Wroblewski et al., 2003) and thus increases in cell area were attributed to cell migration and spreading. Given the importance of active cell protrusion in the process of spreading, the protrusive activity of cell islands was also determined through the measurement of fringe area, normalised for outer edge cell number. The novel measurement of fringe area described in this investigation is representative of cell protrusive activity only, and as such is less likely to be influenced by cell-cell interactions, thus it may provide a more robust representation of spreading activity. Cells spanning the outside edge of epithelial islands respond to a ‘wound-like’ environment and spread over the surrounding substrate. Wounded epithelial cell monolayers have been shown to form cellular outgrowths at the wound edge, with a highly active leader cell located at the tip and the enhanced motility of this cell appears to pull neighbouring cells forwards (Omelchenko et al., 2003). Thus, activity of cells spanning a wound edge, or in this case the outer edge of cell islands, play a key role in driving the spreading of

epithelial cell sheets and as such, measurement of this activity, represented by fringe area in this model, provides important information about the spreading behaviour of cells.

In summary, the protocol described in this chapter enables the reproducible isolation of intact, viable gastric glands, representative of their structure *in vivo*. Thus, this is a physiologically relevant primary culture model that can be used for the study of canine gastric epithelial cell spreading.

Chapter 4 - Characterising the effects of COX antagonism on epithelial cell migration and spreading

4.1 Introduction

COX-2-derived PGE_2 is known to directly regulate cell migration via transactivation of the EGF receptor, leading to activation of the PI3K/Akt pathway (Buchanan et al., 2003) and prostaglandins may also indirectly modulate restitution by maintaining a favourable microenvironment (Morris, 1986). Endogenous prostaglandins have been shown to mediate the promotion of intestinal epithelial restitution by growth factors (Zushi et al., 1996) and non-selective COX inhibition has been shown to impair the migration of human gastric epithelial monolayers via inhibition of stress fibre formation and FAK and tensin phosphorylation (Szabó et al., 2002). COX-2 has an important role in the modulation of HGF-stimulated restitution (Horie-Sakata et al., 1998). HGF was shown to increase COX-2 expression in RGM-1 cells and selective inhibition of COX-2 significantly delayed wound repair (Horie-Sakata et al., 1998). Furthermore, COX-2 and PGE_2 are considered to be important for the migration of cancer cells. PGE_2 was shown to promote the migration of chondrosarcoma cells by up-regulating the expression of α2β1 integrin through activation of the EP1, PLC, PKCα, c-Src and NF-κB –dependant signalling pathway (Liu et al., 2010). COX-2 over-expression is associated with the enhanced motility and invasiveness of breast cancer cells (Singh, 2005).

Whilst the role of COX in cell migration and spreading has been widely studied, little focus has been given to its effects in a multicellular model. The overall aim of this investigation was to test the hypothesis that COX-2-derived PGE_2 mediates epithelial cell migration and spreading *in vitro*. Given the importance of epithelial cell migration and spreading in the repair of gastric damage, this investigation used intact isolated gastric glands, which form multicellular monolayer islands, to determine the role of COX enzymes in gastric epithelial cell migration and spreading. A scratch wound assay, using an immortalised cell line, was used to support the primary cell experiments, enabling a high throughput of reproducible experiments to be performed. Using both kidney and

gastric epithelial cell lines and cells derived from both humans and canines, provides evidence that any observed effects are not cell-type or species specific.

4.2. Materials and methods

4.2.1.Cell line scratch wound assays

4.2.1.1.Cell culture

MDCK, MKN-45 and AGS cells were cultured using conditions described previously (Section 2.5.2).

4.2.1.2.Scratch wound assay

Scratch wounds were performed as described previously (Section 2.7). Cells were serum starved for 12 h prior to scratch wounding, cells were then treated with either 50 µM indomethacin or 10 µM NS-398, made up in serum-free medium, for 24 h. Control wells contained the vehicle only (both chemicals were dissolved in DMSO). The observer was blinded to the treatment used in individual wells.

4.2.1.3.Statistics

A total of six individual experiments were performed, with two separate areas analysed for each scratch wound. The data was presented as the mean difference in scratch wound width, with values normalised to the mean control value within each experiment. Statistical analysis was performed with SPSS 20 (IBM Corp.), using one-way analysis of variance. The criteria for statistical significance was set at $P<0.05$.

4.2.2.Primary cell island spreading experiments

4.2.2.1.Sample collection

Samples were collected as described previously (Section 2.3). All gastric mucosal tissue samples used in this study were obtained from routine canine endoscopic biopsies.

4.2.2.2.Gastric gland isolation

Intact gastric glands were isolated from mucosal tissue samples as described previously (Section 2.4).

4.2.2.3.Gastric gland culture

Isolated glands were cultured in serum-supplemented medium (10% FBS), with either 50 µM indomethacin or 10 µM NS-398. Control wells contained the vehicle only (both chemicals were dissolved in DMSO). Glands were cultured for 48 h at 37°C in a 5% CO_2 incubator, at which point they had fully spread to form monolayer cell islands. The observer was blinded to the treatment used in individual wells.

4.2.2.4.Dose-response experiments

Initially, dose-response experiments were performed for each NSAID in order to characterise the relationship between drug dose and cell spreading and to determine the best dosage to use in subsequent experiments. Briefly, isolated glands were cultured in the appropriate medium plus the NSAID in increasing doses for 48 h. Values for total area and fringe area were taken as described previously (Section 2.6.3). The lowest dose of NSAID at which a visible effect could be seen was used in subsequent experiments.

4.2.2.5.Analysis of epithelial cell island spreading

Cell spreading in individual cell islands was analysed as described previously (Section 2.6.3).

4.2.2.6.PGE_2 ELISA

PGE_2 ELISAs were performed to analyse the effects of NSAID treatment on PGE_2 concentration in cell culture supernatants. ELISAs were performed as

described previously (Section 2.9.3). A commercially available PGE_2 ELISA kit (R&D Systems) was used.

4.2.2.7.Statistics

A total of five experiments, using biopsy samples from five dogs were performed and each experiment consisted of measurements from approximately 75 different cell islands, approximately 25 islands per treatment. There was an inevitable clustering within the observations because each biopsy sample was used in testing the effect of each treatment. The analysis acknowledged this by using a mixed-effects linear regression with the biopsy identity declared as a random effect. The measurements for both total area and fringe area were corrected for by cell number and subjected to a logarithmic transformation. Significant differences were indicated by $P<0.05$ for all data. Statistical analysis was performed using Stata Statistical Software: Release 11 (StataCorp. 2009) and the *xtmixed* command.

For the dose-response experiments, one biopsy sample was analysed per treatment, with approximately 20 glands analysed per concentration. One-way analysis of variance was used to analyse statistical differences between the treatment groups. SPSS 20 (IBM Corp.) was used to perform the analysis and the criteria for statistical significance was set at $P<0.05$.

4.3. Results

4.3.1.Non-selective and COX-2 selective antagonism impairs MDCK scratch wound healing

In this investigation, scratch wound assays were performed on confluent monolayers of MDCK cells in order to assess the effects of indomethacin (a non-selective COX antagonist) and NS-398 (a COX-2 selective antagonist) on immortalised epithelial cell wound healing. In order to negate the effects of serum components, the MDCK cells were serum starved for 12 h prior to scratch wounding. Confluent monolayers of MDCK cells were scratch wounded and cultured in either serum-free medium alone (control) or serum-free medium supplemented with 50 µM indomethacin or 10 µM NS-398. Both non-selective and COX-2 selective antagonism markedly inhibited the healing of scratch wounds, when compared to control (Figure 4.1). Analysis of percentage wound closure, normalised to control, showed that treatment with both indomethacin and NS-398 significantly reduced wound healing over 24 h (Figure 4.2).

4.3.2.Non-selective and COX-2 selective antagonism inhibits PGE_2 production in MDCK cells

In order to assess the effects of both indomethacin and NS-398 treatment on PGE_2 production by serum-starved MDCK cells, a commercially available PGE_2 ELISA kit was used. Indomethacin and NS-398 inhibited PGE_2 concentration by 79.2% and 76.6% respectively (Table 4.1 and Figure 4.3).

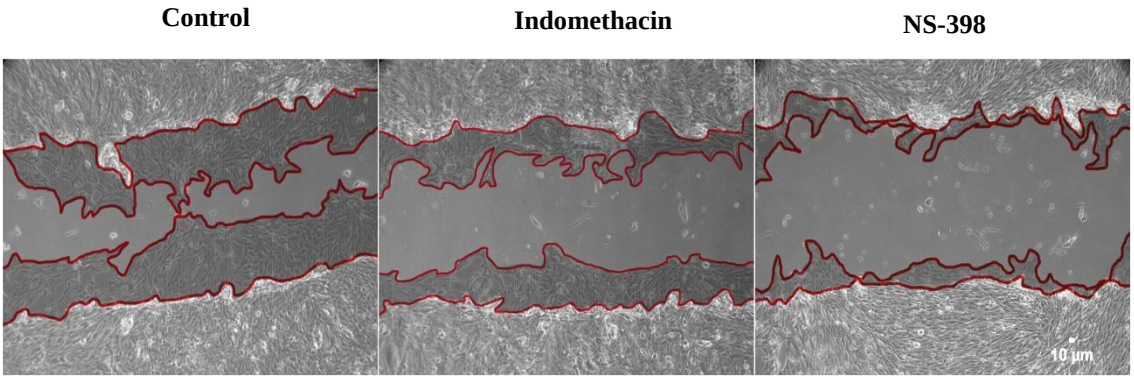

Figure 4.1- Phase contrast photomicrographs highlighting the effect of non-selective (indomethacin, 50µM) and COX-2 selective (NS-398, 10µM) antagonists on MDCK scratch wound healing after 24 h treatment. Images taken at magnification of 10X; scale bar: 10µm

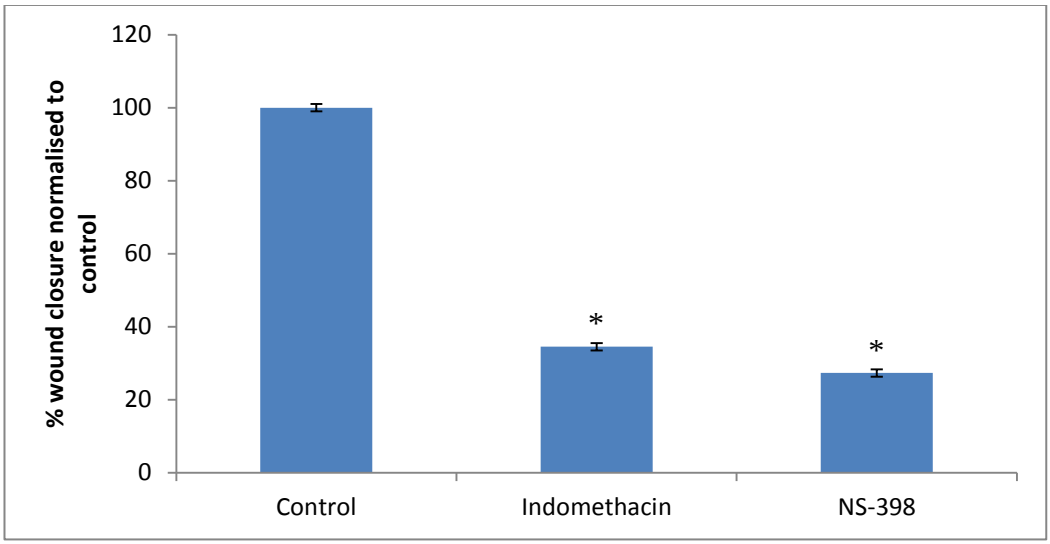

Figure 4.2- Effect of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonists on MDCK scratch wound healing. Data (presented as mean +/- SEM) were normalised to control and analysed using one-way analysis of variance.. There was a significant effect of treatment, with both indomethacin and NS-398 significantly impairing wound healing ($p<0.001$, indicated by *).

Unknowns	O.D	PGE2 concentration (pg/ml)
Control	0.599	253.79
Indomethacin	0.815	52.68
NS-398	0.798	59.34

Table 4.1- O.D values and calculated PGE_2 concentrations for MDCK cell supernatants

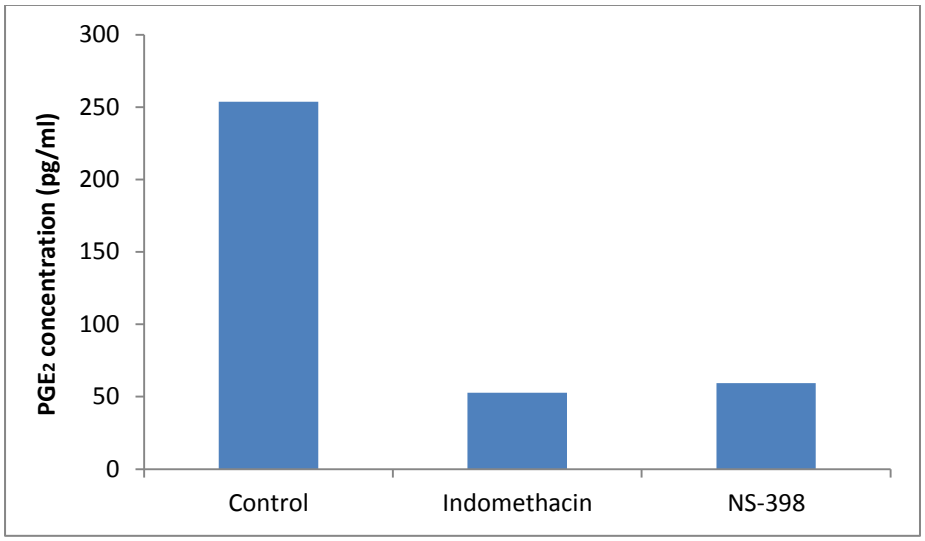

Figure 4.3- Effect of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonism on PGE_2 concentration in supernatants obtained from cultured MDCK cells

4.3.3.Non-selective and COX-2 selective antagonism impairs wound healing in human epithelial cell lines

To ensure that the observed effects were not specific to cells of canine origin, the effects of COX antagonism on scratch wound healing in two human epithelial cell lines, AGS and MKN-45 cells, were analysed. Both of these cell lines are derived from a human gastric adenocarcinoma. Analysis of percentage wound closure normalised to control shows that treatment with both indomethacin and NS-398 significantly reduced wound healing over 24 h in both cell lines (Figure 4.4), thus suggesting that the effects of COX antagonism on epithelial cell migration are not species-specific.

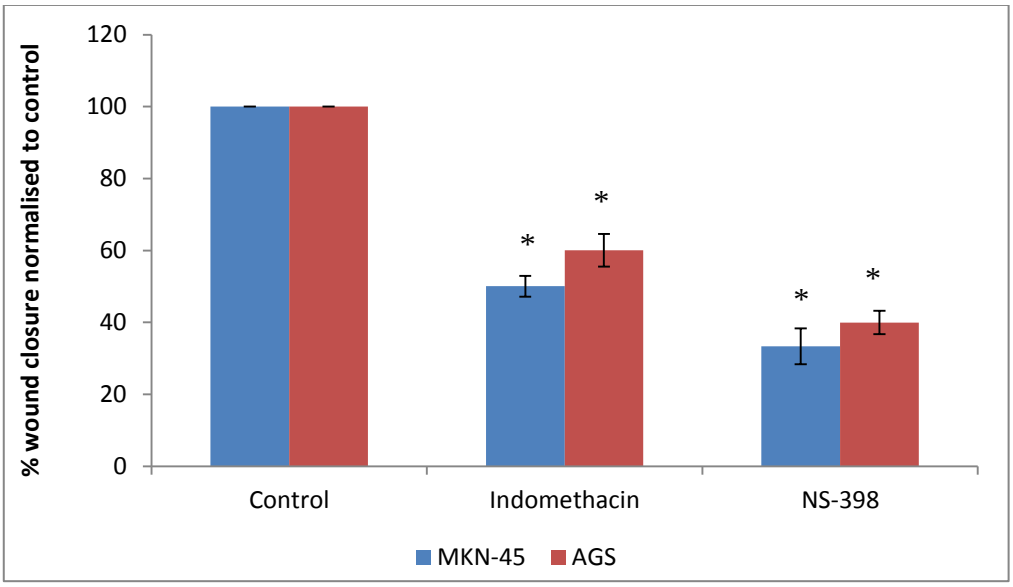

Figure 4.4- Effect of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonists on MKN-45 and AGS cell scratch wound healing. Data (presented as mean +/- SEM) were normalised to control and analysed using one-way analysis of variance. There was a significant effect of treatment for both cell types, with both indomethacin and NS-398 significantly impairing healing ($p<0.001$, indicated by *).

4.3.4.Effect of non-selective and COX-2 selective antagonism on epithelial cell spreading and protrusive activity

4.3.4.1.Dose-response experiments

A dose-response experiment was performed for both indomethacin and NS-398, in order to find the optimal concentration to use in subsequent experiments. Treatment with indomethacin at concentrations above 50 µM was shown to cause a statistically significant decrease in both total area per cell and fringe area per cell (Figure 4.5), thus, in subsequent experiments indomethacin was used at a concentration of 50 µM. Comparison between treatment concentrations of NS-398 showed that treating with concentrations above 10 µM caused a statistically significant decrease in both total area per cell and fringe area per cell (Figure 4.6), thus, in subsequent experiments NS-398 was used at a concentration of 10 µM. At concentrations of 500 µM, NS-398 caused significant cell damage; cell death was confirmed via a trypan blue exclusion assay (data not presented).

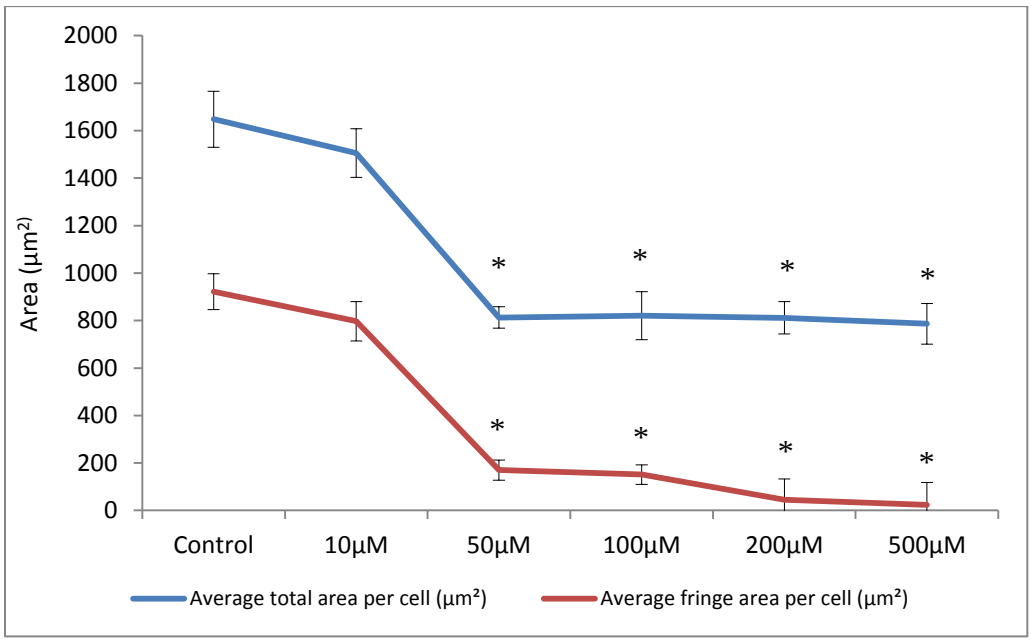

Figure 4.5- Dose response curve for indomethacin (non-selective COX antagonist). Data (presented as mean +/- SEM) was analysed using one-way analysis of variance. Indomethacin, at doses higher than 50 µM, significantly decreased both total area per cell and fringe area per cell ($p<0.001$, indicated by *).

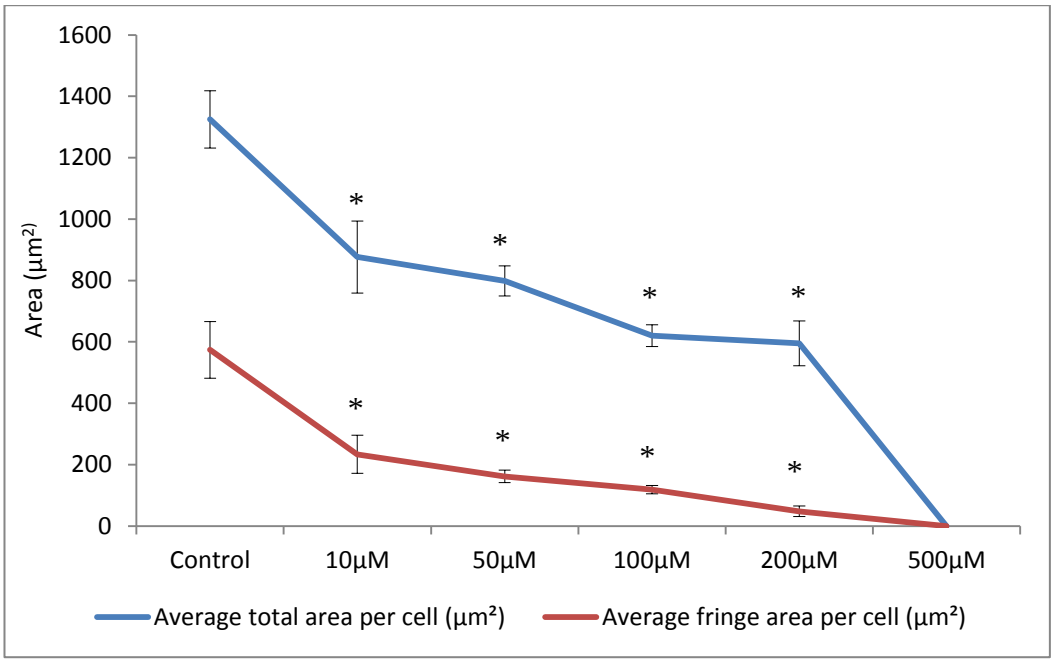

Figure 4.6- Dose response curve for NS-398 (COX-2 selective antagonist). Data (presented as mean +/- SEM) was analysed using one-way analysis of variance. NS-398, at doses higher than 10 µM, significantly decreased both total area per cell and fringe area per cell ($p<0.05$, indicated by *).

4.3.4.2.Non-selective and COX-2 selective antagonism impairs epithelial cell spreading and protrusive activity

Five independent cell spreading experiments, using biopsy samples from five dogs were carried out for the purpose of this study. The dogs varied by breed, age and sex and, as samples were obtained via routine endoscopies, the dogs were presenting at the Small Animal Hospital with varying gastrointestinal symptoms. The effects of indomethacin and NS-398 were assessed by measuring the effect of each drug on 25 individual cell islands in each experiment. Values for mean total spread area corrected for by cell count and mean fringe area corrected for by edge cell count were calculated for each experimental treatment, as well as a control treatment, containing the vehicle only.

Treatment with indomethacin at 50 μM caused a 14.3% inhibition in total spread area per cell and a 63.6% inhibition in fringe area per cell (Figure 4.7). Treatment with the COX-2 selective antagonist, NS-398 at 10 μM caused similar decreases to indomethacin, with a 21.4% inhibition in total spread area and a 65.5% inhibition in fringe area per cell (Figure 4.7). The effects of COX antagonism on cell spreading can also be observed visually (Figure 4.8). Furthermore, analysis of distance between neighbouring cells, reveals that cells within islands treated with both indomethacin and NS-398 remain in closer proximity to each other compared to cells in the control islands (Figure 4.9).

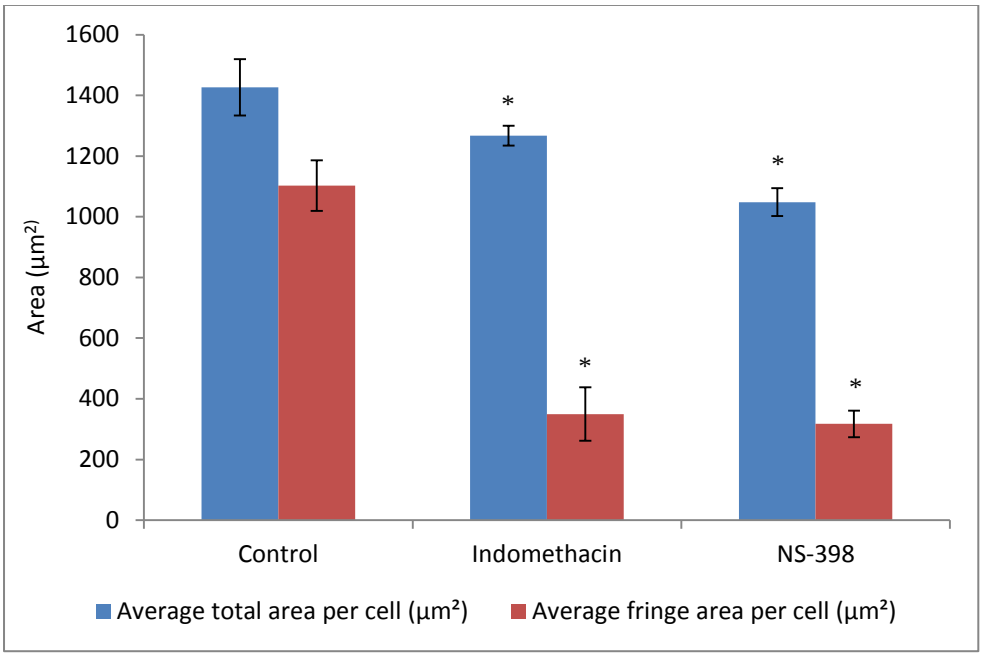

Figure 4.7- Effect of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonists on the migration of primary canine gastric epithelial cell islands. Data (presented as mean +/- SEM) was analysed by mixed-effects regression. There was a significant effect of treatment, with both indomethacin and NS-398 significantly impairing cell spreading and protrusive activity ($p<0.05$, indicated by *).

4.3.4.3.Non-selective and COX-2 selective antagonism inhibits PGE_2 production in canine epithelial cells

In biopsy-derived canine gastric epithelial cells, 48 h treatment with indomethacin and NS-398 inhibited PGE_2 concentration by 93.4% and 89.3% respectively (Table 4.2 and Figure 4.10).

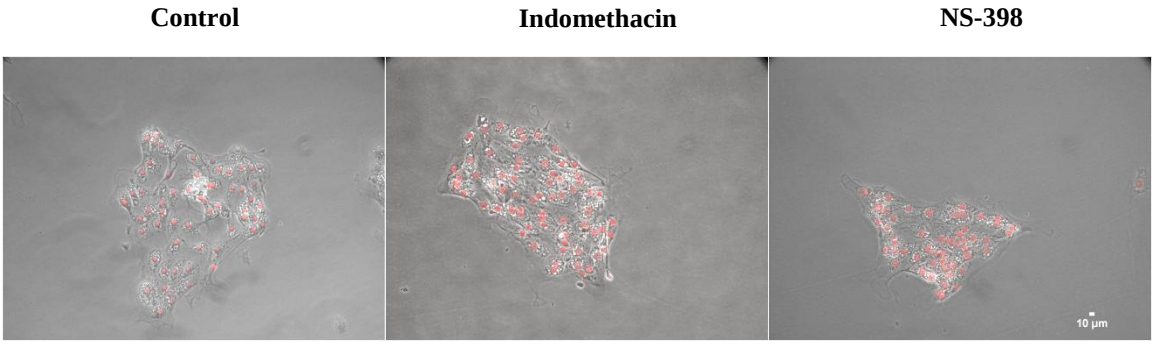

Figure 4.8- Representative photomicrographs highlighting the effects of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonists on the spreading of primary gastric epithelial cell islands. Cell nuclei have been stained with propidium iodide; images taken at magnification of 20X; scale bar: 10µm

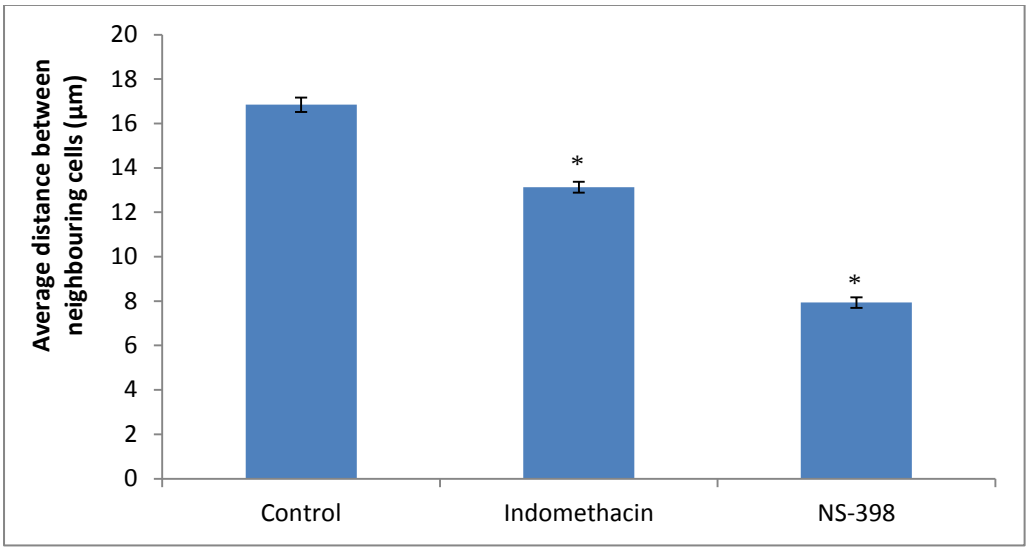

Figure 4.9- Effect of non-selective (indomethacin, 50 µM) and COX-2 selective (NS-398, 10 µM) antagonists on the average distance between neighbouring cells in cultured islands. Data (presented as mean +/- SEM) was analysed using one-way analysis of variance. There was a significant effect of treatment on distance between neighbouring cells, with both indomethacin and NS-398 causing a significant decrease in distance ($p<0.05$, indicated by *).

Unknowns	O.D	PGE2 concentration (pg/ml)
Control	0.582	287.53
Indomethacin	0.955	18.90
NS-398	0.888	30.76

Table 4.2- O.D values and calculated PGE_2 concentrations for canine gastric epithelial cell island supernatants

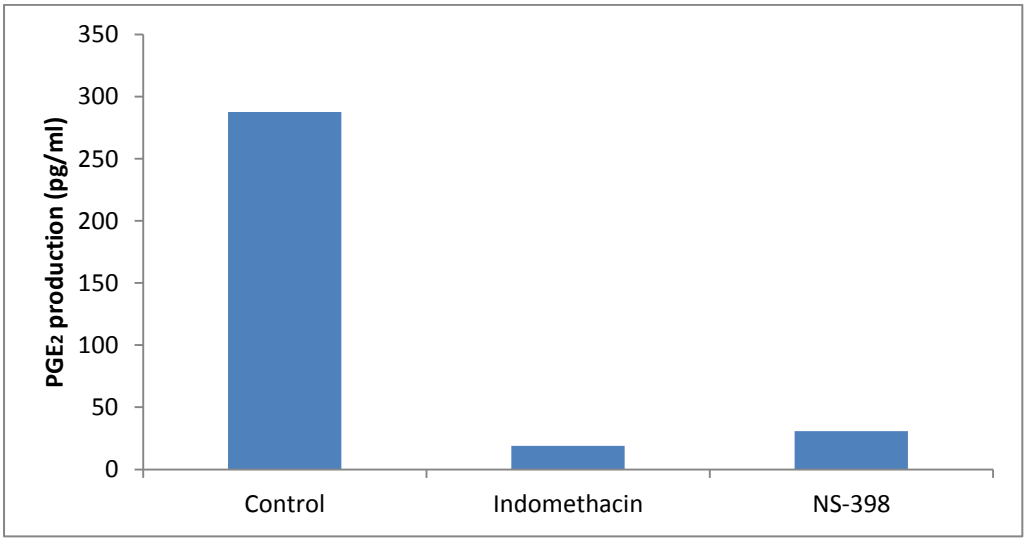

Figure 4.10- Effect of non-selective (indomethacin, 50µM) and COX-2 selective (NS-398, 10µM) antagonists on PGE_2 concentration in supernatants obtained from cultured canine gastric epithelial cell islands

4.4. Discussion

Previous *in vitro* studies of gastric epithelial cell migration and spreading have involved the use of cultures of fully dispersed surface epithelial cells or immortalised cell lines (Murakami et al., 1998; Pai et al., 2001; Kim et al., 2012), however, in these models the cell-cell interactions and cell polarity that are seen *in vivo* are not preserved. The cell spreading model used in this investigation utilised individual intact gastric glands which contain multiple cell types, namely surface epithelial cells, chief cells, parietal cells and endocrine cells (Schubert & Peura, 2008). Normal cell-cell contacts and cell polarity are maintained in this model, thus making it a more representative model for *in vivo* activity. Furthermore, the cell preparation may also contain gastric fibroblasts isolated from mucosal tissue along with the gastric glands, thus any paracrine signalling occurring between mesenchymal and epithelial cells may be maintained. Paracrine signalling may play an important role in the regulation of epithelial cell migration. HGF has been reported to promote gastric epithelial cell migration via a paracrine mechanism (Takahashi et al., 1995). HGF is produced by mesenchymal cells and binds to the Met/HGF receptor present on epithelial cells (Schmassman et al., 1997). Gastrin-mediated paracrine signalling has been shown to induce gastric epithelial cell migration through activation of the EGF receptor, the erbB2 receptor and the MAPK pathway (Noble et al., 2003). TGF-α, produced via the parietal cells and bFGF of stromal origin, have also been shown to enhance canine oxyntic mucosal cell migration, presumably in a paracrine manner (Kato et al., 1999).

The major limitation of using this primary cell culture model is the limited availability of tissue for gland isolation. To overcome this factor, scratch wound assays were performed to support the primary cell experiments. Scratch wound analysis is convenient for performing a large number of highly reproducible experiments (Cory, 2011) and scratch wound models have been successfully used in previous studies to observe the effects of COX antagonism on gastric epithelial cell wound healing (Pai et al., 2001; Giap et al., 2002). Using both kidney and gastric epithelial cell lines and cells derived from both humans and

canines, demonstrated that the observed responses are not cell-type or species specific.

Given the importance of cell migration and spreading in maintaining gastric epithelial integrity (Terano, 2001), the aim of this study was to compare the effects of both a non-selective and a COX-2 selective antagonist on epithelial cell migration and spreading *in vitro*. Clinical studies have shown that treatment with COX-2 selective antagonists leads to less adverse gastrointestinal effects compared with traditional non-selective antagonists (Laine et al., 1999; Simon et al., 1999; Hawkey et al., 2000). The findings presented in this chapter indicate that both non-selective and COX-2 selective antagonism significantly inhibits gastric epithelial cell spreading (total area per cell) and protrusive activity (fringe area per cell). Similar responses were observed with the scratch wound healing assay, with both indomethacin and NS-398 causing a significant delay in wound closure of epithelial cell monolayers of human and canine origin. In a preliminary dose-response experiment, the effects of indomethacin and NS-398 on epithelial cell migration were shown to be dose-dependent. Through the use of a trypan blue exclusion assay, the concentrations of drugs used in this study were determined to have no cytotoxic effect on the cells. ELISA analysis confirmed that PGE_2 production was markedly reduced by treatment with both indomethacin and NS-398 and the extent to which NS-398 treatment inhibited PGE_2 production was similar to that seen with indomethacin, thus suggesting that the majority of PGE_2 produced via epithelial cells in this model is derived from COX-2. NS-398 has previously been shown to be highly selective for COX-2, with a COX-1/COX-2 ratio of 22, compared to 0.029 for indomethacin (Kato et al., 2001). Thus, these findings suggest that COX-2 derived PGE_2 has an important role in the modulation of epithelial cell migration and spreading *in vitro.*

Consistent with our findings, previous studies have shown that both non-selective and COX-2 selective antagonists impair the re-epithelialisation of wounded gastric monolayers *in vitro* (Pai et al., 2001) and gastric ulcer healing in rats *in vivo* (Shigeta et al., 1998). Contrastingly, a study by Giap and colleagues (2002) disagreed with our findings, suggesting that COX-2 specific inhibition

had no effects on basal wound re-epithelialisation of a gastric cell line monolayer, however non-selective COX inhibition significantly reduced re-epithelialisation, indicating the importance of COX-1 in this effect. Notably, in this study cells were not serum-starved prior to scratch wounding as they were in experiments presented here. As reported in this thesis, serum-starvation induces COX-2 expression in cultured gastric epithelial cells (Section 6.3.2), thus this may account for the lack of response. Additionally, COX-2 selective inhibition did significantly reduce bFGF-stimulated wound re-epithelialisation (Giap et al., 2002) and as our primary cell preparation consists of multiple cell types, paracrine bFGF signalling could be taking place.

It has previously been reported that aspirin treatment leads to poorly formed lamellipodia at the wound edge (Yoshizawa et al., 2000), thus suggesting that cytoskeletal disruption may play a role in the suppression of wound repair by COX antagonism. Indomethacin and NS-398 have both been shown to have a direct effect on the cytoskeleton via disruption of actin stress fibre formation, reduction in c-Src activity and a decrease in FAK and tensin phosphorylation (Pai et al., 2001), all of which are very important for cell migration (Ridley et al., 2003). In this investigation, cells treated with both indomethacin and NS-398 were still able to extend protrusions, as observed visually, however, both treatments were shown to significantly decrease the area of cell protrusions around the epithelial islands (fringe area). Thus, this suggests that the inhibitory effect of COX antagonism on epithelial cell migration may occur via changes to the cytoskeletal arrangement, causing a reduction in protrusive activity. This phenomenon could be further investigated through the use of phalloidin staining to detect filamentous actin, which, due to time constraints could not be performed as part of this study.

In summary, the findings presented in this chapter highlight the importance of COX-2 derived prostaglandins in the regulation of epithelial cell migration and spreading *in vitro*. Disruption to the cytoskeletal structure of the cells and inhibition of cellular protrusion formation may explain the inhibitory effects of COX antagonism on epithelial cell migration and spreading.

Chapter 5 - Effects of glucocorticoid receptor agonism and antagonism on epithelial cell migration and spreading

5.1 Introduction

Glucocorticoids exert their effects by binding to the intracellular GR, which is expressed in virtually all cell types (Turner et al., 2006). Once activated, the GR regulates the transcription of responsive target genes (Barnes, 1998). Endogenous glucocorticoids play an important role in a variety of physiological functions, such as the regulation of metabolism, immune response and electrolyte homeostasis (Lu & Cidlowski, 2006). Glucocorticoids are also known to have a suppressive effect on inflammation, via up-regulation of the transcription of genes coding for anti-inflammatory proteins and inhibition of the expression of several pro-inflammatory genes (Barnes, 1998). Glucocorticoids can alter the transcription of certain cytokine genes, such as TNF, IL-1 and IL-6 and inhibit the production of the pro-inflammatory prostaglandins (O'Connor et al., 2000). For this reason, glucocorticoids are widely used in the treatment of inflammatory conditions such as IBD, asthma, rheumatoid arthritis and autoimmune diseases (Barnes, 1998).

Exogenous glucocorticoid administration has been associated with an increased risk of gastric ulceration (Bandyopadhyay et al., 1999) and a delay in ulcer healing (Luo et al., 2004). Treating with NSAIDs and glucocorticoids concurrently causes a greater risk of gastric ulceration compared to using either treatment alone (Rodriguez & Hernández-Diaz, 2001). Exogenous glucocorticoid administration blocks the release of arachidonic acid from phospholipids (Hong & Levine, 1976), thus depriving COX of substrate, this effect is mediated by lipocortin-1 (Croxtall et al., 1995) and as a result, PGE_2 production is decreased. PGE_2 down-regulation at the gastric ulcer margin of rats treated with dexamethasone has been described previously (Luo et al., 2004).

Furthermore, stress has been identified as a predisposing factor for the development of gastric ulceration (Silen et al., 1981). Glucocorticoid secretion is induced in response to stress (Sapolsky et al., 2000), via activation of the HPA axis (O'Connor et al., 2000). Glucocorticoids modulate the stress response by altering gene expression, transcription and translation (O'Connor et al., 2000), leading to inhibition of various pro-inflammatory mediators, such as cytokines. Previous studies have used animal models to show that both physical (Hase et al., 1975; Filaretova et al., 1998) and psychological (Menguy, 1960) stressors induce gastric ulceration. However, more recent studies have shown that acute increases in stress-induced plasma corticosterone levels appear to protect against the development of stress-induced ulcers (Filaretova et al., 1998). The gastroprotective effects of glucocorticoids may be mediated through the maintenance of gastric mucosal blood flow (Filaretova et al., 1999), glucose homeostasis and mucus production and the inhibition of gastric motility and microvascular permeability (Filaretova et al., 2005).

The aim of this study was to test the hypothesis that glucocorticoid receptor signalling modulates epithelial cell migration and spreading *in vitro*. Given the importance of epithelial cell migration and spreading in maintaining the integrity of the gastric mucosa (Terano, 2001), the effects of both GR agonism and antagonism on epithelial cell wound healing and canine gastric epithelial cell spreading were investigated. Previous studies have shown that dexamethasone treatment leads to an inhibition in smooth muscle cell migration via suppression of MMP activity (Pross et al., 2002) and impairs wound healing *in vivo* in rats (Durmus et al., 2003). In particular, dexamethasone treatment has been associated with impaired gastric cell migration of a co-culture of gastric fibroblasts and epithelial cells (Takahashi et al., 2003) and TNF-α stimulated rat gastric epithelial migration (Luo et al., 2009).

5.2. Materials and methods

5.2.1.Cell line scratch wound assays

5.2.1.1.Cell culture

MDCK cells were cultured as described previously (Section 2.5.2).

5.2.1.2.Scratch wound assay

Scratch wounds were performed as described previously (Section 2.7). Cells were serum-starved for 12 h prior to scratch wounding, after which they were treated with either 1 µM dexamethasone, dissolved in DMSO or 1 µM RU-38486, dissolved in ethanol. Both treatments were made up in serum-free medium. Serum-free control wells contained the vehicle only and medium containing 10% FBS was used as a positive control. The observer was blinded to the treatment used in individual wells.

5.2.1.3.Dose-response experiments

Dose-response experiments were performed on scratch wounded monolayers in order to characterise the relationship between drug dose and cell migration. Serum-starved, wounded monolayers were cultured with various concentrations of dexamethasone for 24 h prior to analysis.

5.2.1.4.Statistics

A total of six individual experiments were performed, with two separate areas analysed for each scratch wound. The data was presented as the mean difference in scratch wound width, with values normalised to the mean control value within each experiment. Statistical analysis was performed with SPSS 20 (IBM Corp.), using one-way analysis of variance. The criteria for statistical significance was set at

P<0.05. For the dose-response assays, three individual experiments were performed, analysing two separate areas for each wound.

5.2.2.Primary cell island spreading experiments

5.2.2.1.Sample collection

Samples were collected as described previously (Section 2.3). All gastric mucosal tissue samples used in this study were obtained from routine canine endoscopic biopsies.

5.2.2.2.Gastric gland isolation

Intact gastric glands were isolated from mucosal tissue samples as described previously (Section 2.4).

5.2.2.3.Gastric gland culture

Isolated glands were cultured in serum-supplemented medium (10% FBS), either alone or with 1 µM dexamethasone or 1 µM RU-38486. Glands were cultured for 48 h at 37°C in a 5% CO_2 incubator, at which point they had fully spread to form monolayer cell islands. The observer was blinded to the treatment used in individual wells.

5.2.2.4.Dose-response experiments

Dose-response experiments were performed for both dexamethasone and RU-38486 in order to characterise the relationship between drug dose and cell island spreading. Briefly, isolated glands were cultured in serum-supplemented medium (10% FBS) plus the treatment in increasing doses for 48 h. Values for total area and fringe area were taken as described previously (Section 2.6.3).

5.2.2.5.Analysis of epithelial cell island spreading

Cell spreading in individual cell islands was analysed as described previously (Section 2.6.3).

5.2.2.6.Statistics

A total of five experiments, using biopsy samples from five dogs were performed and each experiment consisted of measurements from approximately 75 different cell islands, approximately 25 islands per treatment. There was an inevitable clustering within the observations because each biopsy sample was used in testing the effect of each treatment. The analysis acknowledged this by using a mixed-effects linear regression with the biopsy identity declared as a random effect. The measurements for both total area and fringe area were corrected for by cell number and subjected to a logarithmic transformation. Significant differences were indicated by $P<0.05$ for all data. Statistical analysis was performed using Stata Statistical Software: Release 11 (StataCorp. 2009) and the *xtmixed* command.

For the dose-response experiments, one biopsy sample was used per treatment, with approximately 20 glands analysed per concentration. One-way analysis of variance was used to analyse statistical differences between the treatment groups. SPSS 20 (IBM Corp.) was used to perform the analysis and the criteria for statistical significance was set at $P<0.05$.

5.3. Results

5.3.1.The effects of GR agonism on MDCK scratch wound healing vary with dose

Given the contrasting evidence available for the role of glucocorticoids in epithelial wound repair, one of the aims of this investigation was to determine the effects of dexamethasone, at varying doses, on MDCK wound healing. For the purpose of this investigation, scratch wound assays were performed on serum-starved confluent monolayers of MDCK cells. As commercially available FBS contains certain growth factors, binding proteins and hormones, as well as mean cortisol levels of 0.5 µg/ml (Price & Gregory, 1982), medium supplemented with 10% FBS served as a positive control.

Physiological cortisol levels were calculated to be approximately 0.1 µM. A pharmacological dose of dexamethasone was calculated as 1 µM, based on the therapeutic anti-inflammatory dose used in dogs. Pharmacological dexamethasone (1 µM) and sub-physiological dexamethasone doses (<0.1 µM) both caused a statistically significant inhibition of MDCK scratch wound healing compared to the serum-free control. In addition, treatment with 10% FBS significantly promoted MDCK scratch wound healing (Figure 5.1).

5.3.2.Both agonism and antagonism of the GR impairs wound healing in MDCK cells

In order to characterise the effects of GR agonism and antagonism on MDCK wound healing, further scratch wound assays were performed on serum-starved confluent cell monolayers. Scratch wounds were made as described previously (Section 2.7) and cells were treated with either serum-free medium or medium containing 1 µM dexamethasone or 1 µM RU-38486. The glucocorticoid receptor antagonist, RU-38486, was used to study the effects of glucocorticoid absence on migration. RU-38486 at a concentration of 1 µM has previously been shown to completely block

apoptosis mediated by 1 μM dexamethasone in rat C6 glioma cells (Morita et al., 1999), thus we chose to use this concentration in our studies.

Both dexamethasone-induced GR agonism and GR antagonism via RU-38486 significantly impaired MDCK wound healing, compared to the serum-free control. After 24 h, cells treated with serum-free medium had migrated to cover approximately 70% of the wound area. However, in cells treated with pharmacological dexamethasone and RU-38486 wounds were only 3% and 5% closed respectively (Figure 5.2). FBS appears to increase wound healing, as cells treated with 10% FBS had migrated to cover approximately 80% of the wound area after 24 h (Figure 5.3). Furthermore, cells along the wound edge appear to form less cellular protrusions when treated with dexamethasone and RU-38486 compared to the serum-free control (Figure 5.4).

Analysis of percentage wound closure normalised to the serum-free control, shows that both pharmacological dexamethasone and RU-38486 cause a statistically significant reduction in MDCK wound healing over 24 h, while FBS significantly increased wound healing (Figure 5.5).

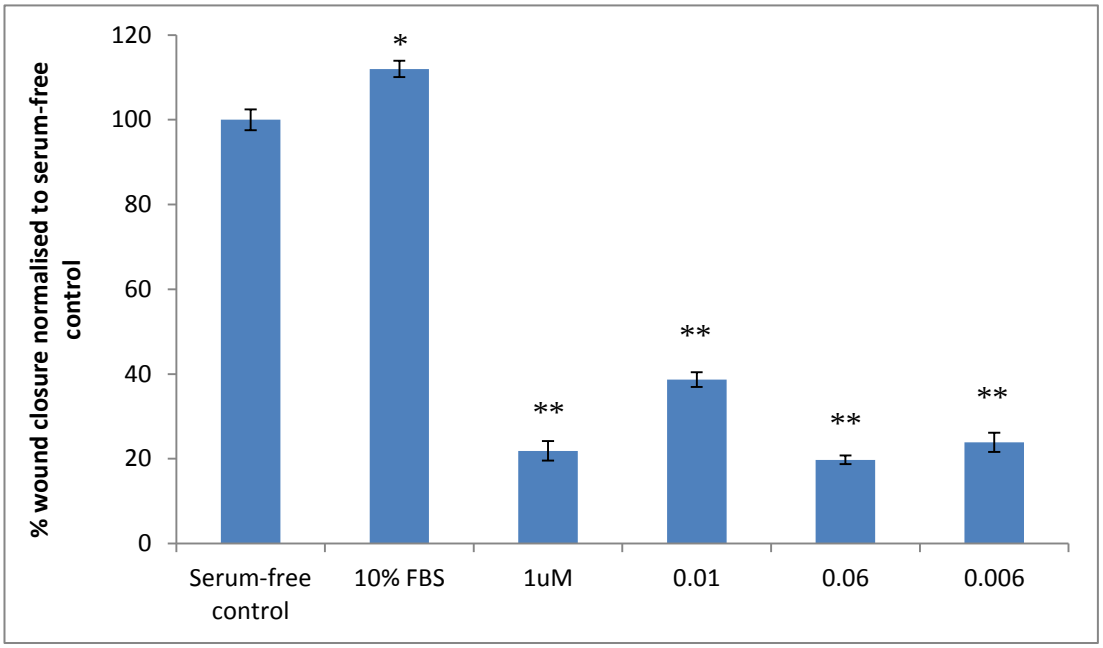

Figure 5.1- Dose-response curve showing the effects of varying dexamethasone concentrations on MDCK cell scratch wound healing. Data (presented as mean +/- SEM) were normalised to a serum-free control and analysed using one-way analysis of variance. There was a significant effect of glucocorticoid concentration, with supra- and sub-physiological concentrations of glucocorticoid significantly impairing healing. Furthermore, treatment with 10% FBS significantly promoted wound healing ($p<0.05$, indicated by *, $p<0.001$, indicated by **).

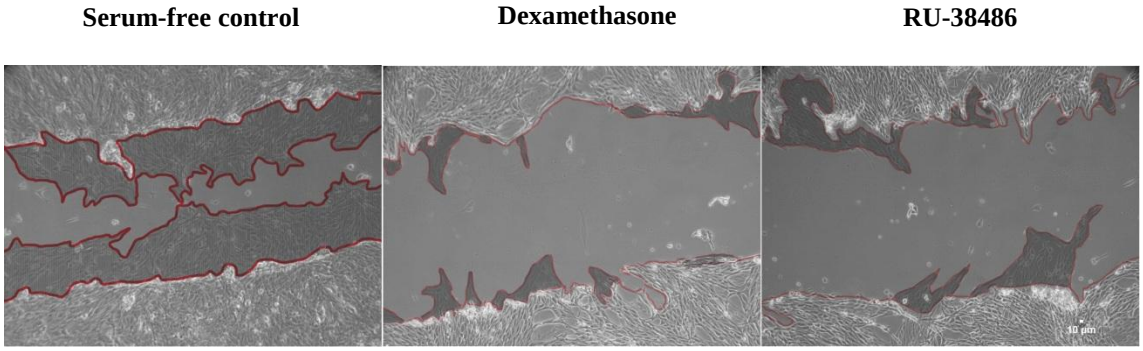

Figure 5.2- Representative photomicrographs highlighting the effects of serum-free medium, pharmacological dexamethasone (1µM) and GR antagonism (RU-38486) (1µM) on MDCK wound healing after 24 h treatment. Images taken at magnification of 10X; scale bar: 10µm

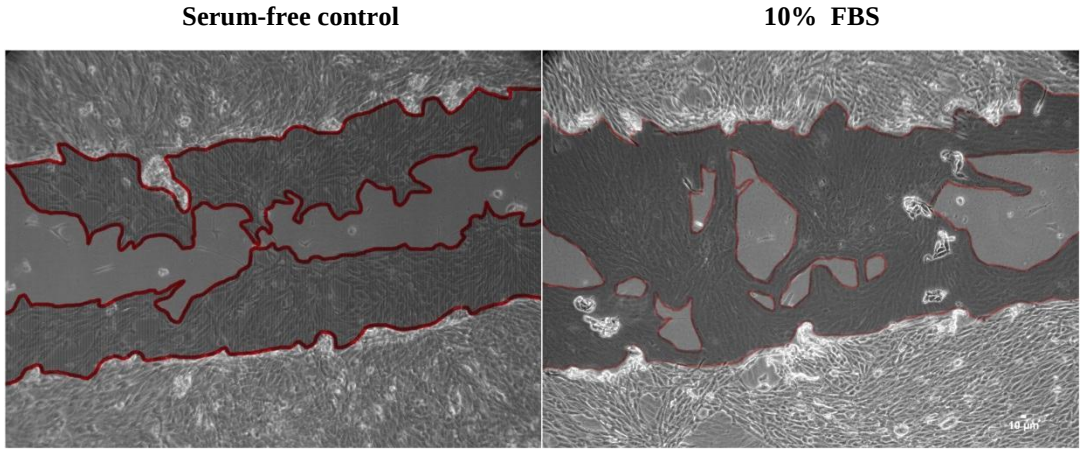

Figure 5.3- Representative photomicrographs highlighting effects of serum-starvation and 10% FBS on MDCK wound healing. Images taken at magnification of 10X; scale bar: 10µm

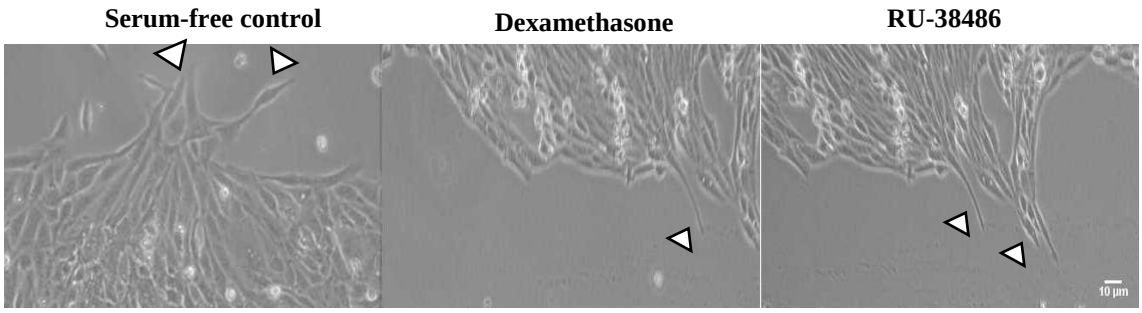

Figure 5.4- Representative photomicrographs of scratch wounded MDCK cells, highlighting cellular protrusions (arrowheads) at the wound edge. Images taken at magnification of 20X; scale bar: 10µm

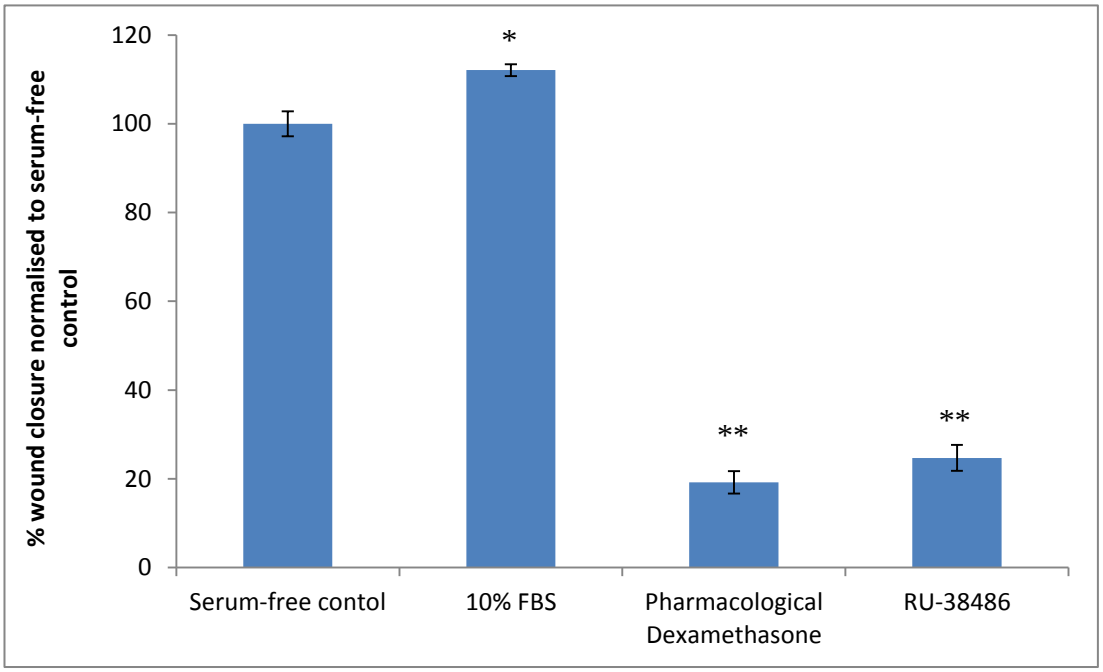

Figure 5.5- Effect of serum-free medium, 10% FBS, pharmacological dexamethasone and GR antagonism (RU-38486) on MDCK scratch wound healing. Data (presented as mean +/- SEM) were normalised to the serum-free control and analysed using one-way analysis of variance. There was a significant effect of treatment, with both pharmacological dexamethasone and RU-38486 significantly impairing healing and FBS significantly promoting wound healing when compared to control ($p<0.05$, indicated by *, $p<0.001$, indicated by **).

5.3.3.Gastric epithelial cell spreading and protrusive activity is not affected by treatment with pharmacological dexamethasone or a GR antagonist

Five independent spreading experiments, involving biopsies from five dogs were carried out for the purpose of this study. The dogs varied by breed, age and sex and as samples were obtained via routine endoscopies, the dogs were presenting at the Small Animal Hospital with varying gastrointestinal symptoms. The effects of dexamethasone and RU-38486 were assessed by measuring the effect of each drug on 25 individual cell islands in each experiment. Values for mean total spread area corrected for by cell count and mean fringe area corrected for by edge cell count were calculated for each experimental treatment, as well as a control treatment, containing FBS only.

Treatment with either pharmacological dexamethasone or RU-38486 had no statistically significant effects on either cell spreading (total area per cell) or the protrusive activity (fringe area per cell) of canine gastric epithelial cell islands (Figure 5.6 and Figure 5.7) when compared to the FBS control. Looking more closely at the individual cell islands (Figure 5.6), neither treatment appeared to prevent cells around the outside edge of islands from projecting normal cellular protrusions.

10% FBS only **Dexamethasone + 10% FBS** **RU-38486 + 10% FBS**

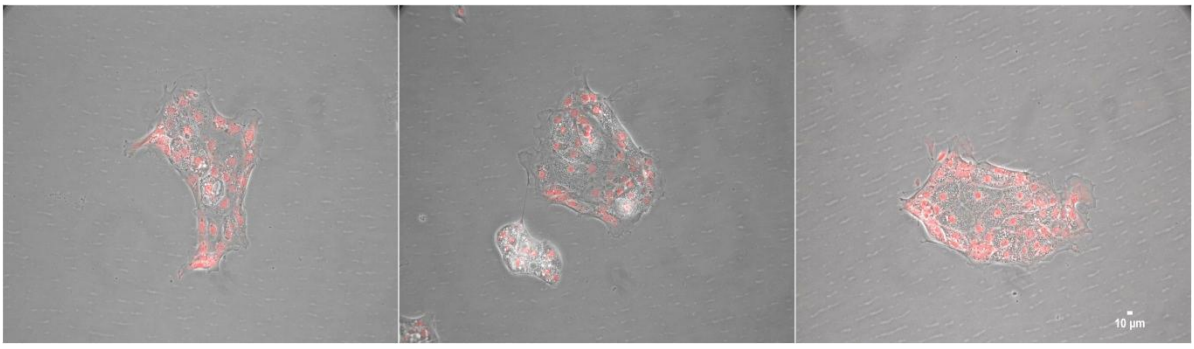

Figure 5.6- Representative photomicrographs highlighting the effects of 10% FBS, pharmacological dexamethasone (1µM) and GR antagonism (RU-38486) (1µM) on the spreading of primary gastric epithelial cell islands. Cell nuclei have been stained with propidium iodide; images taken at magnification of 20X; scale bar: 10µm

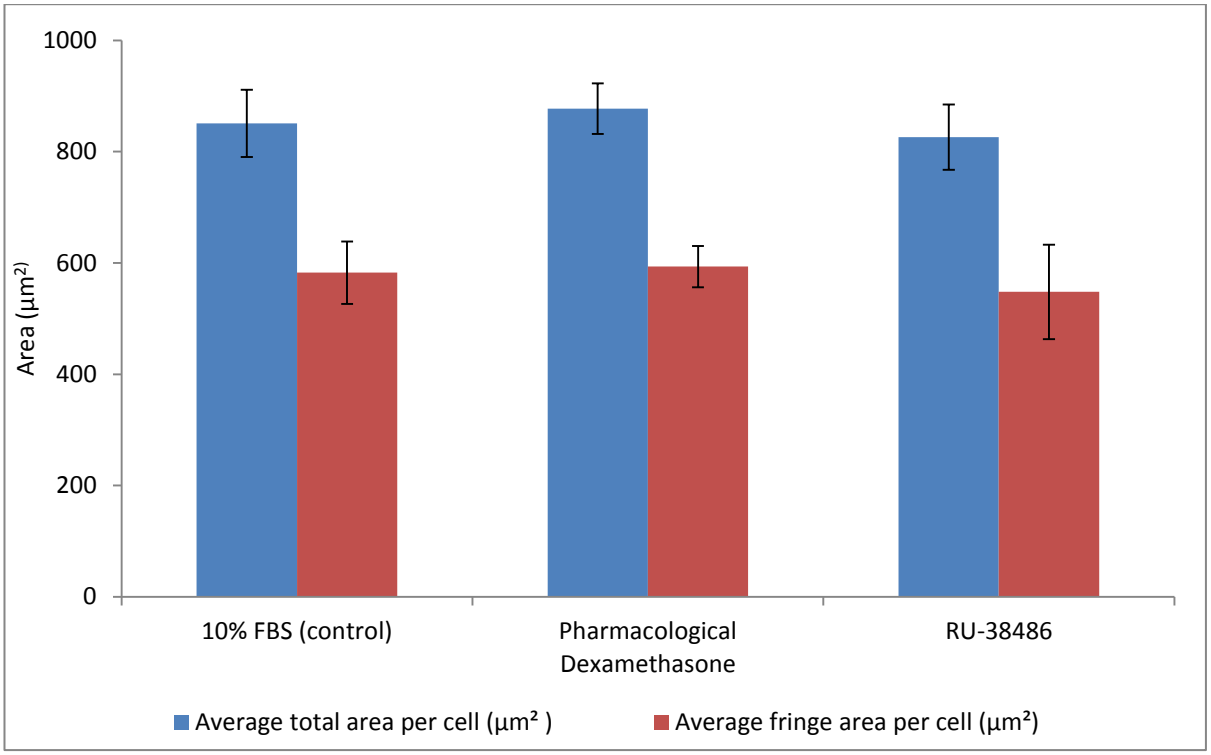

Figure 5.7- Effects of 10% FBS, pharmacological dexamethasone (1µM) and the GR antagonist, RU-38486 (1µM), on the spreading of primary canine gastric epithelial cell islands. Data presented as mean +/- SEM

5.3.4.Varying doses of dexamethasone and RU-38486 have no effect on epithelial cell spreading and protrusive activity

Given our previous findings (Section 5.3.3), we investigated whether higher or lower doses of dexamethasone and RU-38486 would have a statistically significant effect on gastric epithelial cell island spreading. Drug concentrations ranging from 0.1 µM to 10 µM were used for both treatments. The dose-response results show that there was no statistically significant effect for either drug on total spread area per cell or fringe area per cell (Figure 5.8 and Figure 5.9) when compared to the FBS control.

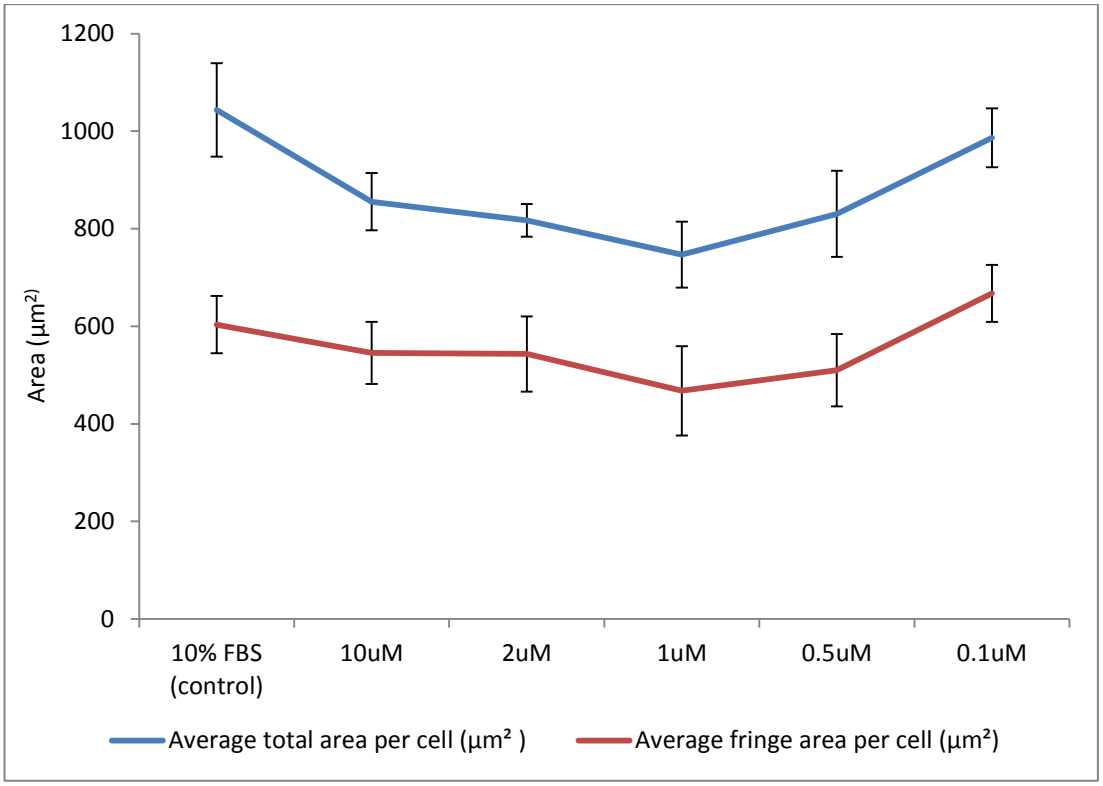

Figure 5.8- Dose response curve for dexamethasone. Data presented as mean +/- SEM

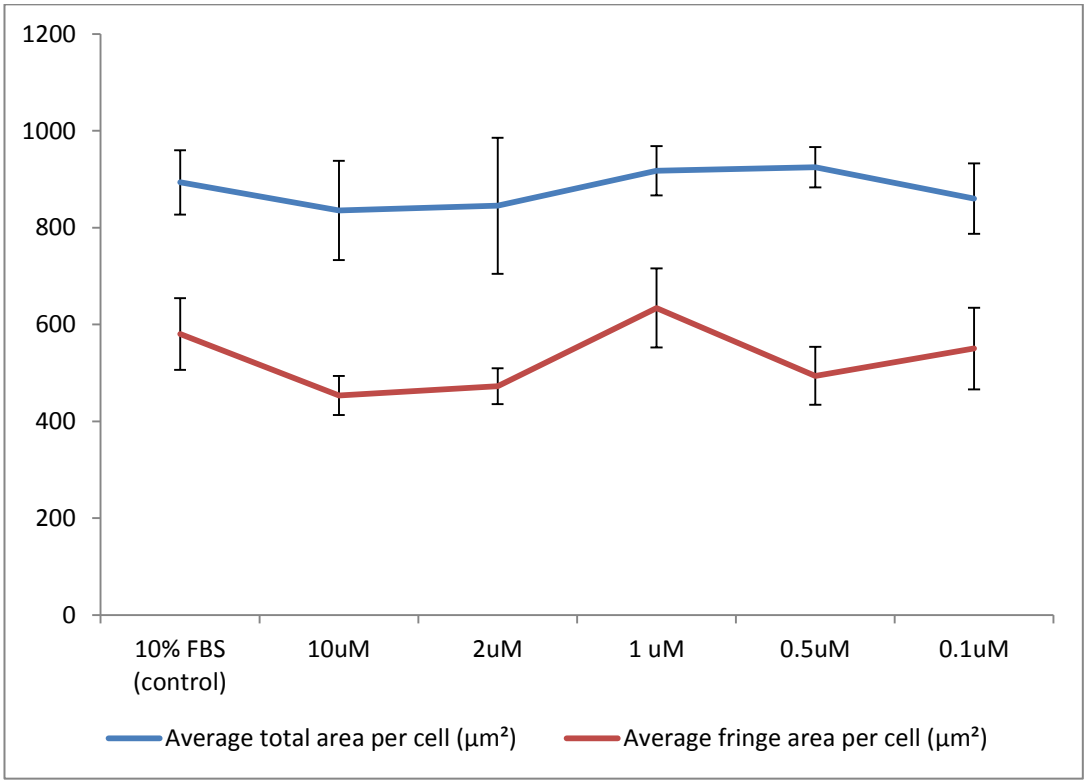

Figure 5.9- Dose response curve for RU-38486 (GR antagonist). Data presented as mean +/- SEM

5.4. Discussion

The aim of this investigation was to characterise the effects of glucocorticoids on epithelial cell migration and spreading *in vitro* by looking at the effects of GR agonism and antagonism. Two different models were used to study these effects, a scratch wound healing assay, using an immortalised epithelial cell line and a primary gastric epithelial cell spreading model, incorporating interactions between multiple cell types.

This study provides evidence that both dexamethasone and RU-38486 impair the healing of scratch wounded MDCK monolayers, thus suggesting that the GR has an important role in the modulation of epithelial cell wound healing. This supports previous studies which have reported an inhibition of epithelial wound healing *in vivo* following dexamethasone treatment (Petroutsos et al., 1982; Durmus et al., 2003). Dexamethasone has been associated with increased cellular adhesion to the ECM and increased expression of α1 and β1 integrin subunits on the cell surface (Murakami et al., 1998). Since interactions with the ECM can influence the migratory properties of cells (Murakami et al., 1998), this could explain the impairment in wound healing observed following dexamethasone treatment. Dexamethasone also inhibits COX-2 activity and thus the production of PGE_2 (Bandyopadhyay et al., 1999). PGE_2 is known to stimulate cell migration via activation of the PI3K/Akt pathway through EGF receptor transactivation (Buchanan et al., 2003). COX-2 derived PGE_2 was previously shown to have an important role in MDCK wound healing (Section 4.3.1).

Whilst exogenous glucocortioids have been reported to inhibit wound repair (Durmus et al., 2003), endogenous glucocorticoids appear to have a role in the regulation of wound repair via increased cell migration and proliferation and increased expression of certain growth factors and ECM proteins (Grose et al., 2002). Due to current evidence highlighting the opposing effects of physiological and pharmacological glucocorticoid levels on wound repair, the effects of varying glucocorticoid concentrations on MDCK scratch wound healing were examined.

Wounded monolayers treated with 10% FBS appeared to re-epithelialise fairly quickly, with approximately 80% of the wound area being covered after 24 h, while wounds in serum-starved monolayers were only 70% covered after 24 h. FBS contains many components which may influence cell migration, including physiological levels of cortisol. Commercially available FBS contains mean cortisol levels of 0.5 µg/ml (Price & Gregory, 1982). Thus when diluted to 10% in media, our cultures contained approximately 0.05 µg/ml of cortisol and the normal canine basal cortisol plasma levels are in the range of 0.006-0.06 µg/ml (Vaden et al., 2009). The results prsesented in this chapter also show that pharmacological (1 µM) and sub-physiological (<0.1 µM) doses of dexamethasone cause a significant inhibition of MDCK wound healing. Previous studies have shown that similar pharmacological concentrations of dexamethasone cause an inhibition of in *vitro* cell migration in a variety of cell types (Pross et al., 2002; Piette et al., 2009; Luo et al., 2009) and wound healing *in vivo* (Durmus et al., 2003). Glucocorticoid deficiency has been shown to aggravate indomethacin-induced gastric lesions (Filaretova et al., 2002), thus suggesting that physiological glucocorticoid levels are involved in gastroprotection.

Given the association between glucocorticoid therapy and the development of gastric ulceration (Bandyopadhyay et al., 1999), the effects of glucocortioids on gastric epithelial cell spreading, an important process involved in the maintenance of gastric epithelial integrity, were determined. A study conducted by Takahashi and colleagues (2003) reported an inhibition of gastric epithelial restitution following dexamethasone treatment. Similarly, under conditions of glucocorticoid deficiency, gastric lesions induced through indomethacin treatment have been shown to be significantly worse (Filaretova et al., 2002), thus the effects of glucocorticoid deficiency in our model were also studied using the GR antagonist, RU-38486. Although there has been extensive work conducted to study the effects of glucocorticoids on cell migration and spreading, there has been little study of their effects in a multicellular model, such as that used in this investigation.

The results presented in this chapter show that both GR agonism by 1µM dexamethasone and GR antagonism by 1 µM RU-38486 had no effect on cell spreading or the protrusive activity of cells in canine gastric epithelial cell islands, when compared to the FBS control. The effects of a wide range of doses of both dexamethasone and RU-38486 were studied, to ensure that the response was not dose dependant, however no statistically significant effect was found at any concentration. These results are suprising given that dexamethasone has previously been shown to impair the migration of gastric epithelial cells alone (Luo et al., 2009) or in coculture with gastric fibroblasts (Takahashi et al., 2003). RU-38486, also known as mifepristone, has also previously been shown to inhibit gastric cancer cell migration *in vitro* (Li et al., 2004).

The major difference between the previous studies and this investigation is the use of a multicellular model. The previous studies examining the effects of dexamethasone and GR on cell migration and spreading involve the use of dispersed cultures of gastric mucosal epithelial cells, discounting other cell types present in the gastric mucosa, such as parietal cells, chief cells and endocrine cells. As this study utilises intact gastric glands, the model should include a representative mixture of all cell types present in the gastric gland *in vivo*, therefore intercellular paracrine signalling between cells in this culture or direct cellular interactions could explain the lack of effects observed. Paracrine signalling has been investigated to some extent, via the use of a co-culture of gastric epithelial cells and gastric fibroblasts and dexamethasone was reported to inhibit gastric epithelial cell migration via the depletion of HGF mRNA expression and release by gastric fibroblasts (Takahashi et al., 2003). However, this study used RGM-1 cells, a cell line made up of a single cell type, thus intercellular signalling networks between different gastric epithelial cell lineages were not evaluated.

Several mechanisms have been reported for the effects of dexamethasone on cell migration. Dexamethasone inhibits migration via GR-dependant inhibition of the ERK1/2/MAPK pathway (Piette et al., 2009) and suppresses the release of HGF (Takahashi et al., 2003) and MMP-2 (Pross et al., 2002). Additionally,

dexamethasone inhibits TNF-α stimulated COX-2 expression, PGE_2 production and cell migration (Luo et al., 2009). These previous studies have all used cells that have either been serum-starved for 24 h prior to assay (Li et al., 2004; Luo et al., 2009) or cultured in serum-free medium during the assay (Li et al., 2004; Pross et al., 2002). HGF activation and secretion can be dose-dependantly stimulated in fibroblasts cultured with FBS at concentrations of 1% to 10% (Ohshima et al., 2002), similarly, FBS can activate ERK-1 and -2 (Lee et al., 2001). Dexamethasone also inhibits COX-2 expression, and thus PGE_2 release (Takahashi et al., 2003) and as reported previously (Section 4.3.4.2), COX-2 derived PGE_2 has a role in the modulation of cell migration in our model. ELISA analysis revealed that FBS contains relatively small amounts of PGE_2.

Furthermore, Yang and colleagues (2008) conducted a study looking at the effects of serum exposure and cell density on the localisation and function of the GR in primary human lung fibroblast cultures. Serum stimulation up-regulated GR mRNA and protein expression in both confluent and subconfluent cells. In subconfluent cells, the GR showed perinuclear localisation, whereas in confluent cells it was expressed both in the cytosol and nucleus and this was not caused by increased GR activation, as dexamethasone increased GR-GRE binding in both confluent and subconfluent cells. Confluent cells expressed 4.2 ± 1.6 times more basal GR protein than subconfluent cells. GR function was also affected by cell density, for instance, in subconfuent cells the GR binds to the transcription factor, C/EBP-α, leading to $p21^{(Waf1/Cip1)}$ expression and suppressed proliferation, whereas in confluent cells, GR binds to C/EBP-β, inducing $p27^{(Kip)}$ expression. As the gastric epithelial cells used in our model are both serum-stimulated and in subconfluent cell islands, it is difficult to compare the effects observed with those seen in previous experiments using serum-starved confluent monolayers. Taking these findings into account, serum stimulation could explain why no effect was observed. Ideally, all assays would be peformed on serum-starved cells in order to induce cell cycle synchronisation and to minimise analytical interference. However, given that treatments were applied immediately after the isolation of gastric glands, serum-free medium was not used because it was assumed to prevent gland spreading.

Another possible reason for the lack of glucocorticoid effect would be if the cells in this model do not express GR. However, GR is known to be expressed ubiquitously in the majority of tissues (Kalinyak et al., 1987). GR expression has previously been described in parietal cells (Kanemasa et al., 1999), chief cells and gastric endocrine cells (Tarasova et al., 1996). GR immunoreactivity in the nuclei of parietal cells was shown to diminsh in adrenolectomised rats (Kanemasa et al., 1999), thus suggesting that GR immunoreactivity is dependant on the presence of its ligand. Activation of GR regulates the expression of GR mRNA in a tissue-specific manner (Kalinyak et al., 1987), thus GR expression can vary greatly among cell types, which may influence cellular responses to glucocorticoids. Immunocytochemical analysis of GR expression in our cultured gastric epithelial cell islands was attempted, however the pattern of fluorescence observed was indistinct and non-specific (data not presented). In future studies it would be useful to to perform double immunofluorescence labelling in order to characterise cell type and GR expression. Primers were designed for the analysis of GR mRNA expression in this model, however due to time constraints RT-PCR analysis was not performed. Other methods considered for analysis of GR expression in this model include, *in situ* mRNA hybridisation and RNase protection analysis. *In situ* hybridisation and RNase protection analysis have been successfully used to study GR mRNA expression in rat tissue (Freeman et al., 2004). The advantage of *in situ* hybridisation is that it enables anatomical localisation of the receptor. Additionally, characterisation of HGF and MMP secretion within our model would be useful in order to characterise the specific effects of dexamethasone on their regulation. HGF secretion by primary gastric fibroblasts has been characterised previously using a specific ELISA kit (Takahashi et al., 2003). MMP sercretion and activity can be effectively analysed using substrate zymography techniques. These methods were also not performed due to time constraints.

Chapter 6 - Characterising the expression of COX-2 and the prostaglandin receptors, EP3 and EP4 in epithelial cells

6.1. Introduction

The actions of COX-derived PGE_2 are mediated by the G-protein-coupled EP receptors (Narumiya et al., 1999) and it has been demonstrated that EP3 and EP4 have important roles in the promotion of both cell migration and wound healing. For cell migration, EP4 activation has been shown to promote endothelial cell migration via ERK activation (Rao et al., 2007), lung cancer cell migration via cellular Src tyrosine phosphorylation (Kim et al., 2010) and to regulate metastatic breast cancer cell migration via cAMP signalling (Timoshenko et al., 2003). Furthermore, activation of the EP3 receptor promotes the migration of human arterial smooth muscle cells and CHO cells, transfected to overexpress the EP3 receptor (Blindt et al., 2002).

For wound healing, PGE_2/EP receptor-mediated signalling has been shown to have an important role in the modulation of scratch wound healing, thus, COX-2 derived PGE_2 stimulates ISMF wound healing via EP2, EP3 and EP4 receptor activation (Iwanaga et al., 2012). PGE_2/EP4 signalling indirectly stimulates ISMF migration by inducing growth factor secretion, while EP3 activation may have a direct effect (Iwanaga et al., 2012). However the mechanism underlying these effects is unclear.

EP receptor activation also modulates gastrointestinal mucosal integrity. PGE_2 promotes the healing of gastric ulcers via EP4 receptor activation, leading to up-regulation of VEGF expression (Hatazawa et al., 2007). Treatment with an EP4 selective agonist alleviates NSAID-induced damage and promotes mucous epithelial regeneration (Jiang et al., 2009). The EP4 agonist also accelerates gastric ulcer healing in the absence of NSAID, suggesting EP4 agonism may promote the healing of both existing and NSAID-aggravated ulceration (Jiang et al., 2009).

An important role for COX-2 derived PGE_2 in epithelial cell spreading and scratch wound healing was described previously (Chapter 4). In view of this it was considered important to study the expression of EP3, EP4 and COX-2 within epithelial cells. Thus, the aim of this study was to test the hypothesis that COX-2, EP3 and EP4 are expressed within the normal canine gastric epithelium and in cultured epithelial cells and that their expression is readily induced in response to acute stressors.

6.2. Materials and methods

6.2.1.PCR

6.2.1.1.RNA isolation

To identify the presence of specific mRNA transcripts, RNA was isolated from cultured epithelial islands and MDCK cells as previously described (Section 2.8.1)

6.2.1.2.Reverse transcription

RNA was transcribed into cDNA as previously described (Section 2.8.2).

6.2.1.3.PCR

Reverse transcription PCR, as described previously (Section 2.8.3) was used to determine gene expression in cDNA samples. Samples were run on a 1.5% agarose gel.

6.2.1.4.Sequencing of PCR products

Where possible, PCR products were sequenced commercially by Eurofins MWG Operon (Eurofins MWG Operon, Ebersberg, Germany), using the same primers that were used for the PCR amplification. A QIAquick® PCR purification kit (Qiagen) was used to purify the amplified products prior to sequencing.

6.2.2.Western blotting

6.2.2.1.Protein extraction

Protein was extracted from cultured cells and mucosal tissue (Section 2.8.7.1) and protein concentration was determined using a BCA protein assay kit, as described previously (Section 2.8.7.2).

6.2.2.2.Western blots

Western blotting was carried out as described previously (see section 2.8.7.3). The primary antibodies used were anti-EP3 goat polyclonal antibody at 1:200, anti-EP4 goat polyclonal antibody at 1:200 and anti-COX-2 goat polyclonal antibody at 1:200. The secondary antibody was a rabbit polyclonal anti-goat IgG (HRP-conjugated) used at 1:10,000. Blots were stripped and re-probed as previously described (Section 2.8.7.4) using an anti-beta actin goat polyclonal antibody at 1:1000 and a rabbit polyclonal anti-goat IgG (HRP-conjugated) at 1:10,000.

6.2.3.Dot blots

Dot blots were performed in order to compare expression in larger sample numbers, using the same antibodies and dilutions used for the Western blot assays, as described previously (see section 2.8.7.6).

6.2.4.Immunohistochemistry

Immunohistochemistry techniques were performed by Veterinary Pathology, University of Liverpool as described previously (Section 2.9.1). The primary antibodies used were anti-EP4 goat polyclonal antibody at 1:50 and anti-COX-2 goat polyclonal antibody at 1:400. The secondary antibody was a biotinylated horse anti-goat IgG, used at 1:100 in both cases.

6.2.5.Immunocytochemistry

Immunocytochemistry techniques were used to localise protein expression in cultured epithelial cell islands, using the primary antibodies described previously at 1:250. The secondary antibody used was a FITC-conjugated donkey anti-goat IgG used at 1:200. The methods for this procedure are described in detail in chapter 2 (Section 2.9.2).

6.3. Results

6.3.1.COX-2 mRNA expression in primary canine gastric epithelial cells

RT-PCR was used to confirm the expression of COX-2 in cultured gastric epithelial cells isolated from biopsy-derived mucosal tissue. The resulting PCR product was of the predicted size (Figure 6.1). Attempts to sequence this product were unsuccessful.

6.3.2.COX-2 protein expression in primary canine gastric epithelial cells, and the cell lines MKN-45 and MDCK

COX-2 expression in cultured gastric epithelial cells from both cadaver and biopsy samples was determined by Western blot analysis. COX-2 protein was detected at very low levels in only one of the three cadaver–derived cell samples and no COX-2 expression was detected in three biopsy-derived cell samples (Figure 6.2). Furthermore, the COX-2 protein was detected in MKN-45 cells but not in MDCK cells (Figure 6.2).

To determine the effects of serum-starvation on COX-2 protein expression, MDCK and gastric epithelial cells were grown in serum-free conditions for 12 h prior to protein extraction. COX-2 protein expression was induced via serum-starvation in all cell types (Figure 6.3).

6.3.3.COX-2 protein expression in tissue from multiple individuals

COX-2 protein expression in whole gastric mucosal tissue samples was determined using dot blot analysis. Tissue samples used for this assay were stored at -80ºC immediately after collection. The results of the dot blot analysis reveal varying COX-2 protein expression between individual samples (Figure 6.4 and Figure 6.6), however, densitometry revealed no statistically significant difference between the mean COX-2 protein expression levels, corrected for beta actin, in biopsy- and cadaver-derived tissue samples (Figure 6.5)

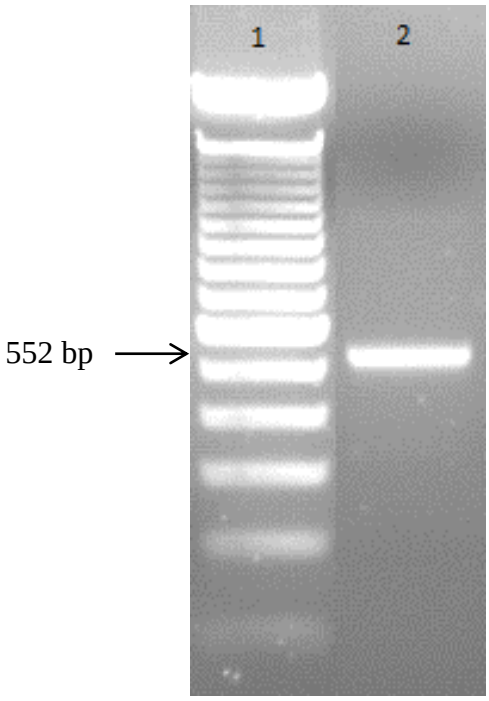

Figure 6.1- RT-PCR analysis of COX-2 mRNA expression in cultured gastric epithelial cells. Lane 1: Track It™ 100bp ladder, Lane 2: cDNA from gastric epithelial cells isolated from biopsy-derived canine mucosal tissue. The predicted size of the PCR product was 552 bp.

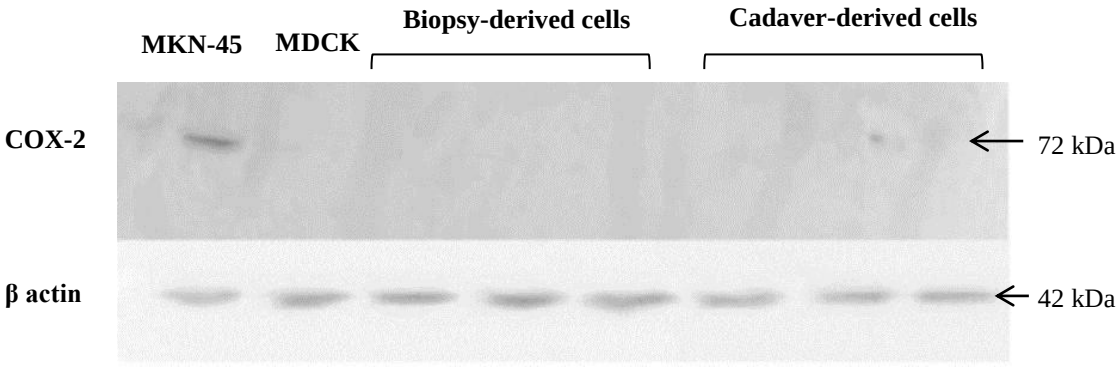

Figure 6.2- Western blot analysis of COX-2 protein expression in MKN-45 (lane 1) and MDCK (lane 2) cells, biopsy-derived cells from three individuals (lanes 3-5) and cadaver-derived cells from three individuals (6-8), all cultured in serum-containing (10% FBS) medium (upper panel). Beta actin was used as a loading control (lower panel).

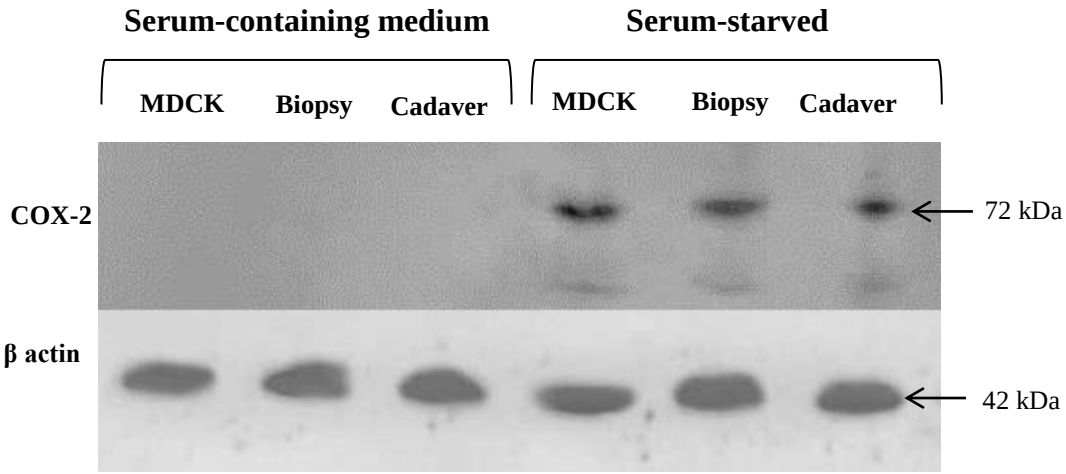

Figure 6.3- Western blot analysis of COX-2 protein expression in cells grown in serum-containing (10% FBS) medium (lanes 1-3) and serum-starved cells (lanes 4-6). Serum starvation leads to increased expression of COX-2 in MDCK, biopsy- and cadaver-derived cells (upper panel); beta actin expression (lower panel) is unaffected.

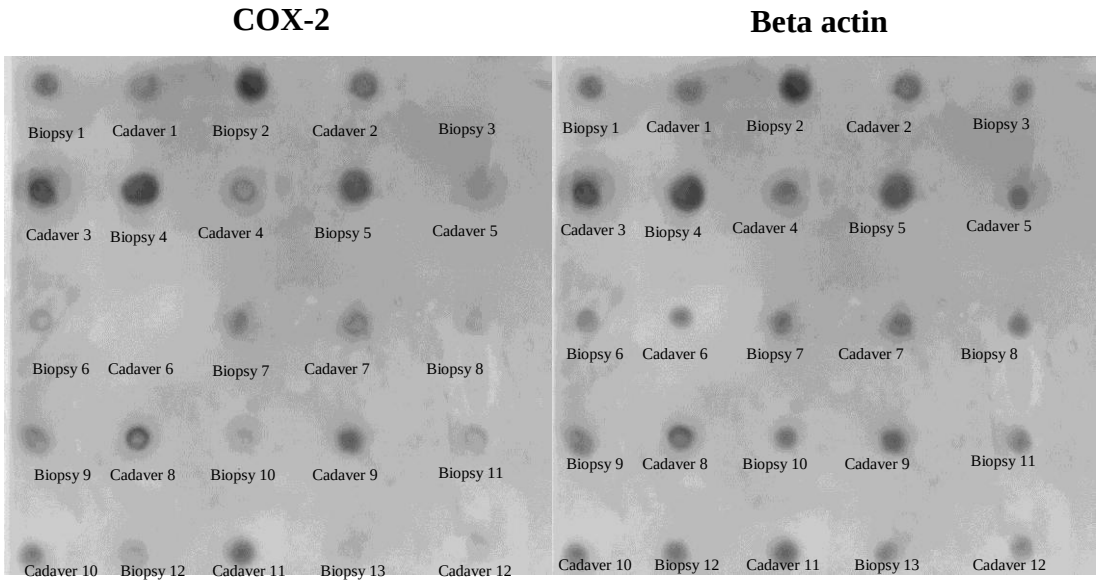

Figure 6.4- Dot blot analysis of COX-2 protein expression in biopsy- and cadaver-derived gastric mucosal tissue (left panel). Beta actin was used as a loading control (right panel).

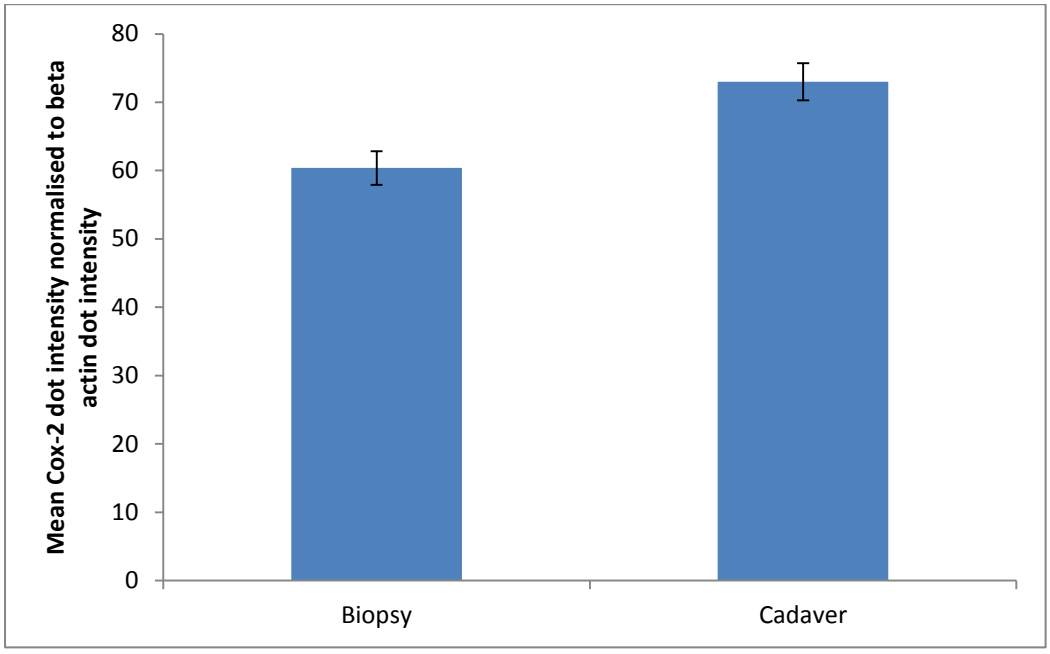

Figure 6.5- Dot blot densitometry quantifying COX-2 protein expression in biopsy- and cadaver-derived gastric mucosal tissue. Values normalised to corresponding beta actin signal.

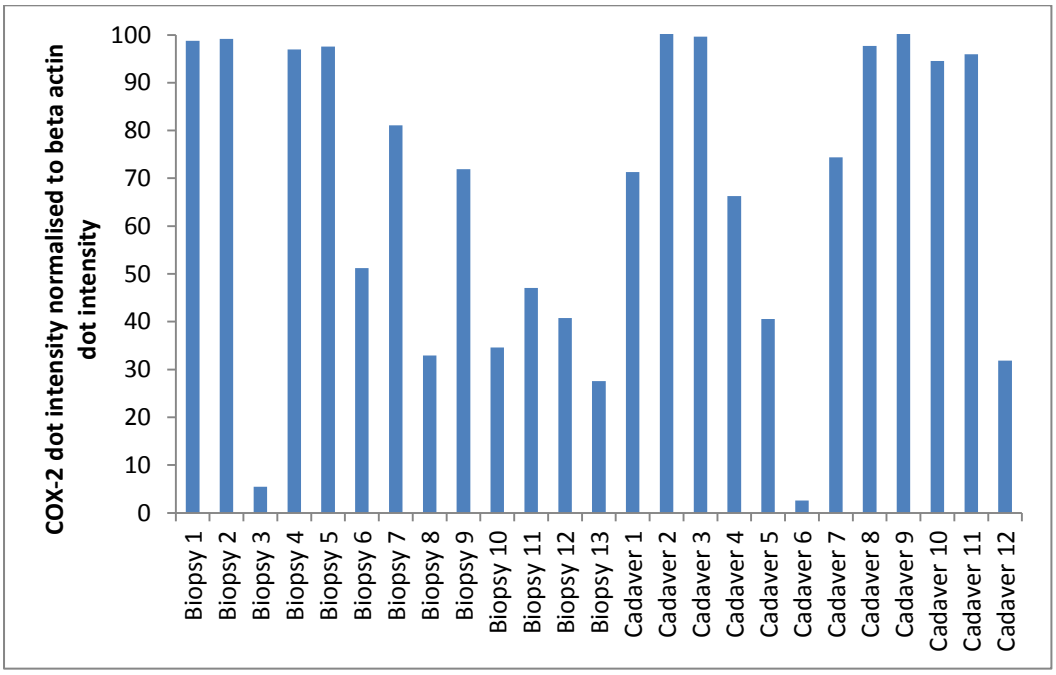

Figure 6.6- Dot blot densitometry quantifying COX-2 protein expression in individual biopsy- and cadaver-derived gastric mucosal tissue samples. Values normalised to corresponding beta actin signal.

6.3.4.Localisation of COX-2 expression in normal canine gastric mucosa

Immunohistochemistry analysis of canine gastric mucosa, revealed COX-2 immunoreactivity in the gastric glands (Figure 6.7). Cells were stained with haematoxylin and eosin and were identified based on their morphology. Strong COX-2 staining was observed for chief cells and weak staining for parietal cells (Figure 6.7).

Furthermore, immunocytochemistry was used to localise COX-2 expression in cultured gastric epithelial cell islands. Approximately 90% of cells within the epithelial islands were shown to strongly express COX-2. In cells that did express COX-2, a granular cytoplasmic staining pattern was apparent, with perinuclear localisation (Figure 6.8). Some cells showed no or very weak COX-2 expression, based on their morphology, these appear to be parietal cells (Figure 6.8).

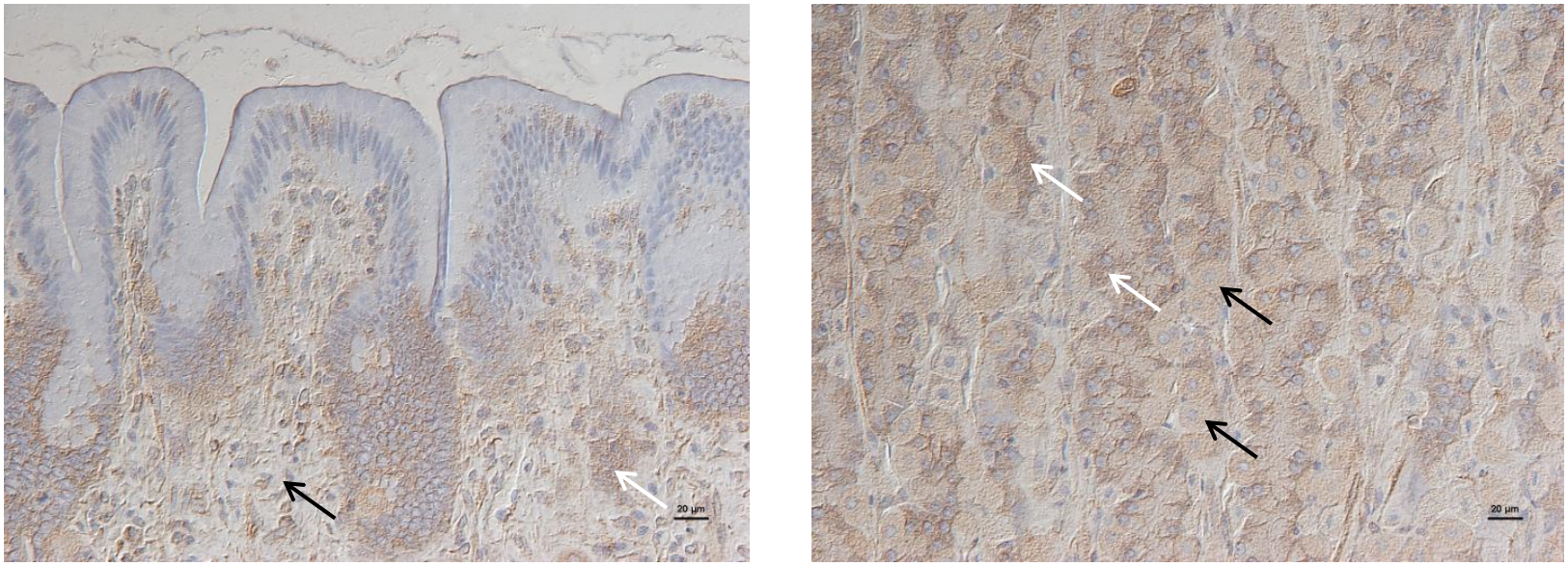

Figure 6.7- Immunohistochemical localisation of COX-2 in the canine gastric mucosa. Black arrows indicate weakly stained parietal cells and white arrows indicate strongly stained chief cells. Scale bar: 20µm

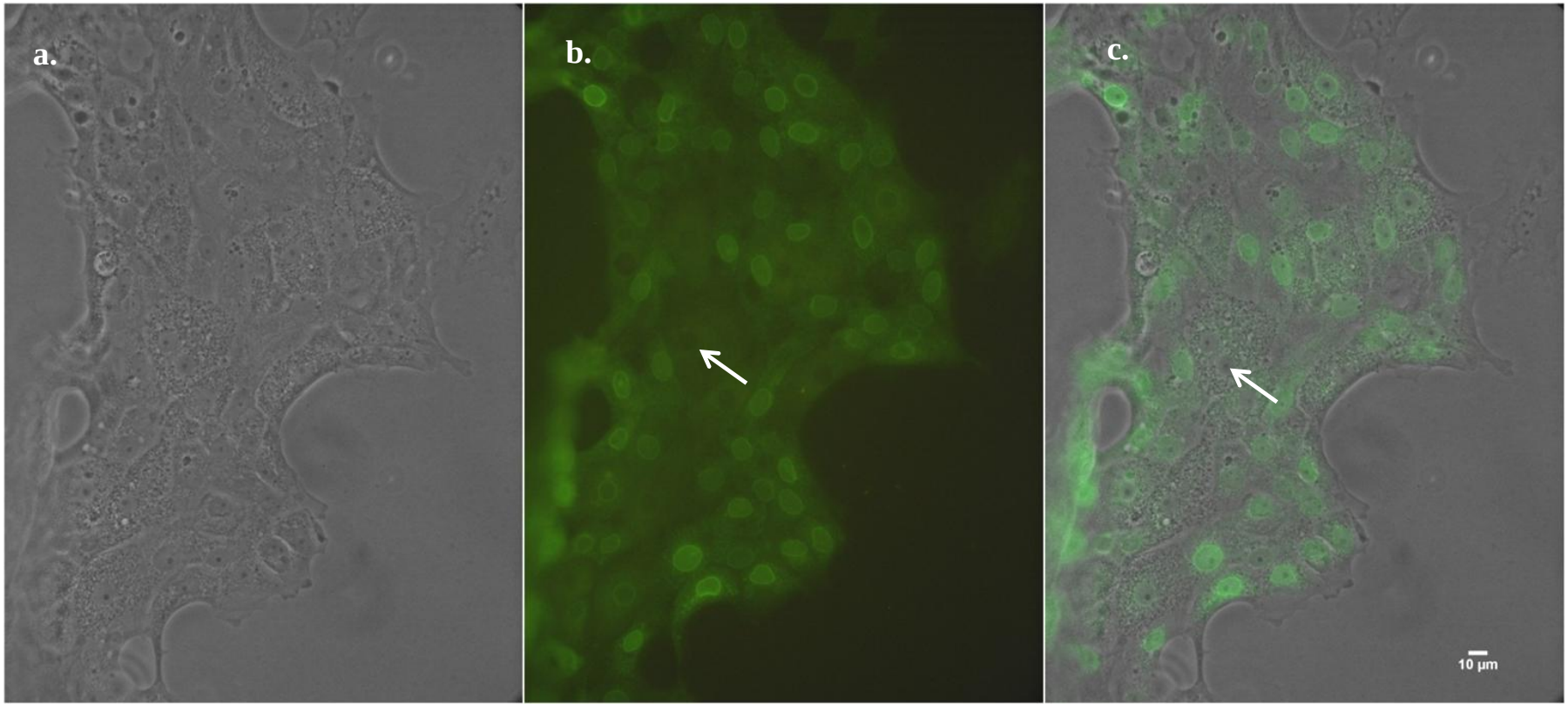

Figure 6.8- Immunocytochemical staining of COX-2 in a cultured canine gastric epithelial cell island; a.) bright field image, b.) fluorescence image, c.) fluorescence image superimposed onto bright field image. Arrow indicates a non-expressing cell. Magnification of 40X; scale bar 10µm

6.3.5.EP3 and EP4 receptor mRNA expression in primary canine gastric epithelial and MDCK cells

Expression of EP3 and EP4 mRNA in MDCK and biopsy-derived canine gastric epithelial cells was determined using RT-PCR. EP3 and EP4 mRNA was expressed in both cell types (Figure 6.9). The housekeeping gene, GAPDH was used as an internal positive control. The PCR products were purified and sent away for sequencing to confirm their identity. The sequencing results showed that both PCR products had 99% homology with the published EP3 and EP4 cDNA sequences (data not presented).

Analysis of EP1 and EP2 mRNA expression was attempted in both cultured gastric epithelial cells and MDCK cells, however no detectable bands were obtained.

6.3.6.EP3 and EP4 protein expression in primary canine gastric epithelial cells, and the cell lines MKN-45 and MDCK

EP3 and EP4 protein expression in canine gastric epithelial, MDCK and MKN-45 cells was determined using Western blot analysis. An initial experiment, using cells grown in serum-containing medium, identified high levels of EP3 expression in MKN-45 cells and cadaver–derived gastric epithelial cells. In contrast, relatively low expression was detected in biopsy-derived gastric epithelial cells and no expression was detected for MDCK cells (Figure 6.10). EP4 protein expression was detected only in cadaver-derived gastric epithelial cells (Figure 6.10). The observed bands of approximately 62 kDa and 53 kDa correspond with the predicted size. A 42 kDa band was identified using a beta actin antibody and confirmed even loading.

In order to simulate an acute stress response *in vitro*, cells were serum-starved for 12 h prior to protein extraction, EP3 and EP4 expression was then analysed and compared to expression in cells maintained in serum-containing medium. Serum-starvation induced EP3 and EP4 expression in all cell types (Figure 6.11). In cells maintained in serum-containing medium, no EP3 expression was detected and EP4 was only expressed in cadaver-derived gastric epithelial cells (Figure 6.11).

Contrastingly, cadaver-derived gastric epithelial cells were previously shown to express EP3 protein when grown in serum-containing medium (Figure 6.10).

Expression of EP3 and EP4 was compared in cultured cells from cadavers and biopsies (n=4 for each). Expression was corrected for loading variation by normalising to beta actin expression. Cadaver-derived cells had a significantly higher expression of EP3 and EP4 (Figure 6.12 and Figure 6.13). There was also some variation in protein expression between individual samples obtained from the same source (Figure 6.14).

6.3.7.EP3 and EP4 protein expression in tissue from multiple individuals

Inter- and intra-group variations in EP3 and EP4 protein expression between the biopsy- and cadaver-derived groups were studied further, via dot blot experiments using tissue samples that were stored at -80ºC immediately after collection. Dot blot analysis confirmed a statistically significant increase in EP3 expression in cadaver-derived tissue (Figure 6.15 and Figure 6.16), compared with biopsy tissue. A slight increase in EP4 protein expression was observed for cadaver-derived tissue; however this difference was not statistically significant (Figure 6.15 and Figure 6.16). Notably, variation in both EP3 and EP4 protein expression between individual samples was observed (Figure 6.17).

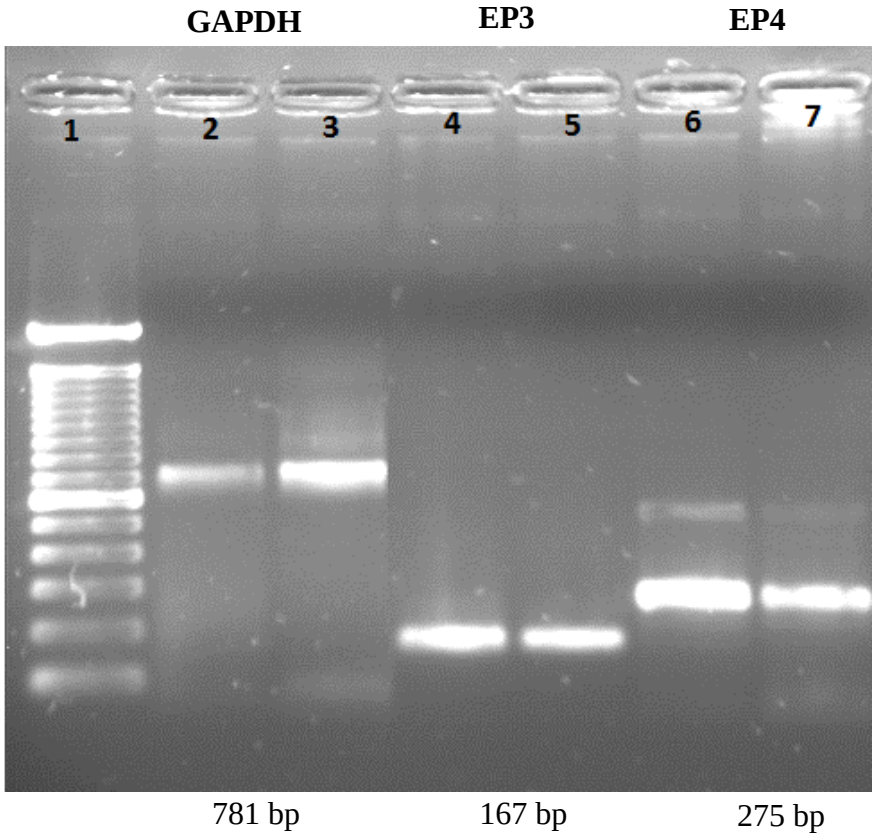

Figure 6.9- RT-PCR analysis of EP3 (lanes 4-5) and EP4 (lanes 6-7) mRNA expression in cultured gastric epithelial and MDCK cells. Lane 1: Track It™ 100bp ladder, Lanes 2, 4 & 6: cDNA from MDCK cells, Lanes 3, 5 and 7: cDNA from gastric epithelial cells isolated from biopsy-derived mucosal tissue. GAPDH was used as a positive control and the product sizes are indicated below the corresponding lanes.

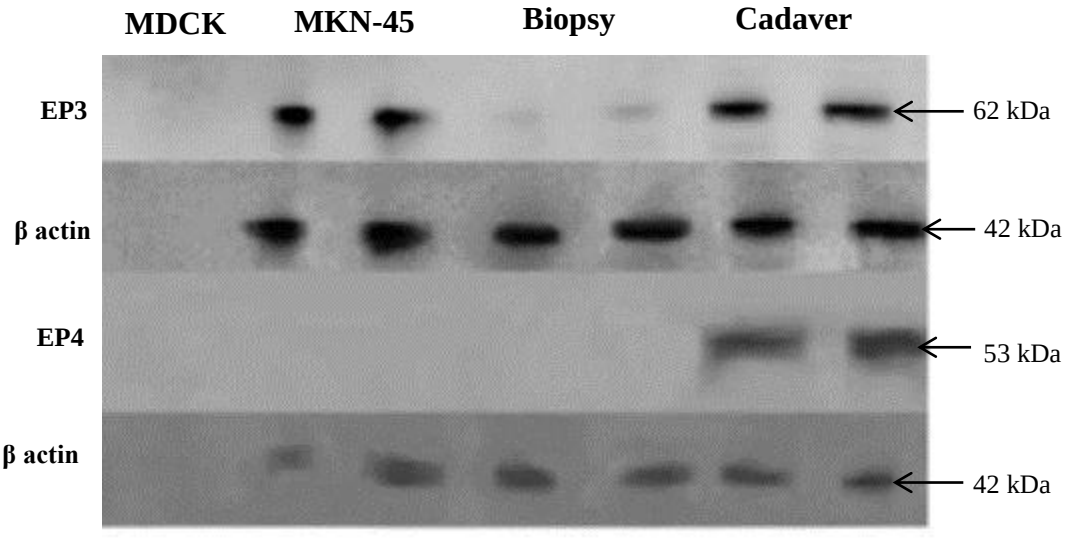

Figure 6.10- Western blot analysis of EP3 and EP4 protein expression in MDCK (lanes 1-2) and MKN-45 (lanes 3-4) cells, biopsy-derived cells from two individuals (lanes 5-6) and cadaver-derived cells from two individuals (lanes 7-8), all cultured in serum-containing (10% FBS) medium (upper panels). Beta actin was used as a loading control (lower panels).

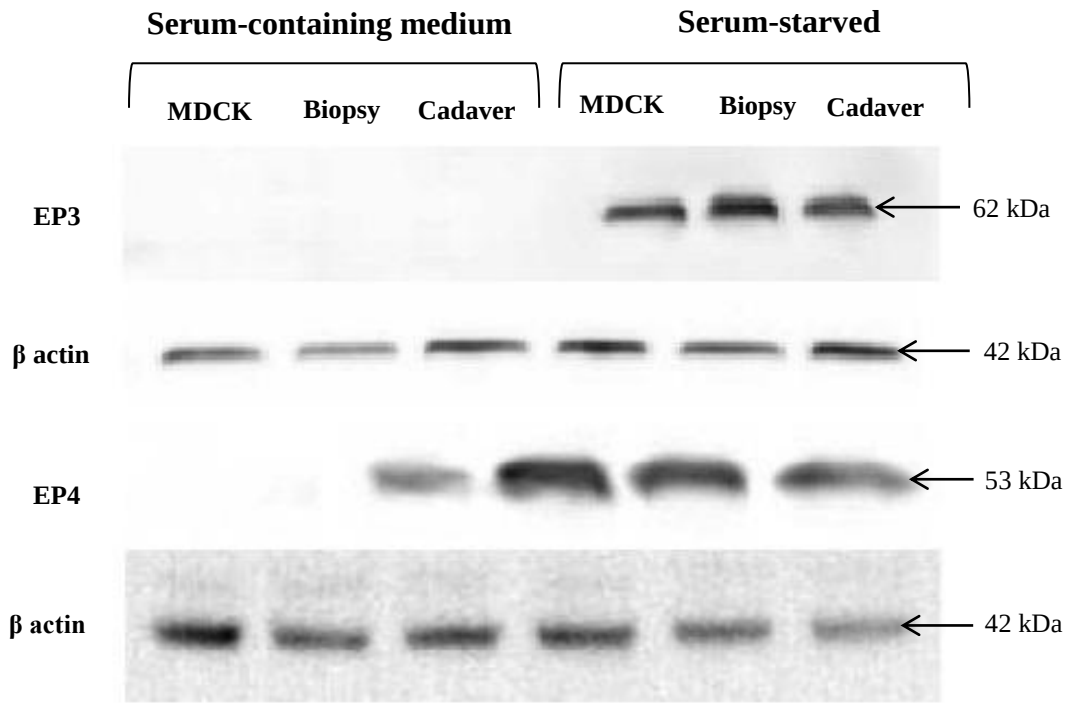

Figure 6.11- Western blot analysis of EP3 and EP4 protein expression in cells grown in serum-containing (10% FBS) medium (lanes 1-3) and serum-starved cells (lanes 4-6). Serum starvation leads to increased expression of EP3 and EP4 in MDCK, biopsy- and cadaver-derived cells (upper panels); beta actin expression (lower panels) is unaffected.

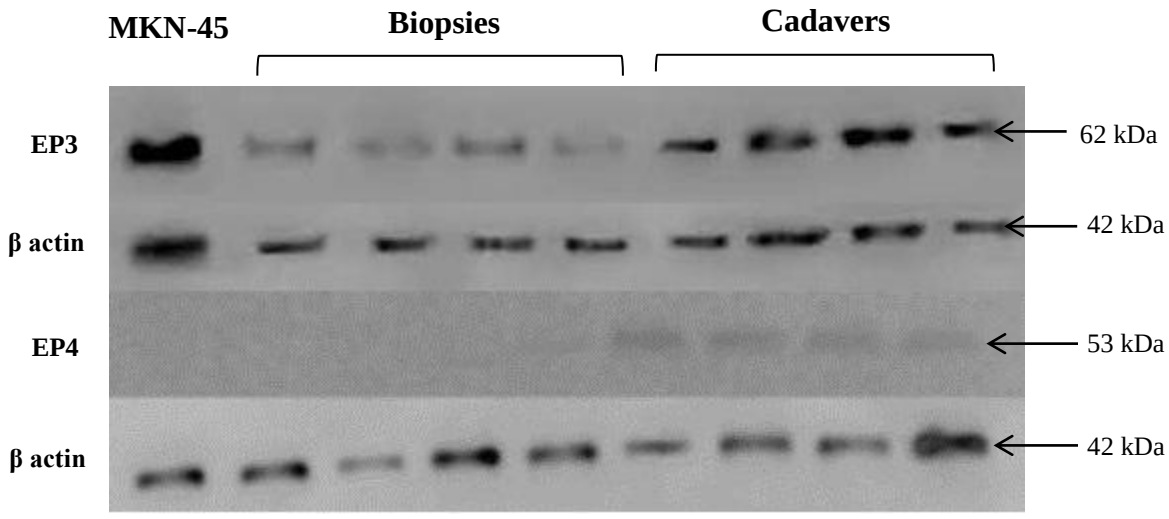

Figure 6.12- Western blot analysis of EP3 and EP4 protein expression in biopsy- (lanes 2-5) and cadaver-derived (lanes 6-9) gastric epithelial cells cultured in serum-containing (10% FBS) medium (upper panels). Cells isolated from four individuals were analysed for each group. MKN-45 cells were used as a positive control (lane 1). Beta actin was used as a loading control (lower panels). Expression of both EP3 and EP4 was significantly higher in cadaver-derived gastric epithelial cells.

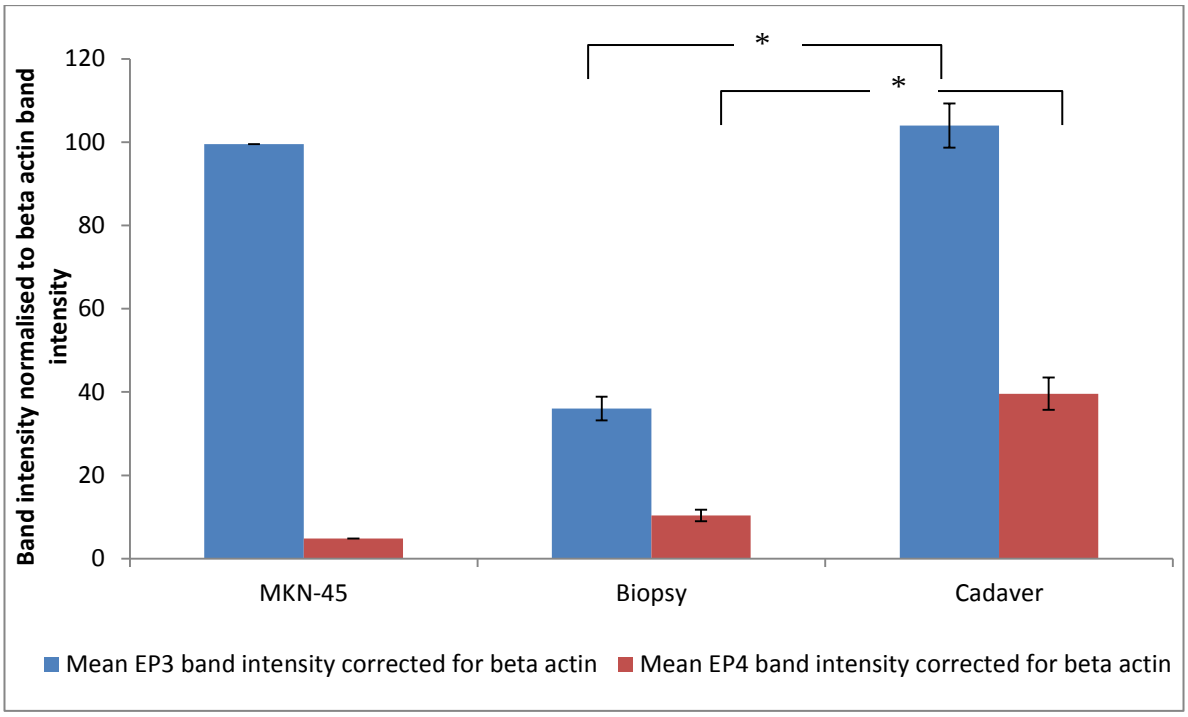

Figure 6.13- Western blot band densitometry quantifying EP3 and EP4 protein expression in gastric epithelial cells isolated from biopsy- and cadaver-derived tissue. Values, corrected for loading using beta actin and analysed using a paired t-test, show increased EP3 and EP4 expression in cadaver-derived cells compared to biopsy-derived cells (*= $P < 0.05$). MKN-45 cells were used as a positive control.

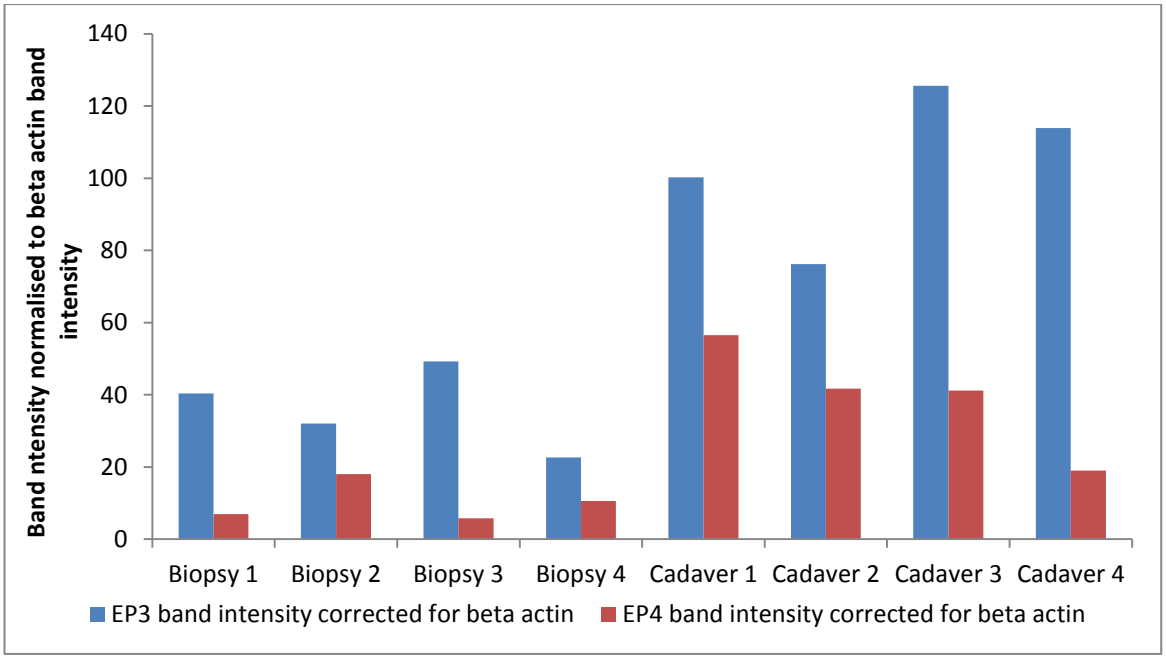

Figure 6.14- Western blot band densitometry quantifying EP3 and EP4 protein expression in cultured gastric epithelial cells isolated from biopsy- or cadaver-derived tissue. Values normalised to corresponding beta actin signal.

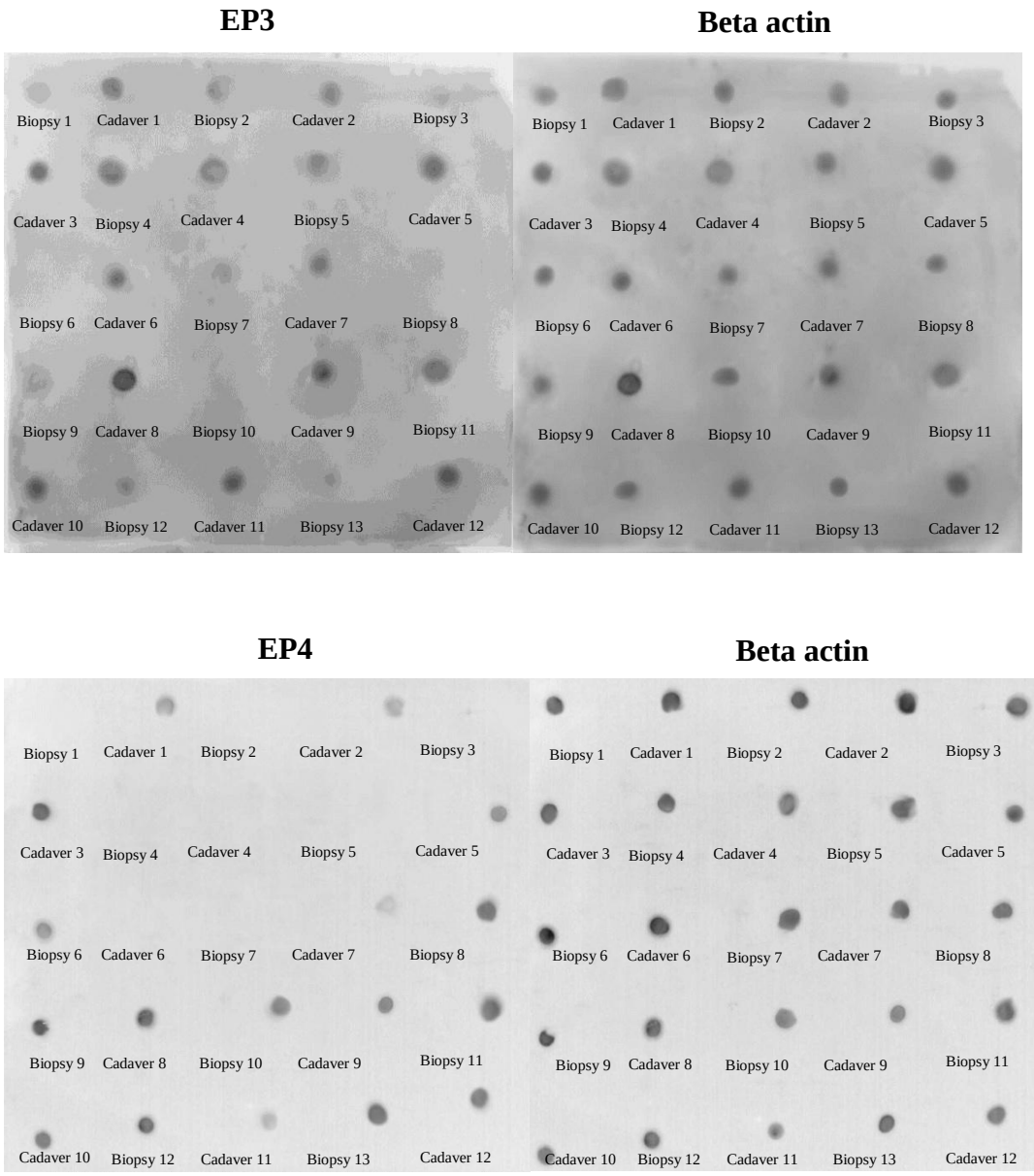

Figure 6.15- Dot blot analysis of EP3 (upper left panel) and EP4 (lower left panel) protein expression in cadaver and biopsy-derived gastric mucosal tissue. Beta actin was used as a loading control (right panels).

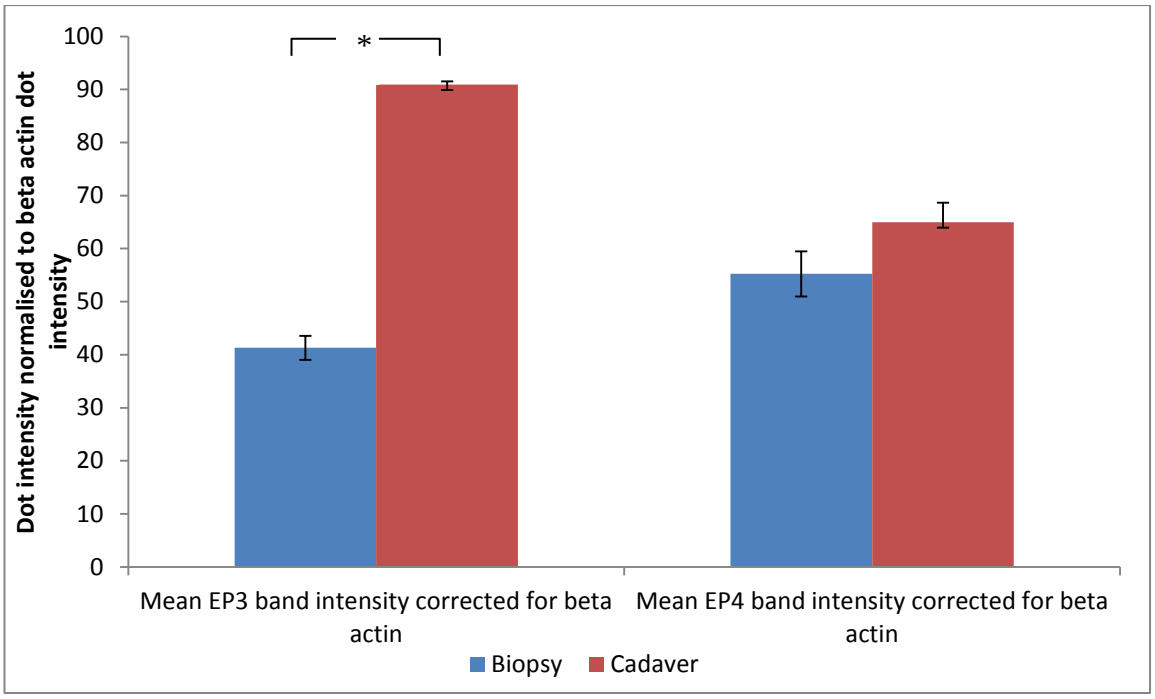

Figure 6.16- Dot blot densitometry quantifying EP3 and EP4 protein expression in biopsy- and cadaver-derived gastric mucosal tissue. Values, corrected for loading using beta actin and analysed using a paired t-test, show increased EP3 expression in cadaver-derived tissue compared to biopsy-derived tissue (*= $P < 0.001$).

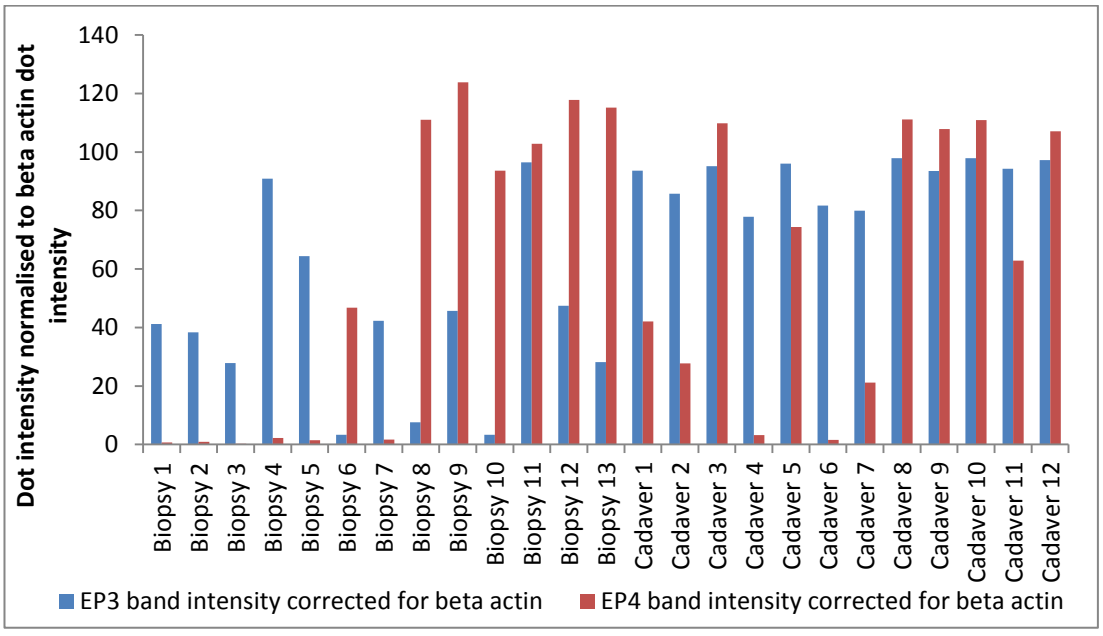

Figure 6.17- Dot blot densitometry quantifying EP3 and EP4 protein expression in individual biopsy- and cadaver-derived gastric mucosal tissue samples. Values normalised to corresponding beta actin signal.

6.3.8.Localisation of EP4 receptor expression in normal canine gastric mucosa

Immunohistochemical localisation of EP4 in cadaver-derived gastric mucosal tissue showed faint positive staining of the parietal cells (Figure 6.18). Immunocytochemistry analysis of cadaver-derived gastric epithelial islands revealed EP4 expression in a single cell. The cell expressing EP4 showed predominately cytoplasmic staining (Figure 6.19).

Immunocytochemical analysis of EP3 expression in cultured gastric epithelial islands was unsuccessful, only non-specific, diffuse cytoplasmic staining was observed in these experiments (data not presented).

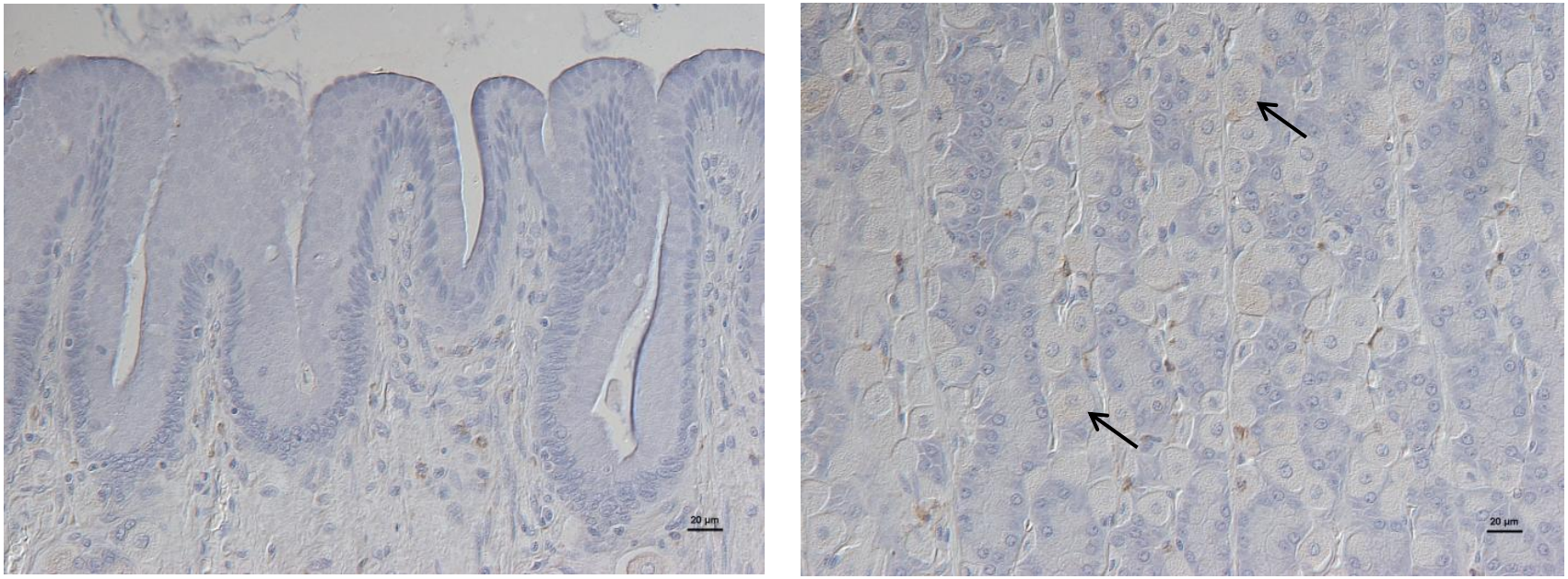

Figure 6.18- Immunohistochemical staining of EP4 in the canine gastric mucosa showing faint positive staining of the parietal cells. Scale bar: 20µm

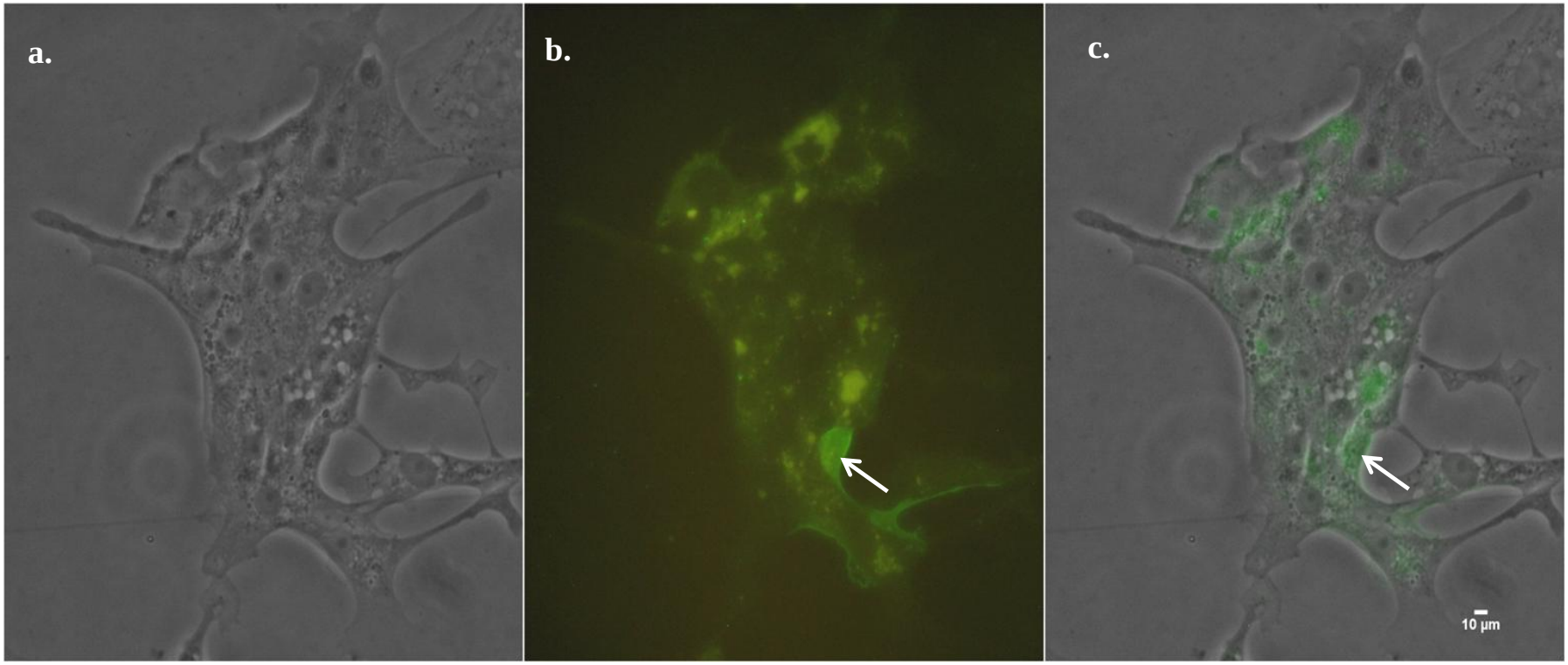

Figure 6.19- Immunocytochemical staining of EP4 in a cultured canine gastric epithelial cell island; a.) bright field image, b.) fluorescence image, c.) fluorescence image superimposed onto bright field image. Arrow indicates an expressing cell. Magnification of 40X; scale bar 10µm

6.4. Discussion

The data presented in this chapter provides evidence for the expression of COX-2 and the prostaglandin receptors, EP3 and EP4 within primary cultured gastric epithelial cell islands and two immortalised epithelial cell lines, commonly used to study cell migration. COX-2 mRNA was shown to be expressed in cultured gastric epithelial cells isolated from biopsy-derived tissue. Although the resulting PCR product could not be sequenced, a single band of the appropriate size was produced. COX-2 expression in normal human and rabbit gastric mucosa has been reported previously (Zimmermann et al., 1998). However, using Western blot techniques, COX-2 protein expression was undetectable in gastric epithelial cells cultured in serum-containing medium. This discrepancy between mRNA and protein expression could be attributed to various factors. The PCR procedure could be amplifying genomic DNA instead of cDNA; however this is unlikely as specific steps to eliminate genomic DNA are incorporated into the RNA extraction protocol. Additionally, protein degradation during the Western blot procedure can be ruled out as bands of the expected sizes were obtained for the loading control. The discrepancies between mRNA and protein expression could indicate decreased protein translation. COX-2 expression is regulated at the transcriptional and post-transcriptional levels (Tanabe & Tohnai, 2002). Post-transcriptional regulation of COX-2 is mediated through the AU-rich element (ARE) within the COX-2 mRNA 3'-untranslated region (3'UTR) (Dixon et al., 2003). The protein, TIA-1 binds to the ARE-containing 3'UTR of COX-2 mRNA and acts as a translational silencer (Dixon et al., 2003). Thus, although COX-2 gene transcription is apparent, the resulting mRNA may not be translated into protein or translation may be highly regulated.

Acute stress through serum starvation induced COX-2 protein expression in both gastric epithelial and MDCK cells. As the protein was detected in serum-starved cells, problems with antibody specificity can be ruled out. This data suggests that serum-starvation causes substantial changes in the regulation of COX-2 protein expression. This result is not surprising, given that COX-2 is known to be highly inducible by a variety of stimuli (Chandrasekharan & Simmons, 2004). The MKN-45 cell line was found to express COX-2 protein even when cultured in serum-

containing medium. This finding is supported by a previous study, which describes over-expression of COX-2 mRNA in MKN-45 cells cultured in serum-containing medium (Tsuji et al., 1996). MKN-45 cells are derived from human gastric carcinomas and COX-2 is known to be up-regulated during carcinogenesis (Cao & Prescott, 2002), thus this could account for the observed differences in expression. Furthermore, dot blot analysis revealed variation in COX-2 protein expression between individual protein samples extracted from mucosal tissue. There was no statistically significant difference in expression between protein samples extracted from biopsy- and cadaver- derived tissue.

Immunohistochemical localisation of COX-2 in the canine gastric mucosa demonstrated specific staining, with strong immunoreactivity detected in chief cells and weak immunoreactivity in parietal cells. Immunocytochemistry revealed strong COX-2 immunostaining in approximately 90% of cells in a cultured gastric epithelial island. Cells expressing COX-2 showed a perinuclear and granular cytoplasmic staining pattern. This evidence supports previous findings that COX-2 functions primarily within the nuclear envelope (Morita et al., 1995). Based on their morphology, the cells showing no COX-2 immunoreactivity appear to be parietal cells, thus conflicting with the immunohistochemistry findings. Given that the tissue samples used in these experiments were obtained from two different cadaver sources, their clinical characteristics will vary and this may influence protein expression. An example of this is the influence of *Helicobacter* infection on COX-2 expression. *H. pylori* is associated with marked COX-2 expression in the parietal cells, which is reduced following successful eradication (McCarthy et al., 1999). Thus, the differences in COX-2 expression observed could be explained by the presence of *Helicobacter* infection in the tissue sample used for immunohistochemical analysis.

As the prostaglandin receptors, EP3 and EP4 are known to be important for the modulation of cell migration (Blindt et al., 2002; Rao et al., 2007) and scratch wound healing (Iwanaga et al., 2012), their expression and localisation in epithelial cells was also characterised. Prior studies have reported EP3 and EP4 mRNA expression in the gastric mucosa (Ding et al., 1997), the kidney (Breyer & Breyer, 2000) and more specifically in cultured rabbit gastric epithelial cells (Takahashi et

al., 1999). EP3 and EP4 mRNA was detected in both MDCK and biopsy-derived gastric epithelial cells and the identity of the resulting PCR products was confirmed through sequencing. For the purpose of this study, gastric epithelial cells were isolated from tissue collected from the body of the canine stomach; both EP3 and EP4 mRNA have previously been found in the body of the rat stomach (Ding et al., 1997). EP3 mRNA was found to be more abundant in the body of the stomach than in the antrum, whereas EP4 mRNA was more abundant in the antrum. Analysis of EP1 and EP2 mRNA expression was attempted; however, no detectable bands were obtained. A previous study reported that EP1 and EP2 mRNA was not expressed in cultured rabbit gastric epithelial cells (Takahashi et al., 1999).

Western blot analysis revealed EP3 and EP4 protein expression in cadaver-derived gastric epithelial cells, maintained in serum-containing medium. Biopsy-derived gastric epithelial cells expressed low levels of EP3 protein and no EP4 protein. Whilst EP3 expression was detected in both biopsy- and cadaver-derived cells grown in serum-containing medium (Figure 6.10 and Figure 6.12), an additional experiment showed no expression (Figure 6.11). The cells assayed in these two experiments were derived from different individuals; thus the differences could be a result of varying expression between individuals or variation in the cell isolation protocol. However, based on sample size (n=2 and n=4 vs. n=1), it seems reasonable to deduce that EP3 is expressed in the majority of cultured gastric epithelial cells. EP3 protein was found to be expressed in MKN-cells maintained in serum-containing medium, thus these cells were used as a positive control in subsequent experiments. Notably MDCK cells maintained in serum-containing medium, showed no expression of EP3 protein, however given that beta actin expression was not detected, degradation of protein or insufficient loading of MDCK protein cannot be ruled out. EP4 was not detected in MDCK, MKN-45 or biopsy-derived gastric epithelial cells grown in serum-containing medium.

Prior to sample collection, cadaver tissue is exposed to a number of stressors, including ischemia and hypoxia. Stressors such as these have been associated with changes in EP receptor expression. Stress-induced ischemia causes changes in the dimer/monomer ratio of all the EP receptors, and changes in the immunoreactivity of

the EP3 receptor (Osborne et al., 2009). Additionally, expression of PTGER4, the gene coding for the EP4 receptor is known to be induced in response to hypoxia, independently of the COX/PG system (Catalano et al., 2011). PTGER4 signalling and hypoxia, in combination, were shown to promote cellular proliferation (Catalano et al., 2011). Thus, it is not surprising that EP3 and EP4 expression is induced in cadaver-derived cells. Similarly, acute stress via serum-starvation induced both EP3 and EP4 expression in MDCK and gastric epithelial cells isolated from biopsy and cadaver tissue. Analysis of protein expression in gastric mucosal tissue, revealed a significant increase in EP3 expression in cadaver-derived tissue, compared with biopsy-derived tissue, however, there was no significant increase in EP4 expression. This difference may be associated with the additional components, such as muscle and connective tissue and microvascular networks, that will be present in gastric mucosal tissue samples.

EP4 expression was localised to parietal cells in the normal gastric mucosa. Tissue sections used for immunohistochemistry were obtained from cadaver sources, thus supporting the previous finding that EP4 is expressed in cadaver-derived gastric epithelial cells. In addition, immunocytochemistry analysis of cadaver-derived cultured gastric epithelial islands, revealed cytoplasmic EP4 staining in a single cell. Additional staining was observed, however, this appeared to represent non-specific cellular staining. Modest EP4 staining of epithelial cells lining the gastric pit has been demonstrated in a previous study (Takafuji et al., 2002). More specifically, parietal cells in the rat gastric mucosa have been shown to express EP4 (Ding et al., 1997).

The findings presented here provide evidence that COX-2 and the prostaglandin receptors, EP3 and EP4 are inducible in primary and immortalised cell types in response to acute stress, such as serum-starvation. In addition, gastric epithelial cells isolated from cadaver-derived mucosal tissue samples show increased expression of the EP3 and EP4 protein. Variation between individual samples suggests that clinical factors may influence EP receptor protein expression.

Chapter 7 - The role of individual EP receptors in epithelial cell migration and spreading

7.1 Introduction

The PGE_2 receptors, EP1-4 are G-protein-coupled receptors that exert their effects via different intracellular signal transduction pathways. Activation of the EP1 receptor induces an increase in intracellular Ca^{2+} and a modest increase in IP3 production (Watabe et al., 1993), EP2 and EP4 induce an increase in intracellular cAMP formation (Regan, 2003), and EP3 primarily signals through the inhibition of adenylate cyclase, leading to decreased cAMP formation (Naruyima et al., 1999). Furthermore, EP2 and EP4 can activate Tcf/Lef signalling, part of the Wnt signalling pathway, through PKA and PI3K dependant pathways respectively (Fujino et al., 2002). The different splice variants of EP3 have been shown to couple to different G proteins and activate different signalling pathways (Namba et al., 1993).

While EP receptor signalling is reported to have an important role in cell migration, the role of individual EP receptor isoforms is unclear. Activation of the EP4 receptor appears to promote migration in various cell types (Rao et al., 2007; Kim et al., 2010), thus EP4 antagonism has been highlighted as a potential target for cancer treatment. EP4 antagonism has been associated with a marked decrease in tumour growth and metastasis via stimulation of VEGF-C and lymphangiogenesis *in situ* (Xin et al., 2012) and inhibition of breast cancer metastasis *in vitro* (Ma et al., 2006). Furthermore, signalling via the EP2, EP3 and EP4 receptors stimulates wound closure in an ISMF cell monolayer (Iwanaga et al., 2012) and EP4 agonism was shown to prevent indomethacin-induced gastric lesions and to promote the healing of existing ulcers by inducing proliferation (Jiang et al., 2009).

Although EP3 and EP4 signalling is known to have an important role in cell migration and spreading, the importance of this with regards to canine gastric epithelial cell migration and spreading has not been investigated. Thus the aim of this investigation was to test the hypothesis that EP3 and EP4 receptor signalling has

a critical role in the modulation of epithelial cell migration and spreading. In order to test this hypothesis, the effects of sub-type specific EP receptor agonists and an EP4 antagonist were assessed.

7.2. Materials and methods

7.2.1.Cell line scratch wound assays

7.2.1.1.Cell culture

MDCK, MKN-45 and AGS cells were cultured as described previously (Section 2.5.2).

7.2.1.2.Scratch wound assay

Scratch wounds were performed as described previously (Section 2.7). Cells were serum starved for 12 h prior to scratch wounding, then treated with either 50 µM indomethacin, 1 µM PGE_2, 10 µM 17-phenyl-trinor-prostaglandin E_2 (EP1 agonist), 10 µM Butaprost (EP2 agonist), 10 µM Sulprostone (EP3 agonist), 10 µM ONO-AE1-329 (EP4 agonist) or 5 µM AH-23848 (an EP4 antagonist) for 24 h at 37°C in a 5% CO_2 incubator. All treatments were made up in serum-free medium. Control wells contained the vehicle only (all dissolved in DMSO) and the observer was blinded to the treatment used in individual wells.

7.2.1.3.Statistics

A total of six individual experiments were performed, with two separate areas analysed for each scratch wound. The data was presented as the mean difference in scratch wound width, with values normalised to the mean control value within each experiment. Statistical analysis was performed with SPSS 20 (IBM Corp.), using one-way analysis of variance. The criteria for statistical significance was set at $P<0.05$.

7.2.2.Primary cell island spreading experiments

7.2.2.1.Sample collection

Samples were collected as described previously (Section 2.4). All gastric mucosal tissue samples used in this study were obtained from canine cadavers.

7.2.2.2.Gastric gland isolation

Intact gastric glands were isolated from mucosal tissue samples as described previously (Section 2.4).

7.2.2.3.Gastric gland culture

Isolated glands were cultured in serum-supplemented medium (10% FBS) immediately after isolation, for 48 h at 37°C in a 5% CO_2 incubator. The cells were then cultured in serum-free conditions for 24 h prior to treatment. After 24 h, the treatments described previously (Section 7.2.1.2) (made up in serum-free medium) were added and the spread cell islands were cultured for a further 24 h. The observer was blinded to the treatment used in individual wells.

7.2.2.4.Analysis of epithelial cell island spreading

Cell spreading in individual cell islands was analysed as previously described (Section 2.6.3).

7.2.2.5.Statistics

A total of six individual experiments, using samples from six canine cadavers were performed. In each individual experiment, measurements were taken from 10 islands for each treatment. There was an inevitable clustering within the observations because each biopsy sample was used in testing the effect of each treatment. The analysis acknowledged this by using a mixed-effects linear regression with the

biopsy identity declared as a random effect. The measurements for both total area and fringe area were corrected for by cell number and subjected to a logarithmic transformation. Significant differences were indicated by $P<0.05$ for all data. Statistical analysis was performed using Stata Statistical Software: Release 11 (StataCorp. 2009) and the *xtmixed* command.

7.2.3.Sirius red staining

Isolated gastric glands were cultured in serum-supplemented (10% FBS) medium for 48 h, until fully spread into monolayer cell islands. Cell islands were fixed with 4% paraformaldehyde and Sirius red staining was carried out by Veterinary Pathology, University of Liverpool.

7.2.4.siRNA transfection

MDCK cells were transfected with EP3, EP4 or scrambled siRNA, as previously described (Section 2.8.6), and transfection success was confirmed by Western blotting.

7.3. Results

7.3.1.Effect of EP receptor agonists and an EP4 antagonist on epithelial cell scratch wound healing

To investigate which EP receptor subtypes are involved in epithelial cell scratch wound healing, the effects of sub-type specific agonists and the EP4 antagonist were assessed (Figure 7.1). To induce EP receptor expression, cells were serum-starved for 12 h prior to treatment. Indomethacin caused a statistically significant decrease in wound healing in all cell lines, as reported previously (Section 4.3.1). PGE_2 and the EP3 receptor agonist, sulprostone, significantly increased wound healing in MDCK cells. Furthermore, the EP4 agonist, ONO-AE1-329, significantly impaired wound healing in MDCK and AGS cells, while AH-23848 (an EP4 antagonist) produced a significant increase in wound healing in all cell lines.

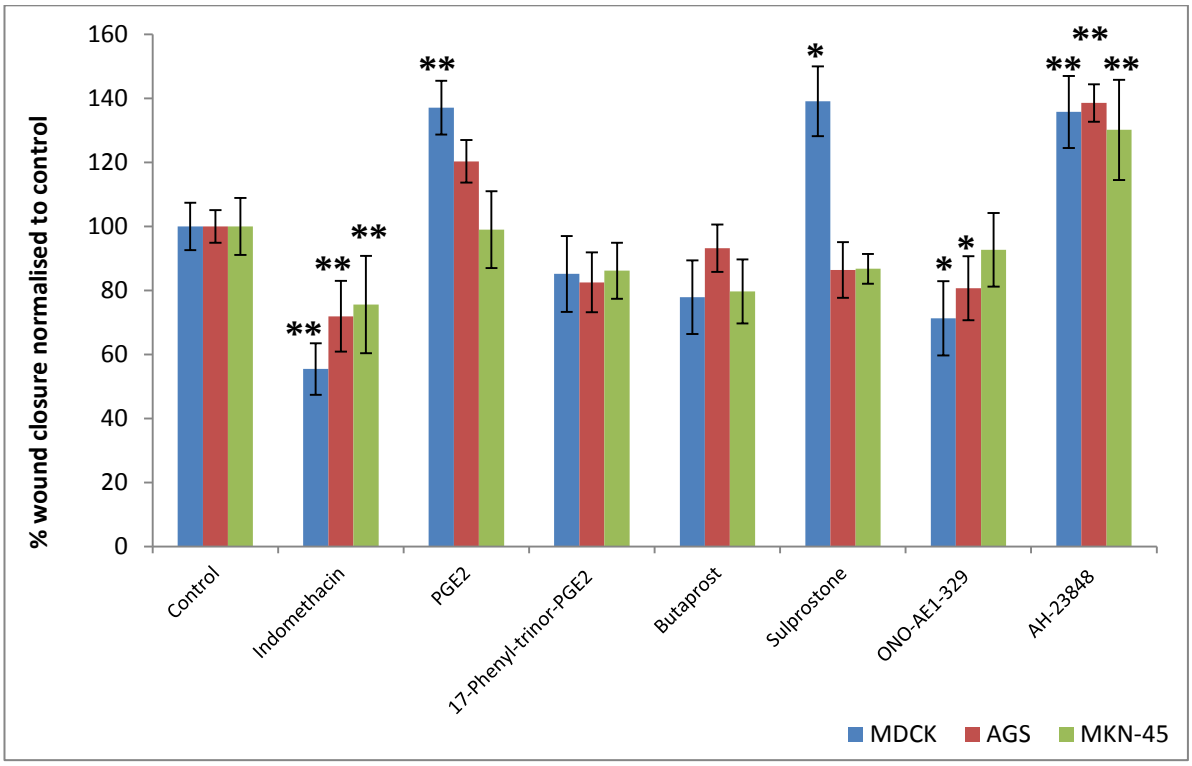

Figure 7.1- Effects of indomethacin, PGE_2, EP 1-4 agonists and an EP4 antagonist on the healing of scratch wounds in serum-starved MDCK, AGS and MKN-45 cell monolayers. Data (presented as mean +/- SEM) were normalised to control and analysed using one-way analysis of variance ($p<0.05$, indicated by *, $p<0.001$, indicated by **).

7.3.2.Effect of EP receptor agonists and an EP4 antagonist on gastric epithelial cell island spreading

The involvement of EP receptor subtypes in gastric epithelial cell island spreading was also assessed using specific EP receptor agonists and an EP4 receptor antagonist. Gastric epithelial cell islands were serum-starved for 12 h prior to agonist/antagonist administration. PGE_2 and ONO-AE1-329 (EP4 agonist) both decreased protrusive activity (fringe area per cell), while AH-23848 (EP4 antagonist) enhanced protrusive activity (Figure 7.2).

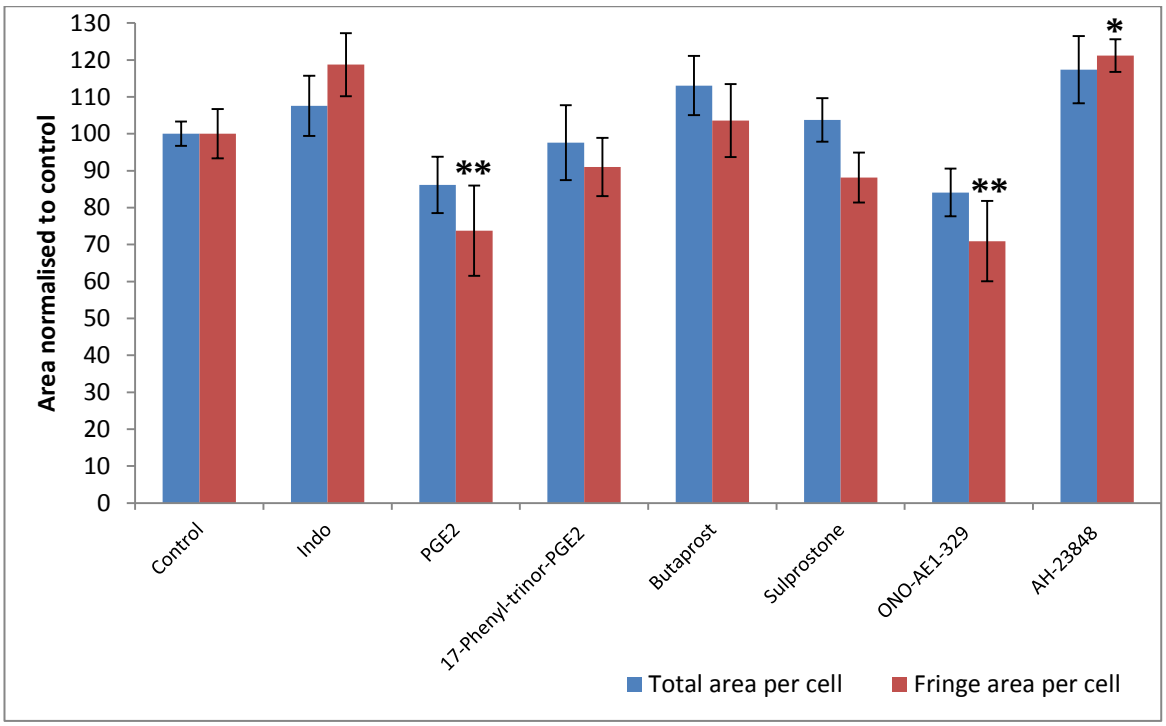

Figure 7.2- Effects of indomethacin, PGE_2, EP 1-4 agonists and an EP4 antagonist on gastric epithelial cell island spreading. Data (presented as mean +/- SEM) were normalised to control and analysed by mixed-effects linear regression ($p<0.05$, indicated by *, $p<0.001$, indicated by **).

Using mixed-effects linear regression, none of the treatments were found to have a statistically significant effect on the total spread area of the cell islands. On closer analysis of the data, it was found that the effects of the EP4 antagonist AH-23848 on total spread area were very variable between samples (Figure 7.3). In 2 of the 6 samples analysed, EP4 antagonism caused a much greater increase in total spread area and the mean cell counts of islands analysed in these two experiments were

found to be significantly smaller, thus the data violates the assumption of equal variance between groups. In contrast, these major variations did not occur when analysing fringe area (Figure 7.4).

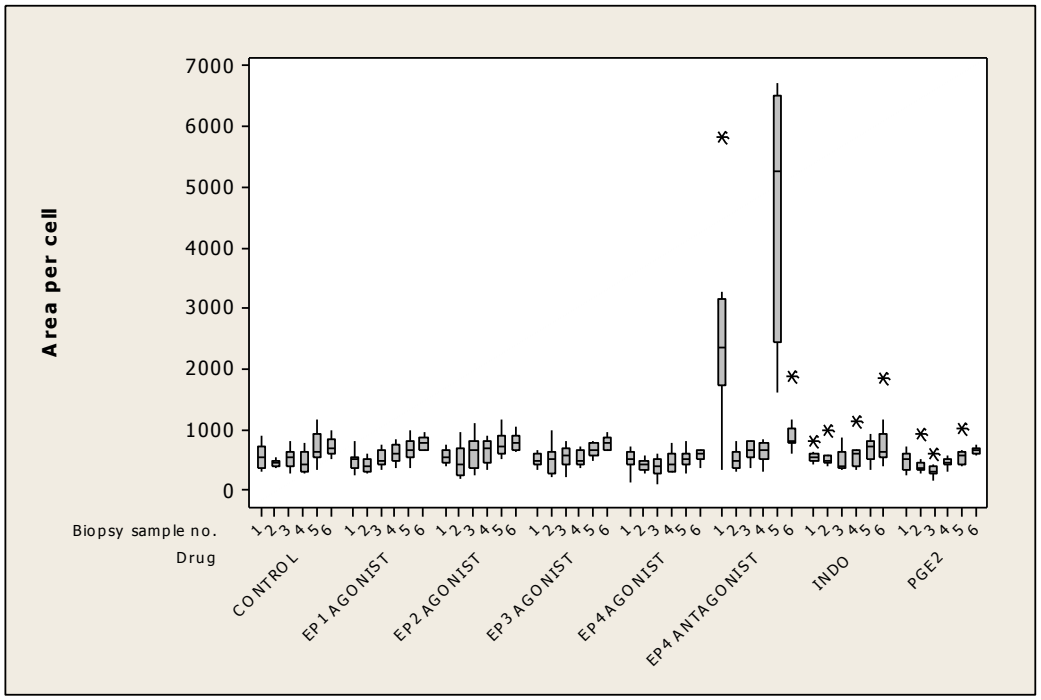

Figure 7.3- Box plot summary of the variation that exists between treatment effects on total spread area per cell; numbers 1-6 represents biopsy number, * indicates outliers. Cell islands derived from biopsies 1 and 5 had a mean cell count of 26, while those from biopsies 2, 3, 4 and 6 had a mean cell count of 49, $p<0.001$ using one-way analysis of variance .

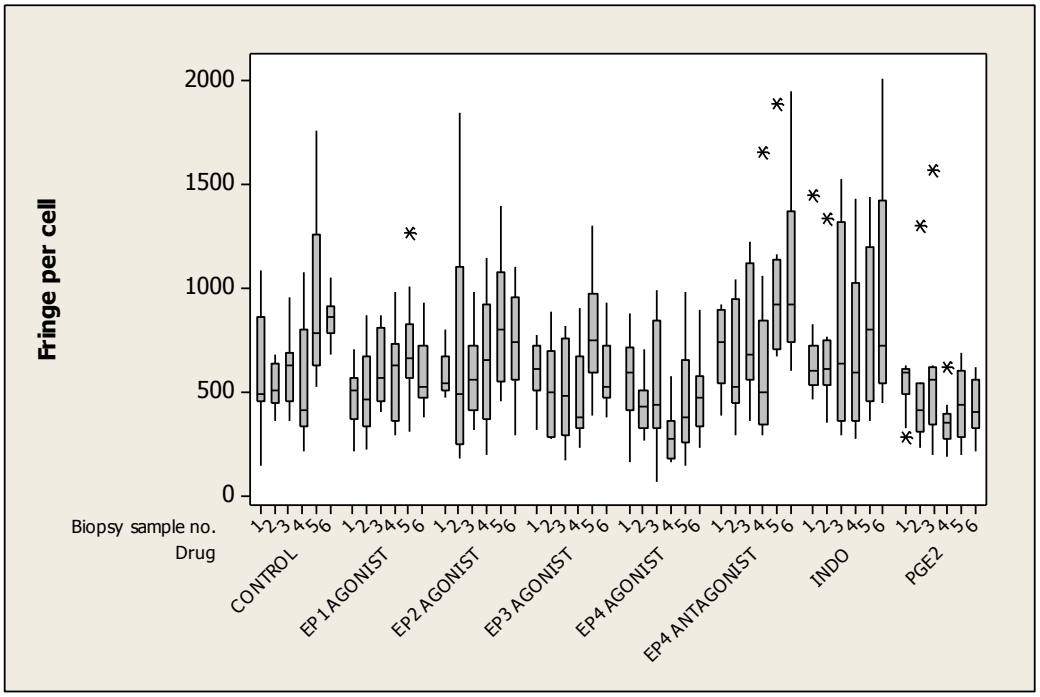

Figure 7.4- Box plot summary showing much less variation between treatment effects on fringe area per cell; numbers 1-6 represents biopsy number, * indicates outliers.

7.3.3.Sirius red staining of mucus-secreting cells

Mucus-secreting cells, identified by Sirius red staining, were primarily located towards the periphery of gastric epithelial cell islands (Figure 7.5).

7.3.4.EP3 and EP4 silenced by siRNA transfection

Given the previous findings, EP3 and EP4 specific siRNAs were designed in order to determine whether siRNA transfection could down regulate EP3 and EP4 protein expression. As shown in Figure 7.6, expression of both EP3 and EP4 in cultured MDCK cells was completely abolished 48 h after transfection with the corresponding siRNA. No off-target effect was seen in either beta-actin or the non-targeted EP receptor for each experiment. Transfection with a scrambled siRNA as a negative control showed no effect on protein expression.

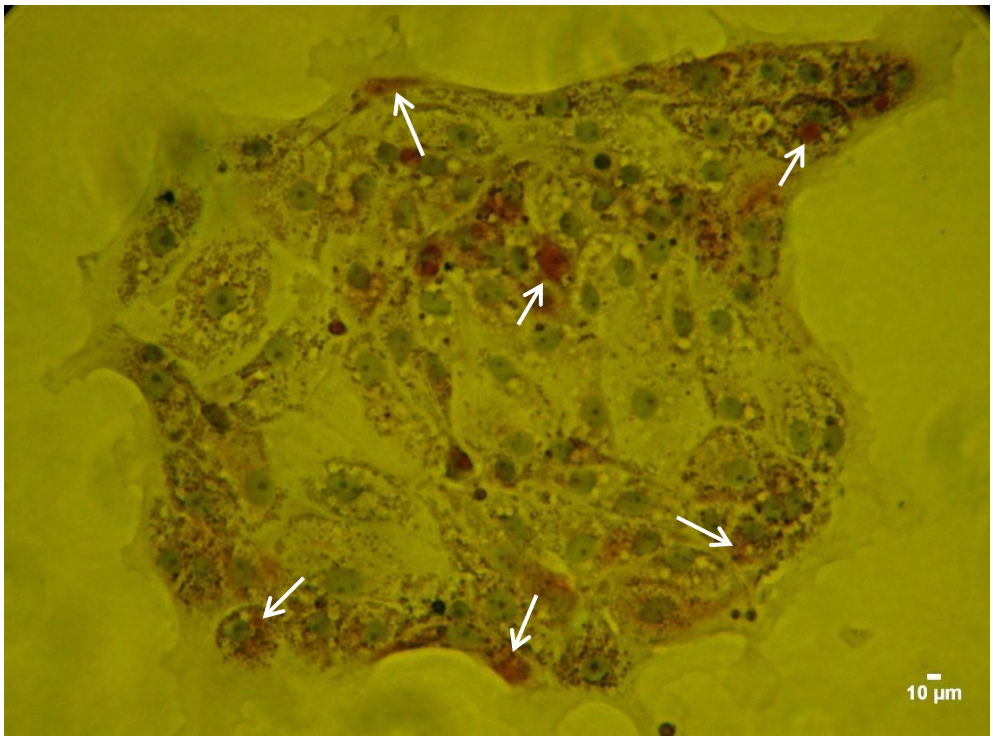

Figure 7.5- Sirius red staining (red) of mucus-secreting cells in a cultured gastric epithelial cell island. Arrows highlight examples of stained cells. Images taken at magnification of 20X; scale bar: 10µm

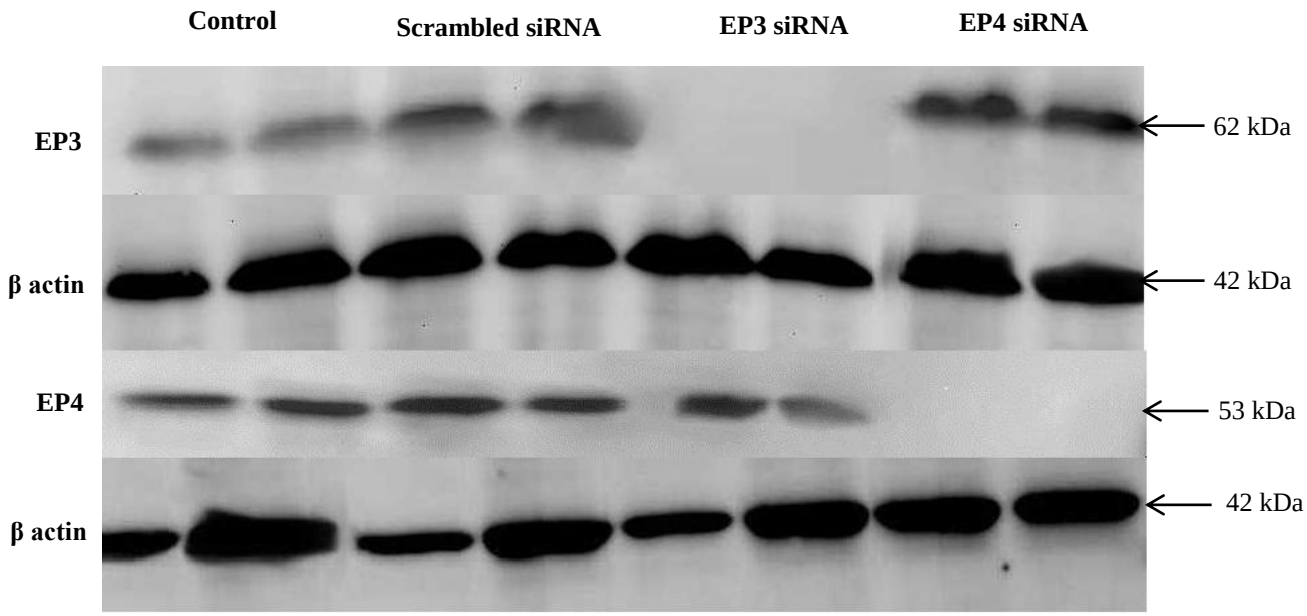

Figure 7.6- Western blot analysis of MDCK cells transfected with EP3 and EP4 siRNA (upper panels). Beta actin was used as a loading control (lower panels). Control cells were not transfected and all samples were run in duplicate, the band sizes are indicated at the side of the image.

7.4. Discussion

The data presented in this chapter provides evidence that PGE_2 signalling via the EP3 and EP4 receptors modulates epithelial cell migration and spreading. Furthermore, EP3 and EP4 signalling elicits specific functional responses in scratch-wounded epithelial monolayers and spreading epithelial cell islands.

As reported in chapter 6 of this thesis, EP3 and EP4 protein expression is induced by serum starvation (Section 6.3.6), thus cultured MDCK, MKN-45 and AGS cell lines were serum starved for 12 h prior to scratch wounding. The treatment responses observed were variable between the different cell lines, however, the observation that indomethacin significantly decreases wound healing in all cell types is consistent with our previous finding that COX-derived PGE_2 plays an important role in epithelial cell wound healing (Section 4.3.1). MDCK wound healing was increased by PGE_2 and the EP3 agonist, sulprostone, while the EP4 agonist, ONO-AE1-329, inhibited wound healing in both MDCK and AGS cells. The selective EP4 antagonist, AH-23848, was shown to promote wound healing in all cell types. Although individual cells presumably express both receptor types, these findings suggest that the effects of PGE_2 on MDCK cell migration in this model are principally mediated by EP3 receptor activation. This is consistent with previous findings that EP3 receptor stimulation promotes cell migration (Blindt et al., 2002; Li et al., 2011).

PGE_2 and sulprostone were found to have no significant effect on the healing of scratch-wounded AGS and MKN-45 cell monolayers. While EP3 expression was previously detected in cultured MKN-45 cells (Section 6.3.6), its expression in AGS cells was not determined. Thus AGS cells may not express EP3, which could explain the absence of an effect. Furthermore, AGS and MKN-45 cells are gastric cancer derived cell lines, whilst MDCK cells are an immortalised cell line derived from the normal canine kidney (Leighton et al., 1970), thus it is not surprising that they respond differently. As G-proteins activated by EP receptors may differ between cell types and, more specifically, the EP3 receptor can exist as multiple splice variants, responses to PGE_2-EP receptor signalling may be cell-type specific. Furthermore, differences in PGE_2 signalling between normal and malignant cells have been

described in a recent study (Tveteraas et al., 2012). PGE_2 stimulation of MH_1C_1 hepatocarcinoma cells was shown to cause EGF receptor phosphorylation and EGF receptor-dependant phosphorylation of ERK and Akt via FP receptor activation and ADAM-mediated release of EGF receptor ligands. However, in normal primary rat hepatocytes, PGE_2 induced the up-regulation of Ras/ERK and PI3K/Akt signalling downstream of the EGF receptor (Tveteraas et al., 2012), mediated primarily though the G_i protein (Dajani et al., 2008), which couples to the EP3 receptor (Narumiya et al., 1999). These findings suggest that different prostanoid receptors and signalling pathways may be involved in PGE_2-mediated effects on normal and malignant cells and that PGE_2 signalling may be mediated by both direct intracellular and indirect intercellular signalling.

Fringe area per cell was measured as a surrogate for lamellipodia formation, a key component of cell spreading, which occurs in leading edge cells in spreading cell sheets. Spread area per cell provides a measure of absolute cell migration. In the current study there was marked inter-assay variation in total area per cell. This may reflect the fact that the largest areas per cell were seen in samples where cell counts per island were lower i.e. where fringe area contributes more to total area leading to a bleed-through of effects on fringe area into total area measurement. Epithelial cells at the periphery of individual cell islands represent leading edge cells of a spreading sheet and thus, significant changes in fringe area were considered to reflect a key change in spreading activity.

In contrast to previous findings (Section 4.3.4.2), PGE_2 was shown to decrease protrusive activity of primary epithelial cells. This may reflect the fact that the cells used in these investigations were derived from a different source, (cadaver-derived in this investigation and biopsy-derived in the previous investigation). Biopsy-derived cells express some EP3 and little or no EP4, whereas, cadaver-derived cells express higher levels of EP3 and a 4-fold increase in EP4 protein (Section 6.3.6). Both PGE_2 and EP4 agonism were shown to inhibit cellular protrusive activity while the EP4 antagonist, AH-23848, increased cell protrusion. Together, these findings suggest that EP4 is the functionally predominant receptor for PGE_2 signals in this system.

Contrastingly, EP3 agonism was found to have no effect on fringe area. Activation of the EP3 and EP4 receptors produces opposing effects on cAMP production, with EP3 and EP4 causing an inhibition and stimulation in production, respectively (Narumiya et al., 1999). Thus if a single cell expressed both EP3 and EP4 the effects of PGE_2 at EP4 may predominate over EP3. Furthermore, a prior study localising EP3 and EP4 mRNA expression in rat gastric epithelial cells, reported that mucus-secreting cells express EP4 mRNA only, while parietal cells express both EP3 and EP4 (Ding et al., 1997). EP4 receptor signalling is known to mediate PGE_2-stimulated mucus secretion (Takahashi et al., 1999), while acid secretion is regulated via the opposing effects of EP3 and EP4 activation (Kato et al., 2005). Chief cells have been shown to express low levels of EP3 and no EP4 (Northey et al., 2000). These observations suggest that surface mucous cells may be the key expressors of EP4 in our system. Sirius red staining suggested a peripheral distribution for mucus-secreting cells in our cell islands and the limited immunohistochemical data (Section 6.3.8) was consistent with expression in peripheral cells. Together, these findings would be consistent with fringe area being primarily influenced by EP4 signalling. This would explain why EP3 agonism had no effect on the fringe area and also why the effects of PGE_2 appear to be mediated by EP4 signalling in this system. In order to confirm which cell types were expressing EP3 and EP4, dual-labelling immunocytochemistry could be used.

The findings presented in this chapter provide evidence that PGE_2-mediated effects on cell migration and spreading can vary significantly, depending on the EP receptor profile of the cell; however, PGE_2 signalling via the EP3 and EP4 receptors does appear to have an important role in the modulation of epithelial cell migration and spreading. PGE_2 stimulation was shown to cause different functional responses in two distinct models of cell migration. In MDCK cells, EP3 signalling appears to positively regulate cell migration, while EP4 activation acts as a negative regulator. Furthermore, in this system EP3 receptor activation appears to be the predominant determinant of PGE_2-induced migration. In primary gastric epithelial cells, protrusive activity is influenced by EP4 signalling. EP4 activation inhibited, while EP4 antagonism stimulated protrusive activity. EP4 activation appears to predominate in mediating the PGE_2 inhibition of protrusive activity in this system. In

order to confirm the role of EP3 and EP4-mediated PGE_2 signalling in the regulation of cell migration, receptor knockdown using siRNA would be the next logical step. Suitable siRNAs have been designed, which successfully and selectively diminished the expression of both EP3 and EP4 in MDCK cells (Section 7.3.4).

Future studies are necessary to determine which signal transduction pathways are involved in the effects reported here. COX-2 derived PGE_2 is known to regulate cell migration via transactivation and phosphorylation of the EGF receptor (Buchanan et al., 2003), thus analysis of EP-dependant activation of the EGF receptor may provide further insight into the signalling cascades involved in these effects. Furthermore, given that EP3 and EP4 signal through the regulation of intracellular cAMP levels (Narumiya et al., 1999), and that the cAMP-dependant PKA pathway has a central role in the regulation of actin cytoskeletal arrangement and migration (Howe, 2004), EP receptor-mediated cAMP activation could be assessed through the use of a gene reporter assay to monitor changes in gene expression.

Chapter 8– Characterising the relationship between various clinical parameters and COX-2, EP3 and EP4 protein expression

8.1 Introduction

GI disease is associated with changes in both COX-2 and EP receptor expression, for instance COX-2 is known to be up-regulated in IBD (Singer et al., 1998) and *Helicobacter pylori*-induced gastritis in humans (Fu et al., 1999) and markedly different EP receptor expression and localisation has been described in normal and inflamed human colonic mucosal tissue (Takafuji et al., 2000). There is also evidence highlighting the association between COX-2/PGE_2 signalling and gastric cancer, with COX-2 overexpression previously reported in colorectal cancer (Maekawa et al., 1998), and gastric adenocarcinoma (Lim et al., 2000). Furthermore, EP4 protein expression has been shown to be increased in colorectal cancer (Chell et al., 2006), whereas EP3 mRNA is down-regulated (Shoji et al., 2004).

Endoscopy is a routinely used tool in the diagnosis of dog and cat GI disease and gastric biopsies may provide important information about both gastric disease and pathologic changes across the whole GI tract (Lidbury et al., 2009). However, considerably different interpretation of clinical biopsy histopathology findings is a problem (Willard et al., 2002) and real efforts have been made to try and standardise endoscopic evaluation at both the gross and histopathological level (Washabau et al., 2010). As such there is potential merit in trying to identify objective markers that might correlate with disease and/or functional histopathological changes. Thus the aim of this study was to test the hypothesis that COX-2, EP3 and EP4 protein expression can be used as objective markers of certain GI diseases.

8.2. Materials and Methods

8.2.1.Dot blotting

8.2.1.1.Protein extraction

Protein was extracted from tissue samples collected during routine endoscopic biopsies and stored at -80°C, as described previously (Section 2.8.7.1).

8.2.1.2.Dot blots

Dot blots were performed as described previously (Section 2.8.7.6) in order to compare protein expression in large sample numbers.

8.2.2.Clinical data

Clinical data was obtained from the hospital clinical record, the endoscopic report and the histopathology report (from either Veterinary Pathology, University of Liverpool or Bridge Pathology, Bristol) and interpreted by a clinician. The histopathological changes used to evaluate gastric inflammation follow the standards of the WSAVA Standardization Group (Day et al., 2008). In addition to WSAVA histopathological scoring, the presence or absence of spiral bacteria was also noted, and if present the localisation of spiral bacteria was categorised as being in the superficial mucus layer (superficial) or the gastric glands (deep) according to the histopathology report.

8.2.3.Cell vacuolation scoring

Cell islands isolated from 9 separate biopsy-derived tissue samples were analysed for vacuolation, with scores given to 3 separate islands per sample. Cell vacuolation was scored on a scale of 0-5, where 0 indicates no vacuolation and 5 indicates severe vacuolation. Scoring was carried out by an observer blinded to the *Helicobacter* status. Immunohistochemical analysis of biopsy samples was carried out by

Veterinary Pathology, University of Liverpool in order to determine the *Helicobacter* status.

8.2.4.Statistics

Individual value plots were used to provide a graphical representation of the effects of each parameter on COX-2, EP3 and EP4 protein expression. The Kruskall-Wallis one-way analysis of variance on ranks was used for analysis of statistical differences. Statistical analysis was performed using the Minitab®15 Statistical Software and the criteria for statistical significance was set at $P<0.05$. The effects of *Helicobacter* status on cell vacuolation were analysed using one-way analysis of variance. SPSS 20 (IBM Corp.) was used to perform the analysis and the criteria for statistical significance was set at $P<0.05$.

8.3. Results

8.3.1.The effects of spiral bacteria on EP3, EP4 and COX-2 protein expression

For the purpose of this investigation, clinical data from 36 patients was analysed, of these there were 19 neutered females, 15 neutered males, 1 non-neutered female and 1 non-neutered male. The mean age was 8 years (range 2-15 years) and none of the patients were taking NSAIDs prior to inclusion in the study. 17 patients were shown to be *Helicobacter*-positive, while 19 patients did not have *Helicobacter* infection. Deep *Helicobacter* infection was noted in 3 cases.

The association between the presence of superficial and deep spiral bacteria in the gastric mucosa and EP3, EP4 and COX-2 expression was analysed. Neither superficial nor deep spiral bacteria had any significant effect on EP3 or EP4 protein expression (Figure 8.1a, b, d and e). Samples positive for superficial spiral bacteria showed significantly higher COX-2 expression, however, there was overlap between COX-2 expression levels in the spiral bacteria positive and negative groups (Figure 8.1c). Deep spiral bacteria did not significantly affect COX-2 expression and samples positive for deep spiral bacteria, showed a similar level of COX-2 protein expression to negative samples (Figure 8.1f).

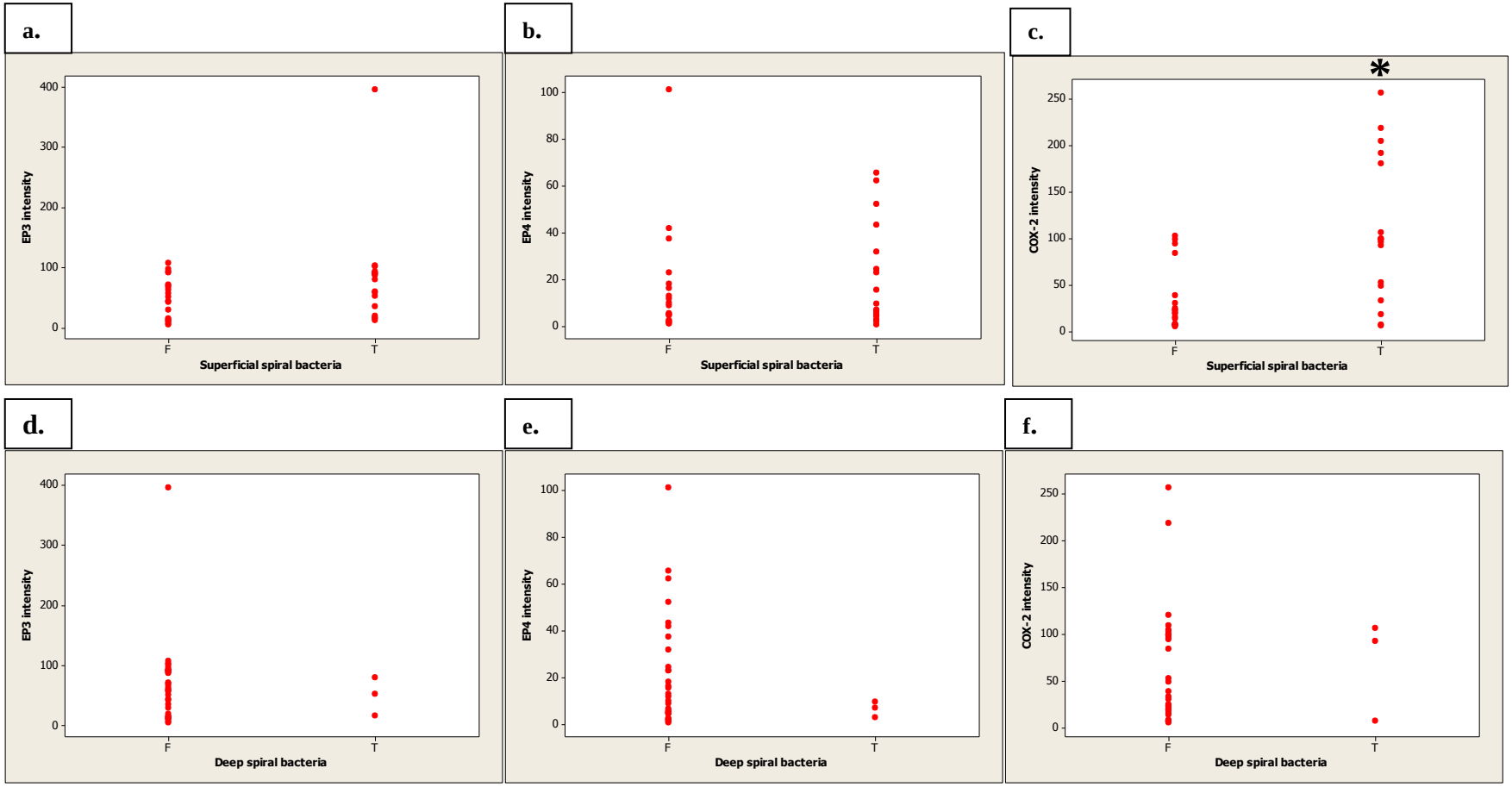

Figure 8.1- Individual value plot analysis of EP3 (a and d), EP4 (b and e) and COX-2 (c and f) expression against the presence of superficial spiral (upper panel) or deep spiral (lower panel) bacteria; F= false, T= True; * represents significantly higher COX-2 intensity values in spiral bacteria-positive samples than in spiral bacteria-negative samples using the Kruskall -Wallis one-way analysis of variance on ranks ($p < 0.05$).

8.3.2.The effects of age, gender, neutering status, breed, inflammation and gastric cancer on EP3, EP4 and COX-2 protein expression

The influence of additional clinical variables on protein expression was also investigated. No significant association was found between EP3, EP4 and COX-2 protein expression and any of the other clinical variables investigated (data not presented). The variables investigated include the age, gender, neutering status and breed of the patient and the impact of gastric inflammation, as indicated by the dominant population of pro-inflammatory cells (intraepithelial lymphocytes, lymphoplasmacytic, lymphofollicular, eosinophillic and neutrophillic). The effects of gastric cancer on protein expression were also considered, however, none of the patients studied were diagnosed with gastric lymphoma and as such an association could not be determined. In addition, only 1 patient of the 36 studied was diagnosed with gastric adenocarcinoma, thus statistical analysis could not be performed; however, relatively low levels of EP3, EP4 and COX-2 protein were expressed in this tissue sample.

8.3.3.The effects of Helicobacter *spp. on gastric epithelial cell vacuolation*

Throughout this investigation, it was noted that certain gastric epithelial cell islands in culture developed intracellular vacuoles (Figure 8.2). The association between vacuolation and the presence of *Helicobacter* spp. in the tissue from which the cells were isolated was investigated. Cell vacuolation was scored on a scale of 0-5, where 0 indicates no vacuolation and 5 indicates severe vacuolation, such as that seen in Figure 8.2. Cells isolated from *Helicobacter*-positive mucosal tissue samples, were found to have significantly higher vacuolation scores than cells isolated from *Helicobacter*-negative samples (Figure 8.3). However, no differences in spreading behaviour were observed in vacuolated cell islands.

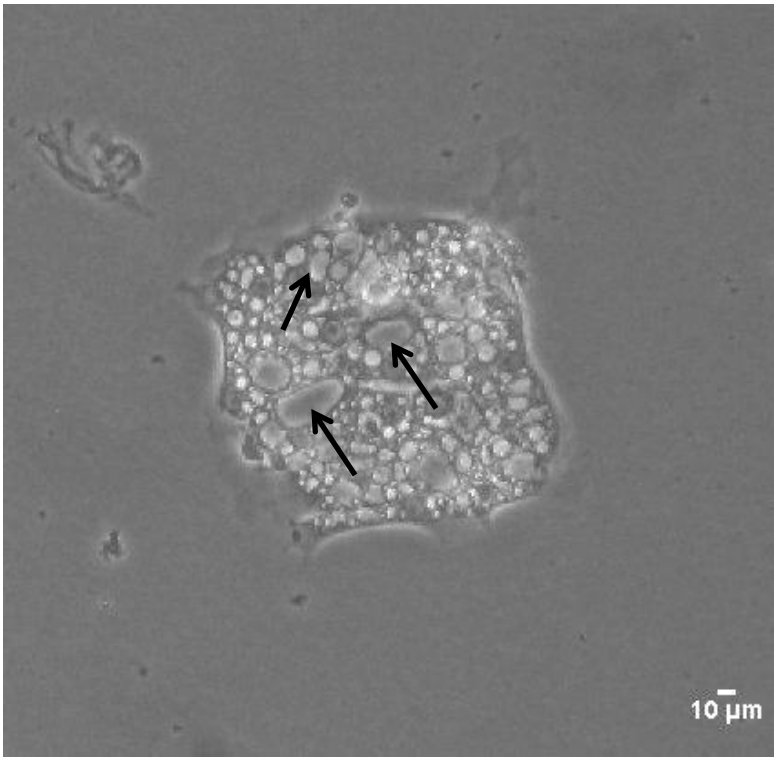

Figure 8.2- Severe vacuolation (score of 5) in a gastric epithelial cell island isolated from *Helicobacter*-positive biopsy-derived tissue. Arrows indicate intracellular vacuoles. Images taken at magnification of 20X; scale bar: 10 µm

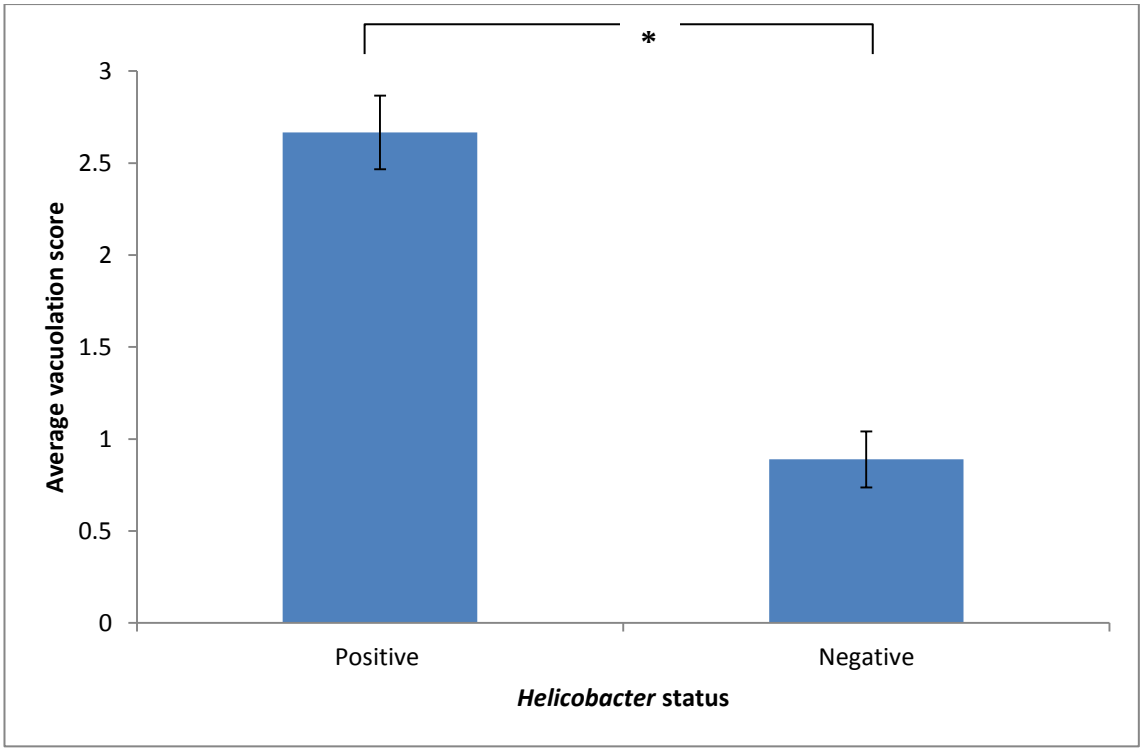

Figure 8.3- Effects of *Helicobacter* status on vacuolation in cultured gastric epithelial cell islands; Vacuolation was scored on a scale of 0-5, where 0 = no vacuolation and 5 = severe vacuolation. Data (presented as mean +/- SEM) was analysed using one-way analysis of variance. Cells isolated from *Helicobacter*-positive tissue samples showed significantly higher levels of vacuolation than cells isolated from *Helicobacter*-negative tissue samples ($p<0.05$, indicated by *).

8.4. Discussion

The data presented in this chapter provides evidence that COX-2 protein expression is up-regulated in the canine gastric mucosa during infection with spiral bacteria, while EP3 and EP4 protein expression is unaffected. The majority of current work has focused on the effects of *H. pylori* infection, given that it is a highly prevalent human pathogen (Khalifa et al., 2010). Although *H. pylori* has not been identified in dogs (Neiger & Simpson, 2000), *Helicobacter* species reported in the canine stomach include *H. felis, H. bilis, Flexispira rappini, H. bizzozeronii, H. salomonis* and *H. Heilmannii* (Eaton et al., 1996; Jalava et al., 1998; Neiger et al., 1999). In previous studies, COX-2 mRNA and protein expression have been shown to be markedly up-regulated in the gastric mucosa of human patients with *H. pylori*-positive gastritis when compared with normal mucosa (Fu et al., 1999). Additionally, COX-2 expression is higher in tissue samples from patients with *H. pylori*-positive gastritis than in *H. pylori*-negative gastritis (Fu et al., 1999), thus induced COX-2 expression may be a direct response to *H. pylori* infection, as opposed to the presence of gastritis. Furthermore, increased COX-2 protein expression is reduced following successful eradication of *H. pylori* (McCarthy et al., 1999). *In vitro* studies have confirmed the effects of *Helicobacter* on COX-2 expression, for instance, culturing *H. pylori* with a normal gastric epithelial cell line for 24 h, was shown to cause a 6-fold increase in COX-2 protein expression and a subsequent increase in PGE_2 production (Shen et al., 2006). Similarly, *H. pylori* up-regulated COX-2 mRNA expression and PGE_2 release in the human adenocarcinoma cell line, MKN-28 *in vitro* (Romano et al., 1998).

H. pylori infection has been shown to activate NF-κB in human gastric epithelial cells both *in vitro* and *in vivo* (Keates et al., 1997), as the COX-2 gene contains an NF-κB binding site in its promoter region (Tanabe & Tohnai, 2002), this could be a potential mechanism for COX-2 induction. Such a mechanism has been described for the induction of COX-2 expression by *H. pylori* in human gastric epithelial cells *in vitro* (Chang et al., 2004). *H. pylori* was reported to act through the toll-like receptors, TLR2 and TLR9 to activate PI/PLCɣ, which induces PKCα and c-Src activation, leading to the tyrosine phosphorylation of IKKα/β. The NIK/IKKα/β pathway is also activated and both pathways converge, resulting in the

phosphorylation and degradation of IκBα and activation of NF-κB in the COX-2 promoter region, leading to the induction of COX-2 gene expression (Chang et al., 2004). Exposure of the AGS gastric cancer cell line to *Helicobacter pylori* has been shown to activate NF-kB signaling, COX-2 expression and paracrine activation of cell migration and invasion (Varro et al., 2004). Furthermore, COX-2 is a potentially important paracrine regulator of PAI-2, a key factor in the regulation of epithelial cell apoptosis and cell migration (Varro et al., 2004).

While it is clear that *Helicobacter* infection induces the expression of COX-2 *in vivo* and *in vitro*, the data obtained during this investigation suggests that other factors may play a role in this. While COX-2 protein expression was markedly increased in some samples positive for spiral bacteria, certain samples expressed similar levels of COX-2 to samples negative for spiral bacteria, thus suggesting that other factors may influence the effects of spiral bacteria on protein expression. No correlation was found between the samples expressing high COX-2 and any of the other clinical parameters, however, due to the small sample size, the effects of breed on protein expression could not be meaningfully investigated. Thus, future studies using a larger sample size are needed to further investigate the association between COX-2 expression and breed. The presence of spiral bacteria was further categorised as colonising deep within the gastric glands, however the presence of deep spiral bacteria was found to have no significant effect on COX-2, EP3 or EP4 protein expression. However, it is interesting that in the small number of cases where deep *Helicobacter* infection was noted, COX-2 and EP4 expression was low.

In this study, cultured gastric epithelial cell islands isolated from mucosal tissue samples positive for *Helicobacter* were shown to have increased cytoplasmic vacuolation. Approximately 50% of *H. pylori* isolates in Western countries produce the cytotoxin, VacA, which induces cytoplasmic vacuolation in eukaryotic cells (Maeda et al., 1998). A previous study reported similar cytoplasmic vacuolation to that seen here, in primary cultures of human gastric epithelial cells, incubated with the *H. pylori* vacuolating cytotoxin and primary cells were significantly more sensitive to the effects of the cytotoxin than cell lines (Smoot et al., 1996). The VacA cytotoxin appears to be unique to the *H. pylori* species (Beswick et al., 2006) and much less is known about the virulence of non-*H. pylori Helicobacters.* However *H.*

trogontum has been shown to cause similar vacuolation in ileal epithelial cells (Moura et al., 1998). Clearly, more work needs to be done in order to characterise virulence factors associated with other *Helicobacter* species. Given the association between *Helicobacter* status and cultured gastric epithelial cells described in this chapter, it seems possible that the *Helicobacter* are colonising intracellularly and as such survive antibiotic treatment in the culture medium. *H. bizzozeronii* and *H. felis* have recently been reported to localise intracellularly in parietal cells and macrophages in the fundic mucosa of Beagle dogs (Lanzoni et al., 2011).

The relationship between protein expression and gastric inflammation, as indicated by various markers, was also investigated. The WSAVA Gastrointestinal Standardization Group has produced a set of standards for the characterisation of inflammatory changes in endoscopic biopsy samples from the gastrointestinal mucosa of small companion animals (Day et al., 2008). These reporting guidelines were used to evaluate inflammatory changes in the biopsy samples used in this investigation. The inflammatory changes reported include the presence of intraepithelial lymphocytes, lymphoplasmacytic, eosinophillic and neutrophillic infiltration and lymphofollicular hyperplasia. No association was found between EP3, EP4 and COX-2 protein expression and gastric mucosal inflammation. Increased COX-2 expression in *H. pylori* gastritis and in tissue adjacent to gastric ulceration has been previously described (Jackson et al., 2000). Additionally, a study comparing PGE_2 receptor expression in normal and inflamed human colonic mucosa, reported a significant increase in EP4 expression in the mucosal T-lymphocytes of inflamed tissue (Cosme et al., 2000) and EP4 epithelial expression in the inflamed mucosa was more intermittent compared with the even distribution seen in the normal mucosa (Cosme et al., 2000). Furthermore, during inflammation non-surface epithelial cells newly and significantly express EP2 and EP3 (Takafuji et al., 2000). As such, the lack of relationship found between protein expression and markers of inflammation is surprising.

The most common neoplasm to affect the canine stomach is gastric carcinoma (Carrasco et al., 2011). Particular breeds show an increased risk for developing gastric carcinoma (Willard, 2012) and little is known about its pathogenesis, but it is assumed to be similar to that of human gastric carcinoma. Many of the markers of

malignancy that are associated with increased COX-2 expression, for instance, invasion and metastasis (Han, 2003) are related to increased cell migration. As only 1 of the 36 patients studied was diagnosed with gastric adenocarcinoma, no meaningful conclusions could be made with regards to its influence on protein expression. Furthermore, none of the patients studied were diagnosed with gastric lymphoma. The over-expression of COX-2 in gastric cancer tissue has been previously reported (Lim et al., 2000; Mao et al., 2007) and was significantly related to metastasis and the depth of invasion (Mao et al., 2007). Increased COX-2 expression has been identified as an independent prognostic marker for poor outcome in gastric cancer and COX-2 is considered an important treatment target in a number of veterinary tumours, particularly transitional cell carcinoma (Doré, 2011). COX-2 over-expression is related to advanced tumour penetration depth, lymph node metastases and non-curative operation (Mrena et al., 2005). COX-2 over-expression is more prevalent in larger tumours and in more invasive cancers, i.e. cancers with more metastatic nodes and a greater invasion depth (Han, 2003). A previous study comparing COX-2 protein expression in gastric carcinoma tissue samples and paired samples from adjacent normal mucosal tissue, found that the carcinoma tissue expressed significantly higher COX-2 levels (Murata et al., 1999). In this investigation, the tissue sample obtained from the patient diagnosed with gastric adenocarcinoma, expressed relatively low levels of EP3, EP4 and COX-2 protein.

Little is known about EP receptor expression in gastric cancer. In veterinary patients, COX-2 and EP1 and EP2 expression are markedly increased in bone tumours (Millanta et al., 2012). In human GI tract tumours, EP3 expression is known to be markedly decreased in colon cancer tissue and its down-regulation is thought to contribute to colon carcinogenesis (Shoji et al., 2004). In contrast, EP4 protein expression has been shown to be increased in colorectal cancers (Chell et al., 2006). Thus, EP3 and EP4 clearly have a role in carcinogenesis and more work needs to be carried out in order to determine the nature of this role in gastric cancer. As samples used for this investigation are obtained from routine endoscopic biopsies and gastric carcinoma is relatively uncommon, the study size was very small. A retrospective evaluation of COX-2, EP3 and EP4 immunoreactivity in canine gastric tumours

using archived material would allow for a larger sample size and would be a valuable staring point to evaluate a potential role for these proteins in canine gastric cancer.

Chapter 9 – Final discussion

9.1 Overview of the major findings of the project

The major findings presented in this thesis are that a.) a previously used primary cell culture model can be reproducibly used to study canine gastric epithelial cell spreading, b.) COX-2-derived PGE_2 has an important role in the modulation of epithelial cell migration and spreading, c.) GR signalling is also involved in epithelial cell migration and spreading; however, its effects are complex and warrant further study, d.) COX-2, EP3 and EP4 expression in cultured epithelial cells is readily induced by acute stressors, d.) EP3 and EP4 signalling is involved in PGE_2-mediated effects on cell migration and spreading and elicits different functional responses in two different cell migration models. Furthermore, activation of EP3 or EP4 was shown to have opposing effects on MDCK cell migration and spreading, and e.) COX-2 expression is induced in dogs infected with spiral bacteria.

9.1.1.Characterisation of a new model for studying cell migration in the canine gastric epithelium

One of the main aims of this project was to develop a reproducible primary cell culture model, enabling the study of the mechanisms and signalling pathways involved in canine gastric epithelial defence. Previous work has established such a model for studying rabbit (Berglindh & Öbrink, 1976), human (Wroblewski et al., 2003), mouse (Pagliocca et al., 2008) and rat (Azerkan et al., 2001) gastric epithelium, however, this model has not previously been used in dogs. Given the difficulties encountered in understanding the correlation between histopathology and disease in canine GI biopsies (Willard et al., 2002), the findings reported here and in further potential studies using this model might provide potentially useful objective criteria by which to assess these biopsies. This model allows isolation of intact multicellular gastric glands, thus more closely resembling the gastric epithelium *in vivo* than cultures of dispersed epithelial cells. For this project, mucosal tissue samples were obtained from dogs undergoing routine endoscopies and, once the techniques for gastric gland isolation were established, cultures of viable glands were

reproducibly obtained. In order to quantify cell migration within this model the rate of gland spreading over 48 h was measured as a surrogate for cell migration speed and lamellipodia protrusion, represented by the measurement of fringe area, was analysed as an index of spreading activity.

9.1.2.COX antagonism and epithelial cell migration and spreading

Non-selective and COX-2 selective antagonism was shown to inhibit epithelial cell migration in scratch wounded monolayers and spreading epithelial cell islands. Furthermore, non-selective and COX-2 selective antagonism decreased PGE_2 in both primary and immortalised epithelial cells to a similar extent. These findings suggest that COX-2 derived PGE_2 is important for the regulation of epithelial cell migration and spreading and support previous findings that COX-2 selective antagonism impairs the re-epithelialisation of wounded gastric monolayers *in vitro* (Pai et al., 2001) and gastric ulcer healing *in vivo* (Shigeta et al., 1998). COX-2 selective antagonists have been associated with less adverse gastrointestinal effects compared with traditional non-selective antagonists (Laine et al., 1999; Simon et al., 1999; Hawkey et al., 2000), however, a requirement for both COX-1 and -2 inhibition in the pathogenesis of NSAID-induced gastric injury has been previously described (Wallace et al., 2000; Takeuchi, 2012). COX-2 expression and PGE_2 production is induced in ulcerated gastric tissue (Takahashi et al., 1998) and COX-2-derived PGE_2 has been shown to promote gastric ulcer healing by mediating the effects of HGF and gastrin (Brzozowski et al., 2000). Thus, COX-2-derived prostaglandins appear to have an important role in gastric mucosal defence and repair and their involvement in the modulation of epithelial cell migration and spreading may contribute to this.

Non-selective and COX-2 selective antagonism was also shown to inhibit the protrusive activity of spreading primary gastric epithelial cells, as indicated through the measurement of fringe area. In agreement with these findings, aspirin treatment has previously been associated with poorly formed lamellipodia at the wound edge (Yoshizawa et al., 2000). Furthermore, non-selective and COX-2 selective antagonism directly affects the cytoskeleton via disruption of actin stress fibre formation, reduction in c-Src activity and a decrease in FAK and tensin phosphorylation (Pai et al., 2001).

Thus, these findings would be consistent with COX antagonism impairing cytoskeletal function, leading to decreased cell protrusion and a subsequent inhibition of cell migration and spreading.

9.1.3.Glucocorticoid receptor agonism and antagonism and epithelial cell migration and spreading

Contrasting results were obtained when investigating the effects of GR agonism and antagonism on epithelial cell migration using scratch wound healing and cell spreading assays. GR agonism and antagonism impaired the healing of scratch wounded monolayers but had no significant effect on the spreading of primary gastric epithelial cell islands. Given that previous studies have described an inhibition of gastric epithelial cell migration following administration of the GR agonist, dexamethasone (Luo et al., 2009) and the GR antagonist, RU-38486 (Mifepristone) (Li et al., 2004), these findings were unexpected. As the primary gastric epithelial cell model, described in this investigation, is a multicellular model, intercellular signalling networks between different epithelial cell lineages could explain the differences observed. Paracrine signalling appears to contribute to GR-mediated effects on epithelial cell migration, for example, dexamethasone was shown to inhibit gastric epithelial cell migration through the depletion of HGF mRNA expression and release by gastric fibroblasts (Takahashi et al., 2003). However, in preparations of biopsy-derived gastric epithelial cells, as used in this investigation, there appears to be few gastric fibroblasts present.

Furthermore, previous studies have all used cells that have either been serum-starved for 24 h prior to assay or cultured in serum-free media during the assay. The presence of serum in cell medium can profoundly influence cell behaviour, for example, HGF activation and secretion can be dose-dependently stimulated in fibroblasts cultured with FBS at concentrations of 1% to 10% (Ohshima et al., 2002), similarly, FBS can activate ERK-1 and -2 (Lee et al., 2001). Given that GR-mediated effects on cell migration involve inhibition of the ERK1/2/MAPK pathway (Piette et al., 2009) and suppression of HGF secretion (Takahashi et al., 2003), performing these experiments in serum-containing medium may significantly alter the cellular responses. As serum also contains relatively low levels of PGE_2 that, as explained

previously, is important for the modulation of epithelial cell migration, this may also influence cell behaviour. GR signalling clearly has a role in epithelial cell migration and spreading, however, more work needs to be performed in this area.

9.1.4.EP receptor expression in the canine gastric epithelium

The expression and localisation of COX-2 and the prostaglandin receptors, EP3 and EP4 in the canine gastric epithelium were characterised in this investigation. COX-2, EP3 and EP4 mRNA was detected in cultured canine gastric epithelial cells. This is in keeping with previous observations that COX-2 mRNA is expressed in the normal human and rabbit gastric mucosa (Zimmerman et al., 1998) and EP3 and EP4 mRNA is expressed in the normal rat mucosa (Ding et al., 1997). In the normal canine gastric mucosa, COX-2 immunoreactivity was found to be localised in the parietal cells and chief cells, while EP4 was localised in the parietal cells only. The pattern of COX-2 expression in cultured gastric epithelial cells was predominately perinuclear, while EP4 staining was cytoplasmic. Perinuclear COX-2 expression has been reported previously in colonic epithelial cells (Singer et al., 1998) and provides support for the finding that COX-2 functions primarily within the nuclear envelope (Morita et al., 1995). In addition, EP4 expression in the parietal cells of the rat gastric mucosa has previously been reported (Ding et al., 1997). Different localisation of COX-2 expression was observed using immunohistochemistry and immunocytochemistry techniques, immunocytochemistry revealed COX-2 immunoreactivity in all cells except for the parietal cells; however immunohistochemistry showed specific staining in the parietal cells. Given that the samples analysed were obtained from two individual cadaver sources, variation in clinical parameters may explain this difference. For instance, infection with *H. pylori* has previously been associated with marked COX-2 immunoreactivity in parietal cells, which is reduced following successful eradication (McCarthy et al., 1999).

COX-2, EP3 and EP4 expression was shown to be induced by serum deprivation of primary and immortalised epithelial cells. Furthermore, cadaver-derived gastric epithelial cells expressed significantly more EP3 and EP4 protein than biopsy-derived cells. Given that tissue samples obtained from a cadaver source will be exposed to stressors, such as ischaemia and hypoxia prior to collection, it is not

surprising that protein expression is affected. Retinal ischaemia, induced through elevation of intraocular pressure, has been shown to change EP receptor expression and localisation and to change the dimer/monomer ratio of all the EP receptors (Osborne et al., 2009). In particular, ischaemia caused the down-regulation of the EP3 and EP4 dimers and the up-regulation of their monomers. Furthermore, expression of PTGER4, the gene encoding the EP4 receptor, is induced in response to hypoxia, independently of the COX/PG system (Catalano et al., 2011) and PTGER4 signalling and hypoxia, in combination, promote cellular proliferation (Catalano et al., 2011). Thus, the observations presented in this thesis suggest that COX-2, EP3 and EP4 protein expression is readily induced in response to acute stressors and indicates a role for these receptors in acute stress responses in the physiologically challenging niche of the gastric epithelium.

9.1.5.Characterisation of the EP receptor subtypes involved in the modulation of epithelial cell migration and spreading

The findings presented in this thesis provide evidence that PGE_2 signalling via the EP3 and EP4 receptors plays an important role in modulating epithelial cell migration and spreading. EP3 and EP4 activation produced markedly different functional responses in two distinct models of cell migration. Using sub-type specific agonists and antagonists, EP3 activation was shown to positively regulate MDCK cell migration, while EP4 acted as a negative regulator. In contrast, EP4 activation inhibited cell protrusion, an indicator of spreading activity, in primary gastric epithelial cells. These findings suggest that the EP receptor profile of a cell may be an important determinant for the outcome of PGE_2 signalling, thus changes to EP receptor expression induced via acute stressors, as described previously, may alter the outcome of PGE_2 signalling.

9.1.6.Relationship between clinical parameters and EP receptor expression in the canine gastric epithelium

Up-regulation of COX-2 expression in the gastric mucosa of *H. pylori*-infected humans has previously been reported (Chan et al., 2001; McCarthy et al., 1999). In addition, *in vitro* studies have shown that COX-2 expression is up-regulated in cell

lines incubated with *H. pylori* (Romano et al., 1998; Shen et al., 2006). Given the role that COX-2-derived prostaglandins have in the promotion of epithelial cell migration and spreading, this up-regulation of COX-2 and subsequent increase in PGE_2 production, could be important for maintaining the gastric mucosal integrity. In this investigation, COX-2 expression was shown to be significantly increased in the gastric mucosal tissue of dogs infected with spiral bacteria. To the best of our knowledge this is the first study to report this finding. Given the role of PGE_2 in regulating epithelial cell migration and spreading, *Helicobacter* status should be taken into account when evaluating the effects of PGE_2 in the canine stomach and may be expected to impact on the effects associated with NSAID treatment.

9.2 Strengths and limitations of the research

Primary cell models utilising intact gastric glands, such as that used in this investigation, have been widely used to study cell migration and spreading. The major difference between this primary cell culture model and *in vivo* experiments is the absence of complex systemic factors, such as blood supply and neural inputs that can influence cell migration, thus eliminating these factors allows analysis of a simpler system. When using this model, results need to be cautiously extrapolated to the whole tissue, preferably by evaluating *in vivo* effects and correlating the findings. However, using the *in vitro* model described in this project, gastric cell spreading can be studied using a minimally invasive approach and, as the cultured gastric glands should comprise all epithelial cell types found *in vivo* and normal cell-cell contacts are maintained, intercellular signalling can be investigated. The use of a model incorporating clinical samples was considered advantageous to the sponsors of this investigation as it enables the influence of clinical variables to be considered. Additionally, using an *in vitro* model enables the quantification of data to be quickly and accurately performed, hence, this model provides a valuable scientific tool for studying the mechanisms involved in gastric cell spreading.

There were some limitations to the use of this model, including a scarcity of sufficient endoscopic mucosal tissue samples. To overcome this limitation, additional mucosal tissue samples were collected from cadaver sources. These, in-turn displayed altered properties which themselves provided valuable insights into

the mechanisms of PGE_2 signalling in the gastric mucosa. Scratch wound assays using immortalised cell lines were also performed to support the primary cell experiments. Scratch wound assays can be quickly and reproducibly performed and have been used previously to study the effects of gastric epithelial cell migration *in vitro* (Pai et al., 2001; Giap et al., 2002). In addition, using clinical samples introduces biological variation as natural heterogeneity exists between individual samples and the sample collection conditions may vary. While clinical data was collected for all endoscopic biopsy samples, this data was not available for tissue samples derived from cadaver sources. Thus, a large amount of the protein expression data could not be compared against clinical findings and as such no meaningful evaluations could be made for the effects of breed and the presence of gastric cancer on protein expression. This investigation was therefore limited by a relatively small sample size, however sampling can continue and the study has highlighted analyses to plan on-going work.

9.3 Future perspectives

This thesis has highlighted key areas where further work is needed. While performing *in vitro* work provides a simplified model for studying the effects of COX antagonism on cell migration and spreading, systemic factors, such as gastric mucosal blood flow may influence these effects. Thus, in order to obtain more clinically relevant information, it would be useful to perform complimentary *in vivo* studies.

Expression of EP3 and EP4 has been partially characterised in this investigation, however, further work is required in this area. Expression of COX-2, EP3 and EP4 in whole gastric mucosal tissue samples was localised to specific cell types using immunohistochemistry techniques, however, while immunocytochemical analysis demonstrated COX-2 and EP4 expression in cadaver-derived cultured gastric epithelial cells, due to time constraints, characterisation of the expressing cells was not performed. As discussed in previous chapters, double immunofluorescence labelling could be utilised in order to identify cell types exhibiting specific immunostaining and identifying cell-type specific protein expression would provide valuable insight into the cell types involved in PGE_2-mediated EP3 and EP4

signalling. Furthermore, expression of EP3 in cultured gastric epithelial cells was not elucidated as immunocytochemical analysis of EP3 expression provided non-specific, diffuse cytoplasmic staining. Given the findings presented in this thesis, demonstrating that EP3 expression is induced through serum-starvation, further studies to investigate EP3 expression in this model may be performed using serum-starved gastric epithelial cells.

In order to better understand the conflicting findings presented regarding GR-mediated effects on epithelial cell migration and spreading, it would be useful to characterise GR expression and localisation in the canine gastric epithelium and in cultured gastric epithelial cells. In addition, given the marked effects of serum-starvation on cellular protein expression presented in this thesis and the effects of serum exposure on GR expression reported previously (Yang et al., 2008), it would be interesting to investigate the effects of GR agonism and antagonism on cell spreading using serum-starved gastric epithelial cell islands.

While COX-2 derived prostaglandins have been shown to be important for regulating epithelial cell migration and spreading, it would be useful to better understand the role of COX-1 through the use of a COX-1 selective antagonist, of which there are many available, including P6, P10 and SC-560 (Calvello et al., 2012). Furthermore, it would be interesting to investigate the influence of gastric fibroblasts on canine gastric epithelial cell spreading using either an *in vitro* co-culture model or *in vitro* culture with gastric fibroblast-conditioned medium. Previous studies have demonstrated, using a co-culture model that prostaglandins target gastric fibroblasts and induce HGF mRNA expression and protein production, which may occur via EP2/EP4-mediated cAMP signalling and that HGF may modulate prostaglandin-induced restitution (Takahashi et al., 1996). In the course of this investigation, putative primary gastric fibroblasts were successfully isolated from mucosal tissue samples in addition to gastric glands; however, communication between mesenchymal and epithelial cells was not studied.

The importance of PGE_2-mediated EP3 and EP4 signalling in the modulation of epithelial cell migration and spreading has been discussed in this thesis. Given that COX-2 derived PGE_2 is known to regulate cell migration via transactivation and

phosphorylation of the EGF receptor (Buchanan, 2003), it would be interesting to determine EP receptor-dependent activation of the EGF receptor. This could be performed using an immunoprecipitation assay followed by Western blot analysis. Blockade of the EGF receptor using EGF receptor-specific tyrosine kinase inhibitors could be used to confirm its role. As EP3 and EP4 are known to signal via inhibition and stimulation of cAMP respectively (Narumiya et al., 1999), a FRET-based cAMP reporter system, such as that used in previous studies (Bagorda et al., 2009), could be used to assess EP receptor-mediated changes in intracellular cAMP levels. Additionally, a gene reporter assay could be performed to evaluate changes in the transcriptional activity of the COX-2 promoter in response to COX antagonism. The canine COX-2 promoter sequence could be cloned from cultured gastric epithelial cells into a luciferase or GFP reporter construct. NF-κB-dependent activation of the canine COX-2 promoter could be assessed through deletion or mutation of the NF-κB binding sites.

The role of EP receptor activation in the modulation of epithelial cell migration and spreading could be confirmed using siRNA transfection experiments to silence the appropriate genes. In the course of this investigation, suitable siRNAs were designed and shown to successfully diminish EP3 and EP4 protein expression in cultured MDCK cells. Future investigation would involve transfection of cultured gastric epithelial cells, in order to determine whether these siRNAs can also silence genes in primary cell cultures. The effect of gene silencing on epithelial cell migration could be assessed using scratch wound assays and analysis of cell spreading in cultured gastric epithelial cell islands.

Finally, the association between specific clinical parameters and COX-2 and EP receptor expression in dogs could have an important clinical relevance. While, this association was investigated in this thesis, a small sample size limited this study and thus these results should be regarded as preliminary findings. Completing this investigation using a larger sample size could provide valuable information.

9.4 Conclusions

In conclusion, this investigation has highlighted an important role for COX-2-derived PGE_2 in the modulation of epithelial cell migration and spreading, which may be related to the formation and maintenance of cellular protrusions. Thus, reduced PGE_2 production in the gastric mucosa may inhibit gastric epithelial migration and spreading and contribute to the ulcerogenic effects associated with COX antagonist therapy. Furthermore, the migratory effects of PGE_2 are mediated through EP3 and EP4 receptor signalling, and given that EP3 and EP4 activation produces different functional responses in two models of cell migration, the EP receptor profile of a cell may be an important determinant of the outcome of PGE_2 signalling. The inhibitory effect of EP4 activation on canine gastric epithelial cell protrusion, an indicator of spreading activity, is a novel finding. Increased expression of EP3, EP4 and COX-2 was found in serum-starved cells, while EP3 and EP4 expression was increased in cadaver-derived cells and COX-2 expression was up-regulated in the gastric mucosal tissue of canines infected with spiral bacteria. Thus, the expression of COX-2, EP3 and EP4 appears to be readily influenced by acute stressors. Further investigation is required in this area in order to determine the clinical relevance of these findings.

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