

**Fibroblast Growth Factors
in Gastrointestinal Development,
Homeostasis and Injury**

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DECLARATION BY CANDIDATE

I hereby declare that this thesis is my own work and effort. Where other sources of information have been used, they have been acknowledged.

Abstract

Fibroblast growth factors (FGFs) and their receptors (FGFRs) are essential for controlling cell growth and proliferation, angiogenesis, wound healing and tumourigenesis. In mammals, there are twenty-three FGFs and five FGFRs, with each FGFR having different ligand binding specificities. FGFs are thought to act in a paracrine manner, in which they are secreted by one cell and activate FGFRs on another cell type, and such interaction helps to establish the fundamental crosstalk between epithelium and mesenchyme during development, homeostasis and tumourigenesis.

This thesis aims to investigate the requirement of FGFR2-isoform IIIb (*Fgfr2b*) and one of its ligands, *Fgf10*, in gastrointestinal (GI) development, homeostasis and injury, using loss-of-function animal models. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} exhibit similar multi-organ defects, including intestinal atresia. While caecal and colonic atresia have been previously described, the mechanism of duodenal atresia and the role of *Fgf* signalling in duodenal development have not been fully established. We demonstrate that absence of *Fgfr2b* or *Fgf10* leads to decreased tissue proliferation and increased apoptosis in the duodenum, contributing to duodenal atresia. These mutants also develop gastric heterotopia in the rostral duodenum due to loss of gastric-intestinal boundary specification. In addition, we demonstrate reduced expression of *Wnt* targets *Tcf1* and *Tcf4* in the small intestine, with corresponding downregulation of *Lgr5*, an intestinal stem cell marker, and *Cdx1*, a homeobox gene involved in anterior-posterior patterning. We show by *in vitro* that *Fgf10-Fgfr2b* signalling is able to regulate *Tcf4* expression via the Grb2/Sos/Ras/MAPK pathway.

In order to study the requirement of *Fgfr2b* in intestinal homeostasis and injury, we crossed a transgenic line bearing a progesterone antagonist (RU486)-dependent *Cre* recombinase (A33-CrePR) expressed under the

control of the intestinal-specific A33 antigen promoter, with an existing conditional *Fgfr2b* line. Following RU486 administration with/without administration of dextran sodium sulphate (DSS) thereafter to induce ulcerative colitis (UC), the A33-CrePR⁺/*Fgfr2b*^{flox/flox} targeted *Fgfr2b* ablation with high efficiency across the small and large intestine. We demonstrate that significant downregulation of *Fgfr2b* does not affect intestinal homeostasis, but significantly increases the susceptibility of colonic epithelium to UC and delays wound healing, contributed by reduced epithelial proliferation.

In order to study the requirement of *Fgfr2b* in gastric homeostasis, we characterised two stomach-specific minimal promoters for trefoil factor 1 (*Tff1*) and H⁺K⁺-ATPase (*Atp4b*) to generate two inducible *Cre* recombinase (CreER^{T2}) lines. Gastric surface mucous cells and acid-producing parietal cells express *Tff1* and *Atp4b* respectively, as well as *Fgfr2b*. We demonstrate by *in vitro* that *Atp4b* nucleotide -1,035bp to +24 bp and *Tff1* nucleotide -641 bp to +28 bp are optimal for driving *Cre* expression in the mouse stomach.

Thus, our novel data provides evidence that *Fgfr2b* and *Fgf10* are required for normal duodenal morphogenesis and differentiation, and *Fgfr2b* confers protection against DSS-induced colonic injury and promotes wound repair.

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Abbreviations

BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-indolyl-phosphatase
BIM-I	bisindolylmaleimide I
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CRC	colorectal cancer
CSP-3	caspase-3
Ct	cycle threshold
DAB	3,3'-diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GI	gastrointestinal
H&E	haematoxylin and eosin
HCl	hydrochloric acid
HRP	horseradish peroxidase
HSPG	heparan sulphate proteoglycan
IBD	inflammatory bowel disease
IEL	intraepithelial lymphocytes
IHC	immunohistochemistry
ISH	<i>in situ</i> hybridisation
KCl	potassium chloride

LB	Luria Bertani
MAPK	mitogen activated protein kinase
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MgCl ₂	magnesium chloride
Min	minute
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
NaCl	sodium chloride
NBF	neutral buffered formalin
NBT	nitroblue tetrazolium
NP-40	nonyl phenoxy polyethoxy ethanol
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of mean
SHH	Sonic hedgehog
TA	transit amplifying
TAE	Tris-acetate-EDTA
Taq	<i>Thermus aquaticus</i>
TBS	Tris buffered saline
TCF4	transcription factor 4
TK	tyrosine kinase
UC	ulcerative colitis
UV	ultraviolet
V	voltage
v/v	volume/volume

w/v	weight/volume
Wt	wildtype
X-gal	bromo-chloro-indolyl-galactopyranoside

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Chapter 1: Introduction

1.1 Fibroblast Growth Factors and their Receptors

1.1.1 Overview

Fibroblast growth factors (FGFs) are a large family of multi-functional intercellular signalling molecules found in organisms across the evolutionary spectrum ranging from nematodes to humans (Ornitz and Itoh, 2001). These molecules play different roles depending on the cell type and context, including controlling cell proliferation and differentiation, angiogenesis, wound healing and tumourigenesis (Grose and Dickson, 2005, Itoh and Ornitz, 2004, Ornitz and Itoh, 2001, Thisse and Thisse, 2005). In the mouse and human, twenty-three FGFs have been identified, designated as FGF1-23 (with FGF15 as the mouse orthologue of human FGF19), sharing a 13-70% homology over a 120 amino acid central core region, with 28 highly conserved and six invariant amino acids (Ornitz and Itoh, 2001). In non-mammals, two additional FGFs, FGF24 and FGF25, were discovered in zebrafish, with FGF25 being an orthologue of FGF10 (Katoh and Katoh, 2005, Draper et al., 2003). Structural studies on FGF1 and FGF2 showed that these proteins adopt a β -trefoil structure, containing twelve β -strands arranged in a triangular array (Zhu et al., 1991, Faham et al., 1998). The molecular weights of mammalian FGFs can range from 17 kDa to 34 kDa (Ornitz and Itoh, 2001).

FGFs exert their activities by binding to and activating fibroblast growth factor receptors (FGFRs), which are a family of receptor tyrosine kinases. Each FGFR is made up of an extracellular ligand-binding domain containing three immunoglobulin (Ig)-like loops, a single transmembrane helix, and a cytoplasmic domain comprising of a split tyrosine kinase

domain (Keegan et al., 1991, Lee et al., 1989) (Figure 1.1). In mammals, there are four FGF transmembrane receptors, designated FGFR1-4, and one soluble receptor known as FGFR5 (also known as FGFR1) that lacks a tyrosine kinase domain (Wiedemann and Trueb, 2001). Alternative splicing of a single exon encoding the second-half of the third Ig-like loop in FGFR1-3 results in each receptor having two isoforms ('IIIb' and 'IIIc'), whilst FGFR4 only exists as the 'IIIc' isoform (Avivi et al., 1993, Chan et al., 1994, Johnson et al., 1991). These isoforms have distinct ligand binding specificities (Figure 1.2). As an example, FGF1, FGF3, FGF7 and FGF10 bind to FGFR2-isoform IIIb (FGFR2b) with high affinity, while FGF2 binds poorly to FGFR2b. Conversely, FGF1 and FGF2 bind with high affinity to FGFR2-isoform IIIc (FGFR2c), whereas FGF7 does not bind to FGFR2c (Miki et al., 1992, Yayon et al., 1992, Dell and Williams, 1992). These isoforms also display cell-type specific expression; the 'IIIb' isoforms are typically expressed in the epithelium, and the 'IIIc' isoforms are typically expressed in the mesenchyme (Ornitz and Itoh, 2001). As an example, FGFR2b is expressed in intestinal epithelium and can be bound and activated by the mesenchymally expressed FGF1, 3, 7, 10 and 22 (Zhang et al., 2006). These differential expression patterns help to establish the fundamental crosstalk between the epithelium and mesenchyme, allowing FGFs and FGFRs to perform their functions during development, homeostasis, injury and tumourigenesis.

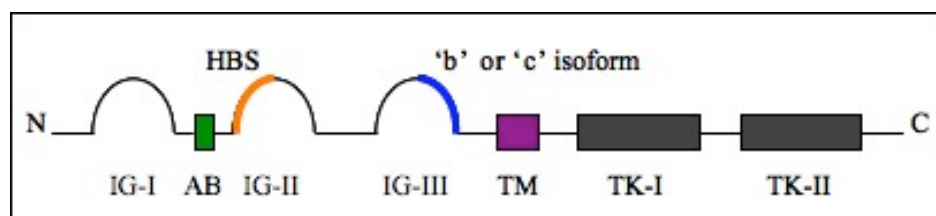


Figure 1.1 Schematic diagram of FGFR (modified from (Mohammadi et al., 2005)).

The heparin binding site (HBS) is coloured orange, and is required for interaction with heparin and heparin sulphate proteoglycan (HSPG) for

FGFR activation (Schlessinger et al., 2000). ‘AB’ stands for acid box, which is required for interactions with neural cell adhesion molecule (NCAM) and N-cadherin (Sanchez-Heras et al., 2006), but not for FGF interaction and function (Shimizu et al., 2001). The single exon that determines the splice variant of FGFR is coloured blue. The N- and C-termini are labelled ‘N’ and ‘C’ respectively. The split tyrosine kinase domains are labelled ‘TK-I’ and ‘TK-II’.

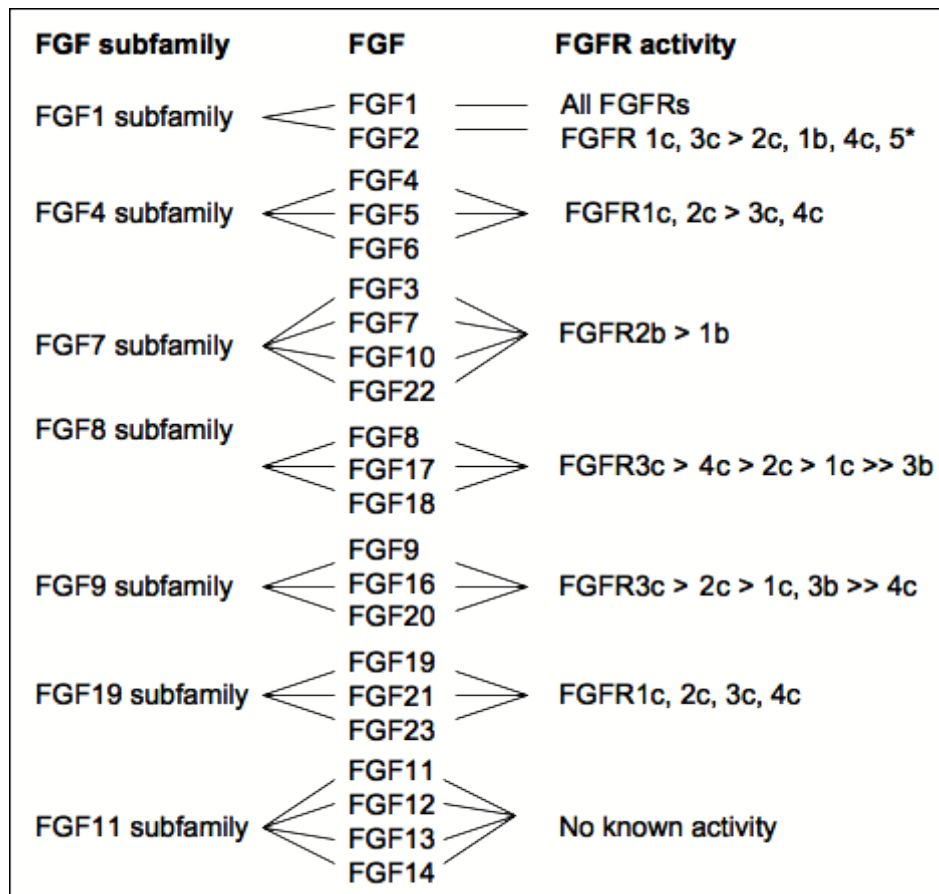


Figure 1.2 Diagram showing binding specificity of FGFRs to FGFs, grouped by FGF subfamilies (modified from (Zhang et al., 2006)).

The recent FGFR5 is added to the list, and marked with asterisk. FGF11-14 are also known as FGF homologous factor (FHF) 1-4 due to their substantial sequence homology to FGFs and can bind to heparin, but fail to activate any of the seven principal FGFRs (Schoorlemmer and Goldfarb, 2001, Olsen et al., 2003).

1.1.2 Activation of FGFRs

Unlike other growth factors, such as platelet-derived growth factor (PDGF), that are dimeric, FGFs are monomeric and are unable to activate FGFRs by themselves (Ornitz et al., 1992). FGFs require the presence of heparin or heparan sulphate proteoglycan (HSPG) to promote FGFR dimerisation, activation and induction of biological responses (Spivak-Kroizman et al., 1994, Ornitz et al., 1992, Schlessinger et al., 1995). Interestingly, different FGF/FGFR interactions display different affinities for various lengths and sulphation patterns of the HSPG (Luo et al., 2006), implying that HSPG may contribute to the regulation of FGF activity and specificity. Structural studies have proposed two models for the FGFR:FGF:HSPG interaction complex. The ‘Pellegrini’ model proposed two FGFR:FGF complexes dimerised by a single HSPG chain connecting the two FGF molecules in a 2:2:1 FGFR:FGF:HSPG fashion (Pellegrini et al., 2000), while the ‘Schlessinger-Mohammadi’ model proposed two FGFR-FGF-HSPG complexes dimerised via the two FGFR molecules in a 2:2:2 FGFR:FGF:HSPG fashion (Mohammadi et al., 2005) (Figure 1.3). In contrast to the canonical FGF/FGFR interactions, FGF19, FGF21 and FGF23 display low binding affinity to heparin and HSPG, and instead they require Klotho proteins as co-receptors to bind to FGFRs (Kurosu and Kuro, 2009, Kuro-o, 2008). The requirement of HSPG and Klotho in FGF/FGFR interactions demonstrates the complex mechanism by which FGF and FGFR activities are tightly regulated.

Following FGFR dimerisation and activation, the tyrosine kinase domains are autophosphorylated (Dibb et al., 2004), and become a binding site for phospholipase C γ (PLC- γ), leading to the formation of two messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Schlessinger, 2004) (Figure 1.4). IP3 releases Ca²⁺ from the endoplasmic reticulum, then acts with DAG to translocate protein kinase C (PKC) to the cell membrane and stimulate its enzymatic activity, regulating the activity of other proteins (Schlessinger, 2004). Additionally, the docking proteins

FGFR substrate 2 (FRS2)- α and FRS2- β , which contain phosphotyrosine binding domains, bind to the activated FGFR tyrosine kinase domains and function as a docking site for multiple Grb2/Sos complexes (Hadari et al., 2001).

Alternatively, activated FRS2 can phosphorylate Shp2 to form Grb2/Sos complexes (Hadari et al., 1998). Sos allows the exchange of GDP to GTP on Ras, leading to the subsequent activation of a cytosolic MAPK cascade. Phosphorylated ERK1 and ERK2 (MAPK) translocate to the nucleus where they phosphorylate transcription factors and other kinases to alter gene transcription (Schlessinger, 2004). Grb2 also recruits a docking protein Gab1, and following tyrosine phosphorylation by FGFRs, leads to recruitment and activation of the PI3K-Akt cell survival pathway (Ong et al., 2001).

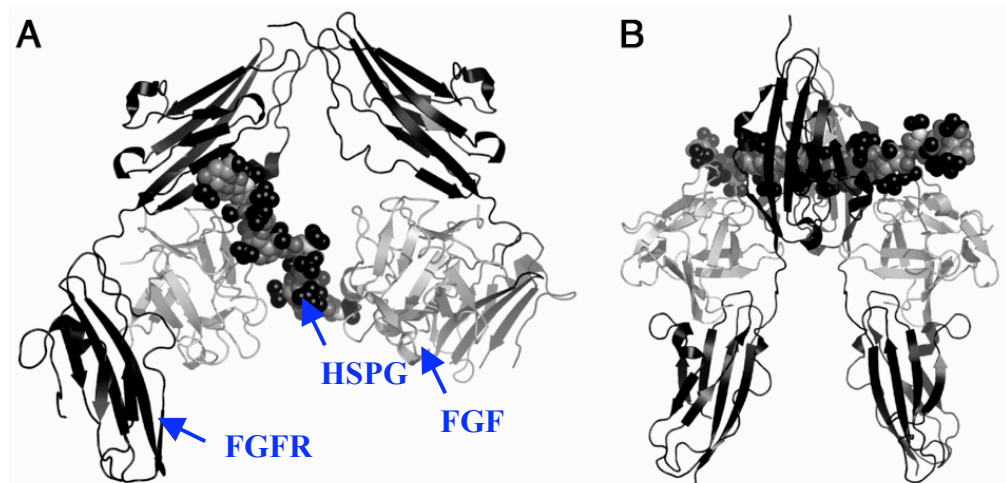


Figure 1.3 Dimerisation of FGFRs by FGFs and HSPGs (modified from (Harmer, 2006)).

Cartoon models depicting the (A) ‘Pellegrini’ model of 2:2:1 FGFR:FGF:HSPG, and (B) ‘Schlessinger-Mohammadi’ model of 2:2:2 FGFR:FGF:HSPG for FGFR activation. FGFR is shown as black cartoon, FGF is shown as light grey cartoon, and HSPG is shown as an atom model.

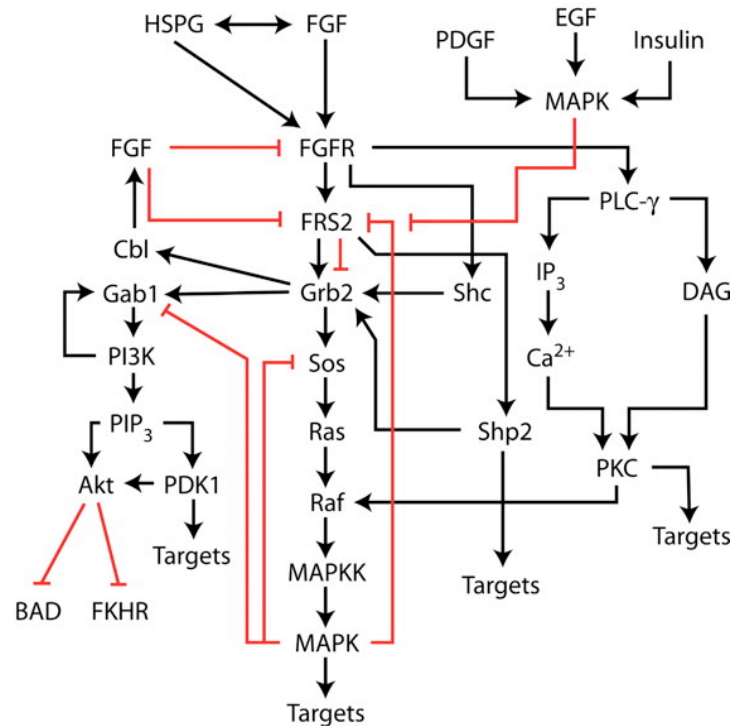


Figure 1.4 Schematic of FGFR signalling (Schlessinger, 2004).

Activation of FGFR leads to direct PLC-γ/PKC and Shc/Grb2/MAPK signalling pathways, or indirect Grb2/Sos/Ras/MAPK and Grb2/Gab1/PI3K/Akt pathways via the FRS2 docking protein.

1.1.3 FGFs and FGFRs during development

Various loss-of-function models in mouse, *Xenopus* and zebrafish have been generated to study the role of FGFs and FGFRs in vertebrate development. Since the work carried out in this thesis was performed mainly in mouse models, Table 1.1 summarises the phenotypes observed in *Fgf* and *Fgfr* knockout mice. From this table, *Fgf4*, 8, 9, 10, 15, 18 and *Fgfr1*, 2 are required for postnatal viability while loss of other Fgfs and Fgfrs leads to postnatal defects, implying that FGFs and FGFRs regulate multiple aspects of processes from embryonic to postnatal development.

Table 1.1 Summary of phenotypes in *Fgf* and *Fgfr* knockout mice.

Gene	Phenotype	Reference
<i>Fgf1</i>	Viable; largely normal	Miller et. al. (2000)
<i>Fgf2</i>	Viable; defects in neuronal and cardiovascular development	Dono et. al. (1998), Ortega et. al. (1998)
<i>Fgf3</i>	Most die at or soon after birth; defects in tail and inner ear development	Mansour et. al. (1994)
<i>Fgf4</i>	Embryonic lethal, E4-5; defects in trophoblast and primitive endoderm	Feldman et. al. (1995), Goldin et. al. (2003)
<i>Fgf5</i>	Viable; defects in hair growth	Hebert et. al. (1994)
<i>Fgf6</i>	Viable; defects in muscle	Floss et. al. (1997)
<i>Fgf7</i>	Viable; largely normal	Guo et. al. (1996)
<i>Fgf8</i>	Embryonic lethal, E9.5; defects in mesoderm and endoderm migration and cell fate determination	Sun et. al. (1999), Meyers et. al. (1998)
<i>Fgf9</i>	Die at birth; defects in lung and gut development	Colvin et. al. (2001), Geske et. al. (2008)
<i>Fgf10</i>	Die at birth; defects in skeletal, gut and lung development	Sekine et. al. (1999), Min et. al. (1998)
<i>Fgf12</i>	Viable; defects in neuromuscular function	Goldfarb et. al. (2007)
<i>Fgf14</i>	Viable; defects in neurological functions	Wang et. al. (2002), Laezza et. al. (2007)
<i>Fgf15</i>	Embryonic lethal, E13.5-P21; defects in heart development and bile acid metabolism	Vincentz et. al. (2005), Inagaki et. al. (2005)
<i>Fgf16</i>	Viable; defects in heart development	Itoh et. al. (2007)
<i>Fgf17</i>	Viable; defects in brain development	Xu et. al. (2000)
<i>Fgf18</i>	Die at birth; defects in skeletal and lung development	Ohbayashi et. al. (2002), Liu et. al. (2002), Usui et. al. (2004)
<i>Fgf21</i>	Viable; largely normal	Itoh et. al. (2007)

<i>Fgf22</i>	Viable; largely normal	Umemori, personal communication
<i>Fgfr1</i>	Embryonic lethal, E7.5-9.5; defects in mesoderm and endoderm migration and cell fate determination	Deng et. al. (1994), Yamaguchi et. al. (1994), Ciruna et. al. (2001)
<i>Fgfr2</i>	Embryonic lethal; E6-8; defects in inner cell mass, and no visceral endoderm formed	Arman et. al. (1998)
<i>Fgfr2b</i>	Die at birth; defects in skeletal, gut and lung development	DeMoerlooze et. al. (2000)
<i>Fgfr3</i>	Viable; defects in skeletal formation, inner ear development and abnormal intestinal crypt proliferation	Colvin et. al. (1996), Vildrich et. al. (2009)
<i>Fgfr4</i>	Viable; defects in cholesterol metabolism and bile acid synthesis	Yu et. al. (2000)

Red and blue boxes indicate *Fgf* genes that are required for viability of mice. The two genes that were investigated further in this thesis are marked by blue boxes.

1.2 Mouse gastrointestinal development

As this thesis investigated the role of *Fgf10* and *Fgfr2b* in GI development, homeostasis and injury, the following sections will provide information on the anatomy of developing and adult mouse gut, and discuss mouse models of ulcerative colitis.

1.2.1 Stomach development

In the mouse, gastrointestinal (GI) development occurs in several sequential steps: differentiation of definitive endoderm, formation and regional specialisation of the primitive gut tube, followed by morphogenesis and differentiation of component cell lineages (Figure 1.5)

(Grapin-Botton and Melton, 2000). At around embryonic (E) day 10.0, the stomach emerges as a bulge from the primitive gut tube (Aubin et al., 2002). At E11.5, stomach epithelial cells proliferate rapidly and the epithelium is pseudo-stratified with little difference between the presumptive forestomach and presumptive glandular stomach (Fukamachi et al., 1979). The epithelium then begins to stratify and form intraepithelial vacuoles from E13.5 onwards. Mesenchymal cells then invade the epithelium and the stratified epithelium becomes a simple columnar structure by E15.5. From E15.5 to E18.5, the epithelial cells differentiate into an epithelial monolayer with numerous invaginations that have morphological features of mucus-producing pit and neck cells, acid-producing parietal cells and hormone-producing enteroendocrine cells observed in adult gastric units (Karam et al., 1997). Cytodifferentiation proceeds postnatally with expansion of these gastric lineages until P28, where distinct pit, isthmus, neck and base compartments are formed (Karam et al., 1997) (see Section 1.3.1 for adult stomach lineages).

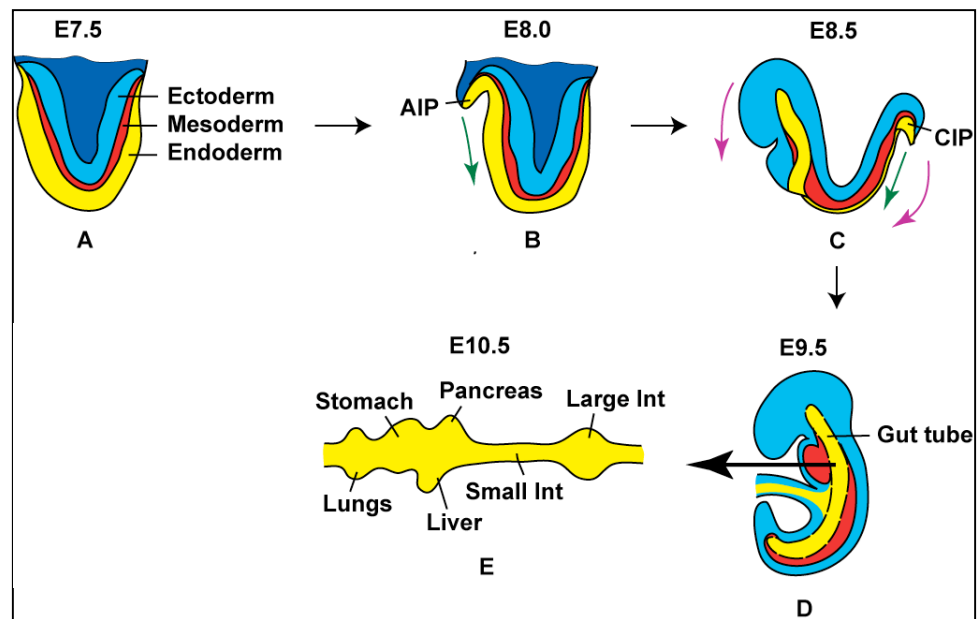


Figure 1.5 Stages in gut tube formation and specification (modified from (Grapin-Botton and Melton, 2000)).

Following gastrulation (A), where all three distinct germ layers (endoderm, mesoderm and ectoderm) are present, the anterior tip of the endoderm forms a fold (anterior intestinal portal, AIP) that travels posteriorly (indicated by the green arrow) to form the initial part of the primitive gut tube (B). This is later followed by the formation of a second fold at the posterior tip of the endoderm (caudal intestinal portal, CIP) that travels anteriorly (indicated by the green arrow) and finally fuses with the AIP at the yolk stalk, which connects the embryo to the yolk sac (C). Fusing of the two folds is also facilitated by ‘turning’ of the embryo, as indicated by the purple arrows in (C). The gut tube (D) then undergoes regionalisation characterised by swelling and budding of different organs: foregut (gives rise to oesophagus, stomach, first part of duodenum until the hepatic duct, pancreas, lungs and liver), midgut (gives rise to small intestine) and hindgut (gives rise to large intestine) (E) (Wells and Melton, 2000). From this point onwards, the different segments of the gut undergo morphogenesis and differentiation.

1.2.2 Intestinal development

The development of the small and large intestines (caecum and colon) is, to some extent, similar to that of the stomach. Intestinal epithelium proliferates rapidly and becomes stratified at E11.5 (Fukamachi et al., 1979). Also at this stage, the caecum develops as a bud that propagates off the main gut tube (hindgut region) at around E11.5. Numerous intraepithelial vacuoles form and fuse with the luminal surface at E13.5. Mesenchymal cells invade the epithelium and the stratified epithelium becomes a simple columnar structure by E15.5. From E15.5 to E18.5, the epithelial cells differentiate into a monolayer of numerous invaginations that have intestinal absorptive cells and mucin-producing goblet cells. In the small intestine, these invaginations lead to the formation of primitive villi. Crypts develop postnatally from P1 to P5 at the base of the invaginations, and cytodifferentiation continues postnatally until twenty

days post weaning (Subramanian et al., 1998). Throughout E12.5 to E18.5, the intestine undergoes a series of rotational events, resulting in the proper positioning of the gut within the abdominal cavity (Takahashi et al., 1998).

1.3 Adult mouse gastrointestinal tract

1.3.1 Anatomy of the stomach

Following ingestion of food by the oral cavity and subsequent transfer into the stomach via the oesophagus, digestion is initiated by mixing the food with gastric juice containing acids and enzymes. During digestion, the food is converted into a mixture called chyme, which is then passed on to the small intestine through a valve known as pyloric sphincter. The adult stomach consists of two distinct parts: forestomach and glandular stomach. The forestomach consists only of stratified squamous epithelium, whereas the glandular stomach consists of gastric units and is further divided into corpus and pyloric antrum. In the corpus region, each gastric unit is composed of gastric precursors in the isthmus, mucus-producing pit and neck cells, acid-producing parietal cells, hormone-producing enteroendocrine cells, and zymogenic chief cells that produce intrinsic factor and pepsinogen (Figure 1.6) (Lorenz and Gordon, 1993).

Using a combination of tritiated thymidine labelling, light microscopy and electron microscopy, the isthmal gastric precursors are thought to consist of undifferentiated granule-free stem cells and three committed daughter cells (granule-free pre-pit precursor cells, granule-free pre-neck cells, and pre-parietal cells) that give rise to the terminally differentiated gastric lineages (Figure 1.7) (Karam et al., 1997). In the pyloric antrum, however, each gastric unit is composed of gastric precursors in the isthmus, and mucus-producing pit and gland cells (Lee et al., 1982). These gastric

precursors are thought to be stem cells and two committed daughter cells (granule-free pit cells and granule-free gland cells) that give rise to pit and gland cells (Figure 1.7) (Karam et al., 2008).

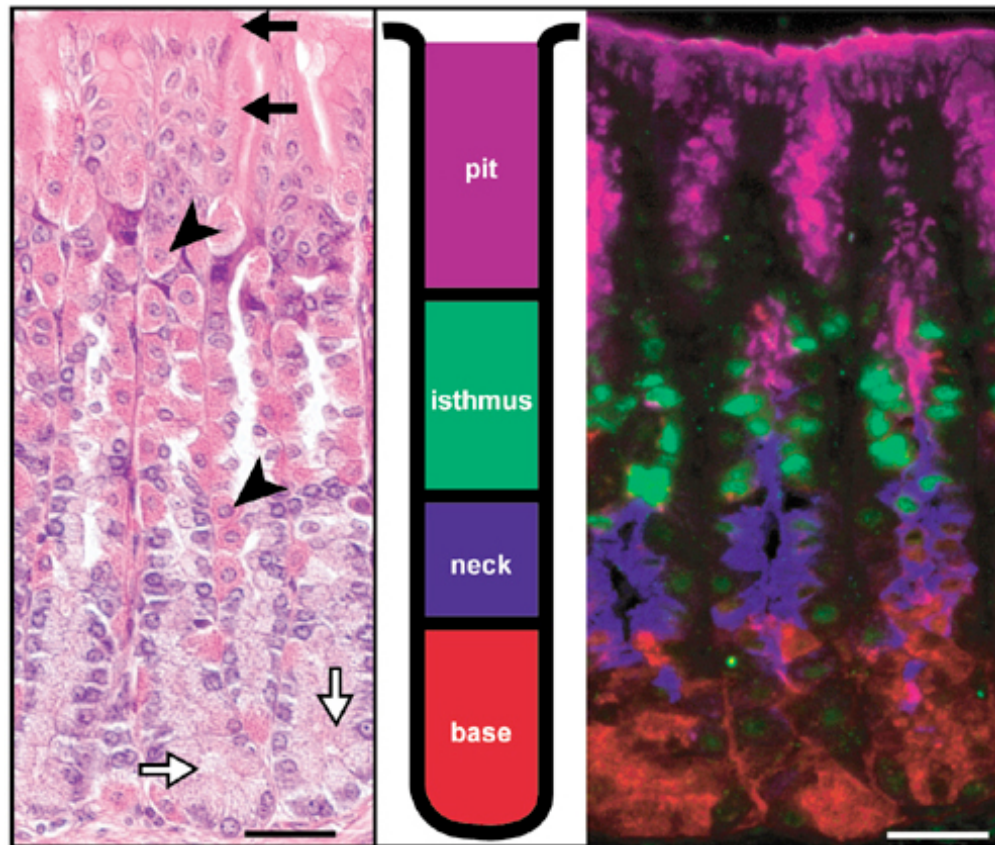


Figure 1.6 Representation of gastric units located in the corpus of a normal mouse stomach epithelium (Syder et al., 2003).

The left image shows a haematoxylin and eosin (H&E)-stained section of the gastric epithelium. Pit cells are marked by filled arrows, parietal cells are marked by arrowheads, and chief cells are marked by open arrows. The middle image shows the four compartments of a gastric unit. The right image shows a multilabel histochemical staining for pit cells (purple), isthmal progenitor cells (green), neck cells (blue), and chief cells (red).

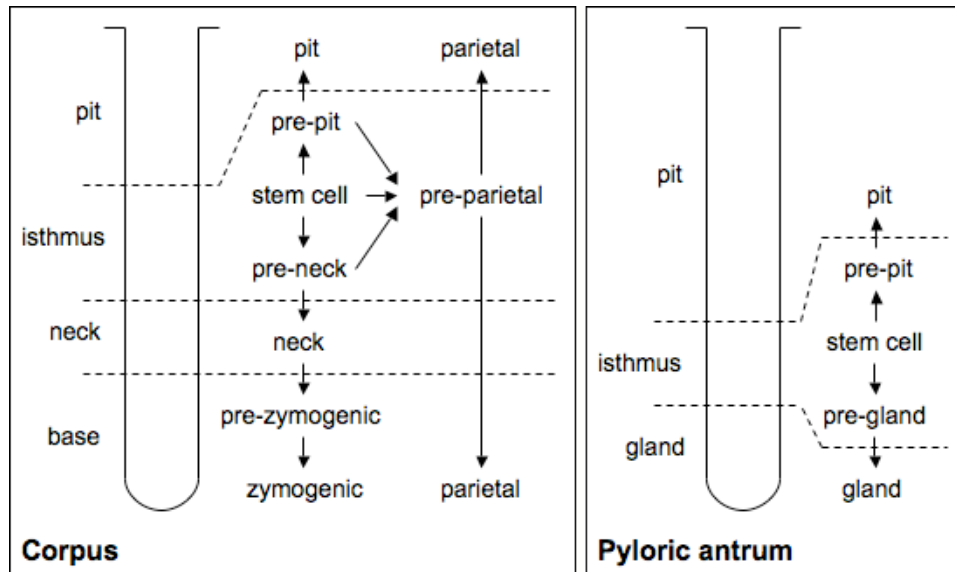


Figure 1.7 Schematic diagram showing the lineage relationships and differentiation programmes of the epithelial cells in the corpus and antrum of mouse stomach (modified from (Li et al., 1998) and (Karam et al., 2008)).

The arrows indicate the direction of migration of various gastric lineages. In the corpus, the pre-parietal cells can derive from three types of granule-free cells: undifferentiated granule-free cells, pre-pit precursor cells and pre-neck precursor cells. In contrast, the pit and zymogenic cell lineages are derived from single committed precursors, which are pre-pit precursor and pre-neck precursor cells respectively. Pre-parietal cells differentiate into parietal cells during bipolar migration, while pit and zymogenic cells arise during unipolar migration. In the pyloric antrum, the isthmal multipotent stem cells give rise to two committed precursors: pre-pit precursors that terminally differentiate into pit cells while migrating upwards, and pre-gland precursors that terminally differentiate into gland mucous cells while migrating downwards.

1.3.2 Anatomy of the intestine

The adult intestine is divided into small and large intestines. The small intestine is further divided into duodenum, jejunum and ileum, while the

large intestine is further divided into caecum, proximal and distal colon. The small intestine absorbs nutrients from the chyme via its surface epithelium. Following removal of nutrients, the chyme, mostly consists of water and indigestible waste, such as cellulose, is then passed through the ileocaecal valve by peristaltic movements into the large intestine. Movement of chyme along the large intestine is slower, and this favours the growth of bacteria that are essential for the health of the digestive tract. These bacteria feed on the chyme and produce essential vitamins, for example Vitamin K and some of the B-complex vitamins, and compete with pathogenic bacteria to colonise the intestinal environment. Caecum and colon mainly absorb water and sodium from the chyme and eliminate wastes, which are composed mainly of undigested or unabsorbed food and bile pigments.

The wall of both the small and large intestines is made up of four layers (Figure 1.8):

- a) mucosa, which lines the digestive tract and consists of epithelial tissue,
- b) submucosa, which lies outside the mucosa and contains connective tissue rich in blood vessels, lymphatics, nerve vessels, and a thin superficial muscular layer (the muscularis mucosa),
- c) muscularis propria, which underlies the submucosa and consists of smooth muscle that induces peristalsis (a wave-like contraction that pushes food along the digestive tract),
- d) serosa (visceral peritoneum), which is the outermost coat of the digestive tract and consists of connective tissue.

In the mucosa of the small intestine, there are numerous finger-like projections, called villi, which increase the surface area of the small intestine to absorb nutrients from food (Figure 1.9). The mucosa also has numerous invaginations known as crypts of Lieberkühn. Within each villus, there is a network of blood capillaries and a central lymph vessel. Nutrients absorbed by the villi are passed to the blood vessels (for amino acids and simple sugars) or the lymphatic system (for fatty acids). In contrast, the caecal and colonic epithelium lacks villi and consists mainly

of crypts. New crypts are thought to form from existing crypts by a process known as 'crypt fission' (Totafurno et al., 1987). The exact mechanism of this process is still under investigation. It is believed that a fissure occurs at the bottom of the crypts and extends across the longitudinal axis, resulting in the formation of two crypts (Figure 1.10) (Totafurno et al., 1987, Wasan et al., 1998). Crypt fission also initiates when the number of stem cells present in a crypt doubles (Loeffler et al., 1993).

There are four main types of cells that populate the intestinal epithelium (Figure 1.11):

- a) absorptive cells (also known as enterocytes in the small intestine, and colonocytes in the large intestine), which are the most abundant cell lineage in the small intestine. These are simple columnar epithelial cells that display microvilli on the apical surface in order to increase their absorptive capacity. Their main functions are to absorb nutrients from the chyme, and secrete enzymes for digestion in the lumen.
- b) goblet cells, which are mucin-producing cells. Their cytoplasm consists mainly of mucinogen granules, while the basal portion of these cells is occupied by the cell organelles. The number of goblet cell increases gradually from the duodenum to the distal colon.
- c) enteroendocrine cells, which are hormone-secreting cells. These cells control gut physiology by secreting serotonin, somatostatin, motilin and secretin.
- d) Paneth cells, which are secretory cells that produce many anti-microbial agents to provide host defence against bacteria. One important agent is cryptidin (alpha-defensins) that preferentially binds to bacterial cells and results in cell lysis by disrupting membrane function (Ouellette and Bevins, 2001). Other anti-microbial agents include lysozyme and phospholipase A2. Paneth cells reside at the bottom of the crypts, in the same vicinity to that of the stem cells, suggesting a critical role of Paneth cells in defending epithelial cell renewal from microbial invasion (Ouellette and Bevins, 2001). Paneth cells are the only lineage absent from the colonic crypts, and the reason for this is currently unclear.

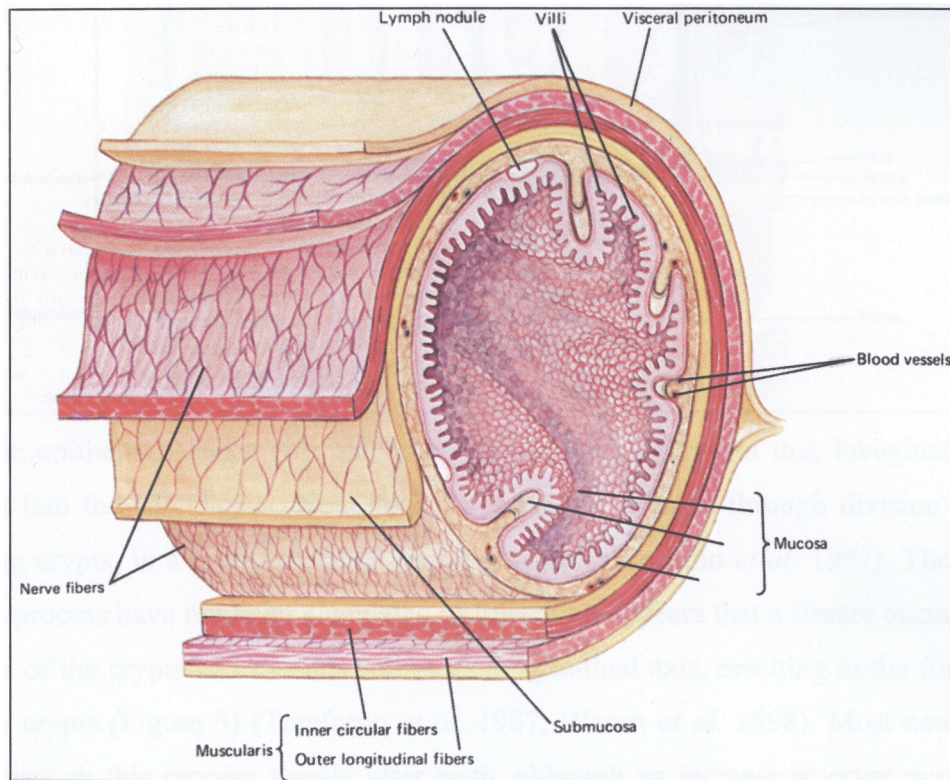


Figure 1.8 Schematic diagram of the layers in a small intestine (Solomon, 1992).

The four layers of the intestine are the mucosa, submucosa, muscularis propria and the visceral peritoneum. In this diagram, the mucosa shows numerous finger-like projections called villi that are present in the small intestine, but the mucosa in the caecum and colon lacks villi and contains only crypts.

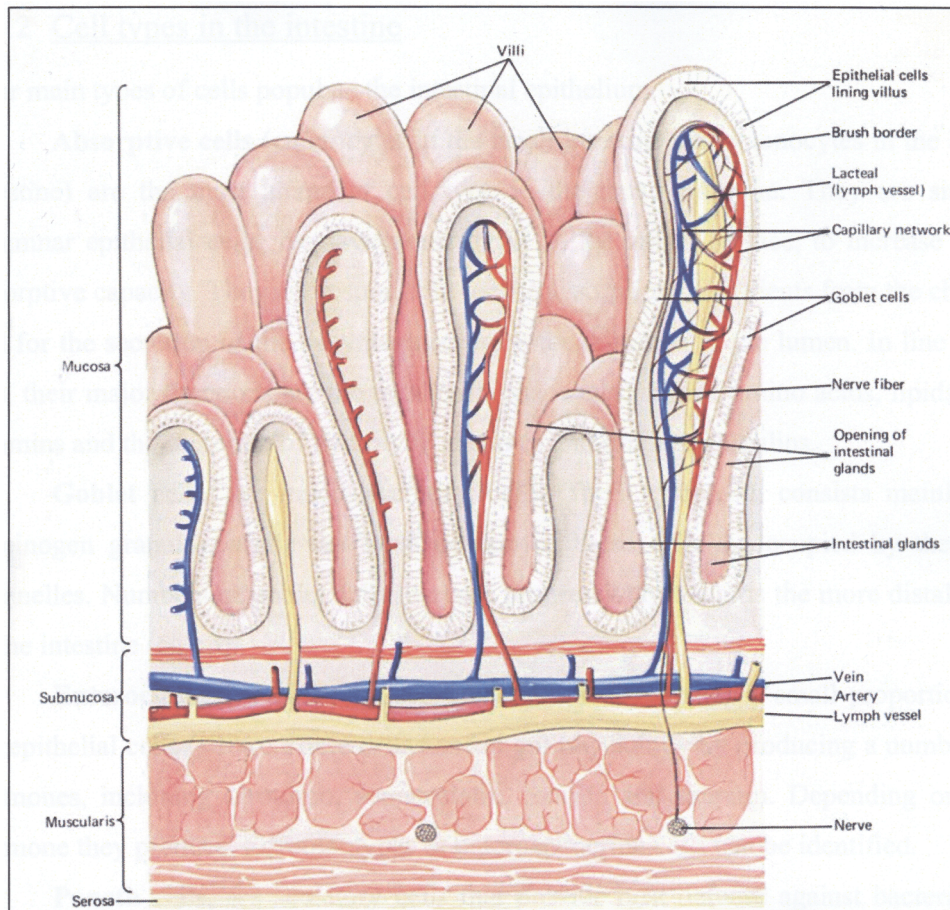


Figure 1.9 Schematic diagram of small intestinal villi (Solomon, 1992).

Each villus has a network of blood capillaries and a central lymph vessel. The base of each intestinal gland contains crypts of Lieberkühn.

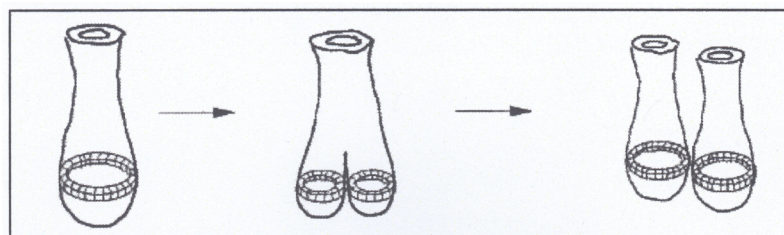


Figure 1.10 Schematic diagram of crypt fission (Wasan et al., 1998).

Each crypt in the small intestine, caecum and colon replicates by this process. As crypts elongate, they bifurcate (divide into two) starting from the bottom.

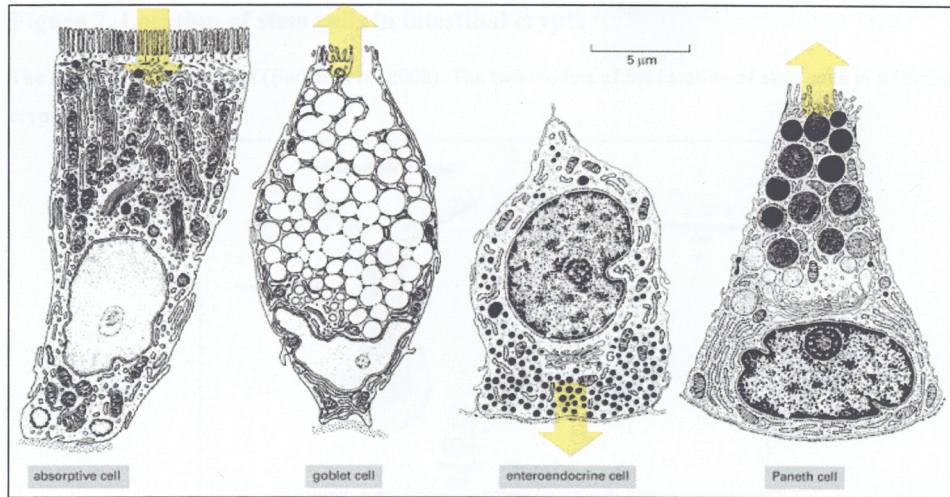


Figure 1.11 Schematic diagram of the four main types of cells in the intestine (Alberts et al., 2007).

These cells represent the different lineages present in the small intestine. The Paneth cells, however, are absent in the caecum and colon. The yellow arrows indicate that three of these cell types are secretory and only one (the enterocyte) is absorptive.

1.3.3 Life cycle of intestinal epithelial cells

Given the highly toxic environment in the intestinal lumen, cells lining the intestinal epithelium are highly proliferative, with crypt turnover taking approximately three days in the mouse (Creamer et al., 1961). Epithelial cells in both small and large intestines are thought to arise from a small population of stem cells that reside at the bottom of the crypts. Intestinal stem cells are elusive in identity, and proposed stem cell markers have been highly debated. The number of stem cells is estimated to be between four and six per crypt (Bjerknes and Cheng, 1999). Long-term tritiated thymidine labelling studies have shown that stem cells are located at the +4 position above the Paneth cells (Figure 1.12) (Potten et al., 1997). An alternative intestinal stem cell hypothesis has suggested that crypt base columnar (CBC) cells interspersed between Paneth cells at the base of the crypts are the true stem cells, as they display longevity and multipotency

characteristics of stem cell nature (Figure 1.12) (Cheng and Leblond, 1974, Bjerknes and Cheng, 1981c, Stappenbeck et al., 2003), although they appear to cycle rapidly.

However, both models agree that stem cells give rise to all other epithelial cell lineages through the proliferation of the transit amplifying (TA) cells. TA cells reside in the lower two thirds of the crypt in the TA compartment, and divide every 12-16 hours to generate approximately 300 cells per crypt per day (Marshman et al., 2002). During their proliferative cycles, TA cells migrate upwards along the crypt-villus axis while terminally differentiating into absorptive, goblet and enteroendocrine cells. However, both models have disagreement on the migration pattern of the Paneth cells. In the '+4 position' stem cell model, the Paneth cells are believed to escape the upward migration flow and instead migrate downwards during the differentiation process, before residing at the crypt base for 3-6 weeks (Ireland et al., 2005, Bjerknes and Cheng, 1981b, Bjerknes and Cheng, 1981a). In the 'CBC-stem cell zone model', the Paneth cells do not migrate during differentiation and stay interspersed between the CBC cells (Cheng and Leblond, 1974, Bjerknes and Cheng, 1981c, Stappenbeck et al., 2003).

Proliferation of TA cells is balanced by apoptosis and shedding of cells that have reached the top of the villus (or the crypt in the colon) into the lumen. Therefore, the intestinal epithelium sheet can be thought of as being in a virtually constant upward movement, except for the Paneth cells in the '+4 position' stem cell model that migrate downwards. In the small intestine, as many as 6-10 crypts can surround a villus, resulting in the villi being polyclonal entities due to the convergence of differentiated cells from multiple crypts (Cheng and Leblond, 1974). The mechanism of controlling the balance between intestinal proliferation and differentiation has been a subject of intense investigation, and an example of a signalling pathway essential in this process is the Wnt/ β -catenin signalling pathway.

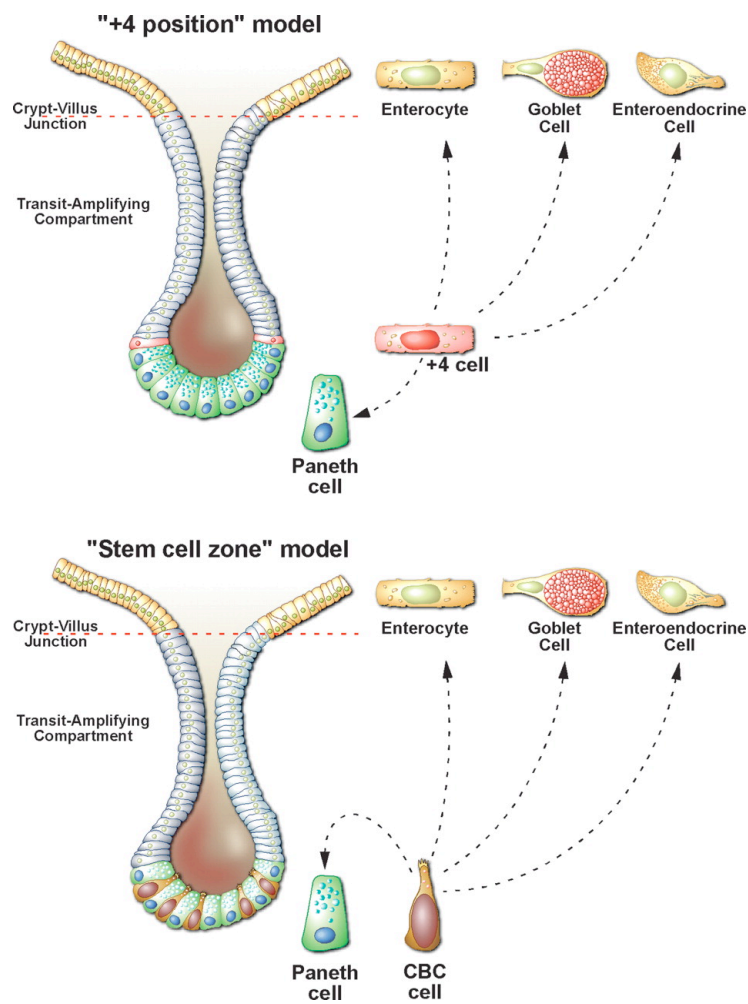


Figure 1.12 Schematic diagram of the location of stem cells in intestinal crypts (Barker et al., 2008).

In the '+4 position' model, the stem cells are located at the +4 position above the Paneth cells, whereas in the 'CBC-stem cell zone', the stem cells are interspersed between the Paneth cells at the base of the crypts.

1.4 Wnt signalling pathway

The Wnt genes encode a family of glycoprotein signalling molecules. The central effector of the pathway activated by these molecules is the β -catenin protein. In the absence of Wnt signalling, β -catenin is

phosphorylated by two serine/threonine kinases: casein kinase 1- α (CK1- α) and glycogen synthase kinase-3 β (GSK-3 β) (Figure 1.13) (Hinoi et al., 2000, Liu et al., 2002). The interaction between these kinases and β -catenin is facilitated by a scaffolding complex, formed by the adenomatous polyposis coli (APC) protein and Axin (Behrens et al., 1998, Ikeda et al., 1998, Yamamoto et al., 1998, Kishida et al., 1998). Together, these proteins form a β -catenin degradation complex by targeting the phosphorylated β -catenin to the 26S proteasome for degradation through ubiquitination by the β -transducin repeat-containing protein (β -TRCP) (Aberle et al., 1997).

Conversely, Wnt signalling is activated when extracellular Wnt ligands bind to one of the Frizzled transmembrane receptors and one of the LRP (low-density lipoprotein receptor-related protein) co-receptors (Fig. 1.13). Axin is then recruited to the membrane, where it interacts with the cytoplasmic domain of the LRP co-receptor. The relocation of Axin causes destabilisation of the β -catenin degradation complex (Mao et al., 2001, Tamai et al., 2000, Pinson et al., 2000). At the same time, hyperphosphorylation of the dishevelled (Dsh) protein occurs, which results in phosphorylation and inactivation of GSK3- β , further repressing β -catenin degradation (Yanagawa et al., 1995). The consequent accumulation of unphosphorylated β -catenin in the cytoplasm results in translocation of free β -catenin to the nucleus, where it recruits the Legless/BCL9 and Pygopus proteins, and interacts with members of the TCF/LEF (T-cell factor/lymphoid enhancer factor) family of transcription factors, activating transcription of a number of *Wnt* target genes, such as the leucine-rich-repeat-containing G-protein-coupled receptor 5 (*Lgr5*) that marks actively cycling intestinal stem cells (Tetsu and McCormick, 1999, Kramps et al., 2002, Belenkaya et al., 2002, Van de Wetering et al., 1996, Korinek et al., 1997, Barker et al., 2007).

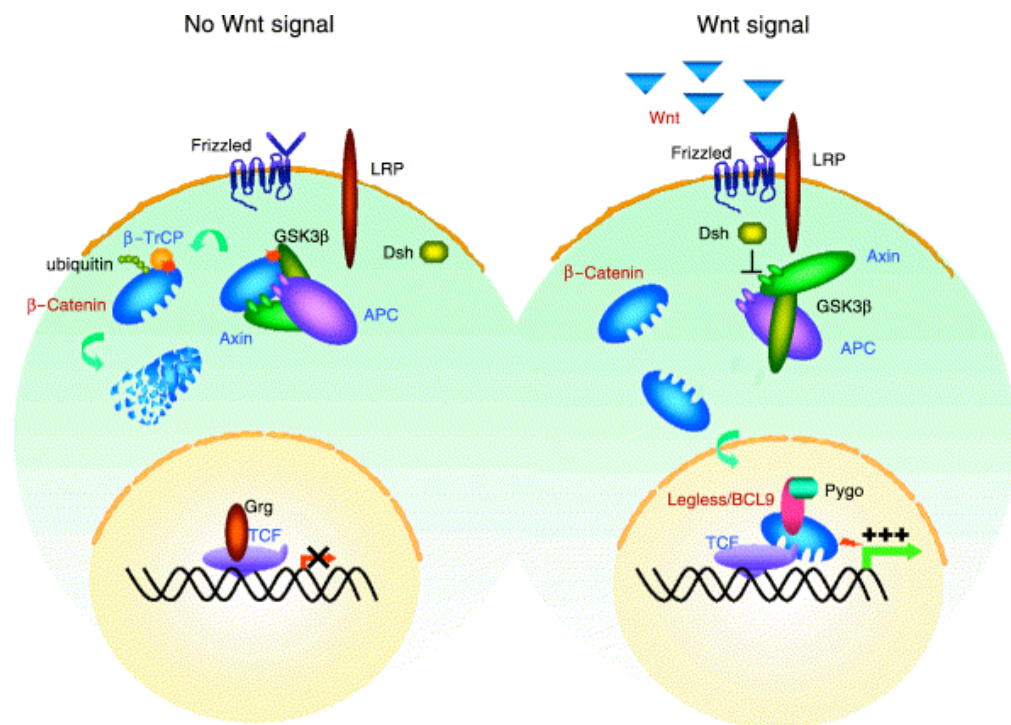


Figure 1.13 Schematic diagram of the Wnt signalling cascade (Giles et al., 2003).

In the absence of a Wnt signal, cytoplasmic β -catenin is bound to a degradation complex, and degraded in the proteasome. In the presence of a Wnt signal, the degradation complex destabilises, and accumulated β -catenin translocates to the nucleus and activates the TCF/LEF transcription factors.

1.5 Inflammatory bowel disease

1.5.1 Overview

Inflammatory bowel disease (IBD) consists primarily of two disorders: ulcerative colitis (UC) and Crohn's disease (CD). The first feature of IBD is chronic and uncontrolled inflammation of the intestinal mucosa that can affect any part of the GI tract (Papadakis and Targan, 2000). Diagnosis is based on the presence of architectural distortion, such as transmural or superficial patchy granulomatous infiltration, and/or acute inflammatory

cells (Hanauer, 2006). The second feature of IBD is the inability to downregulate those responses (Hanauer, 2006). In healthy people, the intestine becomes inflamed in response to pathogen then returns to a state of tolerance once the pathogen is eliminated from the gut. However, in patients with IBD, this inflammation is not downregulated, and the mucosal immune system remains activated, resulting in the intestine being chronically inflamed.

Although UC and CD are recognised as clinically distinct conditions with different anatomical and histological findings (Figure 1.14), accurate diagnosis remains challenging because patients can have a spectrum of conditions with UC and CD at opposite ends (Sands, 2004). In UC, the inflammation affects primarily the mucosal lining of the colon and rectum, whereas in the CD, the inflammation usually affects the whole intestinal wall and can extend to any part of the GI tract (Podolsky, 2002). 10% of patients have indeterminate features between UC and CD that cannot be clearly categorised, and often these patients remain in this category until the diagnostic features of UC or CD become apparent later on (Podolsky, 2002).

In the UK, UC is affecting 120,000 people (about 1:500) with 6,000 to 12,000 new cases being diagnosed each year. In contrast, CD is affecting 60,000 people (about 1:1000) with 3,000 to 6,000 new cases diagnosed each year (statistics from National Association for Colitis and Crohn's Disease (NACC) at <http://www.nacc.org.uk/content/ibd.asp>). There is approximately 3.7% chance that a patient with UC can develop colorectal cancer (CRC) (Eaden et al., 2001).

The great diversity of symptoms within UC and CD suggests a complex interplay of genetic, environmental and immunological factors in the aetiology of IBD, and these factors are currently being studied with numerous mouse models of IBD.

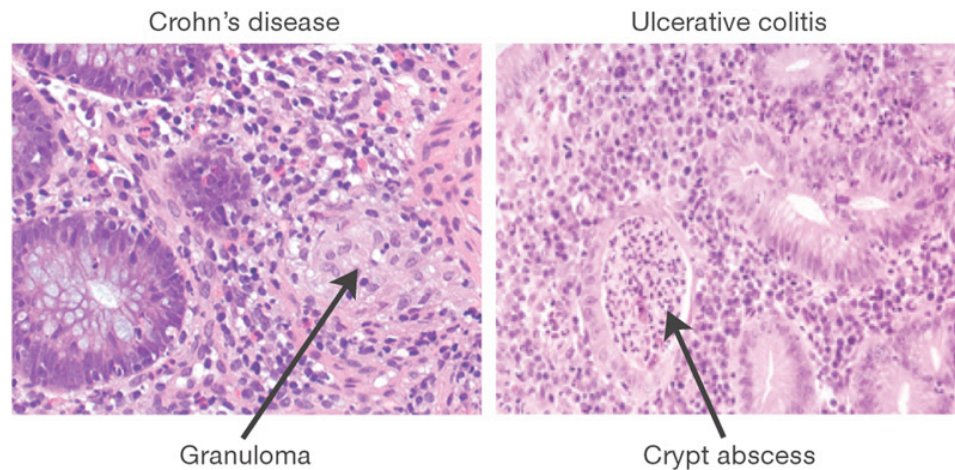


Figure 1.14 Histologic features of human CD and UC (Xavier and Podolsky, 2007).

Key features of CD include formation of discrete granulomas composed of macrophages, and significant infiltration of lymphoid cells and other inflammatory cells surrounding the granuloma. Key features of UC include significant infiltration of neutrophils within the lamina propria, formation of crypt abscesses, and extensive surface mucosal ulceration.

1.5.2 Mouse models of UC

Animal models of UC are valuable tools for investigating the pathogenesis of UC and testing emerging therapeutic strategies in the pre-clinical phase. While there are many types of mouse models of chronic intestinal inflammation, this section will only discuss the use of chemically induced models, as these models are most commonly used to study UC, and they closely resemble some of the immunological and histopathological aspects of human UC (Wirtz and Neurath, 2000). The three most common chemically induced models are TNBS colitis, oxazolone colitis, and DSS colitis.

In the TNBS model, colitis is induced by intrarectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 30-50% ethanol (Wirtz and

Neurath, 2000). Ethanol breaks the mucosal barrier, while TNBS haptensises colonic proteins to make them immunogenic to the host immune system. Chronic TNBS colitis in SJL/L mice has resulted in Th1-mediated granulomatous inflammation with severe diarrhoea, weight loss, thickening of the colonic wall and elevated level of infiltrating lymphocytes, especially the CD4⁺ T-cells (Neurath et al., 1995, Neurath et al., 1996), implying that this model is useful for studying Th1-dependent immune responses. However, the inflammatory process in mice is highly dependent on the strain and the concentration of TNBS administered (Wirtz et al., 2007).

In the oxazolone model, colitis is induced similarly as that of TNBS, and often leads to weight reduction, diarrhoea, significant loss of goblet cells, and high death rates (Ekstrom, 1998, Boirivant et al., 1998). This model is useful for studying the contribution of Th2-dependent immune response to intestinal inflammation, due to its elevated production of Th2 cytokines IL-4 and IL-5, which is similar to those characteristics observed in human UC (Boirivant et al., 1998). Like TNBS, oxazolone colitis is strain-dependent and requires individual optimisation, as a low dose of oxazolone can induce a mixed Th1/Th2-dependent colitis (Iijima et al., 2004).

In the DSS model, colitis is induced by administration of dextran sodium sulphate (DSS) in drinking water for three to seven days, which can lead to bloody diarrhoea, ulcerations and infiltrations with granulocytes (Okayasu et al., 1990). DSS is toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. Several studies have shown that the adaptive immune system does not play a major part in this model (Dieleman et al., 1994), hence DSS colitis is often used to study the contribution of innate immune response to intestinal inflammation. In susceptible strains, such as the C57BL/6 strain, administration of DSS for several cycles results in chronic colitis and when combined with a single initial dose of the genotoxic colon carcinogen azoxymethane (AOM), can

result in inflammation-associated CRC (Tanaka et al., 2003). Hence, DSS colitis is a useful model to study the interplay between colitis and CRC.

1.6 Experimental hypotheses

Given that FGFs and FGFRs are crucial in multiple processes from embryonic development to angiogenesis, wound healing and tumourigenesis (Section 1.1), and both *Fgf10*^{-/-} and *Fgfr2b*^{-/-} embryos have gut defects (Table 1.1), the following hypotheses have been addressed:

- i) Fgf10-Fgfr2b signalling is essential for normal small intestinal development.*
- ii) Fgfr2b is required for maintaining intestinal homeostasis.*
- iii) Fgfr2b plays a protective role in intestinal injury and is required for normal wound repair.*
- iv) Fgfr2b is expressed only within specific adult gastric lineages in the adult mouse.*

Chapter 2: Materials and methods

2.1 *Animal husbandry*

2.1.1 *Animal ethics*

All procedures were approved by the Cancer Research UK and Imperial College School of Medicine Animal Ethics Committees, and covered by the appropriate licences under the Home Office Animal Procedures Act 1986. All mice were culled according to Schedule I of the Animals Procedures Act 1986.

2.1.2 *Animal breeding*

Please see individual chapters for this, as different breeding strategies were adopted for different work.

2.1.3 *Genotyping by PCR*

Yolk sacs or adult ear-snips were incubated in 200 µl of DirectPCR Lysis reagent (401-E; Viagen Biotech) with 0.2 mg/ml Proteinase K (P-6556; Sigma-Aldrich) for 3 hrs at 55°C. Proteinase K was heat inactivated at 85°C for 45 min. Cell debris was pelleted, and 2 µl of supernatant was used for PCR. PCR amplification was performed using a *Taq* DNA Polymerase kit (201203; Qiagen). Each 20 µl PCR reaction contained 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.3 µM of each primer, 1X Q-solution, 2.5 U of *Taq* DNA polymerase, and 2 µl of DNA sample. PCR was performed using a thermal profile starting 2 min denaturation at 94°C, followed by 30 cycles consisting of denaturation at

94°C for 30 s, annealing at $x^{\circ}\text{C}$ for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. X refers to the appropriate annealing temperature for each primer set, which varies for different genes (Table 2.1). PCR products were analysed by agarose gel electrophoresis (Section 2.4.5).

2.2 Preparation of chemicals

2.2.1 RU486

RU486 mix was prepared by dissolving 3 mg of Mifepristone (RU486; M8046; Sigma-Aldrich) per 250 μl of 0.25% (w/v) carboxymethylcellulose/0.5% (v/v) Tween-80 solution, giving a final concentration of 12 mg/ml.

2.2.2 Tamoxifen

Tamoxifen mix was prepared by first dissolving 10 mg of tamoxifen (T5648; Sigma-Aldrich) per 100 μl of 100% ethanol, then adding 900 μl of autoclaved sunflower oil (Tesco) and vortexing the mix until a homogenous suspension was obtained, giving a final concentration of 10 mg/ml.

2.3 In situ hybridisation

2.3.1 Production of DIG-labelled probes

pBluescript IISK+ plasmid carrying a mouse cDNA of the cytoplasmic tyrosine kinase (TK) domain of *Fgfr2* (nucleotides 1726-2714) was

obtained from our laboratory and has been previously described (De Moerlooze et al., 2000). 50 ng of the plasmid was transformed into bacteria, grown as maxi-culture and purified (Section 2.11.7 for full details). 10 µg of purified plasmid was linearised with BamHI (for antisense) and HindIII (for sense) separately.

In vitro transcription was conducted using a DIG RNA Labelling kit (11175025910; Roche) where a 20 µl reaction volume consisting of 1 µg of DNA template, 1X transcription buffer, 30 mM DTT, 1X nucleoside triphosphates mix (ATP, GTP, CTP, and DIG-UTP), 1 U/µl Protector RNase Inhibitor, and 2 U/reaction T7 (for antisense) or T3 polymerase (for sense) was incubated for 2 hrs at 37°C. The sample was treated with 2 U of RNase-free rDNase I (AM1906; Ambion) for 30 min at 37°C to remove DNA template after transcription. rDNase was inactivated with a DNase inactivation reagent provided by the kit. RNA was precipitated in 5 mM EDTA pH 8.0, 120 mM LiCl, and 75% cold ethanol for 2 hrs at -20°C. RNA was pelleted, rinsed with 70% ethanol, and re-suspended in 50-100 µl EDTA 10 mM. Labelled antisense and sense Fgfr2-TK probes were visualised by agarose gel electrophoresis (Section 2.4.5), and extent of DIG-labelling was estimated using a dot blot described in the following section.

2.3.2 Dot blot

Labelled control RNA (from the DIG RNA Labelling kit; 11175025910; Roche) was used to prepare standards of 10 ng/µl, 5 ng/µl, 2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.1 ng/µl using 10X SSC as the diluent. Unlabelled control RNA (from the DIG RNA Labelling kit; 11175025910; Roche) at 10 ng/µl was also prepared with 10X SSC (consisted of 1.5 M NaCl and 0.15 M sodium citrate). 1 µl of antisense and sense probes prepared previously were diluted 1:100 with 10X SSC. All samples were then spot onto an 8 cm x 5 cm nitrocellulose membrane 0.45 µm pore size

(RPN203D; GE Healthcare) and allowed to air-dry for 10 min at room temperature (RT). Membrane was UV cross-linked with 1.2 kJ/m² with a UV Stratalinker 2400 (Stratagene), and incubated in a PBS solution containing 0.1% (v/v) Tween-20 (63158; Sigma-Aldrich) and 1% (v/v) Blocking buffer (1096176; Roche) for 20 min at RT. Membrane was incubated with sheep anti-DIG antibody conjugated with alkaline-phosphatase (11093274910; Roche) at 1:5000 (diluted in 1X TBST buffer, 0.1% Tween-20, and 1% Blocking buffer) for 30 min at RT. Membrane was washed in TBST buffer for 2 x 15 min, in NTMT buffer (containing 100 mM NaCl, 100 mM Tris-Cl pH 9.5, 50 mM MgCl₂, and 0.1% Triton X-100 (T9284; Sigma-Aldrich)) for 2 min, and incubated in a solution containing 4.5 µl/ml NBT (11383213001; Roche) and 3.5 µl BCIP (10760994001; Roche) (made with NTMT) in dark for 5 min. Membrane washed in TBST for 5 min, incubated in 10% neutral-buffered formalin (NBF) for 30 min, and air-dried.

2.3.3 In situ hybridisation (ISH) with labelled probes

Tissues were fixed in 10% NBF, processed and embedded in paraffin (Section 2.5.2), sectioned at 10 µm thickness using gloves and DEPC-treated water, and dried in an oven at 37°C overnight wrapped with aluminium foils. Sections were dewaxed by a 5 min immersion in sulphur-free xylene (BDH), re-hydrated by 5 min incubation in decreasing concentrations of ethanol (100%, 95%, 70%), water and PBS. Sections were treated with 10 µg/ml Proteinase K (P2308; Sigma-Aldrich) for 20 min at 37°C, washed in 0.2% (w/v) glycine/PBS (glycine obtained from 241261; Sigma-Aldrich) for 5 min, and incubated in 4% paraformaldehyde (PFA) for 10 min. Sections were then washed in PBS for 3 x 5 min, 4X SSC for 2 min, and incubated with hybridisation buffer containing 50% (v/v) deionised formamide (F7508; Sigma-Aldrich), 10% (v/v) dextran sulphate (31493; Sigma-Aldrich), 1X Denhardt's solution (53754, Sigma-Aldrich), 10 mM Tris-Cl pH 7.5, 60 mM NaCl, 1mM

EDTA, 0.25% SDS and 1 mg/ml tRNA (R5636, Sigma-Aldrich) for 1 hr at 57°C.

Labelled antisense and sense probes were diluted to 1:100 with the hybridisation buffer, incubated at 75°C for 15 min to denature RNA secondary structures, and cooled on ice for 15 min. Each probe mixture was added to the sections, layered with a parafilm, and placed in tightly sealed humidified chamber at 57°C for overnight. The next day, sections were washed in 5X SSC for 10 min at 60°C, 50% formamide/2X SSC for 30 min at 60°C, 2X SSC for 30 min at 60°C, 0.2X SSC for 30 min at 60°C, 1X maleic buffer for 5 min at RT, and 1X Blocking buffer (1096176; Roche) for 30 min at RT. Sections were incubated with sheep anti-DIG antibody conjugated with alkaline-phosphatase (11093274910; Roche) at 1:1000 (diluted with 1X Blocking buffer) for 2 hrs at 37°C. Sections were washed in 1X maleic buffer for 2 x 15 min at RT, and incubated a detection buffer containing 0.1 M NaCl, 0.04M MgCl₂, 100 mM Tris-HCl pH 9.5, and 2 mM levamisole for 5 min at RT. Antibody binding was developed with a solution containing 4.5 µl/ml NBT (11383213001; Roche) and 3.5 µl BCIP (10760994001; Roche) (made with the detection buffer) in dark at RT until the desired intensity of colorimetric reaction was achieved. Sections were then washed in the detection buffer for 15 min, in water for 15 min, counterstained in Nuclear Fast Red (Vector Laboratories) for 5 min, and mounted in Crystallmount aqueous mounting medium (C0612; Sigma-Aldrich). All buffers were made with DEPC-treated water.

2.4 Gene expression

2.4.1 RNA extraction

Tissues weighing not more than 30 mg were isolated and snap frozen in liquid nitrogen. RNA was extracted with an RNeasy Plus Mini kit (74134; Qiagen) according to the manufacturer's instructions. Briefly, tissues were homogenised in Buffer RLT Plus, passed through a DNA-binding column, added 70% ethanol, and passed through an RNA-binding column. Column membranes were washed with Buffer RW1 and RPE. RNA was eluted with 30 µl RNase-free water. RNA was re-treated with 2 U of RNase-free rDNase I (AM1906; Ambion) for 30 min at 37°C according to the manufacturer's instructions. rDNase was inactivated with a DNase inactivation reagent provided by the kit. The total RNA yield was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and RNA integrity was analysed with a denaturing 2% formaldehyde-agarose gel stained with ethidium bromide as described in the following section.

2.4.2 Denaturing formaldehyde-agarose gel electrophoresis

1.5 g of agarose (A4718; Sigma-Aldrich) was dissolved in 150 ml of 1X NorthernMax Denaturing Gel Buffer (AM8676; Ambion), containing formaldehyde and MOPS buffer, by heating. Ethidium bromide (E7637; Sigma-Aldrich) was added to the melted agarose to a final concentration of 0.5 µg/ml. Agarose mix was then poured into a gel cassette and allowed to cool. 500 ng of RNA was mixed with 2X volumes of Formaldehyde Load Dye, heated at 65°C for 10 min to denature the RNA, and cooled on ice for 2 min. 2 µl of RNA Millennium Markers (AM7150; Ambion) was mixed with 6 µl of NorthernMax Formaldehyde Load Dye (AM8552;

Ambion), heated and cooled as previously. RNA samples, including the markers, were electrophoresed at 100 V constant in 1X NorthernMax Running Buffer (AM8671; Ambion) until the desired migration extent was achieved. Bands were visualised under UV light, and photographed using an EagleEye Still Video system (Stratagene).

2.4.3 First strand cDNA synthesis

cDNA synthesis from RNA template was performed with a Superscript III First Strand Synthesis kit (18080-051; Invitrogen) according to the manufacturer's instructions. Briefly, a 10 µl reaction mix containing 1 µg of RNA, 5 ng/µl random hexamer, and 1 mM dNTP was incubated at 65°C for 5 min, and cooled on ice for 2 min. A separate 10 µl reaction mix containing 2X RT buffer, 10 mM MgCl₂, 20 mM DTT, 4 U/µl RNaseOUT, and 20 U/µl Superscript III was added to the initial reaction mix, giving a total reaction volume of 20 µl. The combined reaction mix was incubated for 10 min at 25°C, for 50 min at 50°C, for 5 min at 85°C, and cooled on ice for 2 min. RNA template was removed by adding 1 µl of RNase H to the reaction, and incubating the reaction mix for 20 min at 37°C. After the reverse transcription, cDNA was purified with a QIAquick PCR purification kit (28104; Qiagen) according to the manufacturer's instructions. Briefly, cDNA was mixed with a Binding buffer, bound to a DNA-binding column, washed with Buffer PE, and eluted with 30 µl RNase-free water. The total cDNA yield was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies), and cDNA integrity was assessed by PCR for *Gapdh* as described in the following section.

2.4.4 Reverse-transcriptase PCR

PCR amplification was performed on the cDNA with various kits due to varying efficiencies of different kits in amplifying the gene of interest (see Table 2.2): *Taq* DNA Polymerase kit (201203; Qiagen), PuReTaq Ready-To-Go PCR beads (407512; GE Healthcare), and MegaMix~Blue (2MMB; Microzone Ltd.).

For the *Taq* DNA Polymerase kit, a 20 μ l PCR reaction contained 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 μ M of each primer, 2.5 U of *Taq* DNA polymerase, and 100 ng of cDNA. Some reactions required an addition of Q-solution, a proprietary mix provided by the kit to enhance efficiency of PCR reactions, to a final concentration of 1X. For the PureTaq Ready-To-Go PCR beads, a 25 μ l PCR reaction contained 0.2 mM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 μ M of each primer, and 100 ng of cDNA. For the MegaMix~Blue, a 25 μ l PCR reaction contained 0.3 μ M of each primer and 100 ng of cDNA, with the remaining volume being the MegaMix solution. This master mix already contained 1.5 mM $MgCl_2$. In all cases, some reactions required additional $MgCl_2$ to achieve a final concentration of 2.0 mM or 2.5 mM.

PCR was performed using a thermal profile starting 2 min denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at x° C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR product was analysed by agarose gel electrophoresis (Section 2.4.5) to confirm the correct amplicon size. X refers to the appropriate annealing temperature for each primer set, which varies for different transcripts (Table 2.2).

2.4.5 Agarose gel electrophoresis

1.5 g of agarose (A4718; Sigma-Aldrich) was dissolved in 150 ml of 1X TAE buffer pH 8.2, containing 4.84 g/L Tris-base, 1.09 g/L glacial acetic acid and 0.3 g/L EDTA, by heating. Ethidium bromide (E7637; Sigma-Aldrich) was added to the melted agarose to a final concentration of 0.5 µg/ml. Agarose mix was then poured into a gel cassette and allowed to cool. 0.1 volume of Orange G 10 mg/ml (prepared in glycerol) (O3756; Sigma-Aldrich) was added to each DNA sample, mixed and added to the sample lanes. 10 µl of appropriate DNA ladder (NEB, UK), pre-mixed with 0.1 volume of Orange G 10 mg/ml, was also added to the sample lanes. All samples were electrophoresed at 100 V constant in 1X TAE buffer until the desired migration extent was achieved. Bands were visualised under UV light, and photographed using an EagleEye Still Video system (Stratagene).

2.4.6 Taqman real-time PCR

Taqman real-time primers were obtained from Applied Biosystems Inventory for the following murine genes: *Fgf1* (Mm00438906_m1), *Fgf3* (Mm00433290_m1), *Fgf7* (Mm00433291_m1), *Fgf10* (Mm00433275_m1), *Fgf22* (Mm00445749_m1), *Fgfr1b* (Mm01215493_m1), *Fgfr2b* (Mm01275521_m1), and β -2 microglobulin (β 2M; Mm00437762_m1). These primers have been carefully checked that they spanned across exon-exon boundary of a gene, and that they amplified the appropriate exon corresponding to the splice variants for *Fgfr1* and *Fgfr2*. The reaction was set up using a Quantitect Probe PCR kit (204341; Qiagen). A 25µl reaction contained 1X PCR master mix and 0.5X primers/probe mix. The master mix consisted HotStarTaq DNA polymerase, Tris-Cl pH 8.7, KCl, (NH₄)₂SO₄, 8 mM MgCl₂, dNTPs (including dUTP), and ROX fluorescent dye. The PCR was performed in an ABI 7900 Sequence Detector (Applied Biosystems) using a thermal

profile starting 95°C for 10 min, followed by 40 cycles consisting of denaturation at 95°C for 15 min, and combined annealing/extension at 60°C for 1 min.

2.4.7 SYBR Green PCR

Primer sequences were obtained from various published sources (see Table 2.3). A 25 µl reaction contained 1X Platinum SYBR Green qPCR SuperMix-UDG w/ROX (11744500; Invitrogen), 0.2 mM of each primer, and 50 ng of cDNA. The PCR was performed in an ABI 7900 Sequence Detector (Applied Biosystems) using a thermal profile starting 50°C for 20 min, 95°C for 10 min, followed by 40 cycles consisting of denaturation at 95°C for 15 s, and combined annealing/extension at 60°C for 1 min. A dissociation curve was run with each PCR (95°C for 15 s, followed by 60°C for 15 s) to ensure no primer-dimer formation.

2.4.8 Data analysis

For each experiment, each cDNA sample was performed in triplicate for a chosen gene, and each plate also contained *Gapdh* or *β2M* as an internal control gene for the cDNA sample. The entire experiment was repeated three times. Average Ct value of the chosen gene for a cDNA sample was normalised to the average Ct value of *β2M* giving a Δ Ct value. The difference between the Δ Ct value of a treated/tested population and that of a control population gave rise to a $\Delta\Delta$ Ct value that was then converted into a 'fold changes' value.

2.5 Immunohistochemical staining

2.5.1 Gut tissue harvesting

Following gut dissection, the caecum was separated from the gut and placed on ice. The small and large intestines were flushed with cold PBS to remove contents. The small intestine was divided into three segments, and all segments including the large intestine were thread onto metal rods. These rods were immobilised on a fibre-free blotting paper (PBP140; Hollingsworth & Vose Co. Ltd.), and the segments were cut open with a sharp scalpel. The segments were then rolled out onto the blotting paper, causing the gut segments to lay flat on the blotting paper with their villi pointing upwards. The blotting paper with the gut segments was immersed in 10% NBF overnight, and rinsed with 70% ethanol the next day. Each gut segment was then rolled up, pinned and processed as described in the following section. For the caecum, the tissue was cut on the outer curvature, flushed with cold PBS, stretched, pinned on a soft board, immersed in 10% NBF overnight, and rinsed with 70% ethanol the next day. Two longitudinal strips were cut from the fixed and stretched tissue, and processed as described in the following section. For the stomach, the tissue was cut on the outer curvature, stretched, pinned, and fixed as previously described for caecum.

2.5.2 Tissue fixation and processing

Tissues were fixed in 10% NBF overnight, dehydrated in serial alcohols, xylene and finally infiltrated overnight with molten paraffin wax (Figure 2.1 for the detailed steps). Tissues were embedded on a cassette, sectioned at 4 μ M thickness and dried in an oven at 37°C overnight. For embryonic work, gut tissues were dissected out under a dissecting microscope, fixed in 4% PFA overnight, and embedded in a 2% agarose before being processed as previously.

2.5.3 Tissue dewaxing and blocking of endogenous peroxidases

Sections were dewaxed by a 5 min immersion in sulphur-free xylene (BDH), re-hydrated by 3 min incubation in decreasing concentrations of ethanol (100%, 70%, 50% in distilled water) and finally in distilled water. Sections were then treated with 1.6% hydrogen peroxide (BDH) in PBS for 10 min to block endogenous peroxidases, rinsed in tap water, and washed in PBS.

2.5.4 Immunohistochemical staining and antigen retrieval

Antigen retrieval was performed dependent on the antibody requirement (Table 2.4), which included 0.01 M citrate buffer pH 6.0, 100 μ M EDTA pH 8.0, 0.1% bovine trypsin pH 7.8 (in 0.1% CaCl_2 /TBS), and 1X GlycA Antigen Retrieval Solution (HK167-5K; Launch Diagnostics, UK). The citrate, EDTA and glycA solutions were pre-heated by a microwave for 10 min at high power (800 W) before adding the sections and microwaving them for a further 10 to 20 min at high power. For the trypsin antigen retrieval, the solutions were preheated to 37°C in a water bath before incubating the sections for a further 10 min.

Sections were then rinsed in tap water, washed in PBS, and pre-incubated in normal serum (DakoCytomation Ltd., UK) from the donor species of the secondary antibody for 30 min, diluted at 1:10 in 1% PBS/BSA. Excess serum was removed, and sections were incubated in primary antibody for 1 hour at RT or overnight at 4°C, followed by incubation in a species-specific biotinylated secondary antibody (Vector Laboratories) for 45 min. Sections were then incubated in a tertiary layer of avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min, and antibody binding was developed with DAB (BioGenex) until the desired staining was achieved. Primary and secondary antibodies were diluted

appropriately with 1% PBS/BSA, and tissue sections were washed in PBS for 3 x 5 min between each layer. Sections were then washed in PBS for 2 x 5 min, rinsed in tap water, and counterstained lightly with H&E as described in the following section.

2.5.5 Counterstaining

After immunohistochemical staining was completed, Harris' haematoxylin and eosin counterstained was applied to sections by immersing sections in Harris' haematoxylin (Pioneer Research Chemicals) for 10 min, followed by a thorough rinse in tap water and immersion in acid alcohol (1% v/v concentrated HCl in 70% ethanol) for approximately 10 s. Following a second rinse in tap water, sections were immersed in Scott's tap water (30 mM NaHCO₃, 160 mM MgSO₄) for 10 s, and rinsed in tap water briefly. Sections were then rapidly dehydrated through graded alcohols to xylene (BDH Laboratories Supplies Inc., UK) and mounted in DePeX (BDH)

2.6 Histochemical staining

2.6.1 Detection of diastase/alcian blue/periodic acid Schiff (D/AB/PAS)

D/AB/PAS is used routinely to stain mucin-producing cells and to identify the nature of the mucin. Sections were dewaxed as previously described (Section 2.5.3), and incubated in 0.5 g/100 ml α -amylase (A6211; Sigma-Aldrich) for 30 min at 37°C. Sections were rinsed in distilled water before being treated with 10 mg/ml alcian blue pH 2.5 (made in 3% acetic acid) for 5 min. Sections were washed in running tap water and rinsed with distilled water. Sections were then treated with 1% periodic acid for 5 min, and washed in distilled water before being treated with Schiff's

reagent (VWR International) for 15 min. Sections were washed in running tap water for 5 min, cleared with 1% acid alcohol (made with concentrated HCl and 70% ethanol), dehydrated through graded alcohols to xylene (BDH) and mounted in DePeX (BDH).

2.6.2 Detection of intestinal alkaline phosphatase (IAP)

Sections were dewaxed as previously described (Section 2.5.3), except the final re-hydration step was performed in TBS, instead of PBS. Sections were washed for 2 x 10 min in NTMT buffer containing 100 mM NaCl, 100 mM Tris-Cl pH 9.5, 50 mM MgCl₂, and 0.1% Triton X-100 (T9284; Sigma-Aldrich). Sections were then incubated with a solution containing 4.5 µl/ml NBT (11383213001; Roche) and 3.5 µl BCIP (10760994001; Roche) (made with NTMT) in dark at RT until the desired intensity of colorimetric reaction was achieved. Sections were washed in TBST for 2 x 10 min, counterstained in Nuclear Fast Red (Vector Laboratories) for 5 min, and mounted in Crytalmount aqueous mounting medium (C0612; Sigma-Aldrich).

2.6.3 Wholemout X-Gal staining

Dissected tissues were rinsed with cold PBS and fixed in a solution containing 0.1 M phosphate buffer pH 7.3, 0.4% PFA and 5 µM EGTA pH 8.0 (E3889; Sigma-Aldrich), 2 mM MgCl₂ for 1.5 hrs at RT. Tissues were rinsed 3 x 15 min in a solution containing 50 mM phosphate buffer pH 7.5, 2 mM MgCl₂, 0.01% sodium deoxycholate (30970; Sigma-Aldrich) and 0.02% NP-40 (74385; Sigma-Aldrich). Finally, tissues were incubated in a solution containing 0.1 M phosphate buffer pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM Fe³⁺, 5 mM Fe²⁺, 2% X-Gal (16664; Sigma-Aldrich) and 20 mM Tris-Cl pH 7.5 at 37°C until the desired colour was achieved.

2.6.4 Wholemount propidium iodide staining

Tissue was dissected and fixed in 4% PFA overnight at 4°C. Tissue was rinsed 2 x 2 min in 1% PBST, and incubated with 10 µg/ml ribonuclease A (RNase A, P6513; Sigma-Aldrich) for 2 hrs at 37°C to remove any RNA. Tissue was rinsed 3 x 5 min with PBS, and incubated in 2.4 µg/ml propidium iodide (P4170; Sigma-Aldrich) at 4°C overnight. Tissue was rinsed 3 x 10 min with PBS, and mounted on a slide in 1:1 PBS/glycerol.

2.6.5 TUNEL staining

TUNEL staining was performed with the DeadEnd Colorimetric TUNEL System (G7130; Promega) according to the manufacturer's instructions. Briefly, sections were dewaxed as previously described (Section 2.5.3), except after the 50% ethanol step, sections were immersed in 0.85% (w/v) NaCl for 5 min, PBS for 5 min, 4% PFA for 15 min and washed 2 x 5 min in PBS. Sections were permeabilised with 20 µg/ml Proteinase K (P2308; Sigma-Aldrich) for 10 min at RT, washed once in PBS for 5 min, and re-fixed in 4% PFA for 5 min. Sections were washed 2 x 5 min in PBS, incubated with 100 µl Equilibration Buffer for 10 min at RT, followed by 100 µl of TdT reaction mix for 60 min at 37°C. Sections were washed with 2X SSC for 15 min, washed 3 x 5 min in PBS, blocked with 0.3% (v/v) hydrogen peroxide (BDH) for 5 min, washed 3 x 5 min in PBS, and incubated with 1:500 Streptavidin-HRP for 30 min at RT. Sections were washed 3 x 5 min in PBS, and developed with DAB (BioGenex) until the desired staining was achieved. Sections were then washed in PBS for 2 x 5 min, rinsed in tap water, and mounted in Crytalmount aqueous mounting medium (C0612; Sigma-Aldrich).

2.7 Morphometry

Morphometry on all immunohistochemically- and histochemically-stained sections, except endomucin staining, was performed by calculating the average percentage of cells that were positively stained for a specific marker from the total number of cells. Endomucin vessel density was scored using a Weibel-1 graticule (Figure 2.2) by calculating the average percentage of lines that intersected with stained vessels (ten random areas per sample). Parametric results are presented as group mean $\pm$ standard error of mean (SEM). The significance of differences between parameters was evaluated by a Student's t-test. All statistics were performed with GraphPad Prism version 5.0 (GraphPad Software Inc.).

2.8 Crypt micro-dissection

2.8.1 Tissue harvesting and fixation

Mice were injected intraperitoneally with vincristine sulphate 1 mg/kg (Mayne Pharma Plc.) 2 hrs before culling. Gut tissues were harvested as previously described (Section 2.5.11), except the blotting paper carrying the intestinal segments was immersed in Carnoy's fixative (made with 6:1:3 of ethanol, acetic acid, and chloroform) for 3 hrs, and stored at 70% ethanol until use.

2.8.2 Assessment of proliferation, crypt fission and crypt area

A small area on the tissue measuring 3 mm x 3 mm was excised with a scalpel, re-hydrated gradually from 70% ethanol to 50%, 25% ethanol and PBS. Tissue was hydrolysed in 1M HCl for 10 min at 60°C, and stained

with Schiff's reagent (VWR International) for 45 min at 4°C. Tissue was then placed on a glass slide in 50% acetic acid, and individual crypts were dissected out using fine needles at x40 magnification. For measuring arrested metaphases as a determination of crypt proliferation, 20 well-orientated crypts per sample were assessed. Using a x200 magnification and focussing up and down through the width of each crypt, a total mitotic count per crypt was obtained. Mitotic nuclei were observed as darkly stained bodies compared to non-mitotic nuclei. Utilising the same preparation, crypt fission was assessed by scanning 200 crypts randomly and counting the number of crypts that were in fission. Crypts were scored only once irrespective of the amount of branching displayed by a single crypt. Fission was recorded as being abnormal if it was asymmetrical or did not originate from the base of the crypts. Using the same preparation, crypt area was assessed by drawing outlines of 20 well-orientated crypts at x25 using a Leitz drawing tube attached to the microscope, cutting out the outlines, obtain an average paper weight of the crypt, and calculating the actual crypt area based on 1 g of paper weight equating to 0.0851 mm² (personal communication with Robert Goodlad, Cancer Research UK).

2.9 Western blot

2.9.1 Protein extraction and measurement

Tissue was dissected and rinsed with cold PBS. Selected segments of the tissue measuring approximately 0.5 cm³ were isolated and snap frozen in liquid nitrogen and stored at -80°C until use. Tissues were homogenised in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% glycerol (G5516; Sigma-Aldrich), 1% Triton X-100 (T9284; Sigma-Aldrich), 1.5 mM MgCl₂, 5 mM EGTA (E3889; Sigma-Aldrich) and 1X Complete, Mini, EDTA-free protease inhibitor cocktail (11836170001, Roche) for 30 s. Lysates were cooled on ice for 15 min before being spun at maximum speed for 15 min at 4°C. Protein concentration was measured

with a BCA Protein Assay kit (2322; Thermo Scientific) according to the manufacturer's instructions. Briefly, a 2 mg/ml BSA stock was used to prepare a series of protein standards ranging from 25 µg/ml to 2 mg/ml using lysis buffer as the diluent. Extracted lysates were diluted 1:100 with the same diluent. Each standard and sample was prepared in triplicates. Following incubation of the protein samples/BCA reagent mix at 37°C for 30 min, absorbance of each sample was measured at A562. A standard curve of average blank-corrected A562 for the standards against its concentration was plotted, and protein concentration in the unknown samples was determined from the standard curve.

2.9.2 SDS-PAGE electrophoresis and western transfer

SDS-PAGE electrophoresis was performed with 4-12% Bis-Tris Novex gels (NO0321BOX; Invitrogen) according to manufacturer's instructions. Briefly, 30 µl samples containing 25 mg of total protein, 1X NuPAGE LDS sample buffer (NP0008; Invitrogen), and with/without NuPAGE Reducing agent (NP0004; Invitrogen) were heated for 10 min at 70°C. Samples were then run on the gel with 1X MES Running buffer (NP0002; Invitrogen) with/without 2.5 µl/ml of NuPAGE antioxidant (NP0005; Invitrogen), alongside with 10 µl of Full-Range Rainbow Molecular Weight Markers (RPN800E; GE Healthcare), at 180V constant for 1 hr. Separated proteins were then transferred to a 0.2 µm pore size PVDF membrane (LC2002; Invitrogen) pre-wet with 100% methanol, in 1X Transfer buffer (NP0006; Invitrogen), for 1.5 hrs at 30V constant.

2.9.3 Western analysis

PVDF membrane (LC2005; Invitrogen) was initially rinsed with PBST, before being incubated in a 5% (w/v) dried milk blocking solution (Marvel) made with PBST for 1 hr at RT. Membrane was then incubated with a primary antibody (diluted in 5% blocking solution) for overnight at 4°C, followed by incubation in a HRP-conjugated secondary antibody for

1 hr at RT. Antibody binding was detected by incubating the membrane in an ECL Plus enhanced chemiluminescence detection system (RON2132; GE Healthcare) for 5 min, removing excess solution, and exposing a chemiluminescence film (RPN3103K; GE Healthcare). Membrane was washed for 3 x 15 min in PBST between each step.

2.10 Cloning

2.10.1 DNA extraction

To obtain a better quality of genomic DNA from mouse tissue, a different DNA extraction protocol was employed. Tissue measuring 0.5 cm³ was incubated in 400 µl lysis buffer containing 100 mM NaCl, 500 mM Tris-Cl pH 8.0, 100 mM EDTA pH 8.0, 1% SDS, and 1.4 mg/ml Proteinase K (P-6556; Sigma-Aldrich) at 55°C overnight with agitation, pelleted by centrifugation, and supernatant was mixed with 400 µl of phenol:chloroform:isoamyl alcohol 24:25:1 (77617; Sigma-Aldrich). The mix was vortexed, spun, and the top aqueous layer was aspirated carefully into a new tube. An equal volume of phenol:chloroform:isoamyl alcohol was added, and the purification procedure was repeated as previously. Isopropanol (I9516; Sigma-Aldrich) was added to the new sample, and the mix was spun to pellet DNA. The pellet was rinsed with 70% ethanol, air-dried, and re-suspended in 50-100 µl of TE buffer, containing 100 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0.

2.10.2 DNA purification

Two methods of DNA purification were employed. The first method purified DNA from a piece of agarose gel using the QIAEX II Gel Extraction kit (20021; Qiagen), and the second method purified DNA in a solution mix (QIAquick PCR Purification kit (28104; Qiagen)). In the first

gel-purification method, a specific DNA band from an agarose gel was excised with a sterile scalpel and weighed not more than 30 mg. Three volumes of Buffer QX1 and 10 µl of QIAEX II were added to the gel slice, mixed and incubated at 50°C for 10 min. Sample was centrifuged briefly, and DNA pellet was washed with Buffer QX1 and Buffer PE before being re-suspended in 30-50 µl of sterile water. In the second method, five volumes of Buffer PB was added to the DNA sample, mixed, and spun briefly to allow DNA to bind to the column. Column was washed with Buffer PE, and DNA was eluted with 30-50 µl of sterile water.

2.10.3 *Restriction digest*

A 50 µl reaction containing 1X restriction digest buffer (appropriate to the restriction enzyme used), 1 U of restriction enzyme per 1 µg of DNA, and 0.1 mg/ml BSA (optional) was incubated at 37°C for 2 hrs. Restriction digest buffers, enzymes and BSA were obtained from NEB. In some cases, double digest with two restriction enzymes simultaneously was performed only when there was a suitable buffer for both restriction enzymes to work efficiently. In some cases where restriction enzymes could be heat inactivated, the reaction was incubated at 65°C for 20 min to inactivate the restriction enzyme. Digested products were confirmed by agarose gel electrophoresis (Section 2.4.5).

2.10.4 *Ligation*

A 20 µl reaction containing 1X Ligation buffer, 30 ng of digested DNA insert, 10 ng of vector, and 1 U of T4 DNA ligase (C1263; Promega) was incubated at 16°C overnight. Ligation buffer and enzyme were obtained from Promega.

2.10.5 *Generation of Escherichia coli (E. coli) DH5 α* ***competent cells***

An aliquot of competent cells was spread onto a Luria-Bertani (LB) agar plate. The plate was incubated at 37°C overnight. The next day, a small culture of 5 ml LB broth was inoculated with a single colony and incubated at 37°C overnight at 200 rpm. 250 ml of LB broth was then inoculated with 2 ml of the mini-culture and incubated at 37°C, 200 rpm until the bacterial density reached OD₆₀₀ of 0.6. Cells were then chilled, pelleted, washed twice with ice-cold distilled water, washed once with 10% glycerol, and finally re-suspended in 10% glycerol. Competent cells were aliquoted and stored at -80°C until use.

2.10.6 *Bacterial transformation*

After thawing an aliquot of competent cells on ice, 1-4 μ l of DNA (containing 10 pg to 100 ng of DNA) was added to the cells and mixed gently. Cells were incubated on ice for 30 min, at 42°C for 30 s exactly, and on ice for 2 min. 250 μ l of pre-warmed S.O.C. medium was added to the cells, and the whole mix was incubated at 37°C, 200 rpm for 1 hr. 50-100 μ l of the mix was plated on an LB plate containing the appropriate antibiotic, and incubated at 37°C overnight.

2.10.7 *Plasmid extraction*

Plasmid extraction was carried out from either a mini-culture of $\leq$ 5 ml or a maxi-culture of 150 ml LB broth. For a mini-culture, 5 ml of LB broth containing an appropriate antibiotic was inoculated with a single colony, and incubated at 37°C, 200 rpm overnight. 1.5 ml of the culture was pelleted, and plasmid was extracted from these cells with a Plasmid Mini kit (12123; Qiagen) according to the manufacturer's protocol. Briefly, the

cell pellet was re-suspended in Buffer P1, Buffer P2, and lysed in Buffer N3. Cell debris was pelleted. The supernatant was passed through a plasmid binding column, washed with Buffer PE, and plasmid was eluted with 30-50 ml of sterile water. For a maxi-culture, 250 ml of LB broth containing an appropriate antibiotic was inoculated with 2 ml of the mini-culture, and incubated at 37°C, 200 rpm overnight. The entire volume of the culture was pelleted, and plasmid was extracted from these cells with a Plasmid Maxi kit (12162; Qiagen) according to the manufacturer's protocol. Briefly, the pellet was re-suspended in Buffer P1, Buffer P2, and lysed in Buffer N3. Cell debris was pelleted. The supernatant was passed through a large plasmid-binding column, washed with Buffer QC, and plasmid was eluted with Buffer QC. The plasmid was mixed with isopropanol, pelleted, washed with 70% ethanol, re-pelleted, air-dried and re-suspended in 500 µl of sterile water.

2.10.8 *DNA sequencing*

To verify the accuracy of DNA bases, 20 µl reaction containing 150 ng of DNA, 3.2 pmol sequencing primer, and 1X Terminator Ready Reaction Mix (4337449; Applied Biosystems) was placed in a thermal cycler with the following thermal profile: 95°C for 5 min, 30 cycles of 95°C for 30 s, 50 - 55°C (depending on template) for 10 s, 60°C for 4 min, and hold at 4°C. Reaction was then cleaned-up with a DyeEx 2.0 Spin kit (63204; Qiagen) according to the manufacturer's protocol. Briefly, the reaction volume was added into a bead column, spun, and dried in a vacuum centrifuge. The sample was then submitted to the Equipment Park, Cancer Research UK for DNA sequencing.

2.11 Cell culture

2.11.1 Maintenance of cell lines

Mouse gastric surface mucous GSM10 and mouse gastric parietal MGE507 cell lines were obtained from NIPPN, Japan. Both cell lines were cultured at 33°C, 5% CO₂ in DMEM/F12 (1:1) medium (Cell Services, Cancer Research UK) containing 10% (v/v) foetal bovine serum (FBS, 16000-044; Invitrogen), 1% (v/v) L-glutamine (G7513; Sigma-Aldrich), 10 µg/ml insulin (I2643; Sigma-Aldrich), 5.5 µg/ml transferrin (T7559; Sigma-Aldrich), 2 µg/ml ethanolamine (E0135; Sigma-Aldrich), 6.7 ng/ml selenite (70153; Sigma-Aldrich) and 10 ng/ml epidermal growth factor (EGF, E4127; Sigma-Aldrich). Embryonic Albino Swiss Mouse fibroblast 3T3 and mouse mammary epithelial cell line HC11 were obtained from Cell Services, Cancer Research UK. Both cell lines were cultured at 37°C, 5% CO₂ in DMEM/L-glutamine medium (11966-025; Invitrogen) containing 10% FBS, 10 ng/ml EGF and 10 µg/ml insulin. Immortalised human keratinocytes nTERT cell line (a kind gift from Dr. Richard Grose, Centre for Tumour Biology, Barts and The London) was cultured at 37°C, 5% CO₂ in DMEM/F12 (1:1) medium (Cell Services, Cancer Research UK) containing 10% FBS, 1% L-glutamine, 400 ng/ml hydrocortisone (H0888; Sigma-Aldrich), 5 µg/ml insulin, 8.4 ng/ml cholera toxin (C8052, Sigma-Aldrich), 10 ng/ml EGF and 5 µg/ml transferrin. Human colorectal cell line SW480 (Cell Services, Cancer Research UK) was cultured at 37°C, 5% CO₂ in DMEM/L-glutamine medium containing 10% FBS.

2.11.2 Passaging of cell lines

Cell lines with 80-90% confluence were split for continuous seeding. Cells were washed twice with versene (Cell Services, Cancer Research

UK), and incubated with 1 ml of 1X trypsin/EDTA (252000-056; Invitrogen) at 37°C until cells were fully dislodged. 5 ml of appropriate medium was added to the cell suspension, and the mix was spun at 2,000 rpm for 5 min. Supernatant was discarded, and cell pellet was re-suspended in the appropriate growth medium before being split into several plates.

2.11.3 *Storage and recovery of cultured cell lines*

Cell lines with 80-90% confluence were trypsinised as previously (Section 2.12.2), except after centrifugation, the cell pellet was re-suspended in a 'freezing' medium containing 8 ml of the appropriate growth medium, 1 ml of FBS, and 1 ml of dimethyl sulfoxide (DMSO, D2650; Sigma-Aldrich) with 1 ml of 'freezing' medium per 60 cm² plate. The cell suspension was aliquoted into freezing vials (Nunc, UK), placed in a Cryo Freezing Container (Nalgene, UK) filled with isopropanol to allow gradual freezing, and stored at -80°C overnight. Freezing vials were transferred to liquid nitrogen the next day for long-term storage. For cell recovery, frozen cells were rapidly thawed using a water bath at 37°C. 5 ml of the appropriate medium was added to the aliquot, and spun at 2,000 rpm for 5 min. Supernatant was discarded, and the cell pellet was re-suspended in the appropriate growth medium before being added onto a plate.

2.11.4 *Transient transfection*

Transient transfection with DNA plasmid, such as luciferase vector, was carried out when the cells 80-90% confluent. For transfection in a 60 cm² plate, a first mix containing 250 µl of Optimem and 30 µl of Lipofectamine 2000 (11668-027; Invitrogen), and a second mix containing 250 µl of Optimem, 10 µg of luciferase vector, and 1 µg of phRL-Renilla plasmid were prepared. Each mix was vortexed briefly and

left at RT for 5 min. Both solutions were mixed, vortexed briefly, and left at RT for another 20 min. During this time, the cells in a 60 cm² plate was rinsed twice with PBS, and 2 ml of the appropriate medium without 10% FBS was added to the plate. 500 µl of the transfection mix was added to the plate, and incubated at 37°C, 5% CO₂ overnight. As a control, a similar experiment was performed, except the transfection mix contained 1 µg of CMV-GFP plasmid instead of luciferase and Renilla constructs.

2.11.5 *Luciferase assay*

Luciferase assay was conducted with a Dual-Luciferase Reporter Assay System (E1910; Promega) according to the manufacturer's protocol. Briefly, cells on the 10 cm² plate was rinsed twice with PBS, and lysed with 500 µl of 1X Passive Lysis Buffer at RT for 15 min. Cell debris was pelleted, and 20 µl of supernatant was aliquoted in triplicate in a 96-well plate (PMX-096-145M; Fischer Scientific). 100 µl of Luciferase Assay Buffer was added to each sample, mixed gently and the luciferase activity was measured by a luminometer. Following this, 100 µl of Stop & Glo Buffer was added to each sample, mixed gently and the renilla activity was measured as previously. Each luciferase reading was normalised to its individual Renilla reading to obtain a more accurate measurement.

Table 2.1 Sequences and annealing temperatures of the primers used for animal genotyping.

Mouse line	Primer label	Sequence (5' to 3')	Annealing temp (°C)	Amplicon size
Fgfr2b ^{+/-}	SE-IIIa AS-loxP	GGCAGTAAATACGGGCCTG CGTAAACTCCTCTTCAGACC	55	Wt (no band) loxP (400bp)
Fgfr2b ^{flox/flox}	SE-IIIb AS-TM	CTGCCTGGCTCACTGTCC CTCAACAGGCATGCAAATGCAAGGTC	55	loxP (410 bp) Wt (320 bp)
Fgf10 ^{+/-}	SE-loxP AS-exon	CACCAAAGAACGGAGCCGGTT ACTCTTTGGCCTCTATCT	55	Wt (no band) loxP (1 kb)
A33-CrePR	SE-cre AS-cre	GGACATGTTTCAGGGATCGCCAGGCG GCA TAA CCA GTG AAA CAG CAT TGC TG	65	Wt (no band) Cre (269 bp)
ROSA26-lacZ- loxP	Rosa1 Rosa2 Rosa3	AAAGTCGCTCTGAGTTGTTAT GCGAAGAGTTTGTCTCAACC GGAGCGGGAGAAATGGATATG	55	Wt (500 bp) Mutant (250 bp)

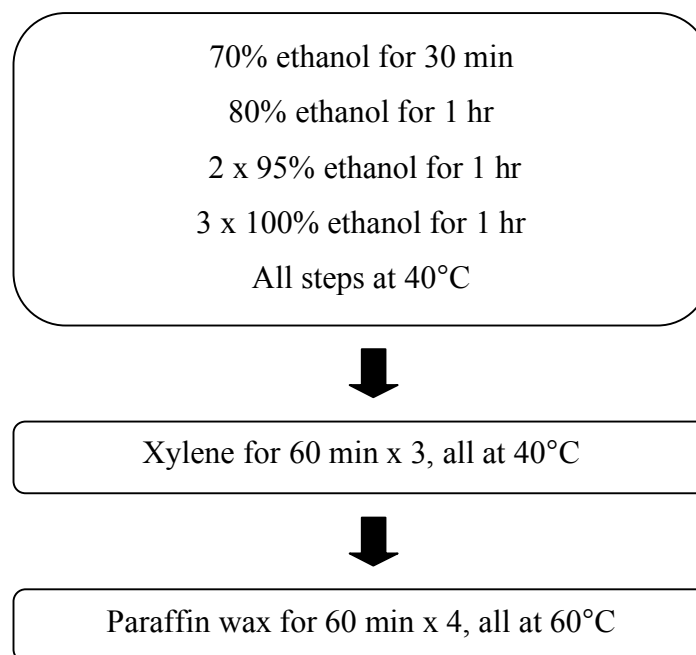


Figure 2.1 Schematic diagram showing the steps involved in tissue processing prior to embedding.

Table 2.2 Sequences and conditions of the primers used for reverse-transcriptase PCR.

Gene	Primer label	Sequence (5' to 3')	Annealing temp (°C)	MgCl ₂ conc (mM)	Kit type	Amplicon size (bp)	Positive control	Reference
Gapdh	SE-gapdh AS-gapdh	TGATGACATCAAGAAGGTGGTGAAG GAGGCCATGTAGGCCATGCGGT	62	1.5	Qiagen Taq	234	Adult liver	
Fgf1	SE-fgf1 AS-fgf1	ACCGAGAGGTTCAACCTGCC GCCATAGTGAGTCCGAGGACC	55	2.0	Qiagen Taq	387	Adult stomach	Ozawa <i>et al.</i> , 1997
Fgf2	SE-fgf2 AS-fgf2	GCCAGCGGCATCACCTCGCT TATGGCCTTCTGTCCAGGTCCCGT	65	2.0	Ready-To-Go	437	Adult brain	Ozawa <i>et al.</i> , 1997
Fgf3	SE-fgf3 AS-fgf3	GATGGGCCTGATCTGGCTTCTGCT CACCATCTCATGGTCCTTGTGGCC	60	2.0	Qiagen Taq	573	Adult brain	Ozawa <i>et al.</i> , 1997
Fgf4	SE-fgf4 AS-fgf4	TACTGCAACGTGGGCATCGGA GTGGGTACCTTCATGGTAGG	60	1.5	Ready-To-Go	344	E10.5 embryo	Herbert <i>et al.</i> , 1990
Fgf5	SE-fgf5 AS-fgf5	CATCTTCTGCAGCCACCTGATCCA AAGTTCCGGTTGCTCGGACTGCTT	65	2.5	Megamix	652	Adult brain	Ozawa <i>et al.</i> , 1997
Fgf6	SE-fgf6 AS-fgf6	CACGCTGCAGGCTCTCGTCTTCTT CAGTCATGATTGGGGACACCTTGC	60	1.5	Megamix	542	Adult eye	Ozawa <i>et al.</i> , 1997
Fgf7	SE-fgf7 AS-fgf7	ATCCTGCCAACTCTGCTCTACAGA CTTCCCTTTGACAGGAATCCCTT	55	1.5	Qiagen Taq	509	Adult brain	Post <i>et al.</i> , 1996
Fgf8	SE-fgf8 AS-fgf8	GTGGAGACCGATACTTTTGG GCCAAGTCCTCTGGCTGCC	55	1.5	Ready-To-Go	371	E14.5 embryo	Kawauchi <i>et al.</i> , 2005

Gene	Primer label	Sequence (5' to 3')	Annealing temp (°C)	MgCl ₂ conc (mM)	Kit type	Amplicon size (bp)	Positive control	Reference
Fgf9	SE-fgf9 AS-fgf9	ATGGCTCCCTTAGGTGAAGTTGG GCCTCCGCCTGAGAATCCCCTTTAAATG	55	1.5	Megamix	196	E14.5 embryo	Hajihosseini <i>et al.</i> , 2002
Fgf10	SE-fgf10 AS-fgf10	CTCTTTTTGGTGTCTTCGTTCCC CGCTGACCTTGCCGTTCTTCTC	55	1.5	Qiagen Taq	228	Adult stomach	Hajihosseini <i>et al.</i> , 2002
Fgf11	SE-fgf11 AS-fgf11	GTCGCTTTAAGGAGTGCGTC CACTGTGGAGAGAAGGCTCC	55	1.5	Ready-To-Go	219	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf12	SE-fgf12 AS-fgf12	TGTATCGCCAACAGGAATCAGG GGAGAGGGAAGAACGGAAGAG	55	1.5	Megamix	278	E14.5 embryo	Hajihosseini <i>et al.</i> , 2002
Fgf13	SE-fgf13 AS-fgf13	CTATCGCCAGCTCGCTCATCC CACCAGAGACGCTTCTGCTC	55	1.5	Qiagen Taq	626	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf14	SE-fgf14 AS-fgf14	GGTGGATATCTTCTCTAAAGTG GGATGGTTCTCGGTACATGG	55	1.5	Qiagen Taq	498	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf15	SE-fgf15 AS-fgf15	GGAACAAAGGACAGCCTAGA CAGAACTGAGAGCCAGGAGC	65	1.5	Megamix + Q-solution	307	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf16	SE-fgf16 AS-fgf16	GAACGAGCGCCTGGGCCAGATCG GGACATGGAGGGCAACTTAGAAGG	65	1.5	Megamix	497	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf17	SE-fgf17 AS-fgf17	CACCCGTCTCCTAATTTTAACC CGAACTGCTTCTGCCTTTCAGC	65	1.5	Ready-To-Go	505	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf18	SE-fgf18 AS-fgf18	CACCACAGTCACCAAGCGATCC CTTTGGTTTCTCGCAGTTTCCTC	55	1.5	Qiagen Taq	340	Adult brain	Hajihosseini <i>et al.</i> , 2002

Gene	Primer label	Sequence (5' to 3')	Annealing temp (°C)	MgCl ₂ conc (mM)	Kit type	Amplicon size (bp)	Positive control	Reference
Fgf20	SE-fgf20 AS-fgf20	ATGGCTCCCTTGACCGAAGTCGG GGCGGCGCAGGATGCCGTCAGG	65	1.5	Megamix	200	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgf21	SE-fgf21 AS-fgf21	CTGGGGGTCTACCAAGCATA CACCCAGGATTTGAATGACC	60	1.5	Ready-To-Go	349	Adult liver	Badman <i>et al.</i> , 2007
Fgf22	SE-fgf22 AS-fgf22	AATTGCTAGCATGCGCAGCCGCCTCTGGCTG AGGCCTCGAGAGACGAGACCAAGACTGGCA	60	1.5	Megamix	226	Adult oesophagus	Zhang <i>et al.</i> , 2006
Fgfr1b	SE-fgfr1b AS-fgfr1b	GCAGGGCTGCCTGCCAACGAGACAGTG GGTCTGGTGCAGTGAGCCACGCAGACTG	60	1.5	Ready-To-Go	318	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgfr1c	SE-fgfr1c AS-fgfr1c	GCAGGGCTGCCTGCCAACGAGACAGTG GAACGGTCAACCATGCAGAGTGATGG	55	1.5	Megamix	300	E14.5 embryo	Hajihosseini <i>et al.</i> , 2002
Fgfr2b	SE-fgfr2b AS-fgfr2b	CCCATCCTCCAAGCTGGACTGCCT CTGTTTGGGCAGGACAGTGAGCCA	55	1.5	Qiagen Taq	239	Adult stomach	Hajihosseini <i>et al.</i> , 2002
Fgfr2c	SE-fgfr2c AS-fgfr2c	CCCATCCTCCAAGCTGGACTGCCT CAGAACTGTCAACAATGCAGAGTG	60	1.5	Ready-To-Go	318	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgfr3b	SE-fgfr3b AS-fgfr3b	GACAGACACACGGATGTGCTGGA GTGAACACGCAGCAAAAGGCTTT	60	1.5	Ready-To-Go	363	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgfr3c	SE-fgfr3c AS-fgfr3c	GACAGACACACGGATGTGCTGGA GCACCACCAGCCACGCAGAG	60	1.5	Ready-To-Go	354	Adult brain	Hajihosseini <i>et al.</i> , 2002
Fgfr4c	SE-fgfr4c AS-fgfr4c	TACAGCTATCTCCTGGATGTGCTG GAAATCGTGCGTGACATTAAGGAG	60	1.5	Megamix	195	Adult brain	Hajihosseini <i>et al.</i> , 2002

Table 2.3 Sequences of the primers used for SYBR Green PCR.

Mouse line	Primer label	Sequence (5' to 3')	Amplicon size
Shh	SE-Shh	AGCCTACAAGCAGTTTATTCCCAA	241 bp
	AS-Shh	CAGCTTCACTCCAGGCCACT	
Tcf1	SE-Tcf1	GCCAGCCTCCACATGGCGTC	161 bp
	AS-Tcf1	GCTGCCTGAGGTCAGAGAATAA	
Tcf4	SE-Tcf4	GAGAGTGCAGCCATCAACCAG	242 bp
	AS-Tcf4	GTGATCGGAGGAAGCGAAAGG	
Cdx1	SE-Cdx1	CTAGGACAAGTAGCTTGCCCTCTT	65 bp
	AS-Cdx1	TCCAACAGGCTCACCACACA	
Lef1	SE-Lef1	AGACACCCTCCAGCTCCTGA	123 bp
	AS-Lef1	CCTGAATCCACCCGTGATG	
Axin2	SE-Ax2	GGTTCCGGCTATGTCTTTGC	66 bp
	AS-Ax2	CAGTGCGTCGCTGGATAACTC	
Beta-catenin	SE-Bcat	TGCTGGTGACAGGGAAGACA	66 bp
	AS-Bcat	TGCCGGCTGGTCAGATG	
Lgr5	SE-Lgr5	TCACACACCCATCTCTGGTAG	76 bp
	AS-Lgr5	CTACTTTGCCCAAGTTTCTGTTGAG	
Gapdh	SE-GAP	TGAAGCAGGCATCTGAGGG	102 bp
	AS-GAP	CGAAGGTGGAAGAGTGGGAG	

Table 2.4 List of antibodies and conditions used for immunohistochemistry.

Antigen	Antigen Retrieval	Primary antibody	Primary antibody dilution and incubation condition	Secondary antibody
BrdU	Citrate buffer for 15 min	Mouse monoclonal anti-BrdU (1585860; Roche, UK)	1:5 dilution for 1 hr at RT	Biotinylated horse anti-mouse used at 1:400
Active (cleaved) Caspase 3	Citrate buffer for 15 min	Rabbit polyclonal anti-Caspase 3 (AF835; R&D Systems, UK)	1:500 dilution for 1 hr at RT	Biotinylated goat anti-rabbit used at 1:250
Endomucin	Not required	Rat monoclonal anti-Endomucin (sc-65495; Santa Cruz Biotechnology)	1:600 dilution for 1hr at RT	Biotinylated rabbit anti-rat used at 1:250
β -catenin	Citrate buffer for 15 min	Mouse monoclonal anti- β -catenin (05-665; Upstate)	1:100 dilution for 1hr at RT	Biotinylated horse anti-mouse used at 1:400
Tff2	Citrate buffer for 10 min	Rabbit polyclonal anti-Tff2 (kind gift from Professor Blin, Tübingen, Germany)	1:500 for 1 hr at RT	Biotinylated goat anti-rabbit used at 1:250
Sox2	Citrate buffer for 20 min	Rabbit polyclonal anti-Sox2 (AB5603; Chemicon, UK)	1:1000 dilution at 4°C overnight	Biotinylated goat anti-rabbit used at 1:250
Cdx2	Citrate buffer for 20 min	Mouse monoclonal anti-Cdx2 (MU392-UC; BoGenex)	neat for 1hr at RT	Biotinylated horse anti-mouse used at 1:400

Antigen	Antigen Retrieval	Primary antibody	Primary antibody dilution and incubation condition	Secondary antibody
Atp4b	Citrate buffer for 10 min	Mouse monoclonal anti-Atp4b (A274; Sigma-Aldrich Co. Ltd.)	1:1000 for 1 hr at RT	Biotinylated horse anti-mouse used at 1:400
Tcf4	Citrate buffer for 15 min	Mouse monoclonal anti-Tcf4 (05-511; Chemicon)	1:500 at 4°C overnight	Biotinylated horse anti-mouse used at 1:400
Muc2	Citrate buffer for 15 min	Rabbit polyclonal anti-Muc2 (sc-15334; Santa Cruz)	1:400 for 1 hr at RT	Biotinylated goat anti-rabbit used at 1:250
Lysozyme	Citrate buffer for 10 min	Rabbit polyclonal anti-Lysozyme (A0099; DAKO)	1:500 for 1 hr at RT	Biotinylated goat anti-rabbit used at 1:250
Pep2	Citrate buffer for 10 min	Sheep polyclonal anti-Pep2 (PC132; The Binding Site)	1:1000 for 1 hr at RT	Biotinylated rabbit anti-sheep used at 1:250
Chromogranin A/B	Citrate buffer for 10 min	Rabbit polyclonal anti-Chromogranin A/B (ab15160; Abcam)	1:1250 for 1 hr at RT	Biotinylated goat anti-rabbit used at 1:250

Antigen	Antigen Retrieval	Primary antibody	Primary antibody dilution and incubation condition	Secondary antibody
Fgfr2b	Citrate buffer for 15 min Glyca buffer for 15 min EDTA buffer for 15 min Trypsin for 10 min	Rat monoclonal anti-Fgfr2b (MAB7161; R&D Systems)	1:100 to 1:1000 at 4°C overnight	Biotinylated goat anti-rat used at 1:250
Fgfr2b	Citrate buffer for 15 min EDTA buffer for 15 min Trypsin for 10 min	Rabbit polyclonal anti-Fgfr2b (from Ishiwata)	1:100 to 1:1000 at 4°C overnight	Biotinylated goat anti-rabbit used at 1:250

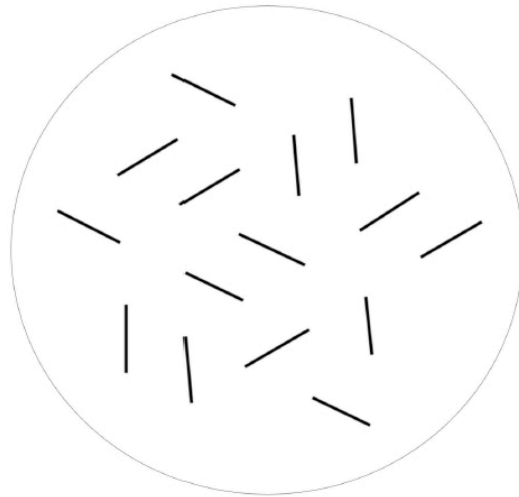


Figure 2.2 Weibel-1 graticule for quantifying endomucin-stained microvessel density.

Chapter 3: *Fgf10-Fgfr2b* signalling in intestinal development

3.1 Introduction

Several studies have demonstrated the requirement for *Fgfr2b* and *Fgf10* in GI development. Embryos null for either *Fgf10* or *Fgfr2b* exhibit similar multi-organ defects (Ohuchi et al., 2000), including GI defects. In the stomach, loss of either *Fgfr2b* or *Fgf10* leads to disproportionately smaller glandular stomach with disrupted mucosal architecture, absence of several early cytodifferentiation markers, including parietal cells, and abnormal expression of members of the Hedgehog family of signalling molecules (Spencer-Dene et al., 2006). In the developing intestine, absence of *Fgfr2b* or *Fgf10* leads to duodenal, caecal (Burns et al., 2004, Fairbanks et al., 2004b) and colonic atresia (Fairbanks et al., 2005, Fairbanks et al., 2006, Sala et al., 2006). These studies demonstrated that elimination of either *Fgfr2b* or *Fgf10* leads to decreased proliferation and increased apoptosis in intestinal epithelium and mesenchyme, resulting in atresia. Amongst the ligands for *Fgfr2b*, *Fgfl^{-/-}* (Miller et al., 2000), *Fgf3^{-/-}* (Mansour, 1994), *Fgf7^{-/-}* (Guo et al., 1996) and *Fgf22^{-/-}* (personal communication with Dr. Hisashi Umemori, Department of Biological Chemistry, University of Michigan) mice have no overt GI defects.

While duodenal atresia has been reported in both *Fgfr2b^{-/-}* and *Fgf10^{-/-}* embryos (Fairbanks et al., 2004a, Kanard et al., 2005), the mechanism and the role of these genes in duodenal development has not been fully established. Duodenal obstruction occurs in 1 of 10000 live births in humans with mortality rate of 34%, and usually requires immediate significant surgical correction (Choudhry et al., 2009). Unlike caecal and colonic morphogenesis that begins with a ‘bud’ that proliferates and subsequently elongates as an embryo develops, the small intestine originally develops from a continuous gut tube. We therefore investigated

whether duodenal atresia in these knockout mutants is caused by an imbalance in cell proliferation and apoptosis at a focal point in the proximal duodenum, and whether loss of *Fgfr2b* or *Fgf10* affects duodenal cytodifferentiation. This knowledge will help us to understand human congenital duodenal atresia and other pathological anomalies, such as gastric heterotopia and paraduodenal heterotopia in adults.

3.2 Materials and methods

3.2.1 Animal breeding

Fgfr2b^{+/-} and *Fgf10*^{+/-} lines have been previously described (De Moerlooze et al., 2000, Min et al., 1998). *Fgfr2b*^{+/-} and *Fgf10*^{+/-} lines were maintained as heterozygotes due to perinatal lethality of *Fgfr2b*^{-/-} and *Fgf10*^{-/-}. Both *Fgfr2b*^{+/-} and *Fgf10*^{+/-} lines were crossed with themselves to generate *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos at different embryonic stages. The morning of the vaginal plug appearance was considered as E0.5. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos were identified by absence of limbs. Wildtype (wt) and heterozygous embryos were identified by genotyping yolk sacs for the presence of a loxP site in the heterozygotes (Section 2.1.3).

3.2.2 Immunohistochemistry

Time-mated mice were injected with 5-bromo-2'-deoxyuridine (BrdU, 550891; BD Biosciences) solution at 50 mg/kg 1 hr before culling. Gut tissues were dissected in cold PBS, fixed, processed and embedded as previously described (Section 2.5.2). Immunohistochemistry was performed with anti-BrdU, anti-Caspase-3 (CSP-3), anti-endomucin, anti- β -catenin, anti-TFF2, anti-SOX2, anti-CDX2, anti-ATP4b, anti-PEP2, anti-TCF4 and anti-chromogranin A/B as previously described (Section 2.5). Morphometry was performed as previously described (Section 2.7).

3.2.3 Histochemistry

Diastase/alcian blue/periodic acid Schiff (D/AB/PAS) and intestinal alkaline phosphatase (IAP) staining was performed as previously described (Section 2.6.1 and 2.6.2). Wholemount propidium iodide

staining was performed as previously described (Section 2.6.4). TUNEL staining was performed as previously described (Section 2.6.5).

3.2.4 RT-PCR

RNA was extracted from small intestine of E18.5 Wt, *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos, and cDNA synthesis was performed as previously described (Section 2.4). Reverse transcriptase PCR was performed for *Fgfr2b* and *Fgf10* as previously described (Section 2.4.4). SYBR Green PCR was performed for *Shh*, *Tcf1*, *Tcf4*, *Cdx1*, *Lef1*, *Axin2*, *β-catenin*, *Lgr5* and *Gapdh* as previously described (Section 2.4.7).

3.2.5 TOPflash/FOPflash reporter assay

SW480 cells (1.5 x 10⁶ cells per 60 cm² area dish) were transfected with a combination of 9.5 µg of either TOP-luciferase or FOP-luciferase plasmid DNA (kind gift from Dr. Marc Rodriguez, Cancer Research UK), and 0.5 µg of pRL-Renilla as previously described (Section 2.11.4). SW480 cell line has been successfully used in this assay in other studies (Hecht et al., 2000, Korinek et al., 1997). The TOP-luciferase construct contains two sets (with the second set in the reverse orientation) of three copies of the wildtype TCF binding site (AGATCAAAGG) upstream of the herpesvirus thymidine kinase minimal promoter and firefly luciferase open reading frame. The FOPflash construct is the same as the TOPflash, except for a double mutation in the TCF binding site (AGGCCAAAGG; mutation underlines). After overnight incubation, cells were split into 24-well plates (50,000 cells per well). Following overnight incubation, the cells were serum-starved for eight hours before being given a combination of 2.5 µg/ml heparin (H2149; Sigma-Aldrich) and rhFGF10 (354-FG; R&D Systems) at 50, 100 and 200 ng/ml. Following 16 hrs of incubation, cells were harvested for luciferase assay as previously described (Section 2.11.5).

In a separate experiment, similar setup was adopted, except following serum-starvation, the cells were given a combination of 2.5 µg/ml heparin, 100 ng/ml rhFGF10, and one of the following drugs: a MAPK inhibitor U0126 (10 µM) (662005; Calbiochem), a PI3K inhibitor LY294002 (20 µM) (440202; Calbiochem) or a PKC inhibitor bisindolylmaleimide I (5 µM) (BIM-I, 203290; Calbiochem). These concentrations have been previously established to be optimal for SW480 cell lines (Graness et al., 1998, Wang et al., 2004, Steinkamp et al., 2003). These inhibitors have also been validated in the human gastric adenocarcinoma AGS cell line to be functionally active. Following 16 hrs of incubation, cells were harvested for luciferase assay as previously described (Section 2.11.5).

3.3 Results

3.3.1 *Fgfr2b* and *Fgf10* are expressed along the GI tract during development

Reverse-transcriptase PCR was used to determine the expression gradient of *Fgfr2b* and *Fgf10* along the developing GI tract in E18.5 Wt embryos. Both *Fgfr2b* and *Fgf10* are expressed with the highest intensity in the caecum and colon, followed by stomach and small intestine (Figure 3.1).

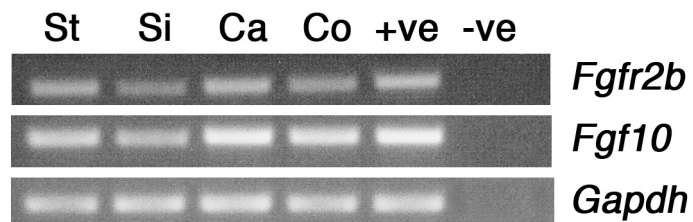


Figure 3.1 Reverse-transcriptase PCR showing *Fgfr2b* and *Fgf10* expression level along the E18.5 mouse GI tract.

This mRNA profile is a representation of the profile shown by three Wt E18.5 embryos. *Fgfr2b* and *Fgf10* are expressed higher in the caecum and colon than in the small intestine. St: stomach, Si: small intestine, Ca: caecum, Co: colon, +ve: positive control, -ve: negative control. Positive control came from adult Wt stomach. The negative control for *Fgfr2b* and *Fgf10* came from E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} small intestine respectively, whereas the negative control for *Gapdh* was a no-template control.

3.3.2 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos have disproportionately short small intestine

Small intestine of Wt, *Fgfr2b*^{-/-}, *Fgf10*^{-/-} embryos at two time-points, E16.5 and E18.5, was examined. While the overall size as measured by crown-rump lengths of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos is reduced by approximately 20% of the Wt littermates at E18.5, these mutants also display disproportional reduction in the small intestinal length by approximately 30% of the Wt littermates (Figure 3.2). The mutants also have poorly developed caecum and colon as previously reported (Burns et al., 2004, Fairbanks et al., 2004b, Fairbanks et al., 2005, Fairbanks et al., 2006, Sala et al., 2006). As in previous studies, Wt, *Fgfr2b*^{+/-} and *Fgf10*^{+/-} embryos are indistinguishable, implying that no significant gene dosage effect has occurred (data not shown). Only data from the Wt group is presented in all cases. The proliferative capacity of the intervillous region, where epithelial progenitors reside, in the intestinal epithelium of these embryos was assessed by BrdU labelling and Ki-67 immunostaining, to determine whether the overall embryonic size reduction is contributed by a reduction in intestinal proliferative capacity. The mutants have significantly reduced epithelial proliferation compared to Wt at both E16.5 and E18.5 (Figure 3.3). Analysis of apoptosis by active caspase-3 (CSP-3) immunostaining and TUNEL labelling show no significant difference in epithelial apoptosis in the mutants compared to Wt (data not shown).

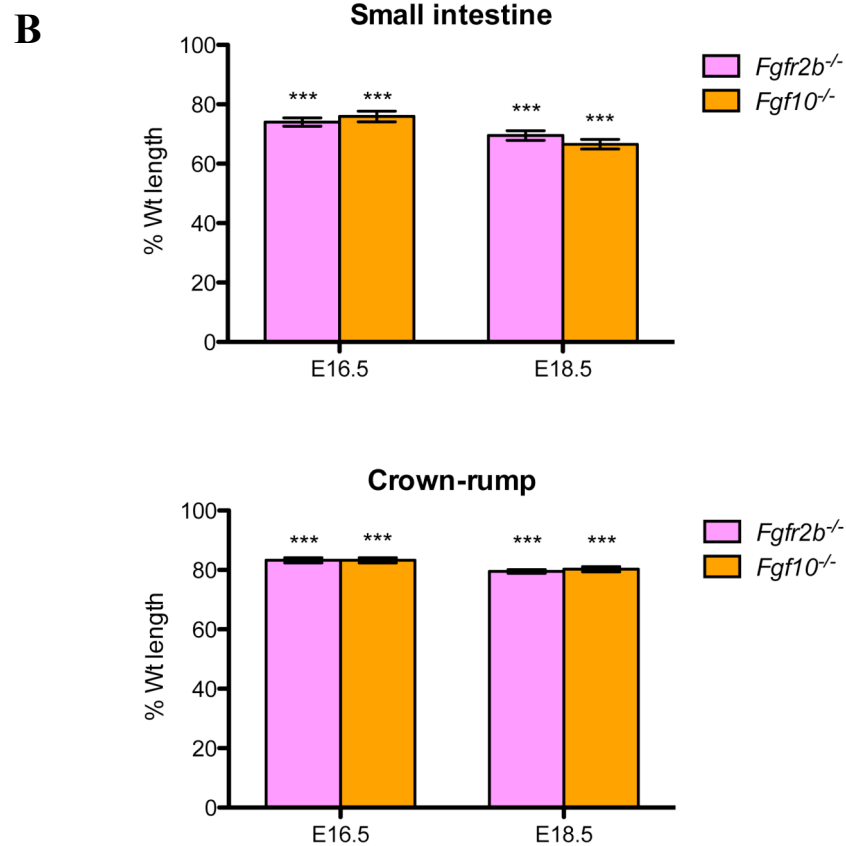
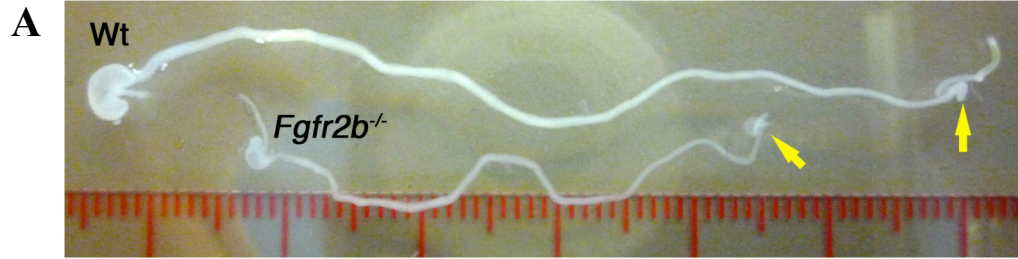


Figure 3.2 Comparison of small intestinal length of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos to Wt littermates.

(A) Wholemount photograph of dissected GI tract of E18.5 Wt and *Fgfr2b*^{-/-} embryos. The yellow arrows indicate the caecum. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} small intestine is shorter than the Wt, but only representation of the *Fgfr2b*^{-/-} is shown.

(B) Quantification of the mean small intestinal length and crown-rump height ($\pm$ SEM) of E16.5 and E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos normalised to Wt littermates (100%). Intestines were stretched out prior to measurement. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos have disproportionately short small intestine. Asterisks indicate that the difference in the means is

statistically significant (when compared with Wt littermates at the same time point) by a Student's t-test; *** $P < 0.001$; $n = 5$ embryos/group.

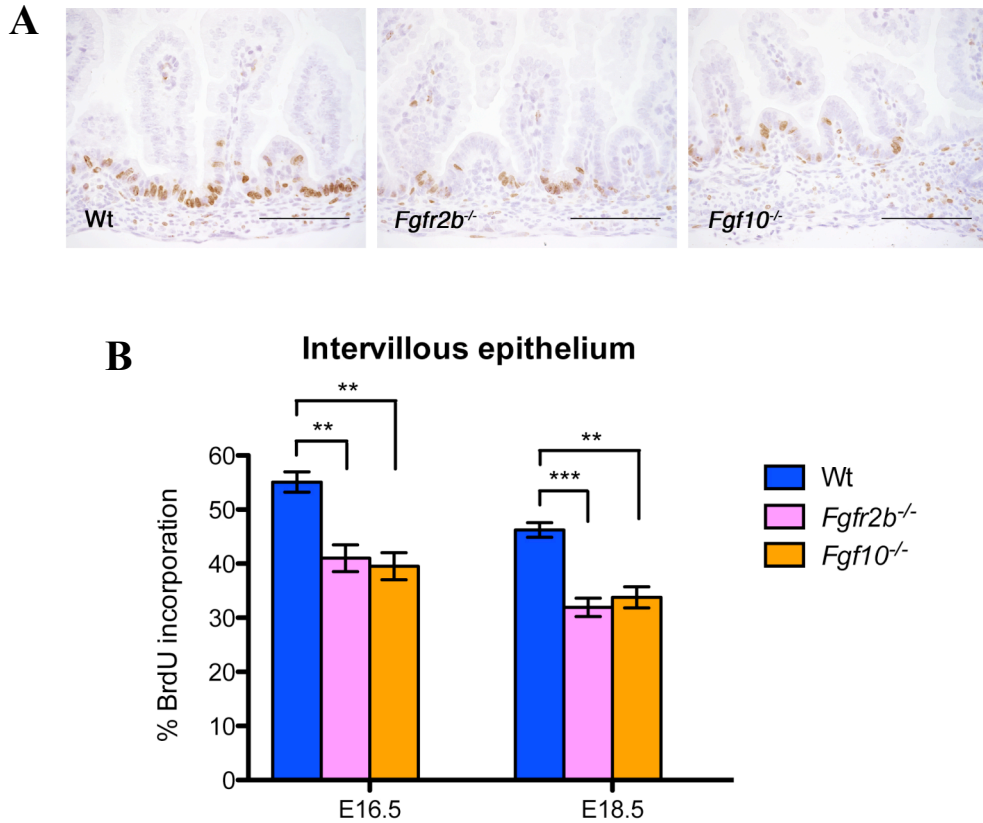


Figure 3.3 Comparison of epithelial proliferation in the proximal small intestine of E16.5 and E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos to Wt littermates.

(A) BrdU immunostaining of E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} proximal small intestine. Scale bars: 100 μ m.

(B) Quantification of the mean percentage BrdU incorporation ($\pm$ SEM) in the intervillous epithelium of E16.5 and E18.5 Wt, *Fgfr2b*^{-/-}, and *Fgf10*^{-/-} embryos. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} have significantly reduced intervillous epithelial proliferation. ** $P < 0.01$, *** $P < 0.001$ by Student's t-test; $n = 6$ embryos/group.

3.3.3 Duodenal atresia in *Fgf10*^{-/-} and *Fgfr2b*^{-/-} is a defect occurring during intestinal morphogenesis

Fgfr2b^{-/-} and *Fgf10*^{-/-} display duodenal atresia with variable penetrance: 16% for *Fgfr2b*^{-/-} and 33% for *Fgf10*^{-/-}. Most of the duodenal atresia is type III (complete separation) while type I (incomplete separation) examples are only occasionally seen. Dilation of the proximal duodenum, characteristic of obstruction, is evident in these cases. Most of the complex intestinal organogenesis occurs between stages E11.5 and E13.5 in the mouse (Ramalho-Santos et al., 2000). The E14.5 stage was examined to determine whether duodenal atresia is the result of an intestinal organogenesis defect or late term regression of a normally developed structure. Duodenal atresia is clearly observed at this stage in both *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos, with the duodenum ending in a blind pouch (Figure 3.4). Further examination of the duodenum at earlier time points of E11.5, E12.5 and E13.5 reveals that duodenal atresia can be observed as early as E12.5, with no obvious defect evident at E11.5 (Figure 3.4).

To determine the mechanism of duodenal atresia, gross morphology of E18.5 small intestine in the mutants was compared to their Wt littermates. The proximal small intestine, the area where duodenal atresia is observed, shows a similar rotation to that seen in Wt, indicating that these mutants display no overt gut malrotation that contributed to the atresia (Figure 3.5). Duodenal stenosis (abnormal narrowing of the intestinal lumen) and overt gut malrotation have been linked to loss of *Shh* (Ramalho-Santos et al., 2000), and our expression data confirm normal *Shh* expression in the small intestine of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos (Figure 3.5). To determine whether duodenal atresia is a consequent of vasculature defects, vascular density in the proximal small intestine of E18.5 mutants was compared to their Wt littermates. Vascular density is unaffected (Figure 3.6).

To establish whether duodenal atresia is caused by proliferative defects in duodenal epithelial and mesenchyme, BrdU labelling and active CSP-3 immunostaining on serial cross-sections at E11.5, the stage before duodenal atresia is initially observed, show no significant decrease in proliferation nor increase in apoptosis at any focal point of the duodenum (data not shown). However, the mutants show a significant general reduction of epithelial proliferation, whilst no corresponding increase in apoptosis along the duodenal length is observed (Figure 3.7). Mesenchymal proliferation and apoptosis are unaffected at this stage. The same parameters were examined at E12.5 on non-atretic and atretic duodena from *Fgfr2b*^{-/-} embryos. A wholemount staining approach using propidium iodide to visualise mitotic and apoptotic cells via confocal microscopy was utilised (Foley and Bard, 2002). Both non-atretic and atretic duodenal tissues (epithelium and mesenchyme) in *Fgfr2b*^{-/-} embryos show significantly reduced proliferation and increased apoptosis compared to Wt (Figure 3.8). However, in the mutants, the atretic ends show a significantly increase in the number of apoptotic cells than in the non-atretic duodenum, and an unexpectedly higher level of proliferation in the atretic ends than in the non-atretic duodenum (Figure 3.8).

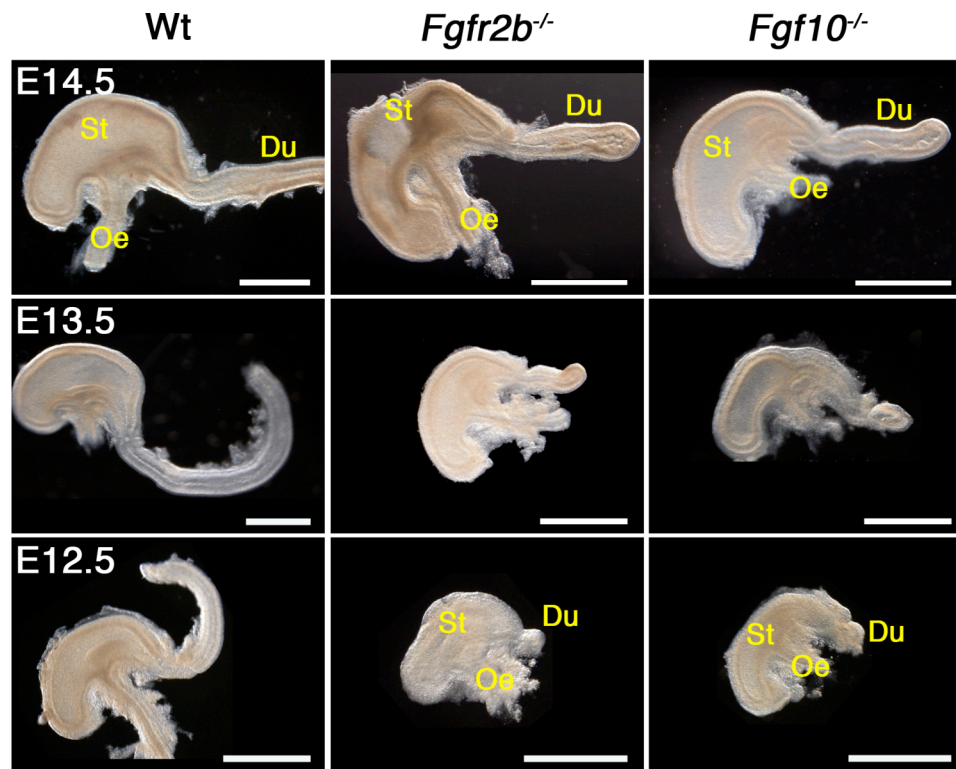


Figure 3.4 Duodenal phenotype of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos at E14.5, E13.5, and E12.5.

Entire GI tract of the embryos was dissected to show type III duodenal atresia, which is characterised by a blind-ending pouch. Duodenal atresia can be detected as early as E12.5, indicating a defect occurring during intestinal morphogenesis. Oe: oesophagus, St: stomach, Du: duodenum, Si: small intestine, Ca: caecum, Co: colon. Scale bars: 1 mm.

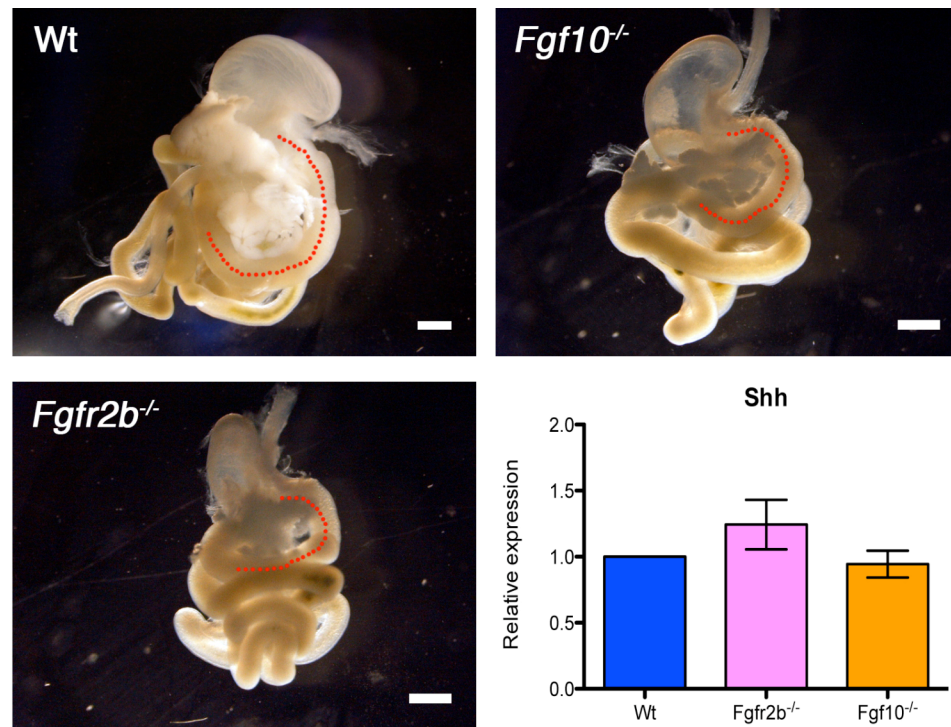


Figure 3.5 Ventral views of E18.5 GI tract of Wt, *Fgf10*^{-/-} and *Fgfr2b*^{-/-} embryos, and comparison of *Shh* expression level in the small intestine.

Red dotted lines indicate the direction of the proximal small intestine rotation from the stomach. The proximal small intestine of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} show similar rotation to the Wt, and has no aberrant expression of *Shh*. Duodenal stenosis and gut malrotation have been implicated in *Shh*^{-/-} mutants (Ramalho-Santos et al., 2000). Scale: 1 mm. *Shh* expression data was normalised to the mean of *Gapdh* expression, and error bars (±SEM) came from three experiments; n = 4 embryos/group.

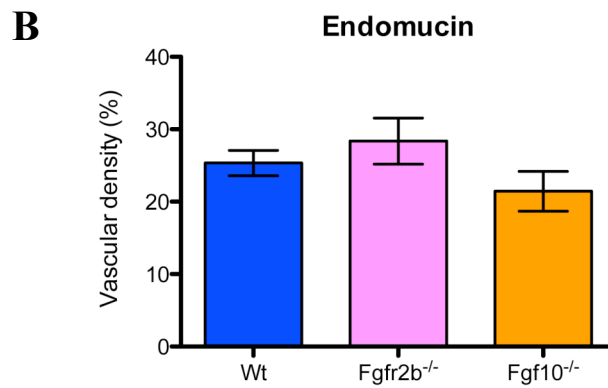
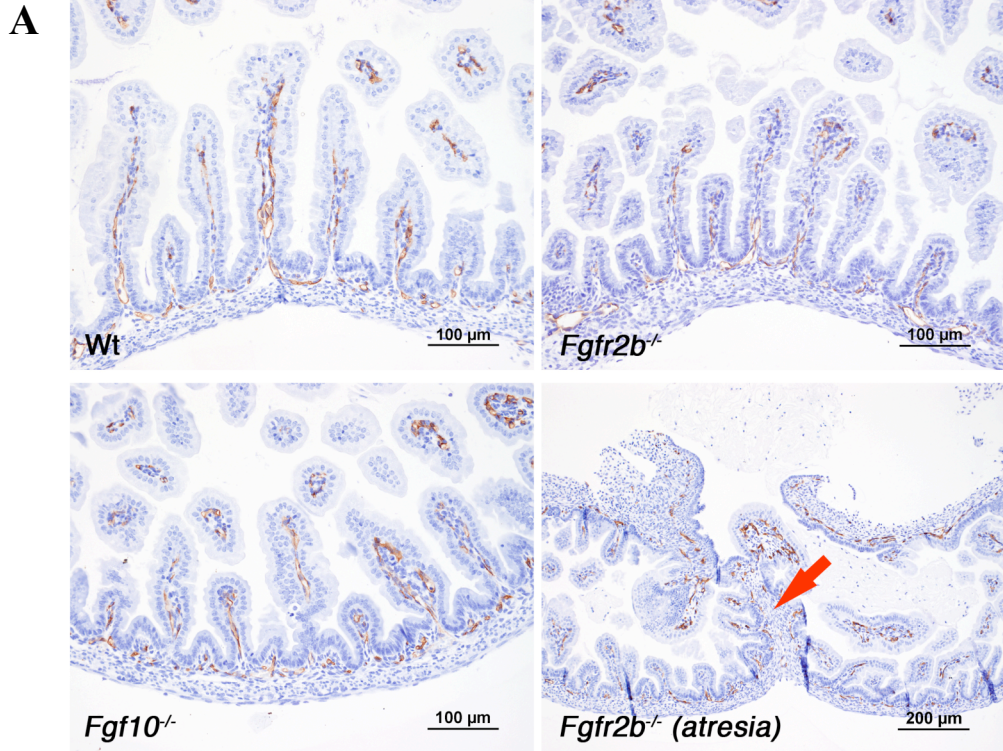


Figure 3.6 Comparison of vascular density in the proximal small intestine of $Fgfr2b^{-/-}$ and $Fgf10^{-/-}$ embryos to Wt littermates.

(A) Endomucin stained longitudinal sections of Wt, $Fgfr2b^{-/-}$, and $Fgf10^{-/-}$ E18.5 proximal small intestine. Red arrow shows the point of atresia.

(B) Quantification of the mean vascular density ($\pm$ SEM) in Wt, $Fgfr2b^{-/-}$, and $Fgf10^{-/-}$ E18.5 proximal small intestine using the Weibel-1 graticule. $Fgfr2b^{-/-}$ and $Fgf10^{-/-}$ show normal vascular density. n = 6 embryos/group.

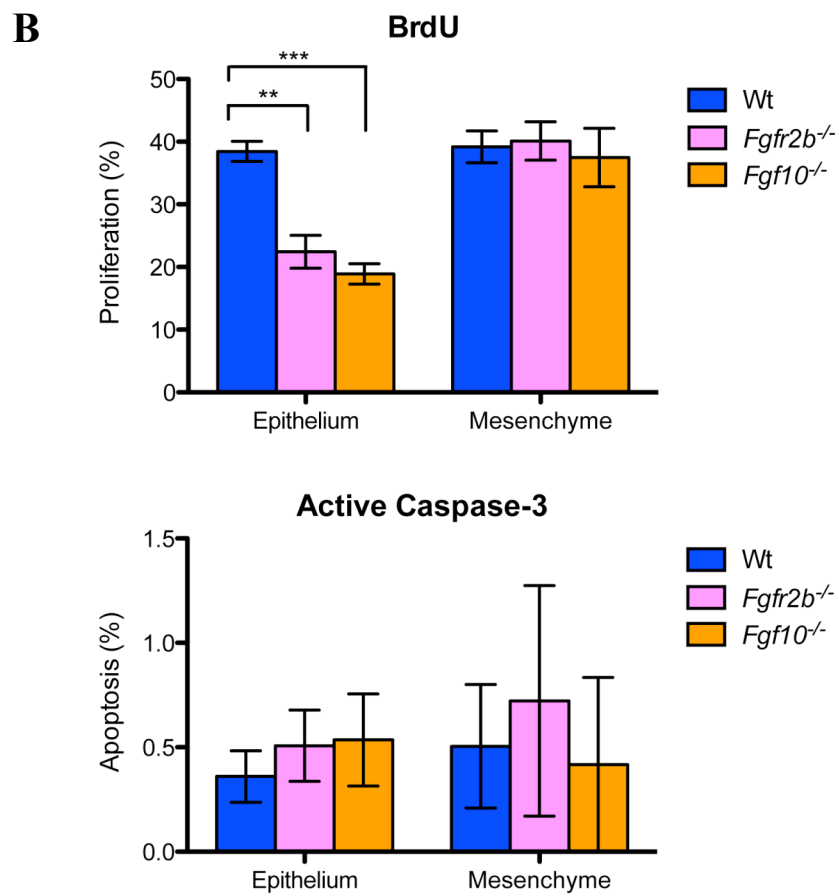
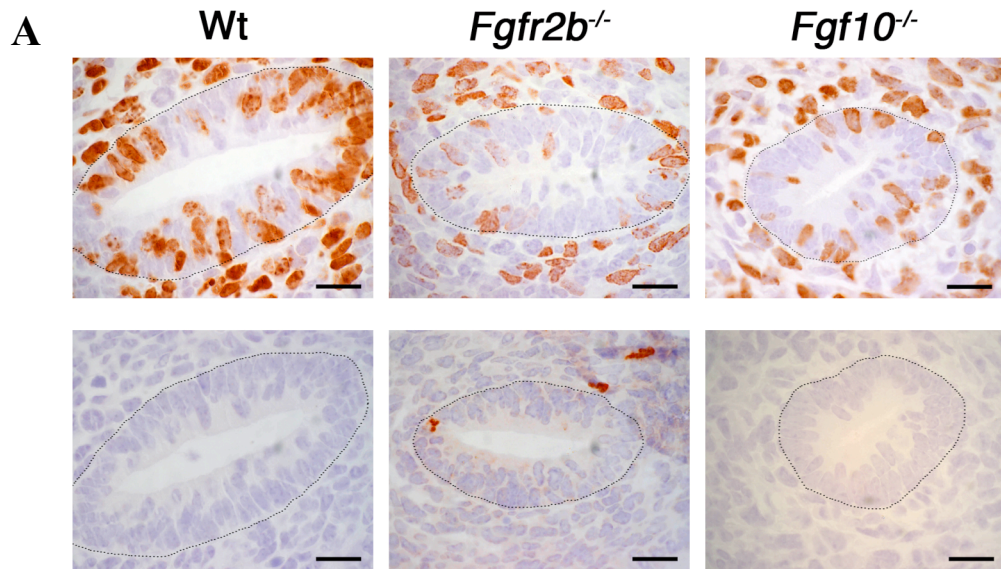


Figure 3.7 Comparison of proliferation and apoptosis in proximal small intestine of E11.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos to Wt littermates.

(A) BrdU and active CSP-3 immunostained sections of Wt, *Fgfr2b*^{-/-}, and *Fgf10*^{-/-} proximal small intestine. The black dotted lines indicate the boundary between the epithelium and mesenchyme. Scale bars: 50 μ m.

(B) Quantification of the mean percentage BrdU incorporation ($\pm$ SEM) and mean active CSP-3 staining ($\pm$ SEM) in the epithelium and mesenchyme of Wt, *Fgfr2b*^{-/-}, and *Fgf10*^{-/-} proximal small intestine. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} have reduced epithelial proliferation in the proximal small intestine. ** P<0.01, *** P<0.001 by Student's t-test; n = 10 embryos/group.

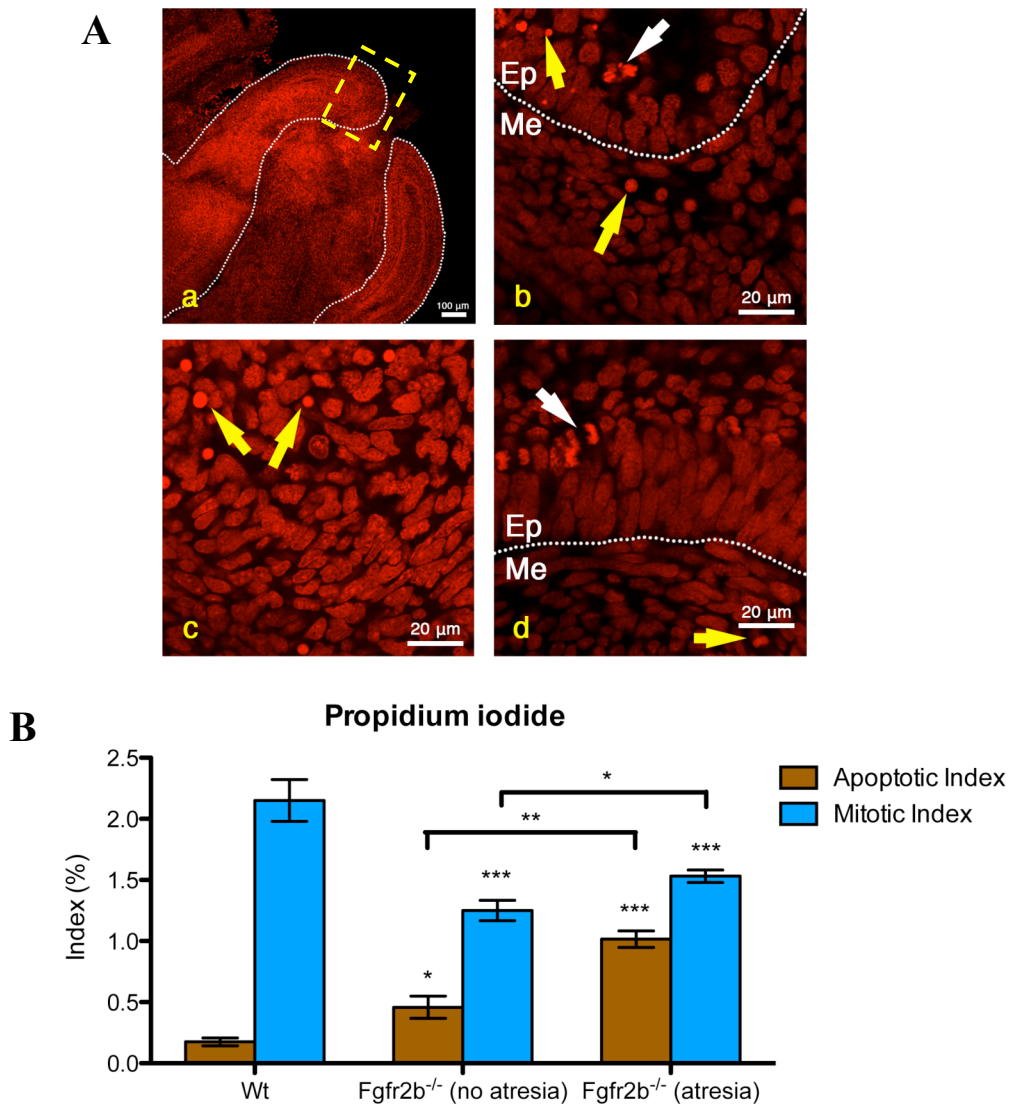


Figure 3.8 Comparison of proliferation and apoptosis in proximal small intestine of E12.5 *Fgfr2b*^{-/-} embryos to Wt littermates.

(A) Wholmount propidium iodide staining of (a) duodenal atresia, (b) higher power view showing the epithelium and mesenchyme of an atretic end (marked by a yellow dotted box in (a)), (c) higher power view of mesenchyme of the atretic end (marked in (a)), and (d) the epithelium and mesenchyme of a corresponding Wt littermate. Yellow arrows show apoptotic nuclei that are smaller and more brightly stained than normal nuclei, and sometimes fragmented. White arrows show mitotic nuclei ranging from pro-metaphase to late anaphase.

(B) Quantification of the mean percentage of mitotic and apoptotic cells ($\pm$ SEM) in the Wt duodenum, *Fgfr2b*^{-/-} duodenum with no atresia, and the atretic duodenum of *Fgfr2b*^{-/-} mutants. Duodenal atresia is contributed by an increase in tissue apoptosis at a focal point on the duodenum. * P<0.05, ** P<0.01, *** P<0.001 by Student's t-test; n = 4 samples/group.

3.3.4 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos develop gastric heterotopia in the GI tract

To determine whether *Fgfr2b* and *Fgf10* play a role in small intestinal differentiation during development, small intestine was examined by H&E staining and immunostained with markers for intestinal and gastric lineages. While enteroendocrine cells stained by chromogranin A/B appear normal in number and distribution in the mutants, the duodena of both *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos show an approximately 30% reduction in the mean number of goblet cells in the proximal small intestine (Figure 3.9). There are heterotopic areas of gastric epithelial cells in the rostral duodenum (Figure 3.10). These gastric cell patches express normal gastric surface mucin shown using combined D/AB/PAS staining. The remaining segments of the small intestine (middle and distal) have normal goblet cell numbers. Further immunohistochemical analysis on the rostral duodenum reveals absence of trefoil factor-2 (TFF2)-expressing Brunner's glands, and partial loss of both intestinal-specific alkaline phosphatase (IAP) and the transcription factor CDX2 (Figure 3.10). Regions devoid of CDX2

abundantly express SOX2, a gastric-specific transcription factor, and H^+,K^+ -ATPase β -subunit (ATP4b), a marker for hydrochloric acid-producing parietal cells (Figure 3.10). This observation is also evident at E14.5, the stage prior to intestinal differentiation, with SOX2 expression extending into the rostral duodenum and interspersing within CDX2-expressing cells (Figure 3.11). The remaining segments of the small intestine (middle and distal) in the E18.5 mutant foetuses also show normal CDX2 and IAP staining with no SOX2 and ATP4b expression (data not shown). Pepsinogen-secreting chief cells cannot be adequately assessed at these stages as pepsinogen is poorly expressed prior to birth (data not shown).

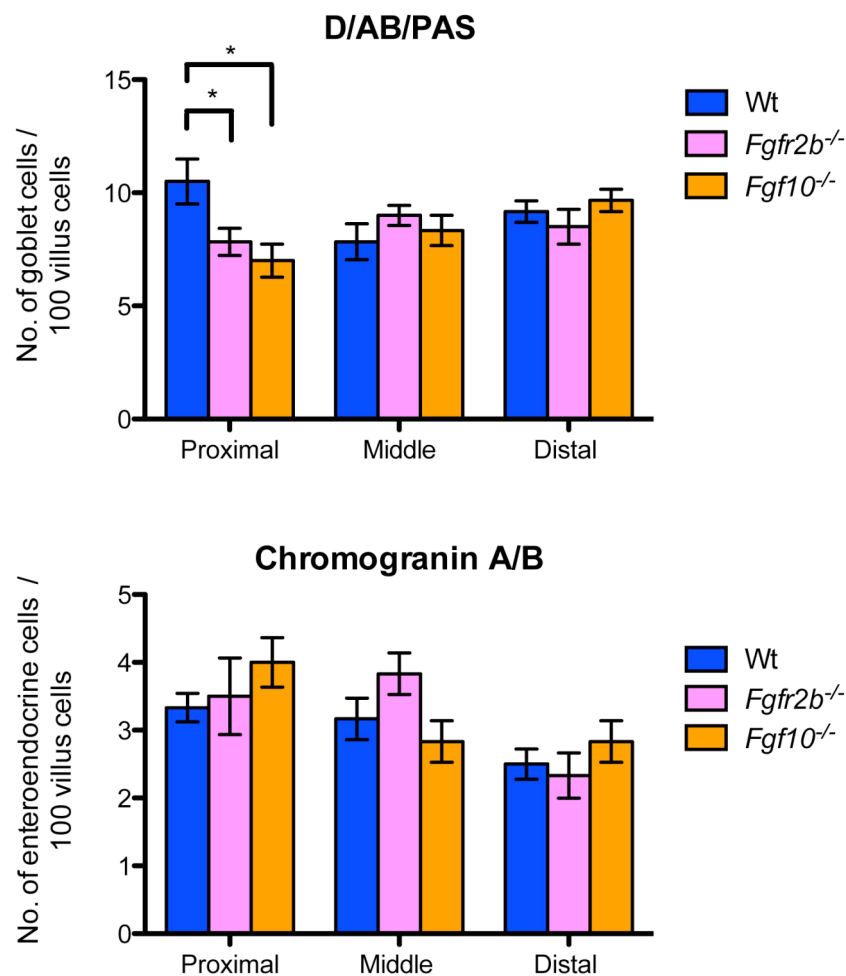
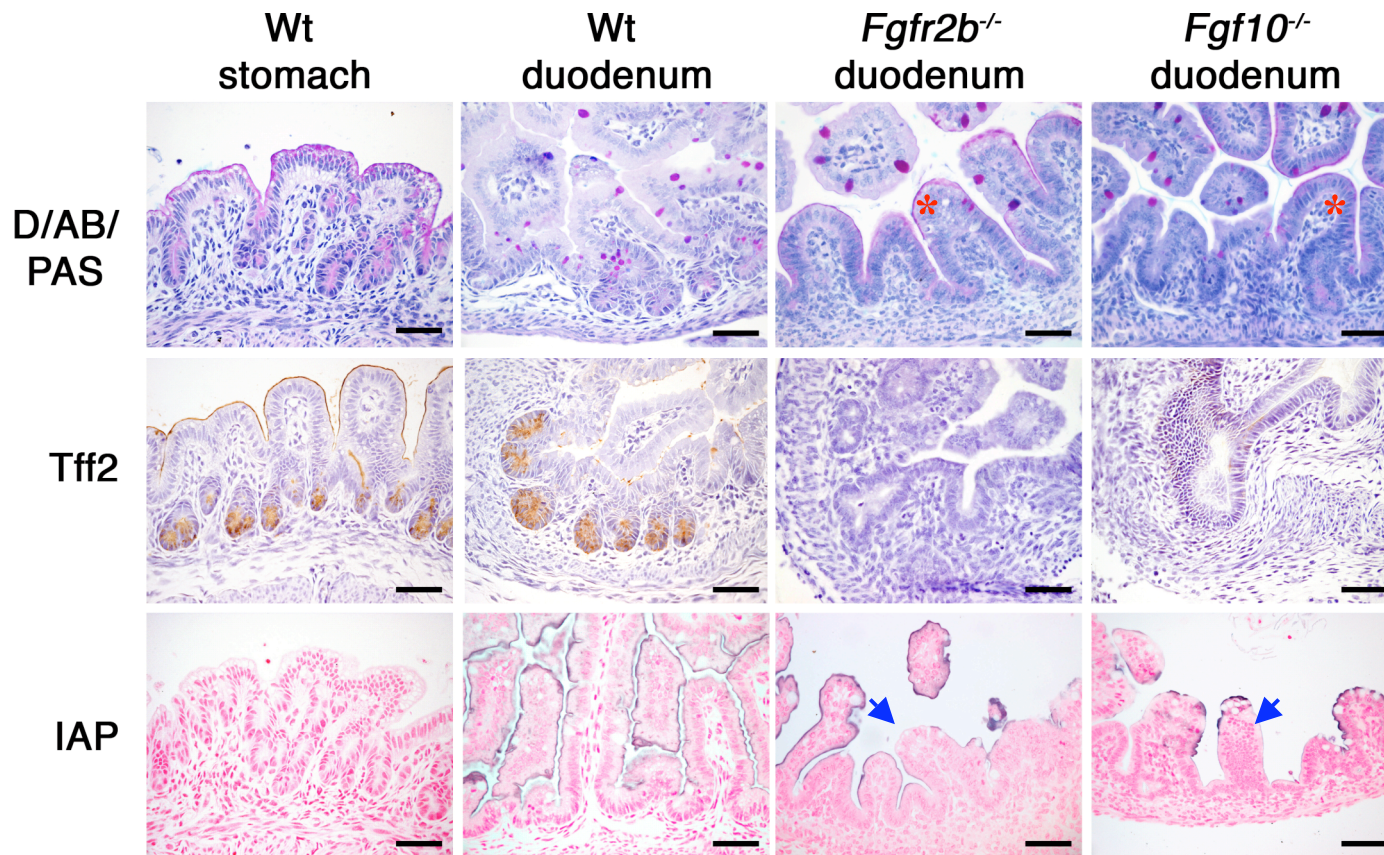


Figure 3.9 Comparison of cytodifferentiation in the small intestine of E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos to Wt littermates.

Quantification of the mean number of goblet (immunostained by D/AB/PAS) and enteroendocrine cells (immunostained by chromogranin A/B) ($\pm$ SEM) in the small intestine of Wt, *Fgfr2b*^{-/-}, and *Fgf10*^{-/-}. *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos have reduced number of goblet cells in the proximal small intestine. * $P < 0.05$ by Student's t-test; n = 6 embryos/group.

Figure 3.10 continue on next page



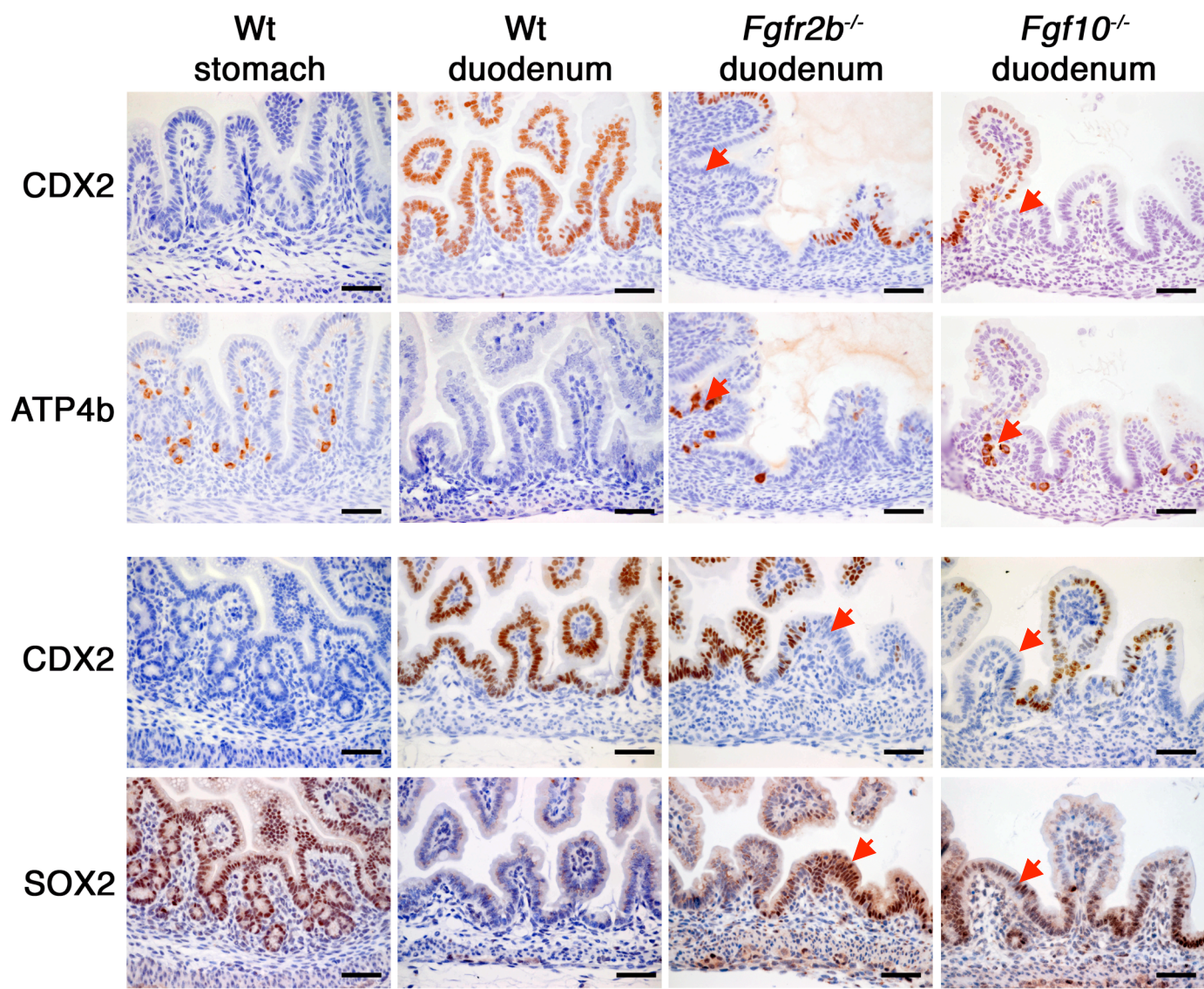


Figure 3.10 Gastric heterotopia of the rostral duodenum in E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos.

Immunostaining of markers for stomach and intestinal cell lineages, including TFF2, CDX2, ATP4b and SOX2, and histochemical staining using D/AB/PAS and IAP. D/AB/PAS staining shows gastric regions in the duodenum of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} (red asterisks). The mutants also have loss of Brunner's glands, and partial loss of IAP expression (blue arrows). Immunostaining images for the pairs CDX2/ATP4b and CDX2/SOX2 were taken from consecutive sections to show that regions devoid of CDX2 express ATP4b and SOX2 (red arrows). Scale bars: 50 μ m.

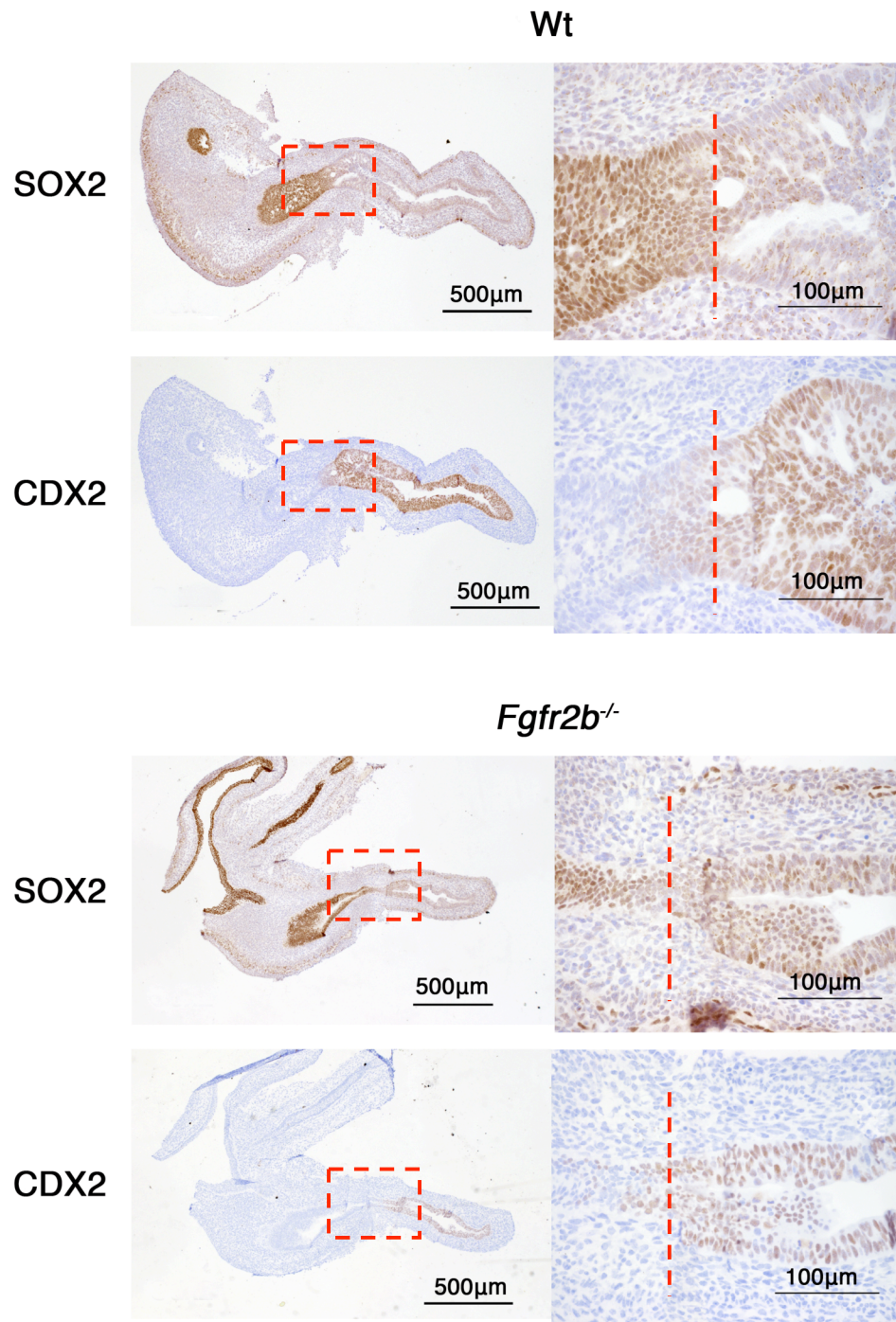


Figure 3.11 Gastric heterotopia of the rostral duodenum in E14.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos.

Immunostaining of SOX2 and CDX2 on consecutive sections of E14.5 Wt and *Fgfr2b*^{-/-} proximal small intestine. Right panels show the magnified version of the region marked by a red dotted box in the left. Red dotted lines show the gastric-intestinal boundary. *Fgfr2b*^{-/-} embryos show extended expression domain of SOX2 into the duodenum, which does not

occur in Wt. *Fgf10*^{-/-} embryos display similar phenotype, therefore only the images for *Fgfr2b*^{-/-} are shown.

3.3.5 *Fgf10*^{-/-} and *Fgfr2b*^{-/-} small intestine has significant downregulation of Wnt signalling

To determine whether *Fgf10-Fgfr2b* signalling may interact with the Wnt pathway, expression levels of major Wnt targets in the small intestine of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos were examined. RT-PCR results show significant downregulation of *Tcf1*, *Tcf4*, *Cdx1*, and *Lgr5* in these mutants compared to Wt (Figure 3.12). On the other hand, *Lef1*, *Axin2* and β -catenin expression levels are unchanged. TCF4 immunostaining confirms the downregulation of this protein throughout the small intestine of the mutants, while β -catenin levels and distribution are normal (Figure 3.13).

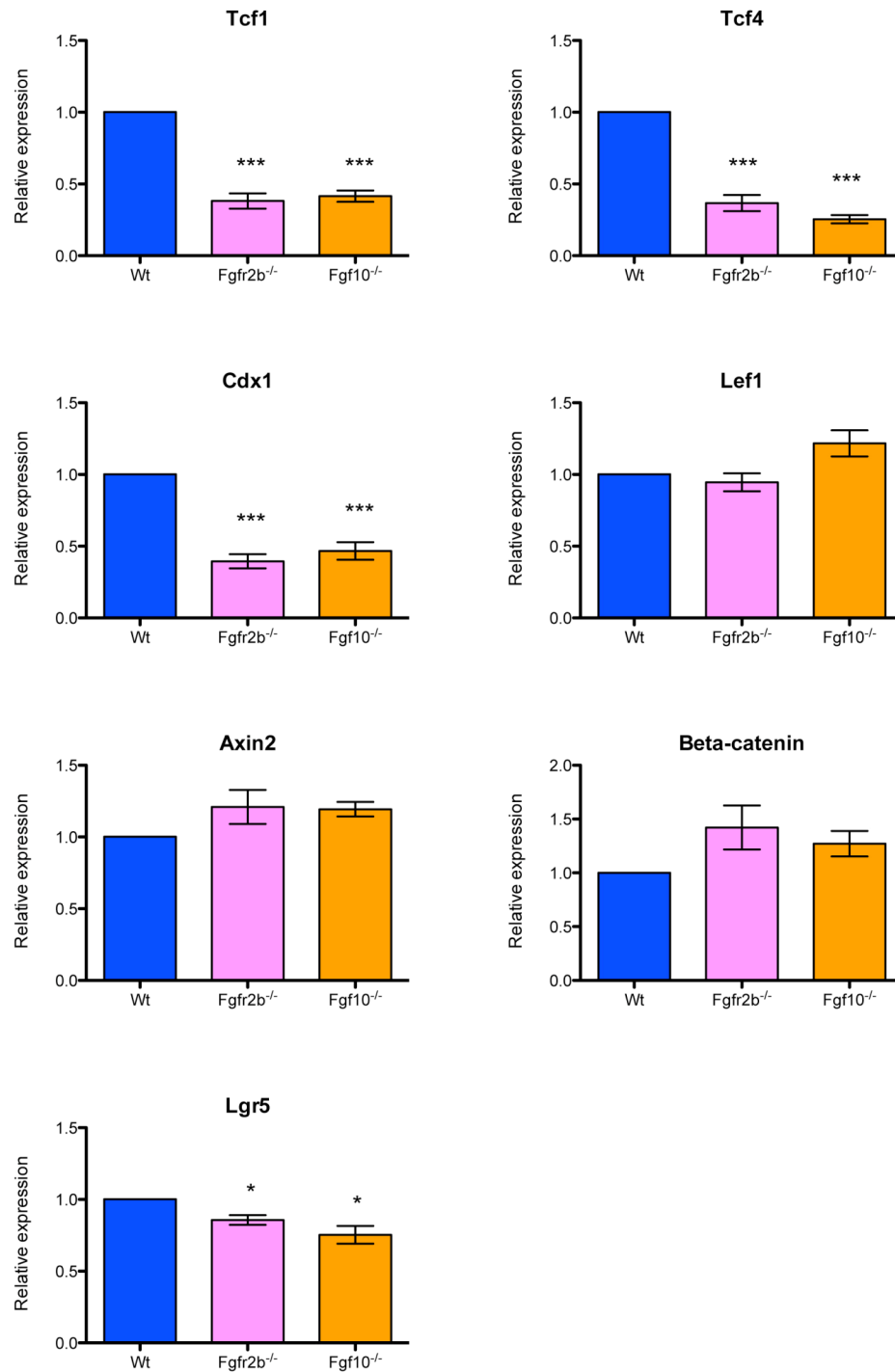


Figure 3.12 Comparison of gene expression in small intestine of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos to Wt littermates.

Fgfr2b^{-/-} and *Fgf10*^{-/-} small intestine has reduced expression of *Tcf1*, *Tcf4*, *Cdx1* and *Lgr5*. RNA was extracted from whole E18.5 small intestine of Wt, *Fgfr2b*^{-/-} and *Fgf10*^{-/-}, and subjected to real-time PCR. All data was normalised to the mean of *Gapdh* expression, and error bars ($\pm$ SEM) came

from three experiments; n = 4 embryos/group. * P<0.05, *** P<0.001 by Student's t-test.

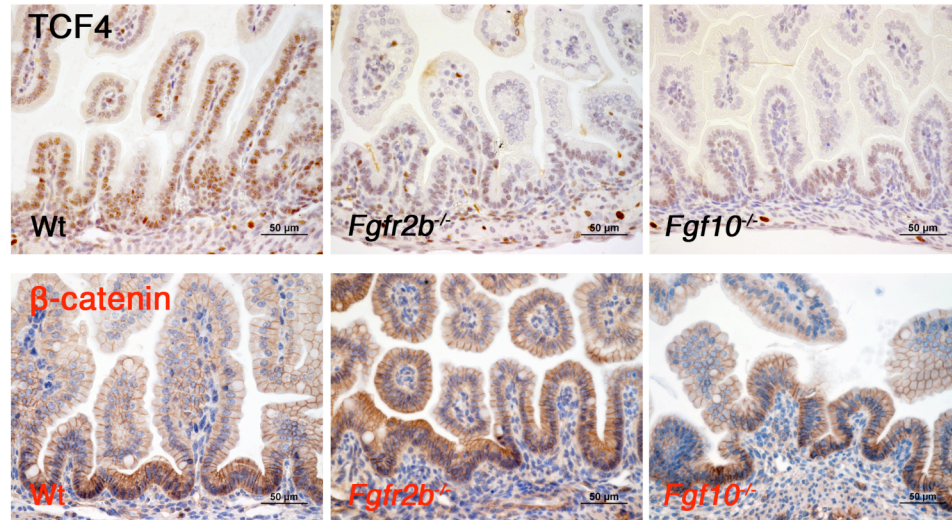


Figure 3.13 Immunohistochemical staining of TCF4 and β -catenin in E18.5 Wt, $Fgfr2b^{-/-}$ and $Fgf10^{-/-}$.

The mutants have reduced TCF4 in the small intestine, with no overt difference in β -catenin localisation and distribution.

3.3.6 *Fgfr2b-Fgf10* signalling pathway regulates *Tcf4* via the MAPK pathway

To determine the regulatory mechanism of *Tcf4* expression by *Fgf10-Fgfr2b* signalling, TOPflash/FOPflash reporter assay was initially used to determine the optimum concentration of rhFGF10 for stimulating Wnt activity in SW480 cells. Luciferase data show that Wnt activity increases with increasing concentration of rhFGF10, with 100 ng/ml being the optimal concentration since addition of 200 ng/ml does not significantly increase Wnt activity (Figure 3.14). RT-PCR for *Tcf4* expression indicates a significant increase in *Tcf4* mRNA level by approximately 3-fold at 50 ng/ml rhFGF10, but not at 100 ng/ml and 200 ng/ml (Figure 3.14).

Following this, the TOPflash/FOPflash reporter assay was performed in combination with 100 ng/ml rhFGF10 and an inhibitor for MAPK (U0126), PI3K (LY294002) or PLC- γ (BIM-1). Luciferase data shows that U0126 and LY294002 markedly downregulates Wnt activity, with highest level of downregulation observed with U0126 (Figure 3.15). RT-PCR for *Tcf4* expression further confirms the downregulation of *Tcf4* mRNA by approximately 75% by U0126, but not by LY294002 and BIM-I (Figure 3.15).

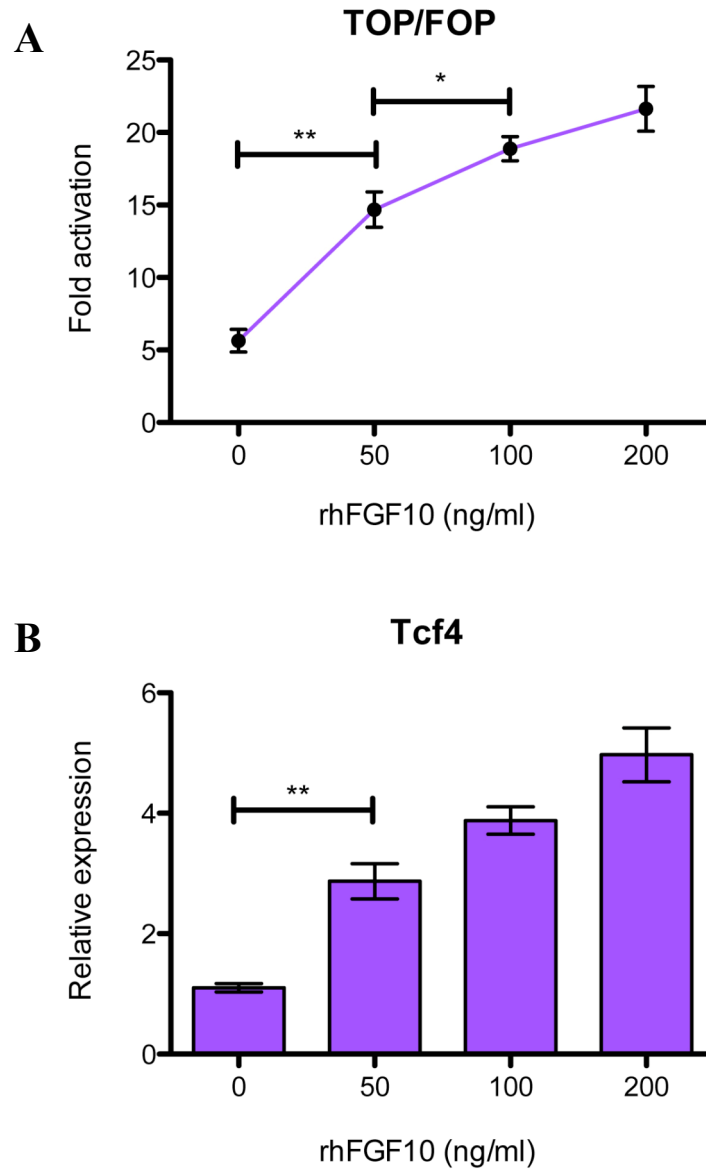


Figure 3.14 Effect of rhFGF10 on Wnt activity and *Tcf4* mRNA expression in SW480 cell line.

(A) The graph shows the rhFGF10-dose response luciferase assay of SW480 cells transfected with the TOP-luciferase or the FOP-luciferase construct. Luciferase activity was measured and normalised to Renilla luciferase activity. Fold activation was calculated as normalised luciferase reading from TOP-/FOP-luciferase constructs. 50 ng/ml and 100 ng/ml rhFGF10 significantly stimulates Wnt activity in SW480 cells. The values ($\pm$ SEM) indicated were the average of three independent experiments. $n = 3$ samples/group. * $P < 0.05$, ** $P < 0.01$ by Student's t-test.

(B) The graph shows a trend of increasing *Tcf4* mRNA level with increasing concentration of rhFGF10. However, only the 50 ng/ml rhFGF10 shows statistically significant increase in *Tcf4* expression. All data was normalised to the mean of *Gapdh* expression, and error bars ($\pm$ SEM) came from three experiments; n = 3 samples/group. ** P<0.01 by Student's t-test.

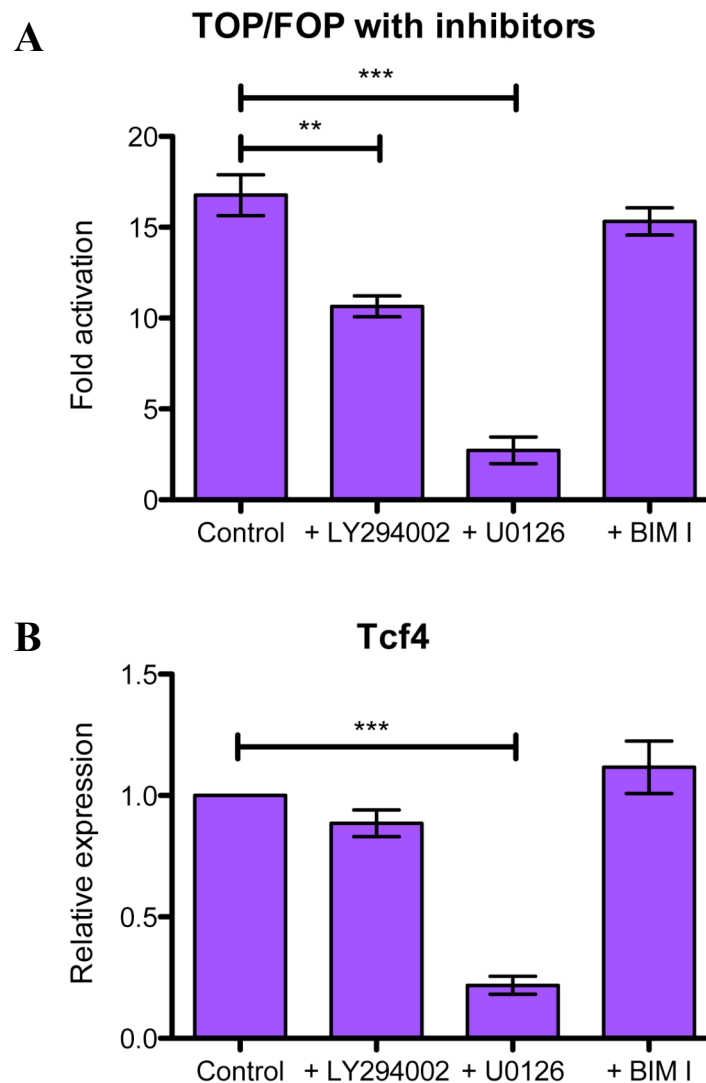


Figure 3.15 Effect of inhibitors on Wnt activity and *Tcf4* mRNA expression in SW480 cell line.

(A) TOPflash/FOPflash reporter assay of SW480 cells treated with a combination of 100 ng/ml rhFGF10 and one of the following inhibitors: U0126 (10 μ M), LY294002 (20 μ M) or BIM-1 (5 μ M). Addition of

U0126 and LY294002 significantly downregulates Wnt activity. Luciferase activity was measured and normalised to Renilla luciferase activity. Fold activation was calculated as normalised luciferase reading from TOP-/FOP-luciferase constructs. The values ($\pm$ SEM) indicated were the average of three independent experiments. n = 3 samples/group * $P < 0.05$, ** $P < 0.01$ by Student's t-test.

(B) The graph shows a significant downregulation of *Tcf4* mRNA level in cell treated with U0126, but not with LY294002 and BIM-I, as evidenced by RT-PCR. All data was normalised to the mean of *Gapdh* expression, and error bars ($\pm$ SEM) came from three experiments; n = 3 samples/group. *** $P < 0.001$ by Student's t-test.

3.4 DISCUSSION

Loss of *Fgfr2b* or *Fgf10* alone is sufficient to cause duodenal atresia with variable penetrance and severity. This variable penetrance may correlate with the expression level of *Fgfr2b* and *Fgf10* along the developing GI tract, where different segments of the GI tract requires different levels of *Fgf10* or *Fgfr2b* for normal development. We have established that *Fgfr2b* and *Fgf10* are expressed in the small intestine during development, although at a lower level compared to the caecum and colon. Additionally, a previous study using an FGF10-*LacZ* reporter line demonstrated a high concentration of FGF10-expressing cells in the rostral duodenum of E18.5 (Kanard et al., 2005), providing more evidence of the importance of this growth factor in normal duodenal development. Our findings also show a higher penetrance of duodenal atresia in *Fgf10*^{-/-} embryos, possibly because in the absence of FGFR2b, FGF10 can signal via the FGFR1-IIIb isoform (FGFR1b) (Zhang et al., 2006, Beer et al., 2000).

One of the main aims of this study is to investigate the mechanism of duodenal atresia occurring in these mutants. Members of the Hedgehog family have been reported to play important roles during gut development, where *Shh* mutants showed duodenal stenosis and overt gut malrotation (Ramalho-Santos et al., 2000). Our results show normal *Shh* expression level in the small intestine, in contrast to the stomach (Spencer-Dene et al., 2006), and no overt gut malrotation in both *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos. A previous study also showed normal *Shh* level by ISH in E12.5 *Fgfr2b*^{-/-} small intestine (Burns et al., 2004). More than a century ago, Tandler proposed that intestinal atresia results from a failure in the recanalisation of the solid cord stage of duodenal development in human (Tandler, 1900). Our data did not support Tandler's theory because duodenal atresia cannot be detected at E11.5 and by this stage the duodenum has already developed an intestinal lumen (Figure 3.7).

Barnard and Louw (1955) alternatively proposed that intestinal atresia arises from an intrauterine mesenteric vascular accident of normally developed intestine late in gestation, resulting in necrosis and subsequent resorption (Barnard and Louw, 1956, Louw and Barnard, 1955). We find no necrosis and normal vascular density in the proximal small intestine of the mutants, including at the site of atresia, confirming that duodenal atresia is unlikely to be a consequence of defects in vasculature. Normal mesenteric vasculature in E11.5 to E13.5 *Fgf10*^{-/-} colon has been previously demonstrated (Fairbanks et al., 2005). However, our data does not preclude the possibility of minor functional differences, such as changes in morphology and location, of intestinal endothelial cells between Wt and mutants.

Another aspect of our investigation concerned small intestinal epithelial and mesenchymal proliferation and apoptosis. Serial cross-sections of E11.5 (the stage before duodenal atresia is first observed) duodenum do not show a focal imbalance of cell proliferation and apoptosis, but instead show a significant reduction of epithelial proliferation along the small intestines of the mutants. This suggests that reduced epithelial proliferation could have contributed to duodenal atresia. This finding is confirmed by our further examination on E12.5 non-atretic and atretic duodena in the *Fgfr2b*^{-/-} embryos via wholemount staining with propidium iodide. Propidium iodide has been used and validated successfully to detect mitotic and apoptotic cells (Coles et al., 1993, Foley and Bard, 2002). Both non-atretic and atretic duodena in the mutants have higher apoptotic activity than in the Wt. The significantly higher apoptotic activity in the atretic duodenum than in the non-atretic duodenum suggests that duodenal atresia is possibly a result of increased apoptosis at a particular focal point in the duodenum. The mechanism that specifies the focal point of atresia is currently unclear. Previous studies on caecal and colonic atresia in *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos have also shown reduced epithelial proliferation and increased apoptosis, further confirming the requirement of *Fgfr2b* and *Fgf10* in normal intestinal morphogenesis (Burns et al., 2004, Sala et al., 2006). The unexpectedly

higher tissue proliferation in the atretic ends compared to the non-atretic duodenum may indicate a response to tissue injury where increased tissue proliferation is required to repair the epithelium and mesenchyme at the atretic ends.

Cytodifferentiation analysis on the small intestine of these mutants reveals a significant reduction of goblet cells in the proximal small intestine, most likely due to gastric heterotopia. This phenotype occurs with complete penetrance regardless of the presence of duodenal atresia. Mucin-producing gastric epithelial cells are present partially in the rostral duodenum, accompanied by loss of Brunner's glands and partial loss of IAP. On the other hand, regions of intestinal epithelium with goblet cells express IAP. Expression of gastric-specific markers, SOX2 and ATP4b, is normally restricted to the stomach epithelium with no transcript detected beyond the pyloric sphincter, which delineates the duodenal boundary, and CDX2 expression is normally restricted to the intestine. In E18.5 *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos, the duodenum partially expresses CDX2, and the regions devoid of CDX2 abundantly express SOX2 and ATP4b.

Further examination on E14.5 mutants, the stage prior to intestinal differentiation, shows that the expression boundary between SOX2-expressing gastric cells and CDX2-expressing intestinal cells in both mutants is poorly defined at the pyloric sphincter. We have previously shown that the development of the pyloric sphincter is normal in both mutants (Spencer-Dene et al., 2006). SOX2 expression extends into the rostral duodenum whereas CDX2 expression is limited to the small intestine. This suggests that *Fgfr2b* and *Fgf10* regulate a signalling mechanism that restricts SOX2 expression into the intestinal region, and loss of *Fgfr2b* or *Fgf10* causes the cells in the intestinal region to commit into gastric cells even before intestinal differentiation has started.

Gastric heterotopia is a result of an anterior-posterior patterning defect in the small intestine, and canonical Wnt signalling has been implicated in specifying anterior-posterior axis of the GI tract (Theodosiou and Tabin,

2003, He, 2003, Mlodzik, 2002, Wodarz and Nusse, 1998, Yamaguchi, 2001). *Tcf4*^{-/-}/*Tcf1*^{-/-} double knockout embryos have been reported to display duodenal atresia with large areas of gastric heterotopia (Gregorieff et al., 2004). Our study also show that *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos develop disproportionately short small intestines (when measured against the overall embryonic size) during late-stage embryogenesis mostly due to reduction in the number of proliferating cells in the intervillous region. A previous study has also shown the absence of intestinal proliferative cells in *Tcf4*^{-/-} embryos, implying a strong link between Wnt signalling and *Fgf10-Fgfr2b* signalling (Korinek et al., 1998).

Expression studies on various Wnt targets in the small intestine of *Fgfr2b*^{-/-} and *Fgf10*^{-/-} embryos reveal significant downregulation of *Tcf1*, *Tcf4*, *Cdx1* and *Lgr5*, with normal expression level of *Lef1*, β -catenin and *Axin2*. While the loss of *Fgfr2b* and *Fgf10* does not fully eliminate *Tcf4* and *Tcf1* expression, the significant reduction of both Wnt targets may be sufficient to generate the patterning defects in the stomach/duodenum region. However, the heterotopia in the *Fgfr2b*^{-/-} and *Fgf10*^{-/-} small intestine is less severe than that of the *Tcf4*^{-/-}/*Tcf1*^{-/-} embryos. Our findings are also consistent with the hypothesis that gastric heterotopia only occurs when both *Tcf4* and *Tcf1* are downregulated, as loss of either *Tcf4* or *Tcf1* alone is not sufficient (Roose et al., 1999, Korinek et al., 1998). Additionally, loss of *Tcf4* leads to depletion of intestinal epithelial stem-cell compartments, and our data suggests that downregulation of *Tcf4* could have contributed to the reduced proliferating capacity of intestinal progenitor cells in the intervillous region of the small intestine, leading to reduced epithelial proliferation observed earlier. This finding is also consistent with the significant downregulation of *Lgr5*, a stem cell marker and a downstream target of the Wnt signalling pathway (Jaks et al., 2008, Korinek et al., 1998).

A previous study on *Fgf10-Fgfr2b* signalling in mouse mammary gland development showed that an ectopic source of recombinant FGF10 failed to induce *Lef1* expression in the mammary epithelium, suggesting that

Lef1 expression is independent of *Fgf10*, consistent with our findings in the small intestine (Mailleux et al., 2002). *Cdx1* expression requires *Tcf4* (Lickert et al., 2000), and the reduced *Cdx1* expression level in these mutants reflects the downregulation of *Tcf4*.

While *Cdx1*^{-/-} mice have no gut abnormality (Subramanian et al., 1995), *Cdx2*^{-/-} embryos die before gastrulation but heterozygous mice are viable (Chawengsaksophak et al., 1997). Several studies have shown that *Cdx2*^{+/-} mice develop gastric heterotopia in paracaecal regions with the lesions expressing SOX2 (Beck et al., 1999, Stringer et al., 2008). Our *Fgfr2b*^{+/-} and *Fgf10*^{+/-} adult mice show absence of gastric heterotopia in the ileum, caecum and proximal colon, with largely normal CDX2 expression (data not shown), indicating that the mechanism of gastric heterotopia in both cases may be different.

FGF2 has been previously shown to reduce GSK3 activity and induce nuclear translocation of β -catenin via the FRS2-GRB2-GAB1-PI3K-AKT signalling cascade (Katoh and Katoh, 2006, Holnthoner et al., 2002). FGF10 hypomorphic (*Fgf10*^{+/-}; *Mlc1v-LacZ*^{+/-}) lungs have been previously shown to have reduced nuclear β -catenin activation (Ramasamy et al., 2007), implying a relationship between *Fgfs* and β -catenin distribution. We did not, however, observe any noticeable change in β -catenin levels or nuclear/cytoplasmic distribution in the small intestine between null and Wt embryos. This suggests that *Fgf10-Fgfr2b* signalling may interact with the Wnt signalling pathway via an alternative signalling cascade to regulate the expression of *Tcf1* and *Tcf4* in the gut.

Indeed, our *in vitro* studies using the human colorectal carcinoma SW480 cell line show that *Fgf10-Fgfr2b* signalling is able to regulate *Tcf4* expression in the developing small intestine via the Grb2/Sos/Ras/MAPK pathway. Inhibition of the MAPK pathway by U0126, an inhibitor of this pathway, leads to significant downregulation of *Tcf4* expression and Wnt activity. We also show that the MAPK pathway cannot be regulated via

the PKC pathway because inhibition of the PKC pathway by BIM-I does not affect Wnt activity and *Tcf4* expression. The question as to whether the regulation of *Tcf4* expression by *Fgf10-Fgfr2b* signalling is direct or indirect requires further investigation. In addition, inhibition of the PI3K pathway leads to downregulation of Wnt activity but not *Tcf4* expression, suggesting that the PI3K pathway is regulating a different component of the Wnt pathway in our *in vitro* experiments.

3.5 Conclusion

In conclusion, we have shown that *Fgf10* and *Fgfr2b* are required for normal duodenal morphogenesis and establishment of the gastric-duodenal differentiation boundary via the canonical Wnt signalling pathway. Loss of either *Fgfr2b* or *Fgf10* leads to disproportionately short small intestine due to reduced proliferation in the intervillous epithelium, duodenal atresia contributed by reduced tissue proliferation and increased apoptosis at a focal point, and gastric heterotopia due to loss of gastric-intestinal boundary specification.

In addition, we have also shown that loss of either *Fgf10* or *Fgfr2b* leads to significant downregulation of Wnt targets *Tcf1* and *Tcf4* in the small intestine, with corresponding downregulation of *Lgr5*, an intestinal stem cell marker, and *Cdx1*, a homeobox gene involved in anterior-posterior patterning. Using *in vitro* studies, we have shown that *Fgf10-Fgfr2b* signalling can regulate *Tcf4* expression via the Grb2/Sos/Ras/MAPK pathway. This knowledge will enhance our understanding on human congenital duodenal atresia and other pathological anomalies, such as gastric heterotopia and paraduodenal heterotopia in adults.

Chapter 4: *Fgfr2b* in intestinal homeostasis

4.1 Introduction

While the previous chapter has demonstrated the requirement of *Fgfr2b* in GI development, the role of *Fgfr2b* in maintaining adult GI homeostasis is not well understood. Expression of *Fgfr2b* and *Fgf7* has been detected throughout the adult rat GI tract from the oesophagus to colon, implying a possible role of *Fgfr2b* and *Fgf7* in maintaining intestinal homeostasis (Housley et al., 1994). Additionally, systemic administration of rhFGF7 rapidly induced the proliferation of epithelial cells in the foregut to the colon, and continued treatment led to the selective induction of mucin-producing cells in a dose-dependent fashion, providing further evidence on the ability of *Fgf7*, which signals via *Fgfr2b*, to activate GI epithelial populations *in vivo* (Housley et al., 1994). Recent studies have also demonstrated the importance of *Fgfr2b* in maintaining other organ homeostasis. Ablation of *Fgfr2b* specifically in the skin resulted in epidermal hyperthickening that developed with age, and 10% of these mutants developed spontaneous papillomas, implying the role of *Fgfr2b* in postnatal skin development and in adult skin homeostasis (Grose et al., 2007). Ubiquitous expression of soluble dominant negative *Fgfr2b* in postnatal mice for six weeks starting immediately after birth led to the formation of rudimentary mammary epithelial tree with completely absent terminal end buds compared to a well-branched structure observed in wild type, demonstrating the role of *Fgfr2b* in postnatal mammary development (Parsa et al., 2008).

Since absence of *Fgfr2b* during early pre-implantation development leads to perinatal lethality (De Moerlooze et al., 2000), we utilise an inducible Cre recombinase-mediated excision, using the progesterone antagonist (RU486) to drive the intestinal-specific A33 antigen promoter. The A33 antigen is expressed on the basolateral surface of intestinal epithelial cells

of all lineages, independent of their position along the rostrocaudal and crypt-villus axis (Johnstone et al., 2000, Johnstone et al., 2002). An alternative approach to this method is the use of Villin-CreER^{T2}, however this line directs high Cre expression in the duodenum that gradually decreases along the intestine with the lowest Cre expression in the colon (el Marjou et al., 2004). In addition, the Cre expression directed by Villin-CreER^{T2} tends to be mosaic (personal communication with Prof. Ian Tomlinson, Oxford University). Conversely, the A33-CrePR line directs high Cre expression in the colon that gradually decreases towards the proximal small intestine (Malaterre et al., 2007), making it an optimal model for this study as the large intestine expresses higher *Fgfr2b* mRNA than the small intestine (see below). Additionally, the A33-CrePR line has been used successfully in a previous study to knock out *c-Myb* in the colonic crypts (Malaterre et al., 2007).

4.2 Materials and Methods

4.2.1 Animal breeding

A33-CrePR line was a kind gift from Dr. Theo Mantamadiotis (in Prof. Joan Heath's laboratory, Peter MacCallum Cancer Centre and Pathology Department, University of Melbourne, Australia) and has been previously described (Malaterre et al., 2007). A33-CrePR⁺ mice were initially crossed with Fgfr2b^{flox/flox} conditional mice to generate an A33-CrePR⁺/Fgfr2b^{flox/wt} line, and this line was subsequently crossed with itself to generate A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} lines.

4.2.2 RU486 administration

RU486 mix was prepared as previously described (Section 2.2.1). Twelve six-week-old males and females for both A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} strains were given 250 µl of RU486 mix daily by gavage for up to 28 days. Following this, mice were either culled the next day or left for another 28 days.

4.2.3 Immunohistochemistry

Mice were injected with BrdU solution (550891; BD Biosciences) at 50 mg/kg 1 hr before culling. Gut tissues were dissected in cold PBS, fixed, processed and embedded as previously described (Section 2.5.2). Immunohistochemistry was performed with anti-BrdU, anti-CSP-3, anti-FGFR2b, anti-MUC2, anti-chromogranin A/B, anti-lysozyme and anti-TCF4 as previously described (Section 2.5). Morphometry was performed as previously described (Section 2.7).

4.2.4 Crypt microdissection

Mice were injected intraperitoneally with vincristine sulphate 1 mg/kg (Mayne Pharma Plc.) 2 hrs before culling. Crypt microdissection was performed as previously described (Section 2.8).

4.2.5 RT-PCR

RNA was extracted from the different GI segments of C57/BL6, A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} mice, and cDNA synthesis was performed as previously described (Section 2.4). Reverse transcriptase PCR was performed for *Fgfr2b* as described in Section 2.4.4, while SYBR Green PCR was performed for *Fgfr1b* and *Tcf4* as described in Section 2.4.7.

4.2.6 Western blot

Protein was extracted from the different GI segments of C57/BL6, A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} mice, and Western blot for Cre and FGFR2b was performed as previously described (Section 1.9).

4.2.7 In situ hybridisation (ISH)

Gut tissues from A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} mice were dissected, fixed, processed and embedded as previously described (Section 2.5.2). ISH for *Fgfr2b* was performed as previously described (Section 2.3.3).

4.3 Results

4.3.1 *Fgfr2b* is expressed in adult mouse GI tract

Reverse transcriptase PCR and ISH were used to determine the expression gradient of *Fgfr2b* along the adult mouse GI tract. *Fgfr2b* is expressed highly in the stomach and distal part of the intestine (ileum, caecum and colon) compared to the proximal small intestine (duodenum and jejunum) (Figure 4.1). *Fgfr2b* mRNA is detected strongly at the crypt-villus junction and in the crypts of the small intestine, and at the bottom of caecal and colonic crypts (Figure 4.4).

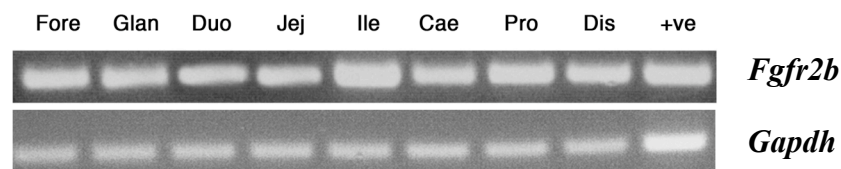


Figure 4.1 Reverse-transcriptase PCR showing *Fgfr2b* expression level in an adult mouse GI tract.

This mRNA profile is a representation of the profile shown by three Wt C57/BL6. *Fgfr2b* is expressed higher in the distal region than the proximal region of the intestine. Fore: forestomach, Glan: glandular stomach, Duo: duodenum, Jej: jejunum, Ile: ileum, Cae: caecum, Pro: proximal colon, Dis: distal colon, +ve: positive control from adult lung mRNA. Negative control (omitted from the above figure) came from E18.5 *Fgfr2b*^{-/-} whole embryo mRNA and show absence of band (data not shown).

4.3.2 RU486 administration leads to reduced *Fgfr2b* mRNA in the GI tract of A33-CrePR⁺/*Fgfr2b*^{flox/flox}

Western blot was used to determine the expression gradient of Cre along the GI tract of A33-CrePR⁺/*Fgfr2b*^{flox/flox} and *Fgfr2b*^{flox/flox} mice. Cre is expressed highly in the caecum and colon, and this expression decreases towards the duodenum (Figure 4.2). RU486 administration on A33-Cre⁺/*Fgfr2b*^{flox/flox} mice for 14 days consecutively show a significant reduction of *Fgfr2b* mRNA by approximately 90% in the colon and 70% in the small intestine compared to the *Fgfr2b*^{flox/flox} mice (Figure 4.2). There is no significant difference in the level of *Fgfr2b* excision between the 14 days and 28 days of RU486 administration. The significant reduction of *Fgfr2b* mRNA is observed globally along the entire GI tract with little mosaicism, as evidenced by ISH (Figure 4.4). These results cannot be validated further by immunohistochemistry and Western blot, as published anti-FGFR2b antibodies are not specific for the mouse FGFR2b antigen despite testing various antigen retrieval conditions, antibody concentrations and on fresh-frozen tissues (Figure 4.3).

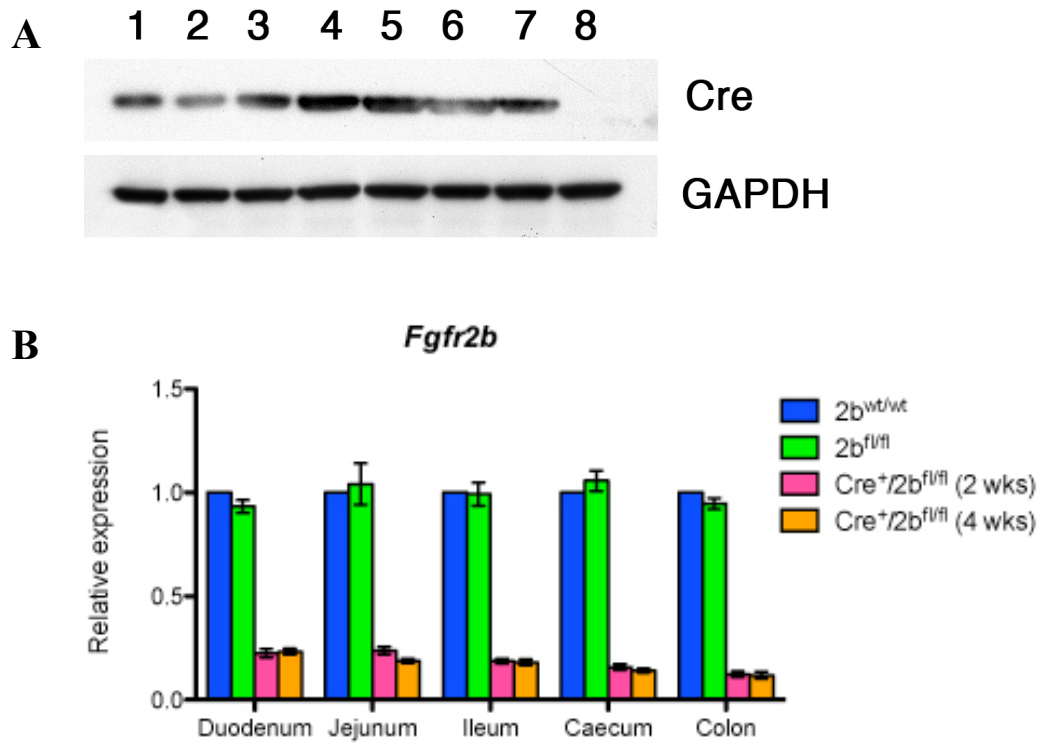


Figure 4.2 Comparison of Cre and *Fgfr2b* expression in the intestine of A33-CrePR⁺/*Fgfr2b*^{flx/flx} and *Fgfr2b*^{flx/flx}.

(A) Cre expression is detectable by immunoblotting in tissue lysates from the gut of A33-CrePR⁺/*Fgfr2b*^{flx/flx} mice, with higher expression in the distal intestine than the proximal intestine. GAPDH was used as a control for equal loading. This result is a representation of the profile shown by three mice. 1: duodenum, 2: jejunum, 3: ileum, 4: caecum, 5: proximal colon, 6: distal colon, 7: positive control from adult duodenum of Villin-CreER^{T2} mouse, 8: negative control from lung tissue of A33-CrePR mouse.

(B) RU486 was administered on *Fgfr2b*^{flx/flx} (labelled as '2b^{fl/fl}') for four weeks, and A33-CrePR⁺/*Fgfr2b*^{flx/flx} (labelled as 'Cre⁺/2b^{fl/fl}') for two and four weeks. Mice were culled one day after RU486 administration. The A33-CrePR system excises *Fgfr2b* alleles with higher efficiency in the distal intestine than in the proximal intestine. Data was normalised to the mean of *Gapdh* expression, and error bars ($\pm$ SEM) came from three experiments; n = 4 animals/group.

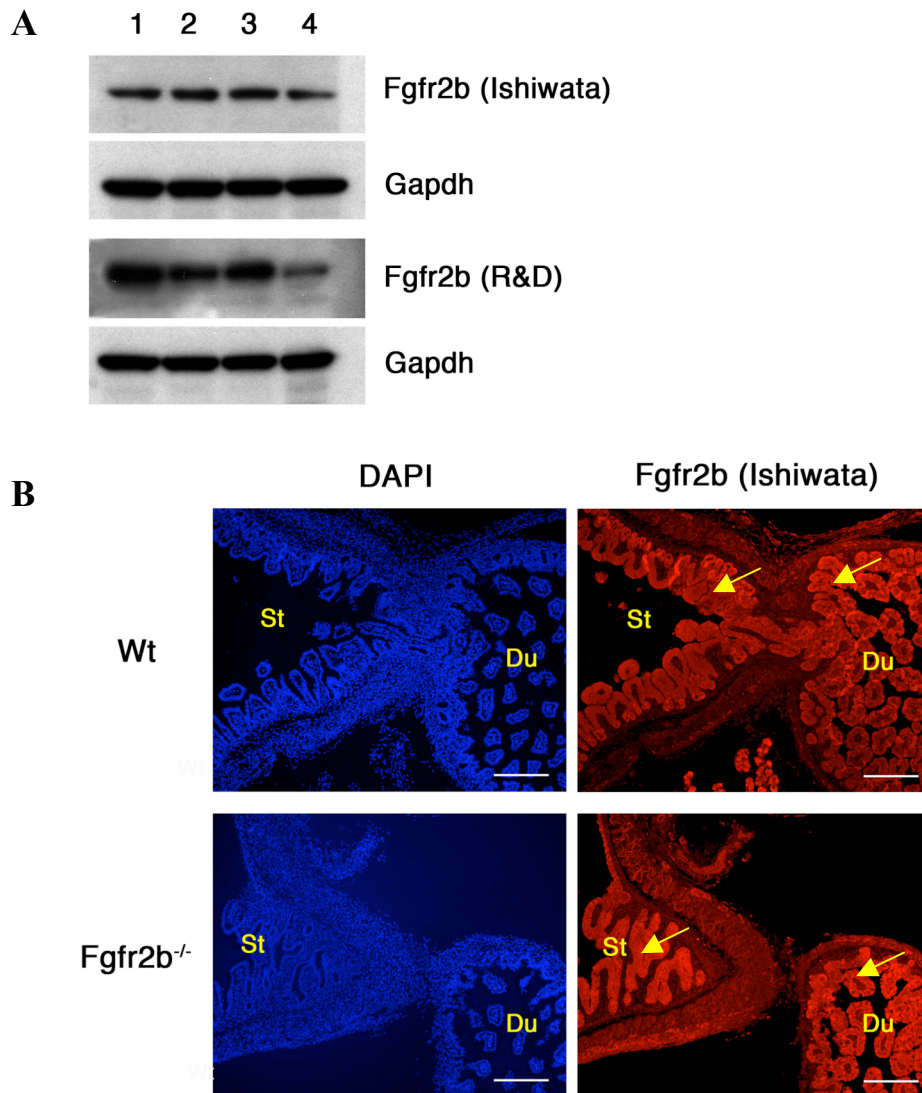


Figure 4.3 Demonstration of anti-FGFR2b antibodies in Western blot and immunofluorescence staining.

(A) Protein lysate was obtained from two whole E14.5 Wt (labelled as ‘1’ and ‘2’) and *Fgfr2b*^{-/-} (labelled as ‘3’ and ‘4’) embryos, and subjected to western transfer and probed with published anti-FGFR2b antibodies from Ishiwata (Ishiwata et al., 2002) and R&D Systems. GAPDH was used as a control for equal loading. Both antibodies detect FGFR2b expression in Wt and mutant tissues, implying that the antibodies are not specific for the mouse FGFR2b antigen.

(B) Immunofluorescence was performed on E18.5 stomach and duodenal sections from Wt and *Fgfr2b*^{-/-} embryos using antibodies as described previously. The antibody from R&D Systems show absence of positive

staining even in Wt tissue, hence is omitted from the above figure. The antibody from Ishiwata show apparently positive staining in *Fgfr2b*^{-/-} stomach and duodenum (yellow arrow), largely indistinguishable to that of the Wt.

Figure 4.4 Continue on next page

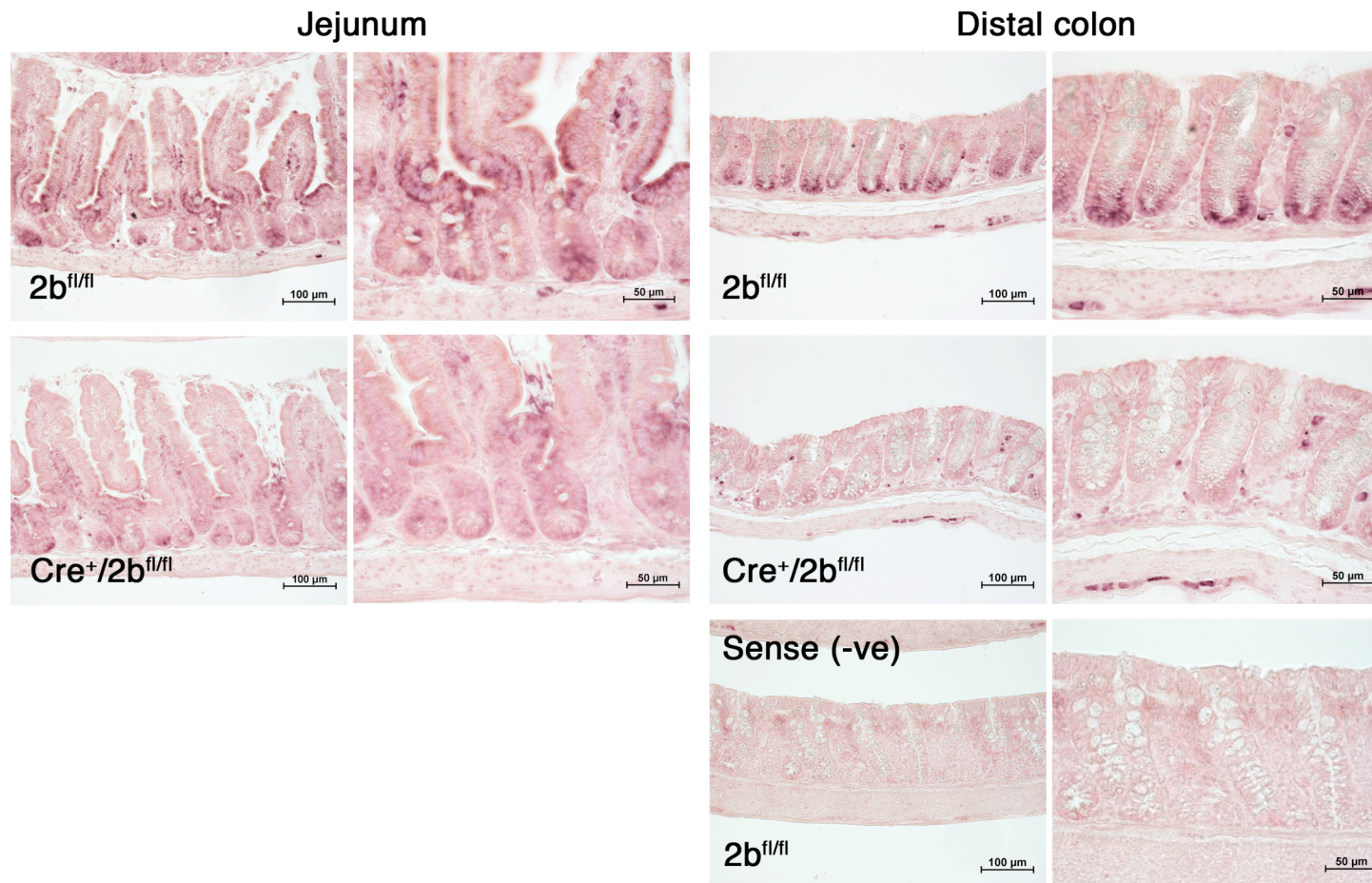


Figure 4.4 Comparison of *Fgfr2b* mRNA expression in the GI tract of A33-CrePR⁺/*Fgfr2b*^{flx/flx} and *Fgfr2b*^{flx/flx} following RU486 administration for 28 days.

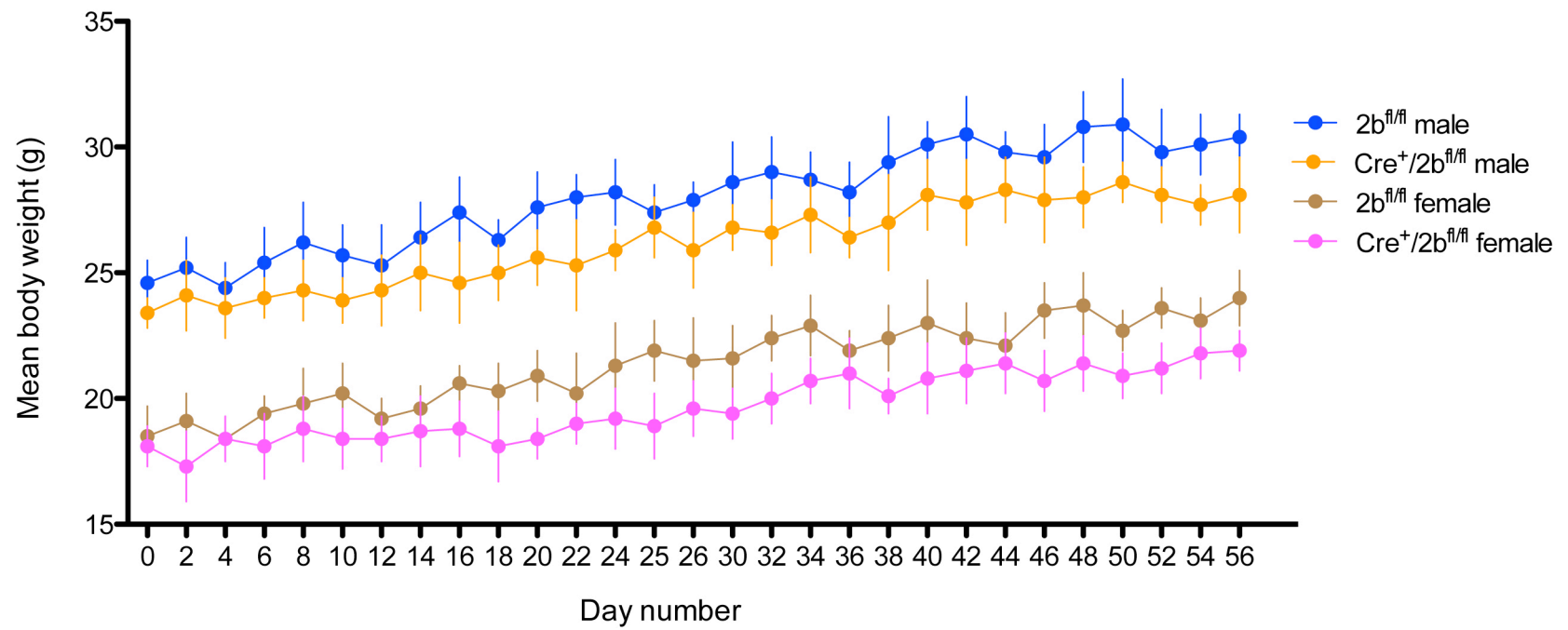
ISH for *Fgfr2b* mRNA was performed with tissue sections from the different segments of the GI tract from both mice groups, however only results from jejunum and ileum are shown. Blue arrows indicate the location of *Fgfr2b* mRNA at the crypt-villus junction and the bottom of crypts. The A33-CrePR⁺/*Fgfr2b*^{flx/flx} mice show reduced *Fgfr2b* mRNA level.

4.3.3 Reduced *Fgfr2b* does not lead to overt change in body weight

To determine whether downregulation of *Fgfr2b* can affect body weight as a parameter for measuring intestinal homeostasis, mice were given four weeks of RU486 and left for another four weeks, with body weight measured every two days. The A33-Cre⁺/*Fgfr2b*^{flxflx} mice show a consistent trend of reduced body weight compared to the *Fgfr2b*^{flxflx} mice regardless of the gender (Figure 4.5). However, both mice groups show no significant difference in their body weight when data at two separate time points were compared: the first time point was immediately after the four weeks of RU486 administration (28th day), and the second time point was immediately after the entire experiment (56th day) (Figure 4.5).

Figure 4.5 (continue on next page)

A



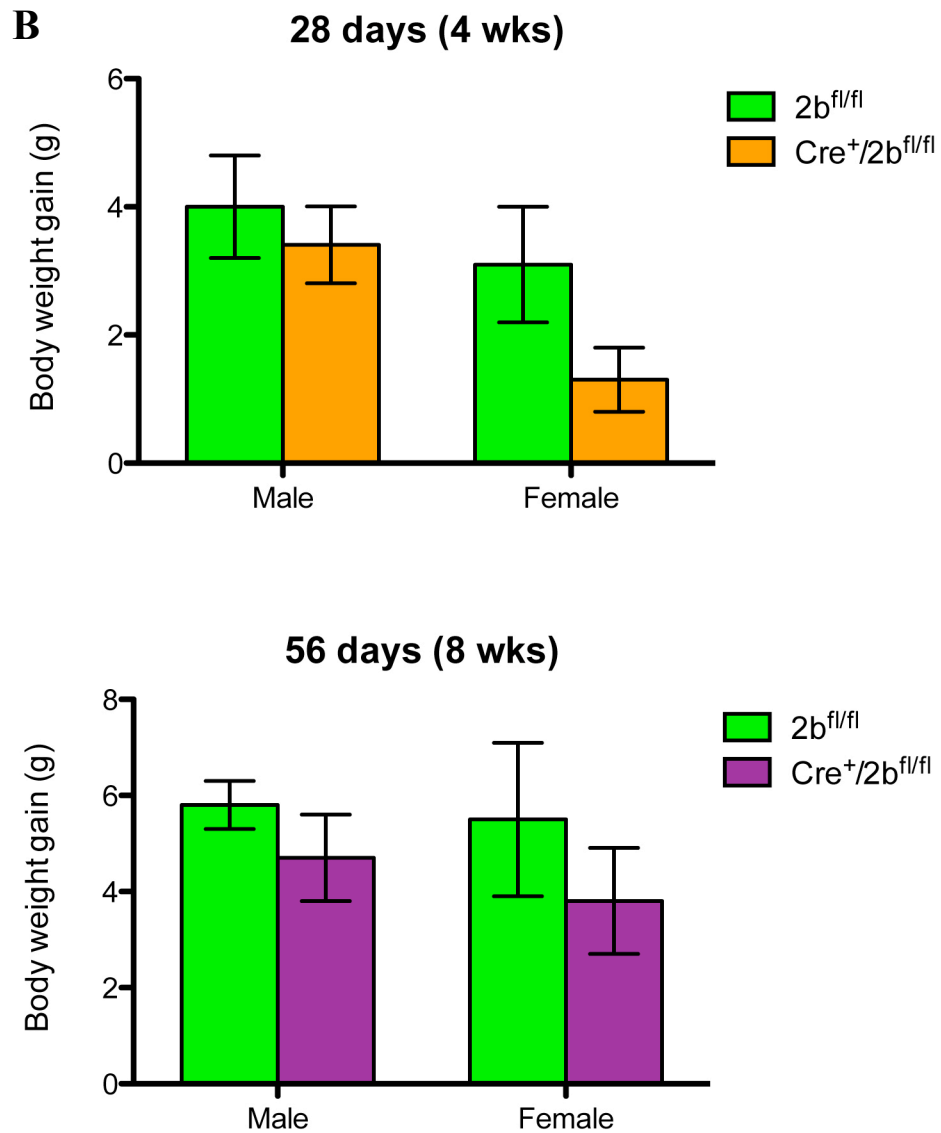


Figure 4.5 Comparison of body weight between A33-CrePR⁺/Fgfr2b^{flx/flx} and Fgfr2b^{flx/flx} mice following RU486 administration.

(A) Both mice groups were given RU486 for the first 28 days, and left for another 28 days. Body weight was measured every two days for eight weeks. The Fgfr2b^{flx/flx} mice consistently show a trend of reduced body weight compared to the controls. All data was presented as mean ($\pm$ SEM) body weight; n = 6 animals/group/gender.

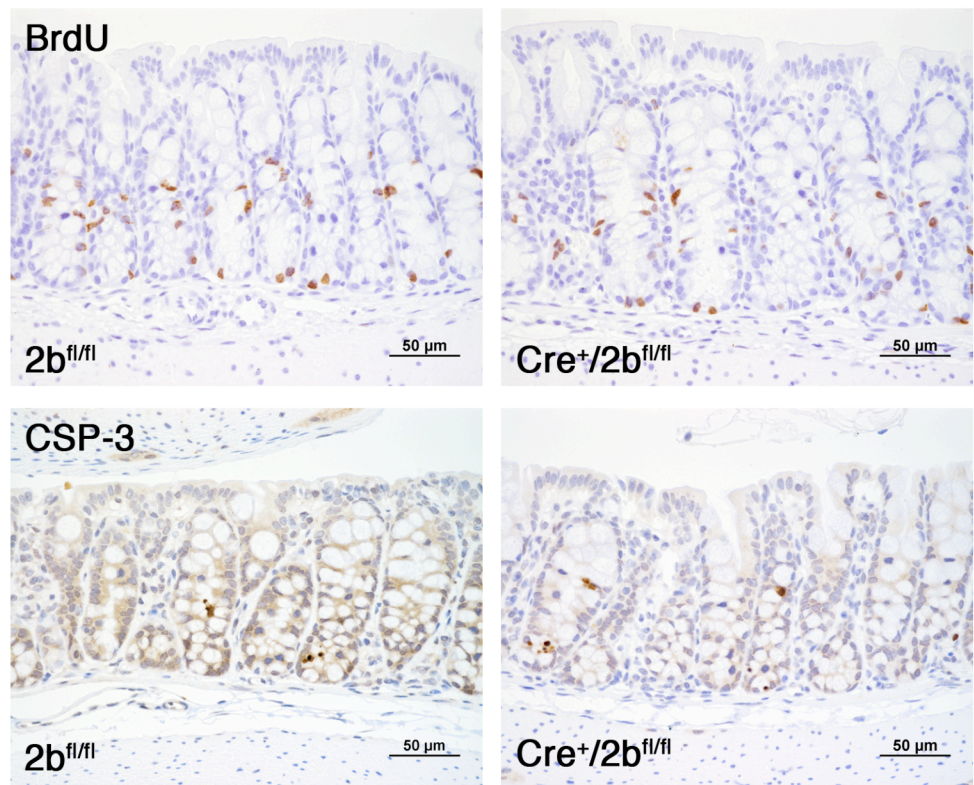
(B) Average gain in body weight between both mice groups was calculated at the end of 28 and 56 days separately. Body weight between both groups was largely similar. All data was presented as mean ($\pm$ SEM) body weight; n = 6 animals/group/gender.

4.3.4 Reduced *Fgfr2b* does not lead to overt change in intestinal crypt proliferation, apoptosis and differentiation

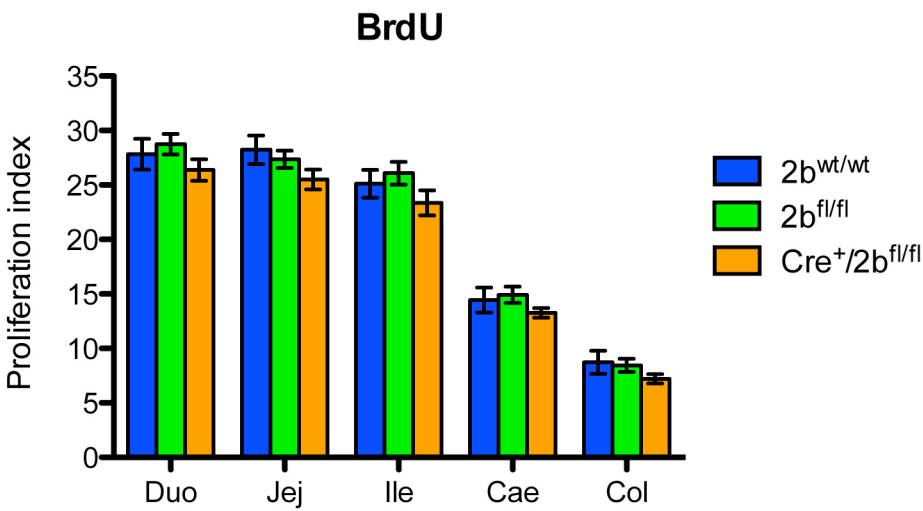
Various markers for proliferation, apoptosis and differentiation were used to determine the effect of reduced *Fgfr2b* mRNA on intestinal homeostasis. Both A33-CrePR⁺/*Fgfr2b*^{flox/flox} and *Fgfr2b*^{flox/flox} mice have largely similar degree of epithelial proliferation as measured by BrdU immunostaining and crypt microdissection (Figure 4.6 and 4.7). The crypt microdissection technique assessed number of cells in metaphase, number of crypts undergoing fission and crypt volume in a three-dimensional aspect. Both groups also have largely similar rate of apoptosis as measured by active CSP-3 immunostaining (Figure 4.6), and differentiated cells as evidenced by immunostaining for goblet cells (MUC2), enteroendocrine cells (chromogranin A/B) and Paneth cells (lysozyme) (Figure 4.8). To establish whether reduced *Fgfr2b* mRNA can affect the expression of intestinal transcription factors, expression level of *Tcf4* was compared between A33-CrePR⁺/*Fgfr2b*^{flox/flox} and *Fgfr2b*^{flox/flox} mice. Both mice groups show no significant difference in the *Tcf4* mRNA and protein expression (Figure 4.9). The A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice also show largely normal CDX2 immunostaining (Figure 4.9).

Figure 4.6 Continue on next page

A



B



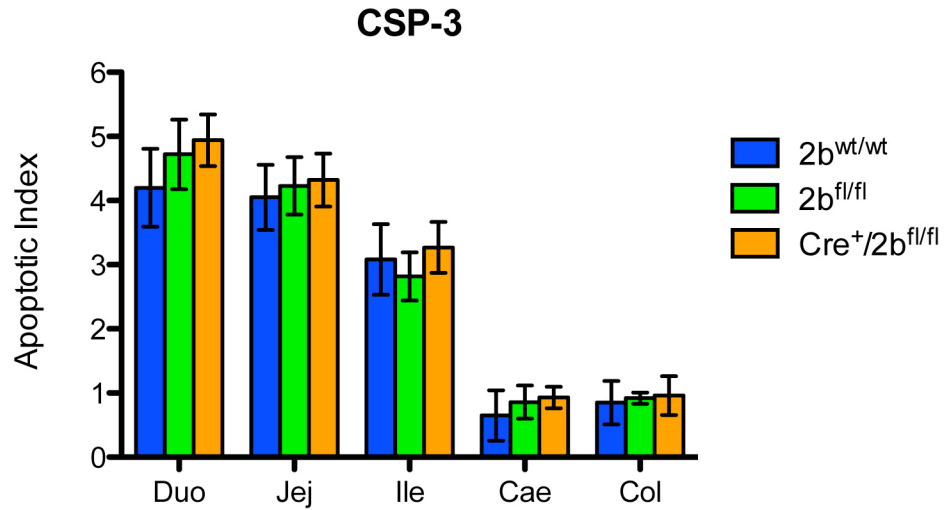


Figure 4.6 Comparison of proliferation and apoptosis in the GI tract of $Fgfr2b^{wt/wt}$, $Fgfr2b^{flx/flx}$ and $A33CrePR^+/Fgfr2b^{flx/flx}$ following RU486 administration.

(A) All three groups of mice were given RU486 for 28 days consecutively and culled after another 28 days. Representative images for BrdU and CSP-3 IHC came from distal colon.

(B) Quantification of the mean number of cells positively stained cells in 20 random fields of intestinal epithelium viewed at x40 magnification. All three groups show largely similar epithelial proliferation and apoptosis. All data was presented as mean ($\pm$ SEM); n=12 animals/per group. Duo: duodenum, Jej: jejunum, Ile: ileum, Cae: caecum, Col: distal colon.

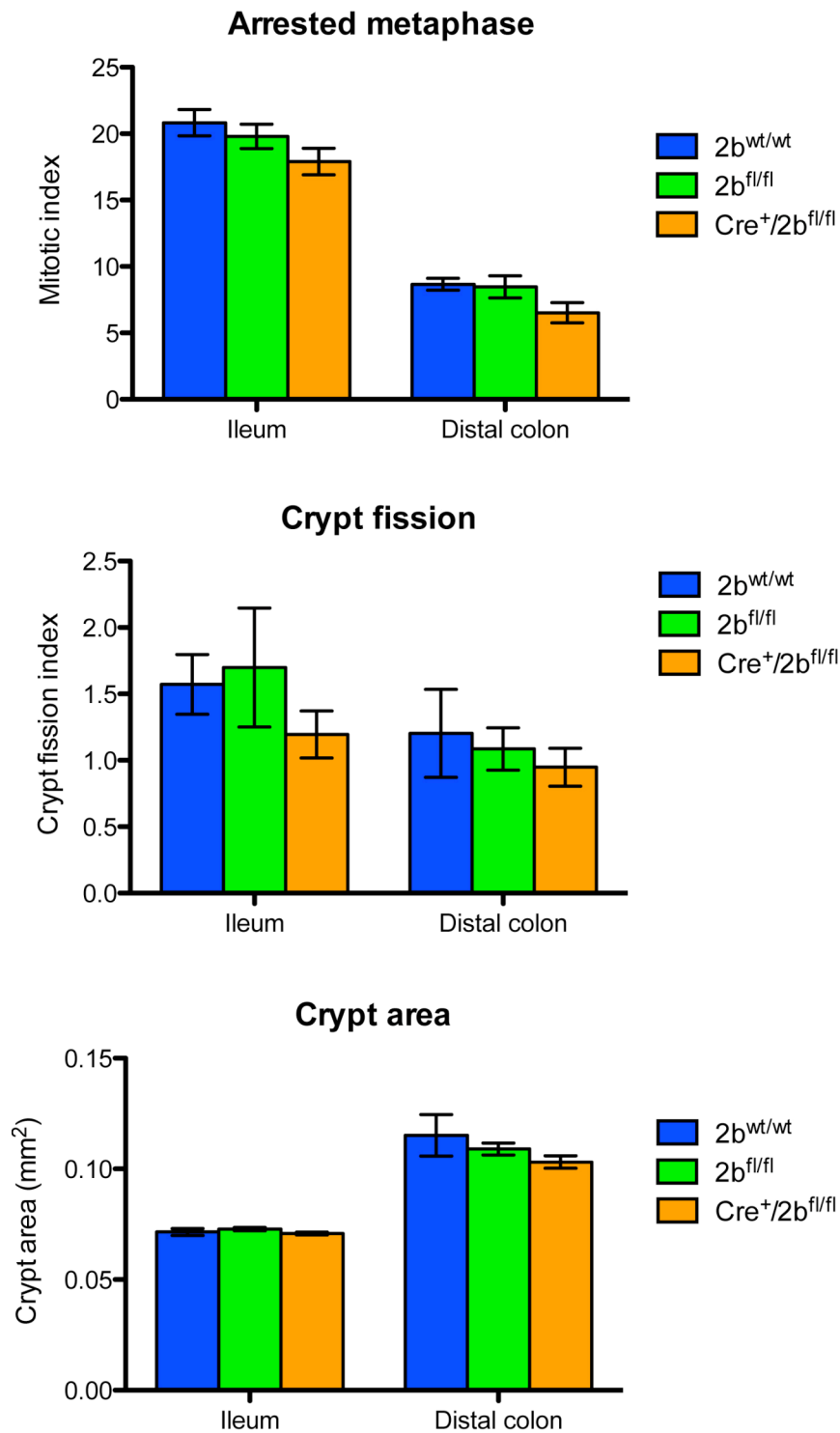


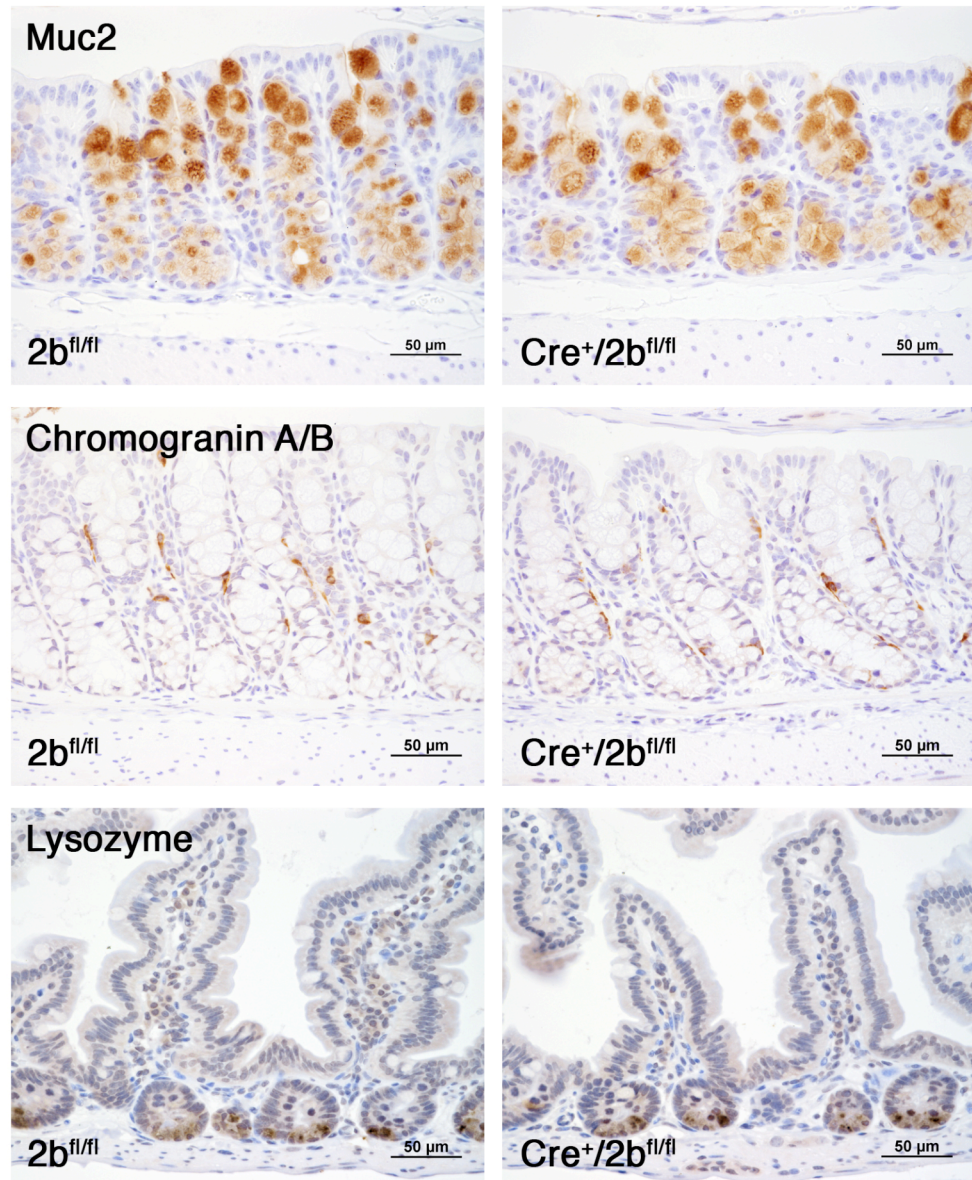
Figure 4.7 Comparison of proliferation by crypt microdissection in the GI tract of $Fgfr2b^{wt/wt}$, $Fgfr2b^{flx/flx}$ and $A33CrePR^+/Fgfr2b^{flx/flx}$ following RU486 administration.

All three groups of mice were given RU486 for 28 days. Quantification was performed as described in Section 2.8.2. All three groups show

largely similar crypt proliferation. All data was presented as mean ($\pm$ SEM); n = 8 animals/per group.

Figure 4.8 Continue on next page

A



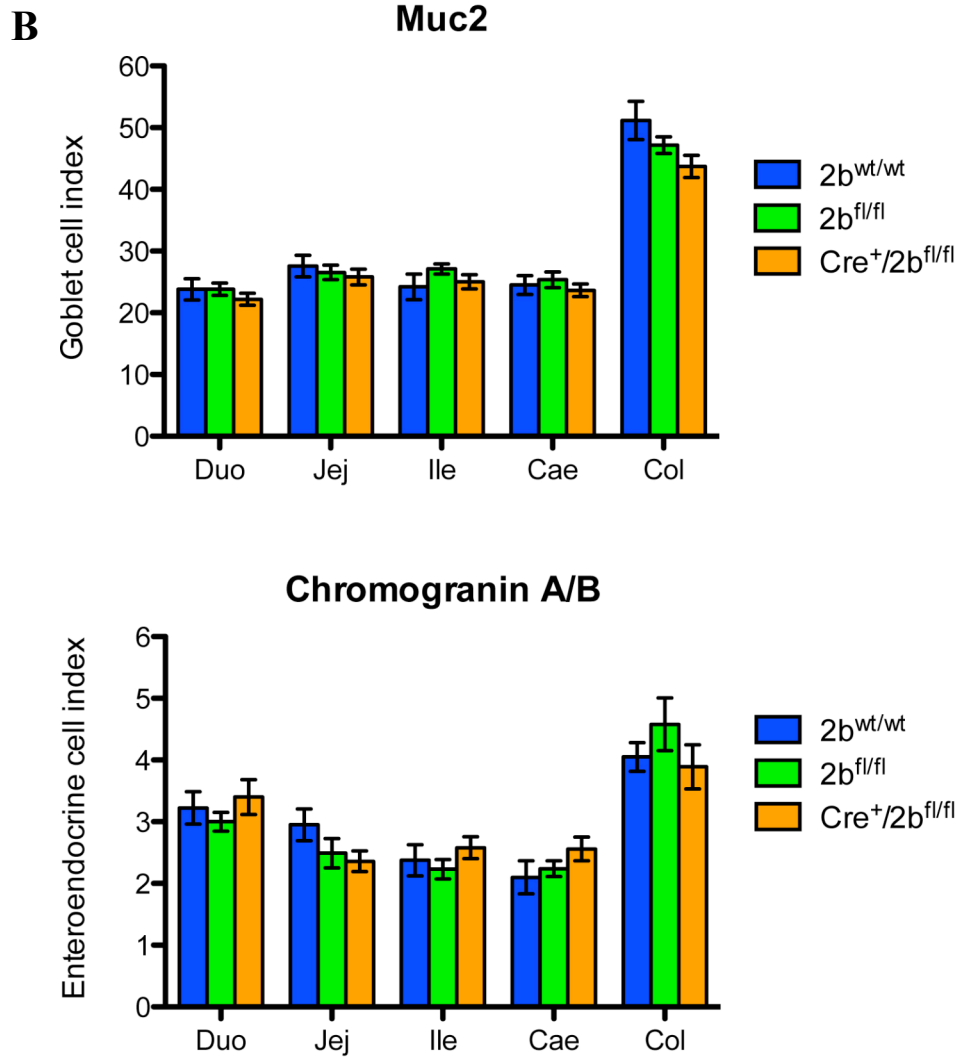


Figure 4.8 Comparison of differentiation markers in the GI tract of $Fgfr2b^{wt/wt}$, $Fgfr2b^{flx/flx}$ and $A33CrePR^+/Fgfr2b^{flx/flx}$ following RU486 administration.

(A) Representative images for MUC2 and chromogranin A/B IHC came from distal colon, whereas representative images for lysozyme came from jejunum as the colon does not have Paneth cells.

(B) Quantification of the mean number of cells positively stained cells in 20 random fields of intestinal epithelium viewed at x40 magnification. The $A33CrePR^+/Fgfr2b^{flx/flx}$ mice show largely normal cytodifferentiation. All data was presented as mean ($\pm$ SEM); n=12 animals/per group. Duo: duodenum, Jej: jejunum, Ile: ileum, Cae: caecum, Col: distal colon.

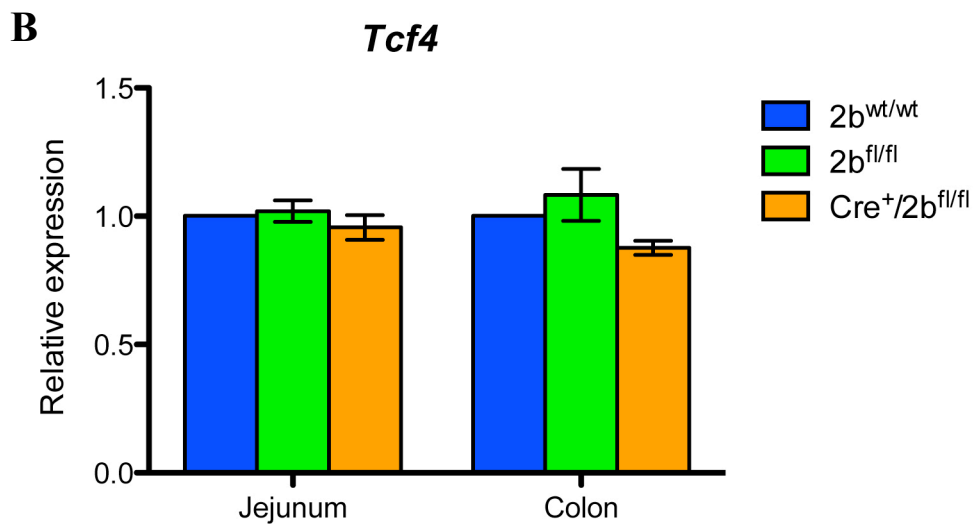
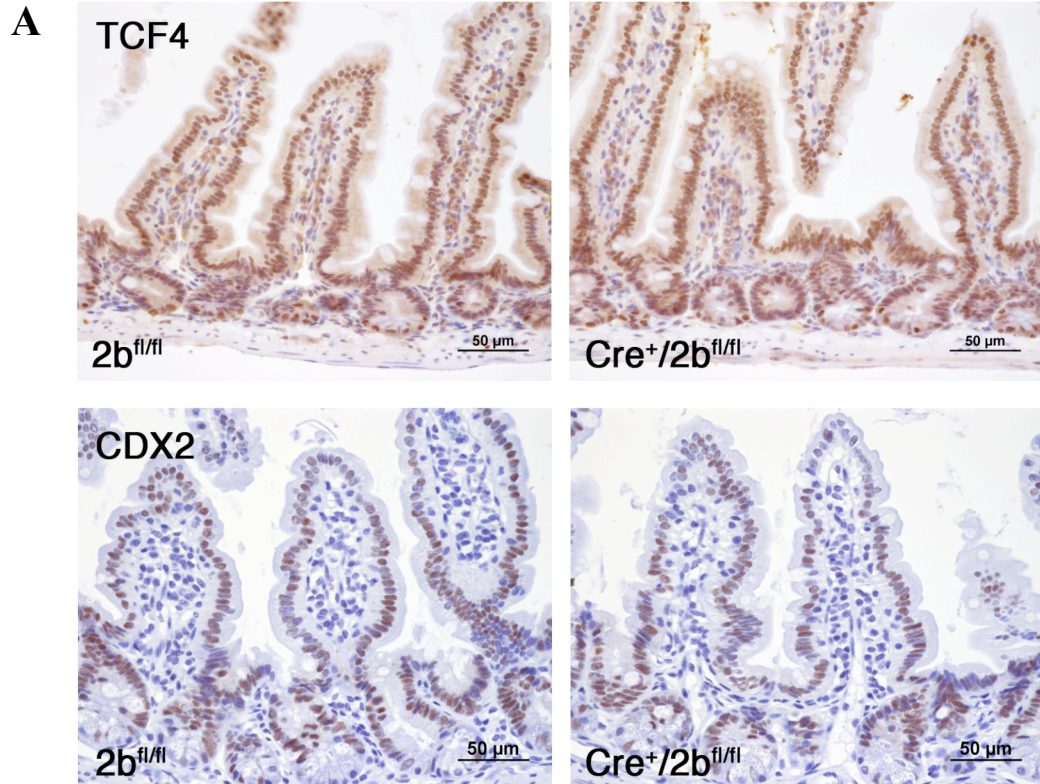


Figure 4.9 Comparison of TCF4 and CDX2 expression in the GI tract of $Fgfr2b^{wt/wt}$, $Fgfr2b^{lox/lox}$ and $A33CrePR^+/Fgfr2b^{lox/lox}$ following RU486 administration.

(A) Representative images for TCF4 and CDX2 IHC came from jejunum.

(B) RNA was extracted from jejunum and distal colon, and subjected to SYBR Green RT-PCR for *Tcf4*. The $A33CrePR^+/Fgfr2b^{lox/lox}$ mice show largely normal *Tcf4* expression. All data was normalised to the mean of

Gapdh expression, and error bars ($\pm$ SEM) came from three experiments; n = 4 mice/group.

4.3.5 *Fgfr2b* may be functionally redundant in maintaining adult intestinal homeostasis

Reverse-transcriptase PCR was used to determine whether other *Fgfs* and *Fgfrs* are expressed in the adult GI tract. RT-PCR results show that the GI tract expresses various *Fgfs* and *Fgfrs* with different expression gradient along the GI tract (Figure 4.10). Of the *Fgf* receptors, all *Fgfrs*, except *Fgfr2c*, are expressed in the intestinal region. *Fgfr1b* expression level was compared between A33-CrePR⁺/*Fgfr2b*^{flox/flox} and *Fgfr2b*^{flox/flox} mice to determine whether *Fgfr1b* may compensate for the downregulation of *Fgfr2b*. RT-PCR data show largely normal *Fgfr1b* expression in both mice groups (Figure 4.11).

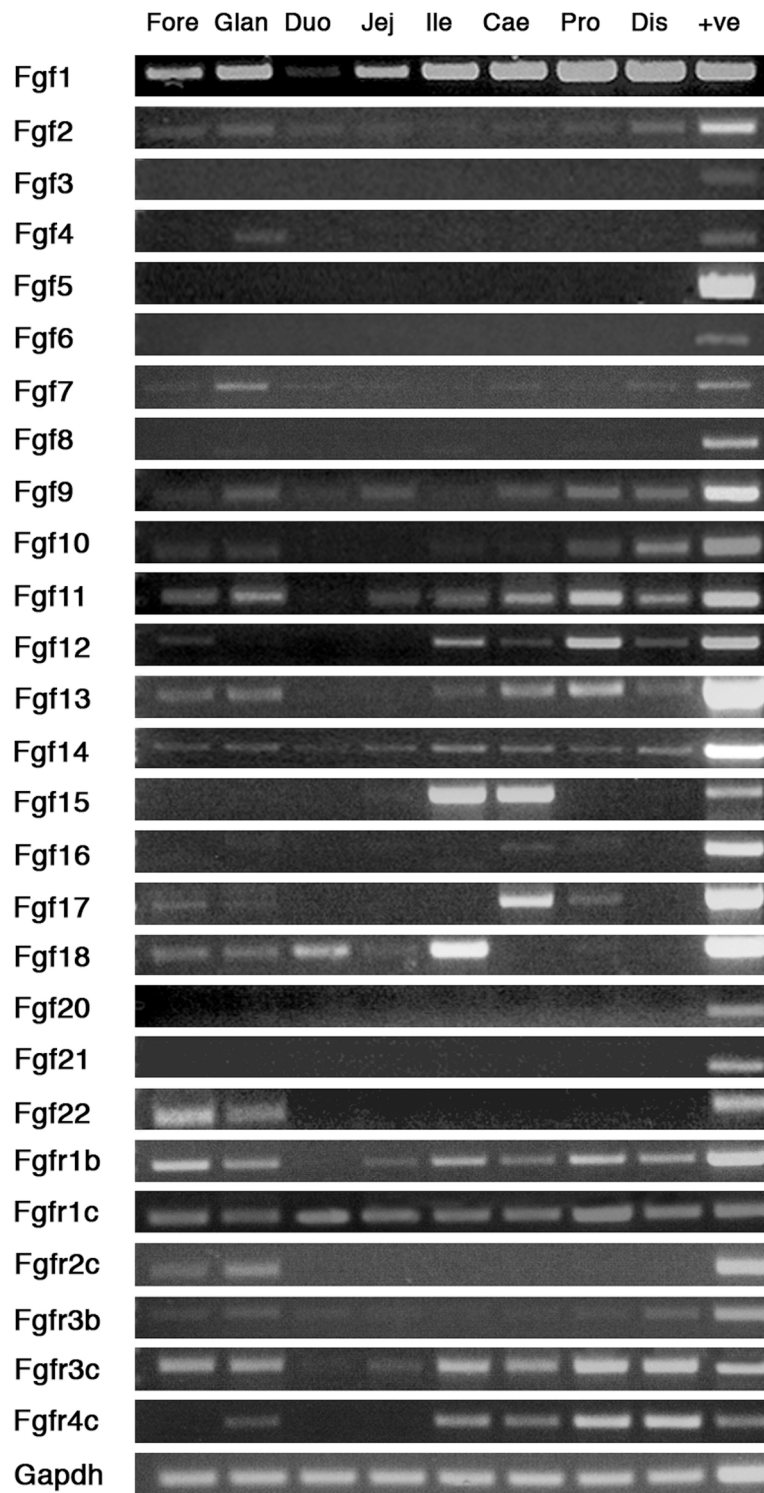


Figure 4.10 Reverse-transcriptase PCR showing expression of *Fgfs* and *Fgfrs* in adult mouse GI tract.

This mRNA profile is a representation of the profile shown by three Wt C57/BL6 mice of approximately six weeks old. *Fgf19* and *Fgf23* could not be optimised. *Fgfr2b* mRNA profile has been shown in Figure 4.1,

hence was omitted from the above figure. *Gapdh* was used as a loading control. Positive controls used are listed in Table 1.2, and negative controls came from omitted cDNA template from the reaction (data not shown). All RNA samples have been checked for DNA contamination by *Gapdh* PCR.

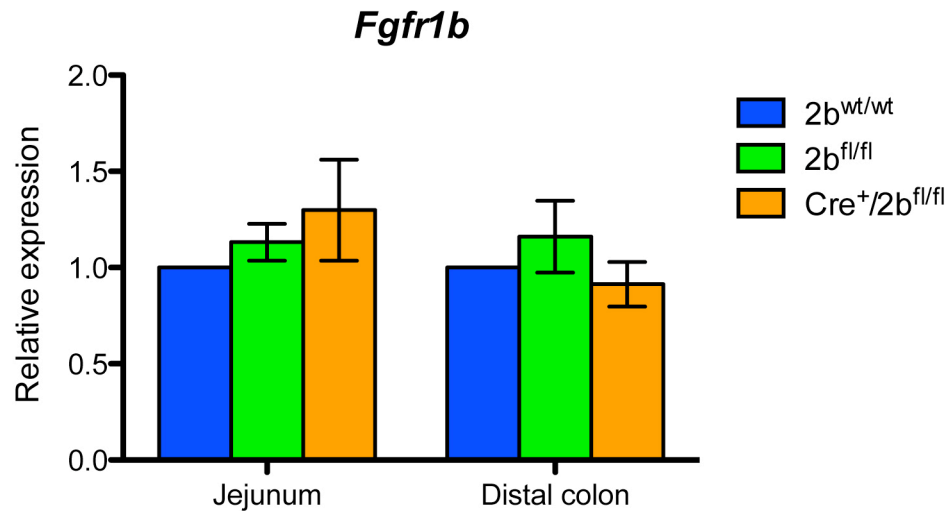


Figure 4.11 Comparison of *Fgfr1b* expression in the GI tract of *Fgfr2b*^{wt/wt}, *Fgfr2b*^{flx/flx} and A33CrePR⁺/*Fgfr2b*^{flx/flx} following RU486 administration.

RNA was extracted from jejunum and distal colon, and subjected to RT-PCR. The A33CrePR⁺/*Fgfr2b*^{flx/flx} mice show largely normal *Fgfr1b* expression. All data was normalised to the mean of *Gapdh* expression, and error bars ($\pm$ SEM) came from three experiments; n = 4 mice/group.

4.4 Discussion

The adult mouse GI tract expresses *Fgfr2b* mRNA more strongly in the distal region of the GI tract, especially in the ileum, than in the stomach and proximal small intestine. The results are in contrast to the mRNA profile shown in adult rats where *Fgfr2b* is expressed highest in the forestomach and glandular stomach (Housley et al., 1994), suggesting that the difference in *Fgfr2b* mRNA level can be accounted by the difference in animal species.

This study utilises the A33-CrePR mouse line that expresses Cre recombinase preferentially in the distal region of the GI tract (Malaterre et al., 2007) where *Fgfr2b* is expressed highly, and the Cre is activated upon RU486 administration. Western blot for Cre protein in the GI tract of A33-CrePR mice confirms this preferential expression. Additionally, RU486 administration for two or four weeks consecutively results in significant downregulation of *Fgfr2b* mRNA across the GI tract of A33-CrePR⁺/*Fgfr2b*^{flox/flox}, with the strongest downregulation observed in the distal colon, further providing evidence that the A33-CrePR line gives better Cre-mediated excision efficiency in the distal intestine than in the proximal small intestine. There is no significant difference in excision efficiency between two-week and four-week induction periods, although the four-week induction period displays a trend of stronger *Fgfr2b* downregulation. ISH with DIG-labelled *Fgfr2* probe show that downregulation of *Fgfr2b* is accounted by the global reduction of *Fgfr2b* transcript across the intestine rather than mosaic deletion of *Fgfr2b* alleles.

For this study, RU486 was administered for four weeks to ensure a higher extent of *Fgfr2b* downregulation while at the same time allowing the mice to develop any intestinal phenotypic abnormality. Body weight measurement was used as a preliminary parameter to determine whether downregulation of *Fgfr2b* affects the nutrient absorption function of small

and large intestines. A33-CrePR⁺/Fgfr2b^{flox/flox} mice show a consistent trend of, but insignificant, lower body weight compared to the control mice, suggesting that downregulation of *Fgfr2b* has minor effects on the overall absorption function of the intestines.

This study, however, does not preclude changes in other factors, such as the absorption capacity of the villi, and level of plasma hormones that regulate appetite. Cholecystokinin (CCK), for example, is produced by endocrine cells of the duodenum and jejunum, and increased CCK expression is associated with decreased appetite (Naslund and Hellstrom, 2007). Glucagon-like peptide-1 (GLP-1) and GLP-2 are secreted by the enteroendocrine cells in the ileum and colon, and function similarly as CCK (Naslund and Hellstrom, 2007). Therefore, it will be informative to investigate whether the A33-CrePR⁺/Fgfr2b^{flox/flox} mice have a higher level of plasma CCK, GLP-1 and GLP-2 that could have contributed to the trend of lower body weight observed in these mice compared to the controls.

BrdU labelling and active CSP-3 immunostaining on cross sections along the different intestinal segments show normal level of proliferation and apoptosis in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice. An alternative method of measuring proliferation using crypt microdissection also demonstrates normal crypt proliferation in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice. In contrast to BrdU that labels cells in the S-phase (DNA replication phase) of a cell cycle, the vincristine sulphate used in the crypt microdissection technique causes mitotic cells to be arrested at metaphase, hence allowing accurate quantification on the number of mitotic cells in a full crypt volume (Alferez and Goodlad, 2007). However, one disadvantage associated with this technique is that the crypts in the duodenum are much smaller and have numerous mitotic figures compared to the crypts in the distal colon, resulting in frequent overlapping of one crypt with another and can result in inaccurate quantification. To overcome this, crypt microdissection was only performed on tissues from ileum and colon.

The intestinal epithelium of the A33-CrePR⁺/Fgfr2b^{flox/flox} mice also show largely normal differentiation markers, including MUC2-producing goblet cells, the chromogranin A/B-expressing enteroendocrine cells, and the lysozyme-expressing Paneth cells, suggesting that downregulation of *Fgfr2b* does not affect normal intestinal differentiation. Previous chapter has shown that loss of *Fgfr2b* during intestinal development leads to significant reduction of intestinal transcription factors, such as *Tcf1* and *Tcf4* (Chapter 3). In this study, *Tcf4* mRNA expression is largely unperturbed in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice, suggesting that total *Fgfr2b* ablation may be required to see an affect on *Tcf4* mRNA expression, or an alternative signalling pathway may be regulating *Tcf4* mRNA expression in the adult gut.

So far, our data show that downregulation of *Fgfr2b* does not affect intestinal proliferation, survival and differentiation, which is not an expected observation when a growth factor receptor is significantly downregulated. It is possible that total *Fgfr2b* ablation in the intestine may be needed to observe a phenotypic abnormality, and there is currently no inducible intestinal-specific Cre recombinase line that has 100% excision efficiency. Alternatively, *Fgfr2b* may not be essential in maintaining intestinal homeostasis due to possible functional redundancy. Expression analysis of the entire *Fgfs* and *Fgfrs*, except *Fgf19* and *Fgf23*, show presence of numerous *Fgfs* and *Fgfrs* along the adult GI tract, suggesting that other *Fgfs* and *Fgfrs* may be required for maintaining intestinal homeostasis.

Fgf18, for example, is expressed in the duodenum, jejunum and ileum as shown from our data. Interestingly, administration of recombinant mouse FGF18 into normal mice resulted in significant increased of duodenal, jejunal and ileal wet weights due to intestinal hypertrophy (Hu et al., 1998), suggesting a role for *Fgf18* in the small intestine. To date, no study has reported any intestinal defect in *Fgf18*^{-/-} mice, however absence of *Fgf18* leads to perinatal lethality, and proliferation and differentiation defects during osteogenesis, chondrogenesis and lung development (Usui

et al., 2004, Ohbayashi et al., 2002). In addition, the requirement of *Fgfr3*, the receptor for *Fgf18*, has been demonstrated to play a role in intestinal homeostasis.

Fgfr3^{-/-} mice, in which both isoforms of this receptor were ablated, when analysed between postnatal day 1 and 28 showed 50% reduction in the number of intestinal crypts and reduced β -catenin/*Tcf4* signalling (Vidrich et al., 2009). However, when analysed at ten weeks of age, loss of *Fgfr3*^{-/-} led to a modest increase in the number of replicating TA cells and the size of crypts (Arnaud-Dabernat et al., 2008). The difference in results suggests that *Fgfr3* plays different functions during postnatal day 1 to 28, a period that still requires active stem cell proliferation and crypt morphogenesis, and ten weeks of age, a period where the number of crypts is static and crypt stem cells are relatively quiescent compared with the suckling mouse intestine. While we have shown that *Fgfr2b* is not required in the intestine of six-week-old mice, a recent study expressing ubiquitous soluble dominant negative FGFR2b from birth to six weeks postnatally reported no overt gut defect in the GI tract (Parsa et al., 2008), providing further evidence that *Fgfr2b* is not essential for postnatal intestinal development and in the unperturbed adult intestine.

In both murine and human prostate carcinoma, decreased expression of FGFR2 resulted in overexpression of FGFR1 (Jin et al., 2003, Yasumoto et al., 2004). However, our data showed that *Fgfr1b* does not compensate for the significant downregulation of *Fgfr2b*, suggesting that the compensatory mechanism of FGFR1-FGFR2 may be context-dependent.

4.5 Conclusion

In conclusion, we have shown that *Fgfr2b* and *Fgf10* are expressed throughout the adult mouse GI tract from forestomach to distal colon, with a higher expression towards the distal region of the intestine. The A33-CrePR/*Fgfr2b*^{flox/flox} model is ideal for this study due to its higher efficiency of excising the *Fgfr2b* alleles in the distal region of the intestine than in the proximal intestine.

We have also shown that *Fgfr2b* is not required for maintaining intestinal homeostasis, as severe downregulation of *Fgfr2b* across the adult intestine gives little or no effect in intestinal epithelial proliferation, survival and differentiation. This can be accounted by functional redundancy of *Fgfr2b* since the adult GI tract also expresses a wide variety of other *Fgfs* and *Fgfrs*. *Fgfr2b* may also be essential in other contexts, such as intestinal injury and wound healing, which are explored in the next chapter.

Chapter 5: *Fgfr2b* in intestinal injury

5.1 Introduction

Fgfs and *Fgfrs* have been implicated in tissue injury and wound healing. In the GI tract specifically, *Fgfr2b* and *Fgf10* mRNA expression was massively upregulated at the base of the intestinal crypts following small bowel resection, accompanied by significant increase in epithelial proliferation, suggesting a role for *Fgfr2b* and *Fgf10* in enhancing the process of gut adaptation (Tai et al., 2009). *Fgfr2b* and *Fgf7* mRNA was overexpressed in intestinal tissues obtained from patients with IBD, implying a role for *Fgfr2b* and *Fgf7* in intestinal injury (Finch et al., 1996, Brauchle et al., 1996). Similar overexpression was also observed in inflamed and adapting mucosa of patients with UC and familial adenomatous polyposis (FAP), further suggesting an involvement of *Fgfr2b* and *Fgf7* in intestinal repair and adaptive changes (Otte et al., 2005). Administration of either recombinant human (rh) FGF10 or rhFGF7 to DSS-induced colitis models of IBD reduced the severity of colitis injury, promoted intestinal healing, and increased epithelial proliferation (Byrne et al., 2002, Egger et al., 1999, Miceli et al., 1999, Zeeh et al., 1996). Similar observations were also reported in a small intestinal ulceration model in rat following administration of either rhFGF7 or rhFGF10, further suggesting that *Fgfr2b* and its ligands promote wound healing by stimulating epithelial proliferation (Han et al., 2000, Playford et al., 1998).

While these studies demonstrated the therapeutic roles of *Fgf7*, *Fgf10* and *Fgfr2b* in intestinal injury and wound healing, the requirement for these growth factors and receptor in these models has not been established. This study therefore utilises the A33-CrePR⁺/*Fgfr2b*^{flox/flox} line to study the requirement of *Fgfr2b* in DSS-induced UC. The A33-CrePR⁺/*Fgfr2b*^{flox/flox} line provides an excellent model for this study due to

the high excision efficiency of the conditional *Fgfr2b* alleles in the adult colon. We hypothesise that substantial downregulation of *Fgfr2b* in colonic epithelium leads to increased severity of colitis, and delayed wound healing.

5.2 Materials and methods

5.2.1 RT-PCR

Eighteen six-week old male $Fgfr2b^{flox/flox}$ mice were administered 4% (w/v) DSS (31403; Sigma-Aldrich) in their drinking water for five days consecutively, followed by normal drinking water. Mice were culled in batches of three at the following time-points; prior to DSS administration (Day 0), followed by one day (Day 1), three days (Day 3), five days (Day 5), seven days (Day 7), and thirteen days (Day 13) after DSS administration. RNA was extracted from proximal and distal colon respectively, and cDNA synthesis was performed as previously described (Section 2.4). Taqman RT-PCR was performed for *Fgf1*, *Fgf3*, *Fgf7*, *Fgf10*, *Fgf22*, *Fgfr1b*, and *Fgfr2b* as previously described (Section 2.4.6).

5.2.2 In situ hybridisation

Similar setup of experiment as Section 5.2.2 was performed, except twelve mice were used and culled in batches of two at each time-point. Gut tissues were dissected, fixed, processed and embedded as previously described (Section 2.5.2). ISH for *Fgfr2b* was performed as previously described (Section 2.3.3).

5.2.3 RU486 and DSS administration

RU486 mix was prepared as previously described (Section 2.2.1). Forty six-week-old male $A33-CrePR^+/Fgfr2b^{flox/flox}$ and $Fgfr2b^{flox/flox}$ mice were given 250 μ l of RU486 mix until the mice were culled. After the initial fourteen days of RU486 administration, mice were given 4% (w/v) DSS in drinking water for five days consecutively, followed by normal drinking water. Mice were culled in batches of six (three $Fgfr2b^{flox/flox}$ and

three A33-CrePR⁺/Fgfr2b^{flox/flox} mice) on one day (Day 1), three days (Day 3), five days (Day 5), seven days (Day 7), and thirteen days (Day 13) after DSS administration.

5.2.4 Histology scoring

Mice were injected with BrdU solution (550891; BD Biosciences) at 50 mg/kg 1 hr before culling. Gut tissues were dissected in cold PBS, fixed, processed and embedded as previously described (Section 2.5.2). Tissue sections were stained with H&E as previously described (Section 2.5.5). Histology scoring was performed in a blinded fashion by a pathologist according to the parameters shown in Table 5.1. The scores ranged from 0 (normal), 1 (mild), 2 (moderate) to 3 (severe).

5.2.5 Immunohistochemistry

Immunohistochemistry was performed with anti-BrdU and anti-CSP-3 antibodies as previously described (Section 2.5). Morphometry was performed as previously described (Section 2.7).

Table 5.1 Histologic scoring colitis method.

Feature scored	Score	Description
Mononuclear infiltration	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Neutrophil infiltration	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Loss of crypts	0	None
	1	1-2 crypts
	2	3-5 crypts
	3	>5 crypts
Ulceration	0	None
	1	0-10 crypts
	2	>10 crypts
	3	>10 crypts and lamina propria
Hyperplasia	0	None
	1	up to 3-fold increase
	2	3- to 6-fold increase
	3	>6-fold increase

5.3 Results

5.3.1 *Fgf7* is the main ligand for *Fgfr2b* during intestinal injury and wound healing

RT-PCR was used to determine the expression levels of *Fgfr2b*, *Fgfr1b* and their ligands during intestinal injury and wound healing. Proximal and distal colon was assessed separately to determine any differential expression level between them. While some time points show differential expression of *Fgfs* and *Fgfrs* between proximal and distal colon, most time points show closely correlated expression levels (Figure 5.1). Expression data show a substantial 80-fold upregulation of *Fgf7* expression one day following DSS administration, and this level decreases over the duration of the experiment to 50-fold, thirteen days after DSS administration (Figure 5.1). *Fgf10* mRNA levels, in contrast, is initially upregulated by 5-fold, and this level gradually peaks to 12-fold after one week, then subsequently decreases to 9-fold after three days. *Fgf1* mRNA level is also initially upregulated by 5-fold, and gradually decreases to 3.5-fold thirteen days after DSS administration. *Fgfr2b* mRNA is initially upregulated by 40-fold, and this level gradually increases to 50-fold over the thirteen days duration of the DSS treatment. *Fgf3*, *Fgf22* and *Fgfr1b* mRNA levels, in contrast, are upregulated less than 3-fold initially, and the level stays relatively unchanged throughout the thirteen days duration. ISH on colonic gut rolls at all time points using DIG-labelled *Fgfr2* probe show increased expression of *Fgfr2b* mRNA in injured crypts, epithelial cells undergoing re-epithelialisation, restituting crypts, and basal crypts of hyperplastic epithelium (Figure 5.2).

Figure 5.1 Continue on next page

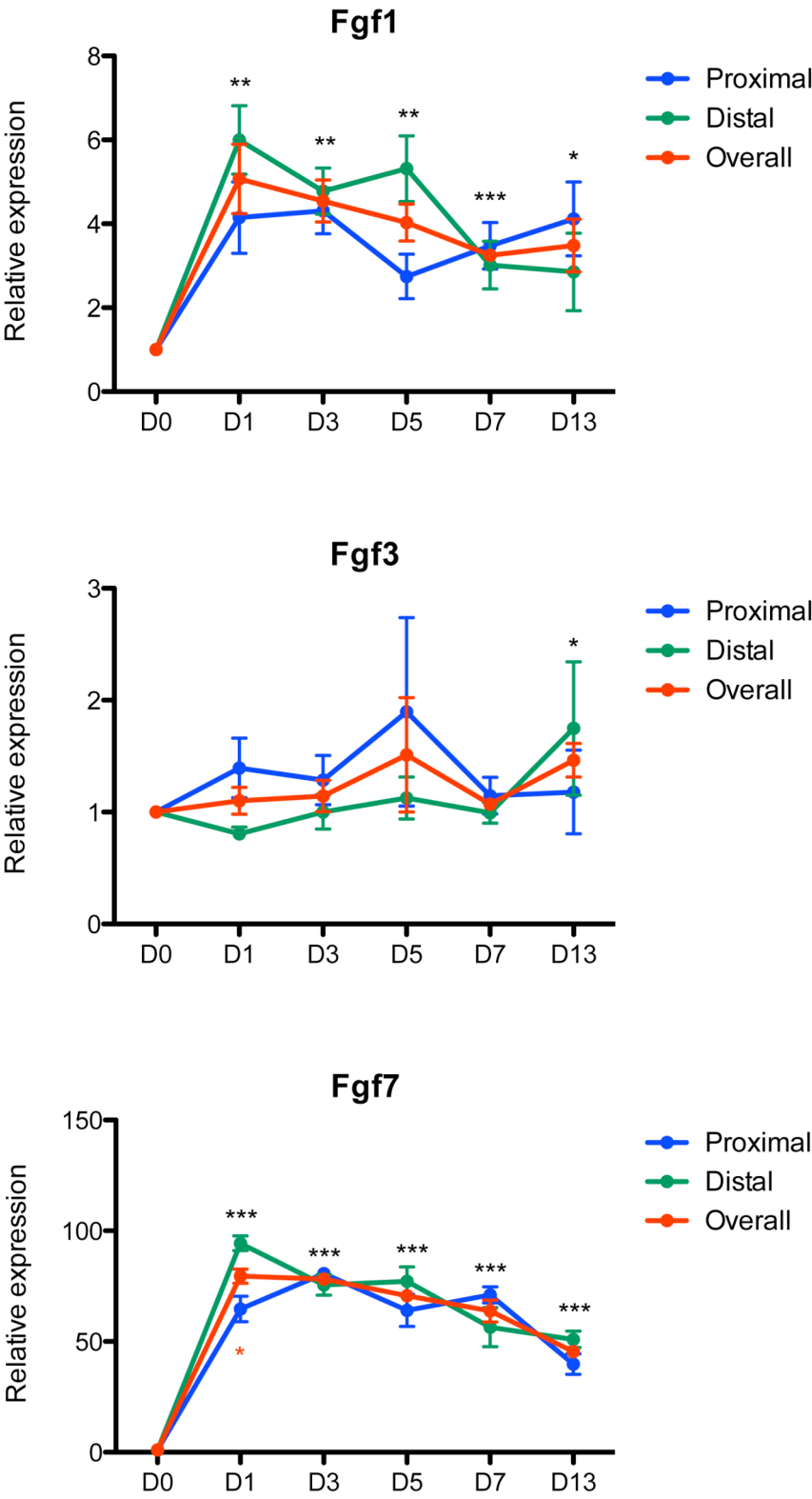
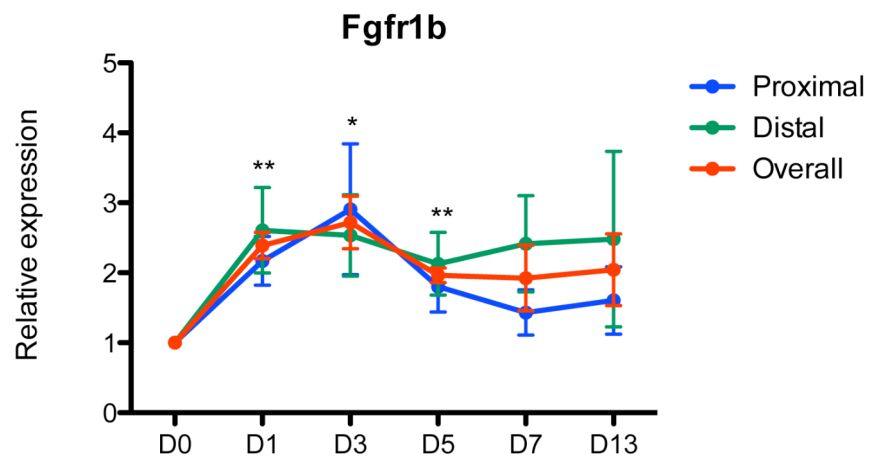
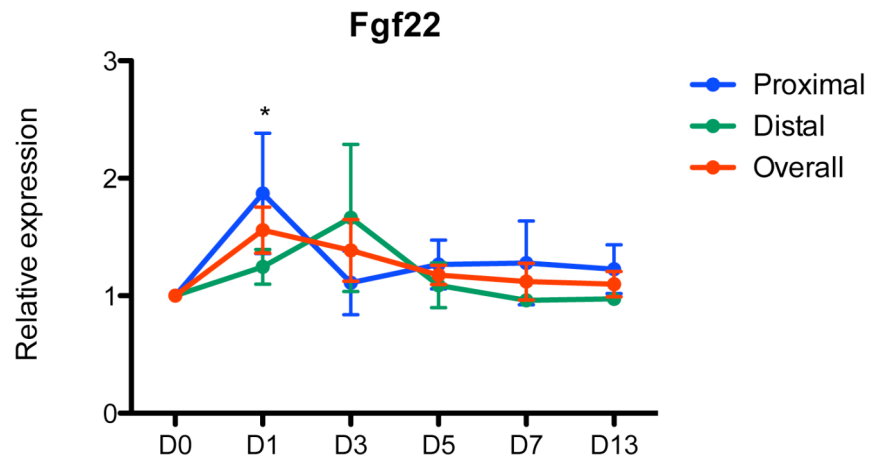
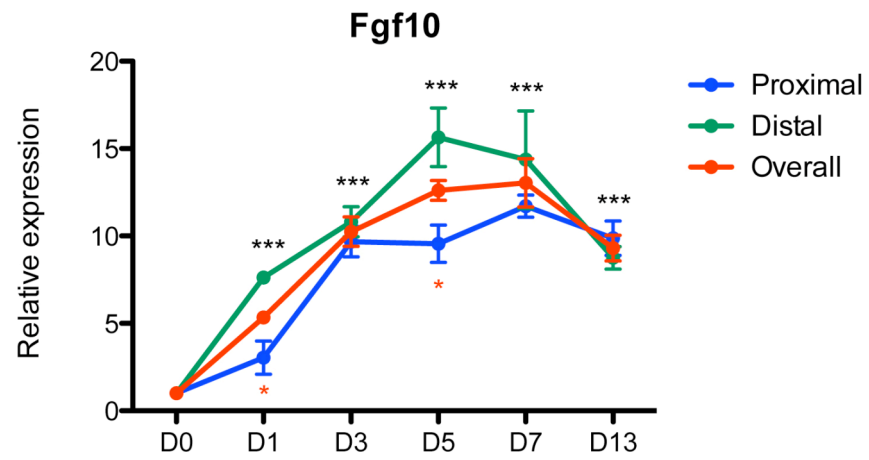


Figure 5.1 Continue on next page



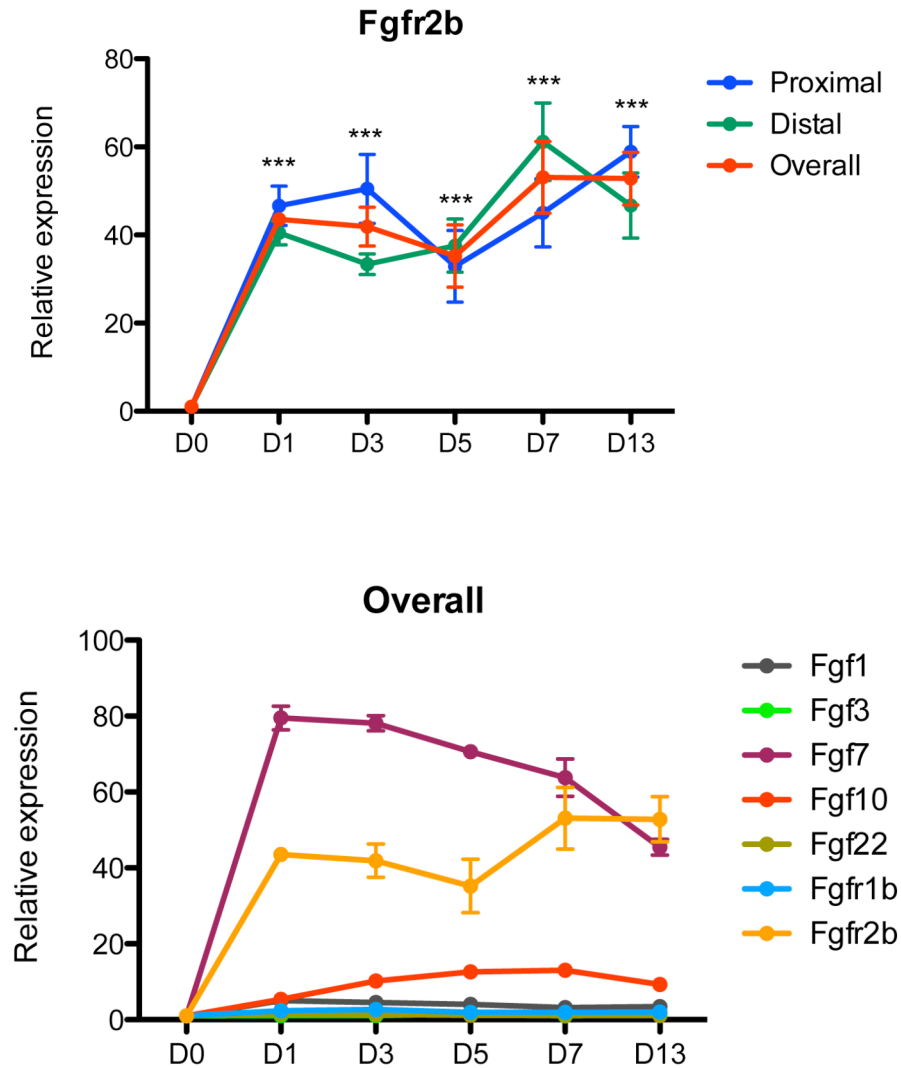
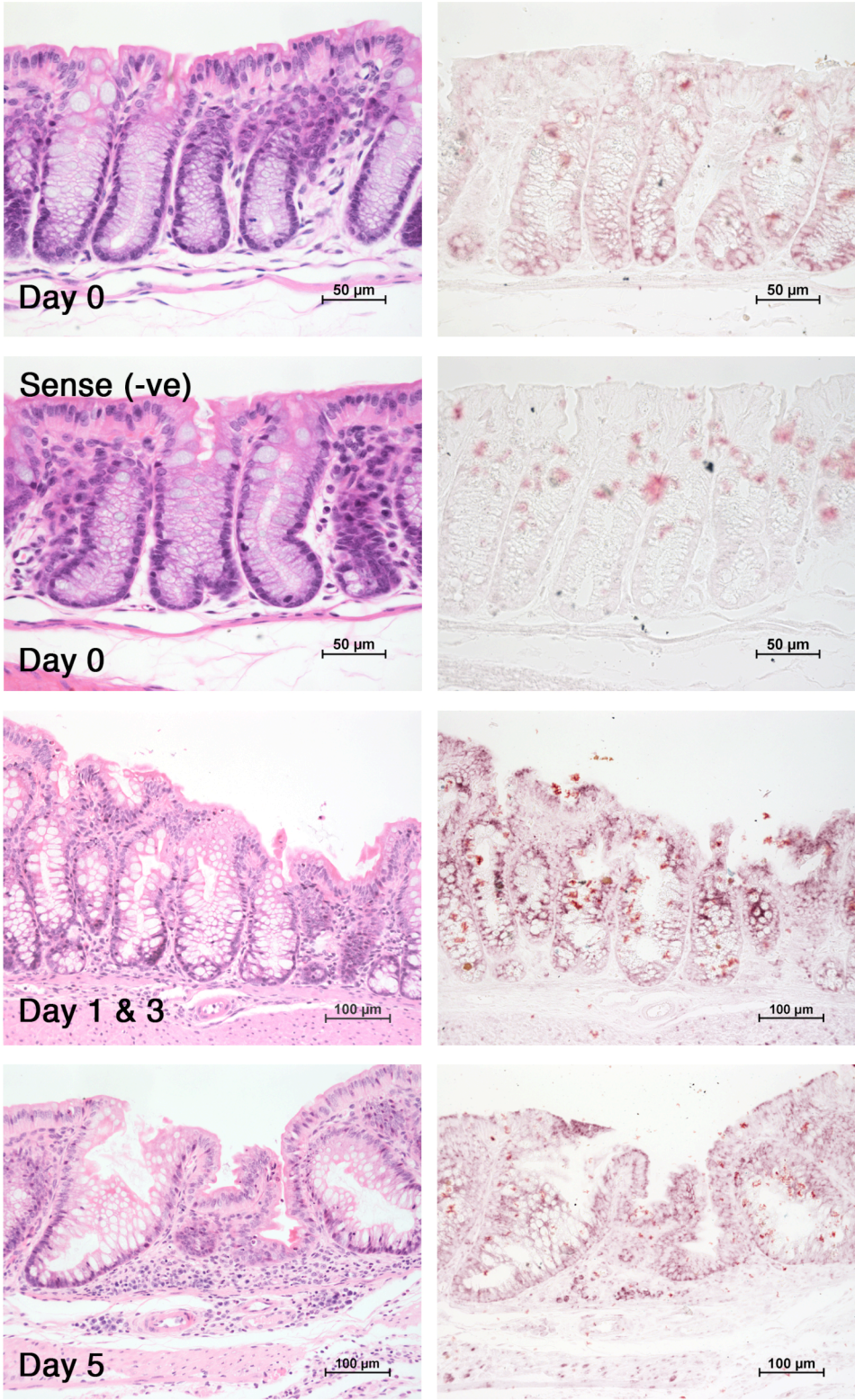


Figure 5.1 Expression levels of *Fgfr2b*, *Fgfr1b* and their ligands in proximal and distal colon of *Fgfr2b*^{flox/flox} mice with colitis.

RNA was extracted from proximal and distal colon at various time points, and subjected to Taqman RT-PCR. D0: Day 0, D1: Day 1, D3: Day 3, D5: Day 5, D7: Day 7, D13: Day 13. ‘D0’ represents the time-point prior to DSS administration. All data was normalised to the mean of *Gapdh* expression, and expression level was relative to that of ‘D0’. Black asterisks indicate significance calculated between the ‘overall proximal and distal value’ and the value at ‘D0’, whereas red asterisks indicate significance calculated between the proximal and distal intestine values. Error bars ($\pm$ SEM) came from three experiments; n = 3 mice/group. *P<0.05, **P<0.01, ***P<0.001 by Student’s t-test.

Figure 5.2 Continue on next page



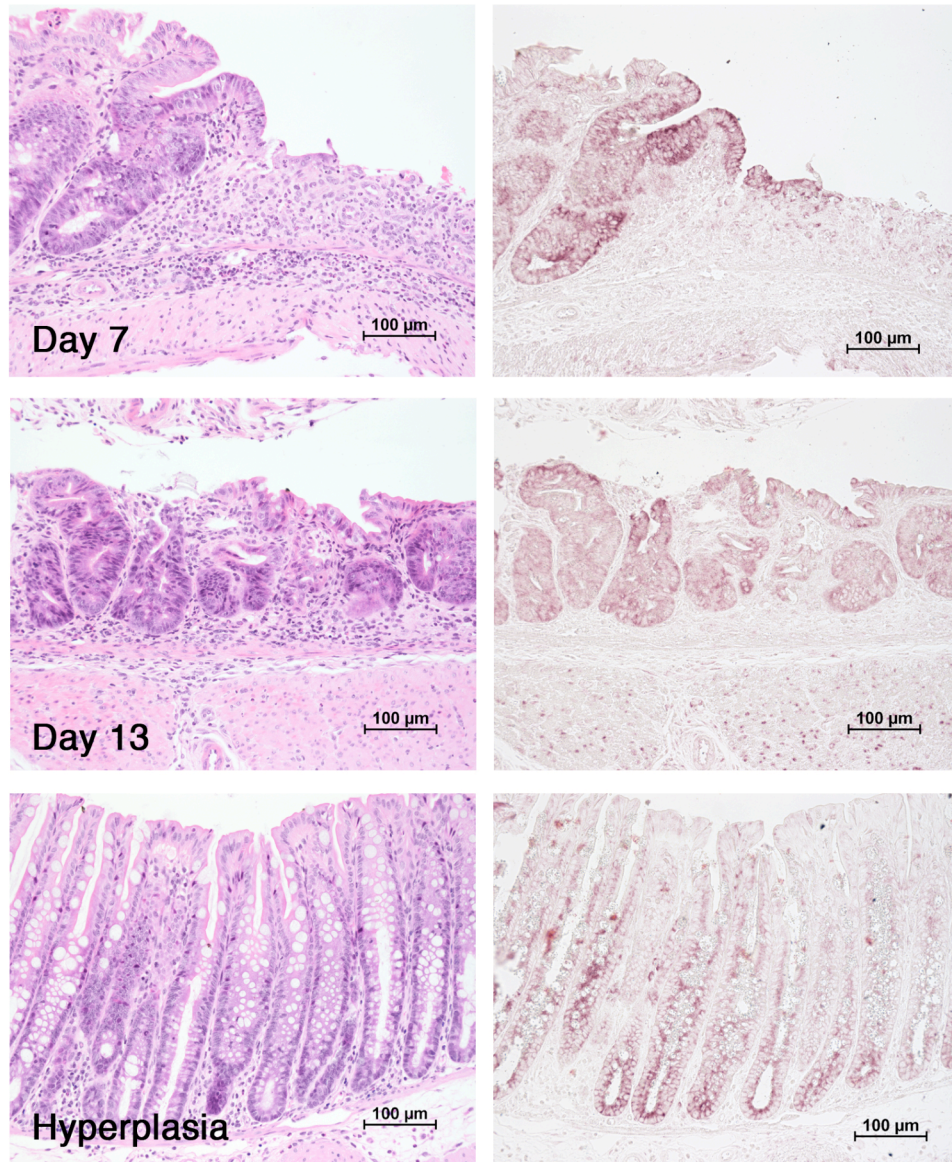


Figure 5.2 Representative images of H&E-stained (left panel) and *Fgfr2* ISH (right panel) distal colon with ulcerative colitis.

These images are a representation of the progression of colitis shown by two *Fgfr2b*^{flox/flox} mice at each time point. Images came from Day 0 (no DSS treatment), Day 1 and 3 (representative image from two time points), Day 5, Day 7 and Day 13. Hyperplasia is seen throughout all the time points with various degrees of severity. Negative control came from DIG-labelled sense *Fgfr2* probe on Day 0 tissue sections.

5.3.2 Characterisation of DSS-induced colitis

Microscopic examination on the GI tract of $Fgfr2b^{flox/flox}$ mice, following DSS administration for five days consecutively, shows reproducible ulcerations of the large intestine (Fig. 5.2). At Day 1 and 3 following DSS administration, the colon shows signs of mucosal erosion including epithelial loss, crypt shortening, and inflammation. Lesions to the intestinal mucosa occurs as discrete areas surrounded by normal epithelium, consistent with a previous study in this model system (Wirtz et al., 2007). Epithelial cell damage is confined to the colon and does not involve other regions of the GI tract, such as the small intestine (data not shown). Crypt loss and oedema are evident by Day 5. Clear signs of mucosal healing, including surface re-epithelialisation and hyperplastic crypts, are evident as early as three days after DSS administration.

5.3.3 Downregulation of *Fgfr2b* increases severity of colitis and delays wound healing

The requirement of *Fgfr2b* in intestinal injury and wound repair in DSS-induced colitis was assessed using the $A33-CrePR^{+}/Fgfr2b^{flox/flox}$ mice. Following a combination of RU486 and DSS administration regime, the $A33-CrePR^{+}/Fgfr2b^{flox/flox}$ mice show several major differences in disease severity compared to the control $Fgfr2b^{flox/flox}$ mice. The $A33-CrePR^{+}/Fgfr2b^{flox/flox}$ mice show a higher degree of crypt loss and ulceration three days after DSS administration (Figure 5.3). The lesions are more widespread, and often include the caecum (data not shown). Mononuclear and neutrophil infiltrations appear to be largely similar between the two groups at all time points. Cryptitis, crypt abscesses, goblet cell depletion and fibrosis are rarely observed in both groups at all time points.

Interestingly, repair of damaged epithelium is also dramatically impaired in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice with minimal surface re-epithelialisation observed three days after DSS administration. The A33-CrePR⁺/Fgfr2b^{flox/flox} mice also maintain higher hyperplasia, loss of crypts and ulceration scores at Day 7 and Day 13 after DSS administration, while at these time points, the control mice have significantly lower corresponding scores. The overall score, which only takes account of mononuclear and neutrophil infiltrations, loss of crypts, ulceration, and hyperplasia, shows a consistently high severity of injury and delayed wound healing in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice compared to the Fgfr2b^{flox/flox} mice (Figure 5.3).

Figure 5.3 Continue on next page

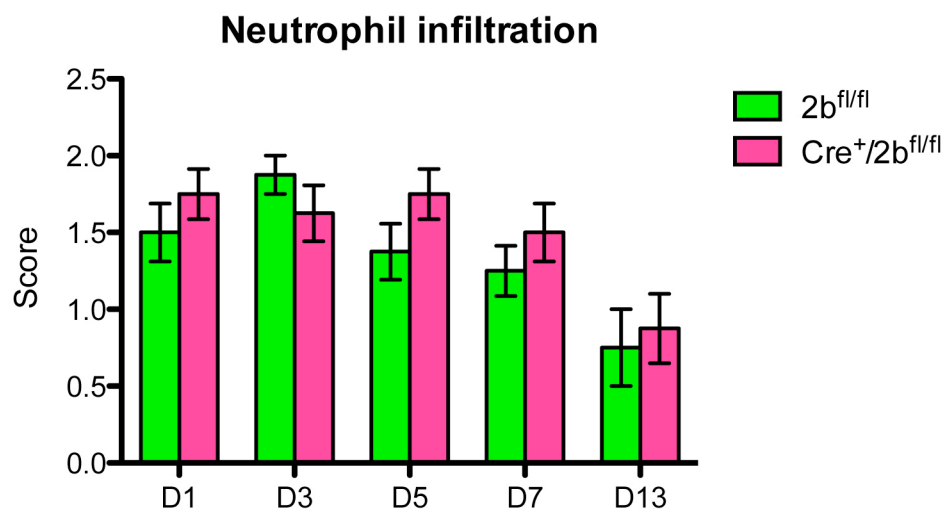
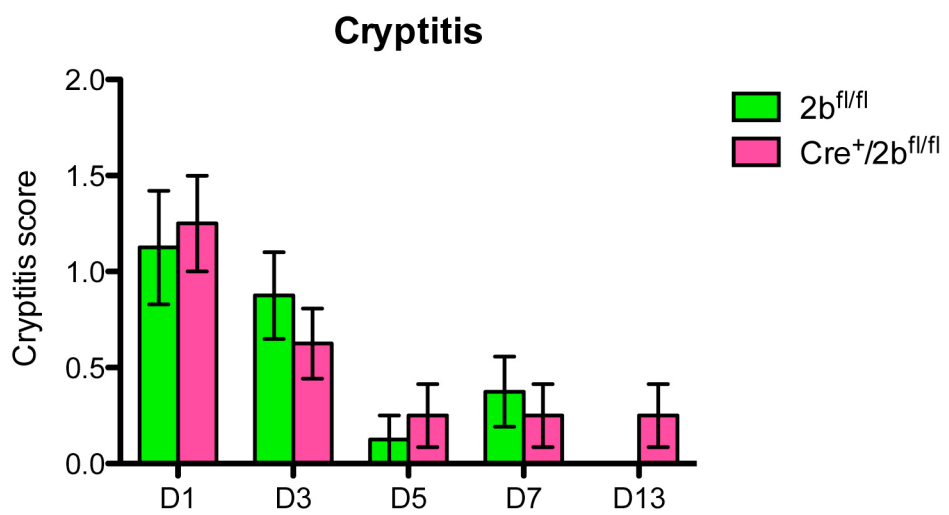
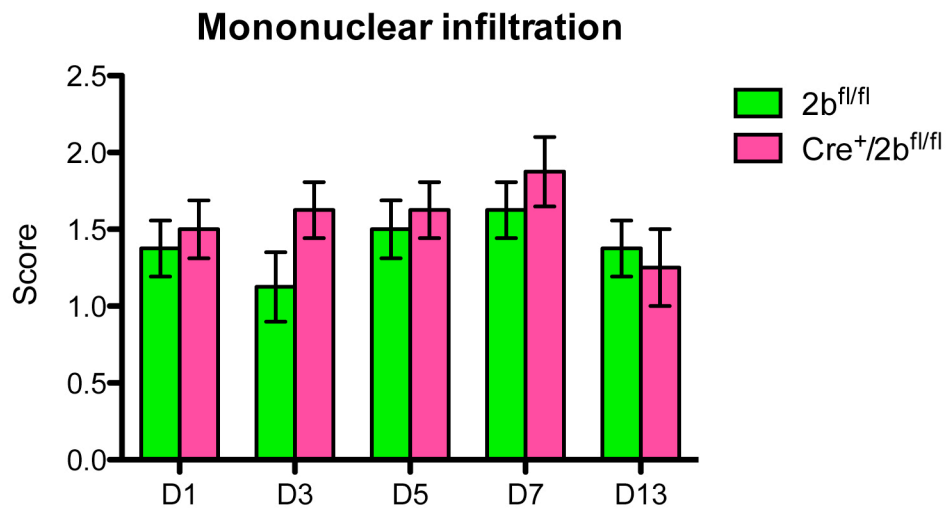


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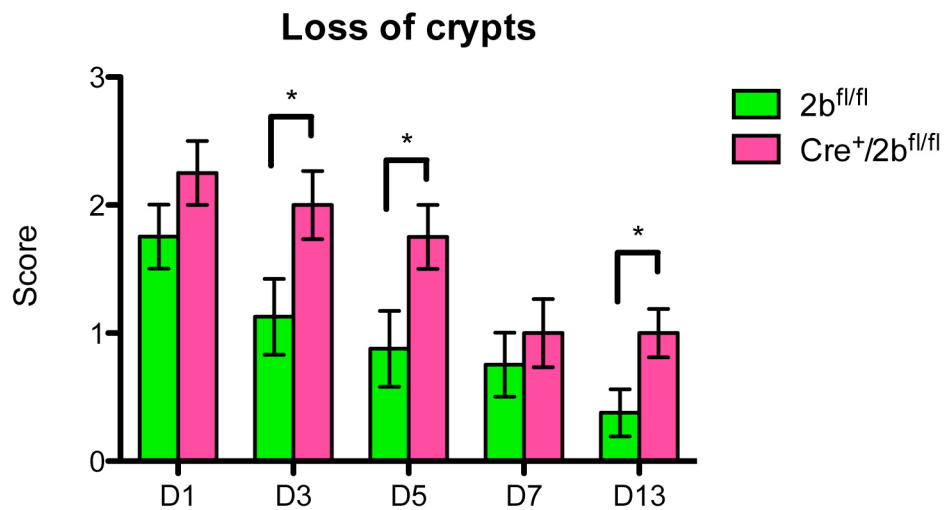
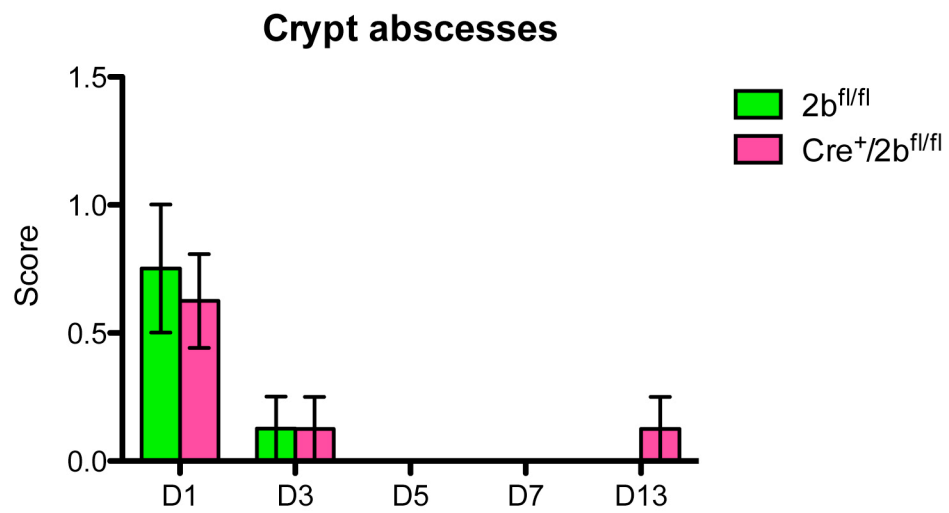
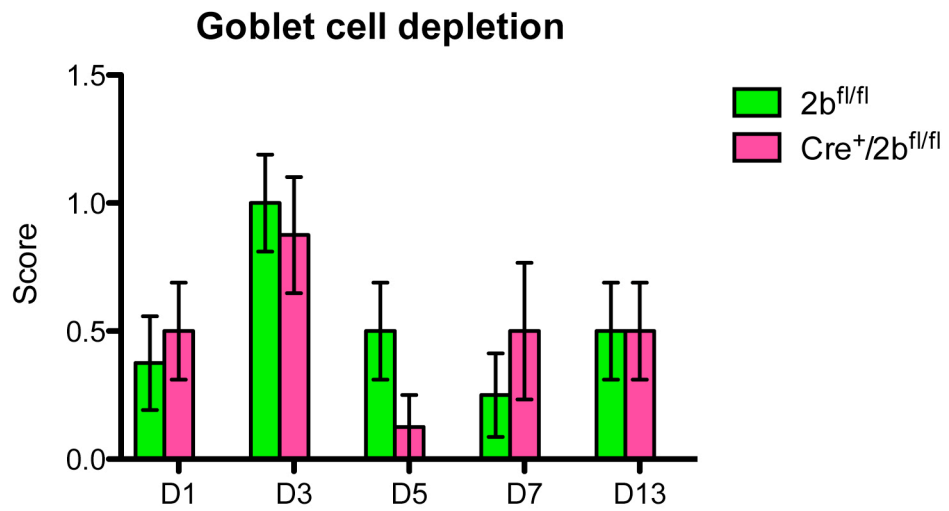
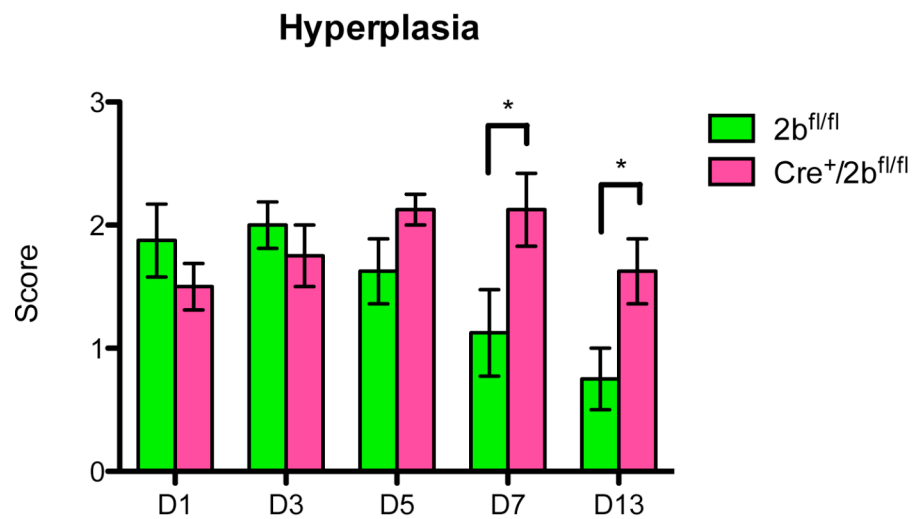
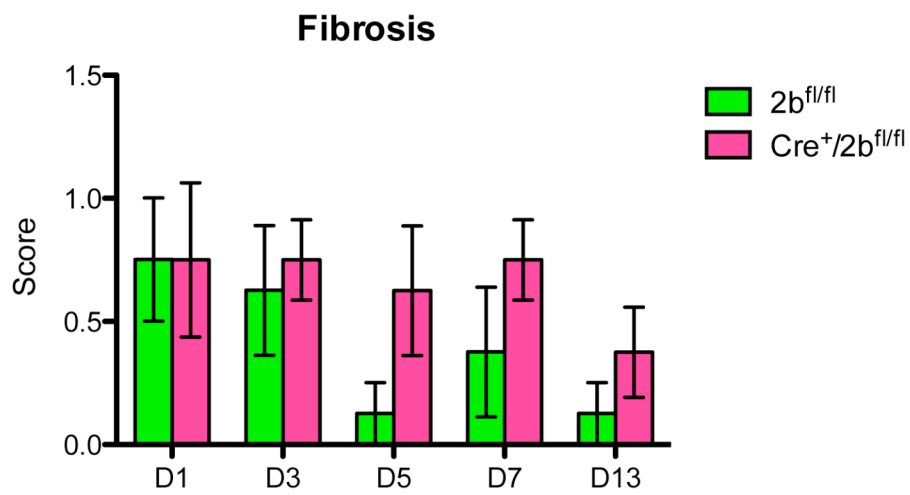
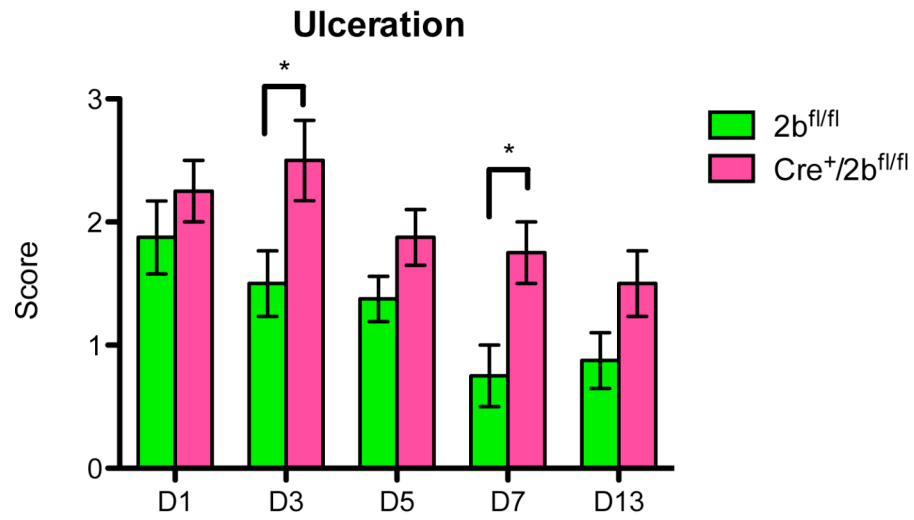


Figure 5.3 Continue on next page



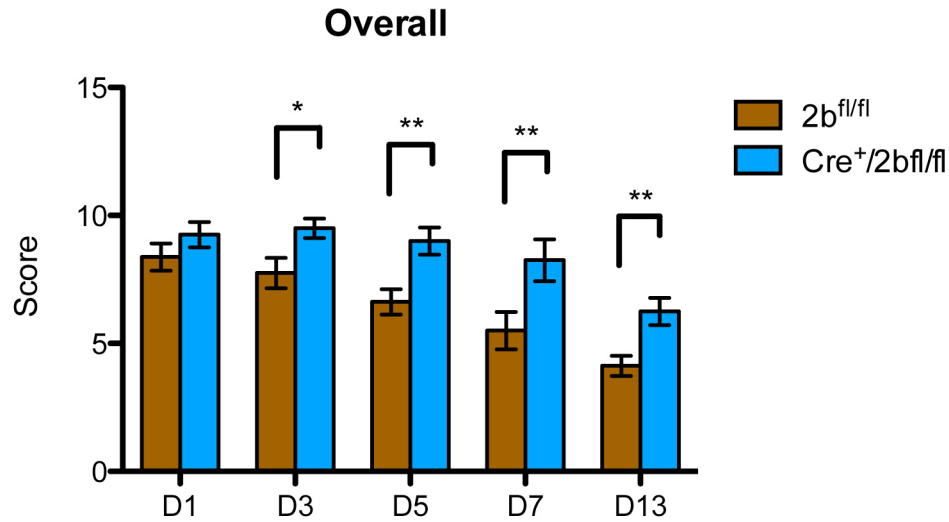


Figure 5.3 Histological scoring of the large intestine of $Fgfr2b^{flx/flx}$ and $A33CrePR^+/Fgfr2b^{flx/flx}$ mice following combination of RU486 and DSS administration.

Scoring was based on overall assessment of the proximal and distal colon. D1: Day 1, D3: Day 3, D5: Day 5, D7: Day 7, D13: Day 13. The $A33CrePR^+/Fgfr2b^{flx/flx}$ mice have more severe injury with delayed wound healing. Error bars ($\pm$ SEM) came from 8 mice/group. The overall data only took account of mononuclear and neutrophil infiltrations, loss of crypts, ulceration and hyperplasia. * $P < 0.05$; ** $P < 0.01$ by Student's t-test.

5.3.4 Downregulation of *Fgfr2b* significantly reduces epithelial proliferation

To determine whether the $A33-CrePR^+/Fgfr2b^{flx/flx}$ mice have an imbalance epithelial proliferation and apoptosis, tissues were immunostained for BrdU and CSP-3, and the positive staining was quantified. $A33-CrePR^+/Fgfr2b^{flx/flx}$ mice consistently show significant reduction of epithelial proliferation at all time points, except Day 13, in the colonic crypts, compared to the $Fgfr2b^{flx/flx}$ mice (Figure 5.4). In contrast, both mice groups rarely show apoptosis, and quantification of apoptotic cells show no significant difference between them (Figure 5.4).

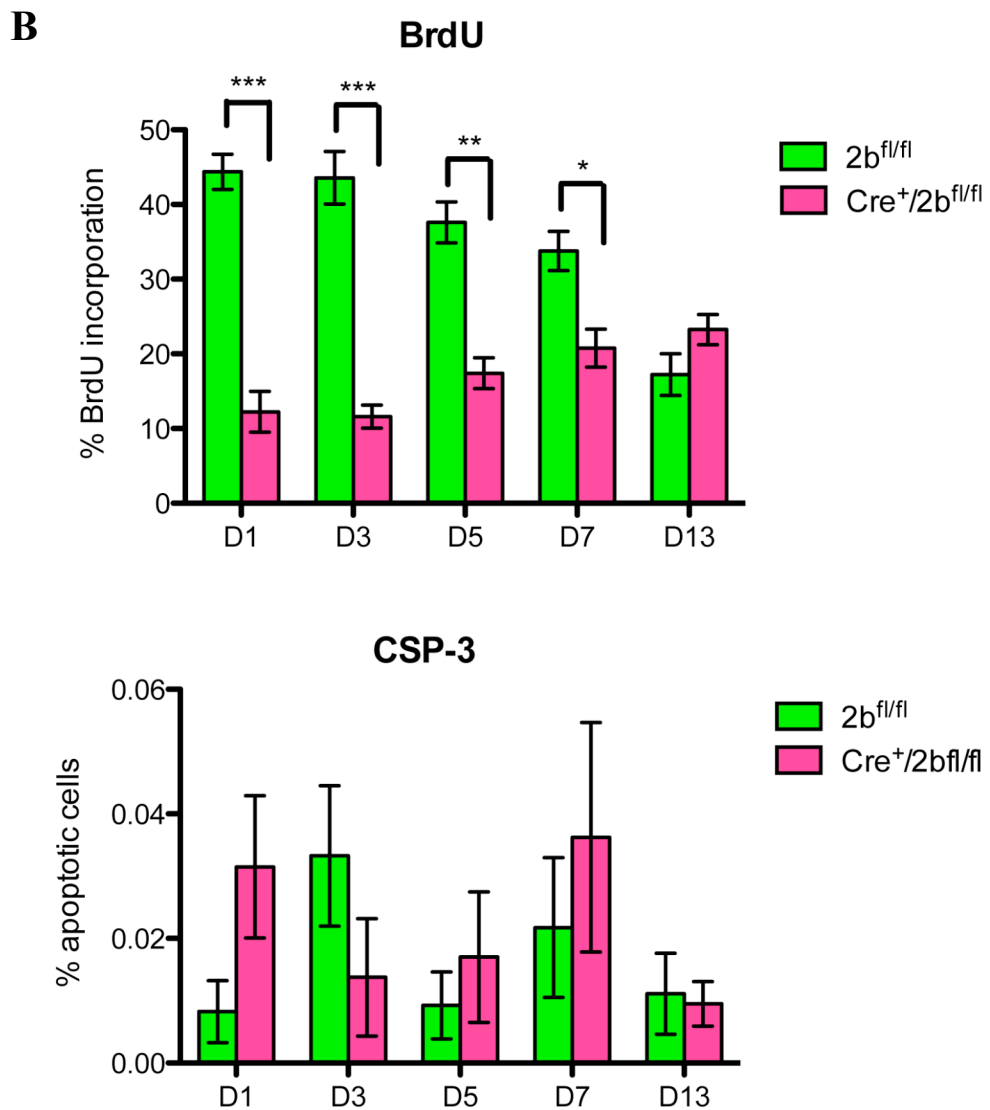
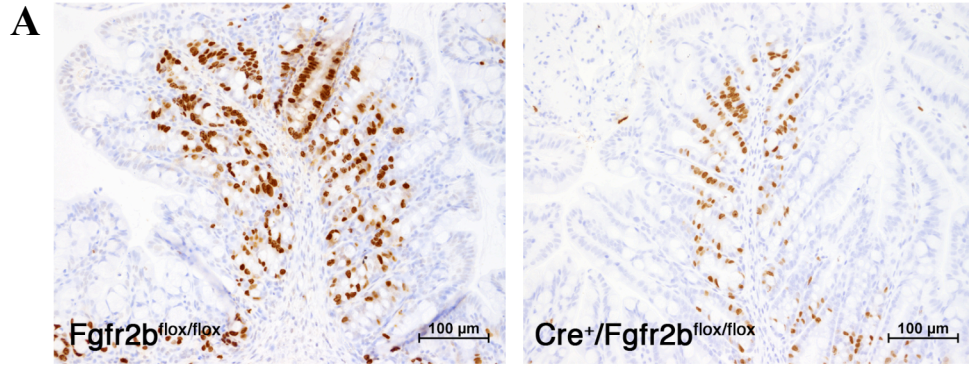


Figure 5.4 Comparison of proliferation and apoptosis in colon of $Fgfr2b^{flox/flox}$ and $A33CrePR^+/Fgfr2b^{flox/flox}$ following combination of RU486 and DSS administration.

(A) Representation of BrdU immunostaining in proximal colon epithelium between both mice groups.

(B) Quantification of the mean percentage of cells positively stained cells in 20 longitudinally sectioned colonic crypts. D1: Day 1, D3: Day 3, D5: Day 5, D7: Day 7, D13: Day 13. The A33CrePR⁺/Fgfr2b^{flox/flox} mice show reduced epithelial proliferation compared to the Fgfr2b^{flox/flox} mice. All data was presented as mean ($\pm$ SEM). * P<0.05; ** P<0.01; *** P<0.001 by Student's t-test; n = 8 animals/group.

5.3.5 Fgfr1b can compensate for the significant downregulation of Fgfr2b during injury and wound repair

Fgfr1b expression level was compared between A33-CrePR⁺/Fgfr2b^{flox/flox} and Fgfr2b^{flox/flox} mice to determine whether *Fgfr1b* may compensate for the downregulation of *Fgfr2b* during tissue injury and wound healing. RT-PCR data shows a trend of upregulation of *Fgfr1b* mRNA expression at all time points, with significance noted at Day 3 and Day 7 in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice compared to the Fgfr2b^{flox/flox} mice (Figure 5.6).

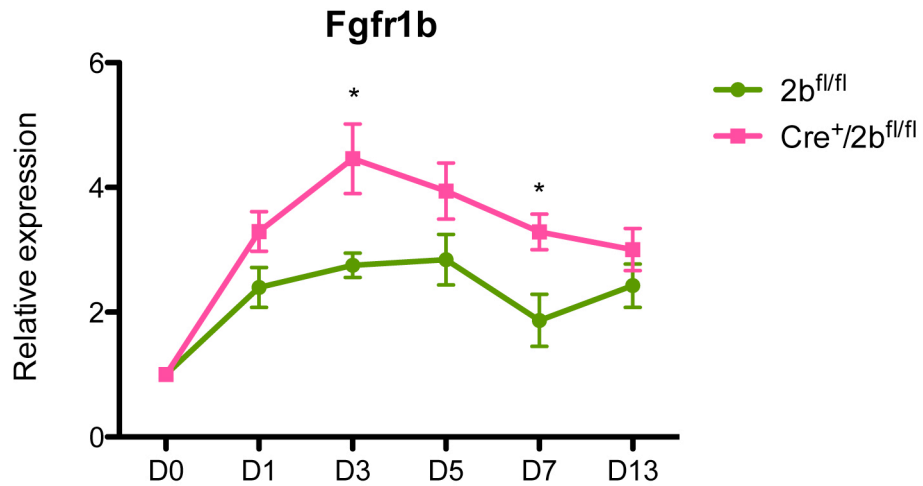


Figure 5.5 Relative expression of *Fgfr1b* in the distal colon of *Fgfr2b*^{flx/flx} and A33CrePR⁺/*Fgfr2b*^{flx/flx} following combination of RU486 and DSS administration.

Fgfr1b can compensate for the downregulation of *Fgfr2b* with significance observed at Day 3 and Day 7. All data was normalised to the means of *Gapdh* expression, and error bars ($\pm$ SEM) came from three experiments; n = 3 animals/group. *P<0.05 by Student's t-test.

5.4 Discussion

There is currently no study that compares expression of multiple *Fgfs* and *Fgfrs* in a colitis model, and this study not only provides such comparison but also the progression of the gene expression across a set of time points. In our colitis model, *Fgfr2b*, *Fgf7* and *Fgf10* are the top three genes that are significantly upregulated at all time points. Interestingly, *Fgf7* shows the highest mRNA expression (approximately 90-fold) starting from one day after DSS administration compared to all other *Fgfs*, suggesting that *Fgf7* is the main ligand for *Fgfr2b* during injury. Such high expression level correlates with previous studies showing upregulation of *Fgf7* mRNA by 50 to 150-fold higher in patients with IBD (Brauchle et al., 1996), and by 160-fold within 24 hours in skin injury in rats (Werner et al., 1992).

While *Fgf7* expression decreases over time, *Fgf10* expression is upregulated at later time points even though the level of expression is nowhere near the level of *Fgf7*, indicating that *Fgf10* may play a more important role in the wound healing process. Administration of rhFGF10 has been shown to stimulate epithelial proliferation in small intestinal ulceration models, and induce *in vitro* migration in human epithelial colorectal adenocarcinoma Caco-2 cell line (Han et al., 2000), suggesting that *Fgf10* acts as a wound-healing agent to stimulate epithelial cell proliferation and hastens wound closure.

The higher *Fgfr2b* expression level than *Fgfr1b* throughout the time points suggests that *Fgfr2b* is the more important receptor during intestinal injury and wound healing. While the requirement of *Fgfr1b* in intestinal injury and wound healing has not been investigated, *Fgfr1b*^{-/-} mice have no obvious phenotype, but *Fgfr1c*^{-/-} embryos died at E9.5 during gastrulation due to defect in cell migration through the primitive streak (Partanen et al., 1998). Mice deficient in both *Fgfr1* isoforms displayed phenotypes similar to that of *Fgfr1c*^{-/-} embryos (Deng et al.,

1994, Yamaguchi et al., 1994), demonstrating the possible functional redundancy of *Fgfr1b*.

The expression level of *Fgfr2b*, however, does not match the expression level of *Fgf7*. In normal human intestine, *Fgf7* expression has been demonstrated in stromal fibroblasts underlying the mature enterocytes in the colon, but in human IBD cases, *Fgf7* expression can also be contributed by activated (but not resting) intestinal $\gamma\delta$ intraepithelial T lymphocytes (IELs) (Finch et al., 1996, Rubin et al., 1989, Aaronson et al., 1991, Boismenu and Havran, 1994). This suggests that the high *Fgf7* expression observed earlier is contributed by the additional source of *Fgf7* expression, and the activation of T-lymphocytes may be independent of the level of *Fgfr2b*. Mice deficient of $\gamma\delta$ ($\text{TCR}\delta^{-/-}$) have increased susceptibility to DSS-induced mucosal injury and delayed wound repair, suggesting that $\gamma\delta$ IEL cells help preserve the integrity of damaged epithelium by providing localised delivery of KGF (Chen et al., 2002).

ISH with DIG-labelled antisense *Fgfr2* probe shows an upregulation of *Fgfr2b* mRNA in epithelial crypts at all time points, including damaged crypts, basal hyperplastic crypts, and regenerating crypts, demonstrating that *Fgfr2b* is expressed by cells that require a strong proliferation impulse. Our data is in agreement with a previous study demonstrating overexpression of *Fgfr2b* in the mucosal epithelial cells lining the colonic crypts of IBD patients (Finch et al., 1996, Brauchle et al., 1996).

To better understand the requirement of *Fgfr2b* in intestinal injury and wound healing, both $\text{Fgfr2b}^{\text{flox/flox}}$ and $\text{A33-CrePR}^+/\text{Fgfr2b}^{\text{flox/flox}}$ mice were given a combination of RU486 and DSS to ensure constant downregulation of *Fgfr2b* during colitis. The colon of these mice was monitored over a period of thirteen days. Histological scoring data shows a more severe loss of crypts and ulceration in the $\text{A33-CrePR}^+/\text{Fgfr2b}^{\text{flox/flox}}$ mice than in the control $\text{Fgfr2b}^{\text{flox/flox}}$ mice, suggesting that downregulation of *Fgfr2b* increases the susceptibility to intestinal injury.

Surprisingly, the mononuclear and neutrophil infiltration scores are largely similar between both mice groups, indicating that downregulation of *Fgfr2b* does not affect the infiltration ability of mononuclear and neutrophils at the site of injury. A previous study on *Fgfr2b*-null skin showed a significant increase of $\gamma\delta$ IELs in *Fgfr2b*-null skin (Grose et al., 2007), however we were not able to assess the level of $\gamma\delta$ IELs in our models as the antibody failed to recognise the antigen in both formalin-fixed paraffin-embedded and fresh frozen tissue sections by IHC in our hands. There is little evidence of cryptitis, crypt abscesses, goblet cell depletion and fibrosis, which are phenotypes associated with more advanced stages of colitis, indicating that the level of *Fgfr2b* downregulation in our models may not be sufficient to observe these advanced stages.

Our data also shows that regeneration of the intestinal mucosa is significantly delayed but not abrogated in the A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice. While the control A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice show high extent of hyperplasia at Day 3 that gradually decreases over time, the A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice show a peak hyperplasia score at Day 7. Additionally, A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice have significantly higher severity score of loss of crypts and ulceration at Day 7 and Day 13, implying that downregulation of *Fgfr2b* leads to delayed wound healing.

Assessment of epithelial proliferation and apoptosis on the colonic crypts shows a significant reduction of proliferation in the A33-CrePR⁺/*Fgfr2b*^{flox/flox} mice, suggesting that this observation contributes to the increased susceptibility to injury and delayed wound healing observed in these mice. Our findings are consistent with a previous study in a skin injury model showing that mice expressing dominant-negative *Fgfr2b* transgene displayed substantially delayed re-epithelialisation in wounded skin due to reduced proliferation rate of epidermal keratinocytes at the wound edge (Werner et al., 1994). Interestingly, while epithelial proliferation in the controls decreases over the thirteen days period, the

epithelial proliferation in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice increases slowly and gradually with a peak proliferation rate achieved at Day 13, even though the peak rate is nowhere near to that of the controls, indicating that the epithelial cells in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice are attempting to proliferate as much as possible even at Day 13. It will be interesting to extend the duration of the experiment beyond thirteen days to determine whether the A33-CrePR⁺/Fgfr2b^{flox/flox} mice will achieve similar peak proliferation rate as the controls, and the length of time needed for these mice to complete the wound healing process. Additionally, the level of apoptosis is unchanged in the A33-CrePR⁺/Fgfr2b^{flox/flox} mice compared to the controls, suggesting that downregulation of *Fgfr2b* does not affect cell survival during injury and wound healing.

The existence of parallel or compensatory mechanisms operating to maintain intestinal homeostasis may account for the ability of the A33-CrePR⁺/Fgfr2b^{flox/flox} mice to eventually repair intestinal lesions. In both murine and human prostate carcinoma, decreased expression of FGFR2 resulted in overexpression of FGFR1 (Jin et al., 2003, Yasumoto et al., 2004). Indeed, our data show an upregulation trend of *Fgfr1b* expression in the colitis model of A33-CrePR⁺/Fgfr2b^{flox/flox} mice, with significance observed at Day 3 and 7 after DSS administration, suggesting that *Fgfr1b* can, to some extent, compensate for the downregulation of *Fgfr2b*. Despite this, the A33-CrePR⁺/Fgfr2b^{flox/flox} mice continue to have more severe injury and delayed wound healing than the controls, suggesting that *Fgfr1b* compensation is insufficient to induce the protective effect and tissue repair in the absence of *Fgfr2b*, and other cytokines including other *Fgfs* and *Fgfrs* may contribute to the wound repair process.

Fgf2, for example, has been shown to be upregulated within 12 hours following radiation of the small intestine, and administration of rhFGF2 prior to irradiation significantly enhanced crypt stem cell survival (Houchen et al., 1999). Administration of rhFGF20 to DSS-colitis mice significantly reduced the severity and extent of mucosal damage, and

enhanced wound repair (Jeffers et al., 2002). This was an interesting finding, given that *Fgf20* is not expressed in the normal mouse GI tract as shown by our data (Chapter 4, Figure 4.10). This is also confirmed by another study showing *Fgf20* mRNA being absent in the normal intestinal mucosa but present in adenomas of *Apc*^{Min/+} mice (Chamorro et al., 2005), indicating a complex mechanism by which *Fgfs* and *Fgfrs* act during tissue injury and wound healing, including for members that are not expressed in normal GI tissue.

Numerous studies have so far shown the therapeutic roles of *Fgf7* and *Fgf10* in intestinal injury and wound healing, but no study to date has investigated the specific roles/requirement of *Fgf7* or *Fgf10* in this scenario. *Fgf7*^{-/-} mice have no overt phenotype, and *Fgf7* is not required for skin injury and healing, suggesting a possible functional redundancy of *Fgf7* (Guo et al., 1996). However, the *Fgf7*^{-/-} mice will need to be challenged with DSS to ascertain its requirement in intestinal injury and wound healing. Due to perinatal lethality associated with *Fgf10*^{-/-}, investigation on the requirement of this growth factor in intestinal injury and wound healing will require a conditional *Fgf10* line, and this line has recently been generated (Abler et al., 2009). Subsequent crossing of conditional *Fgf10* line with the A33-CrePR generating the A33-CrePR/*Fgf10*^{lox/lox} line will enable the investigation on the requirement of *Fgf10* in DSS-induced colitis.

5.5 Conclusion

In conclusion, we have shown that *Fgf7* is the main ligand for *Fgfr2b* during colonic injury and wound healing. *Fgf10* may play a role in the wound healing process, but its requirement in colonic injury and wound healing will need the use of a conditional *Fgf10* line crossed to an inducible intestinal-specific *Cre* line. Epithelial cells that require strong proliferation impulse, such as injured and regenerating crypts, heavily express *Fgfr2b*.

We have also shown that *Fgfr2b* is required for reducing colonic susceptibility to injury, and promoting wound repair. Downregulation of *Fgfr2b* leads to a greater extent of injury and delays wound healing in DSS-induced colitis, contributed by reduced epithelial proliferation. This knowledge will contribute to our understanding of the roles played by *Fgfs* and *Fgfrs* in human IBD.

Chapter 6: Characterisation of stomach-specific promoters

6.1 Introduction

Normal gastric development in the mouse requires *Fgfr2b* and *Fgf10*, as a previous study examining *Fgfr2b*^{-/-} or *Fgf10*^{-/-} embryos showed disproportionately reduced glandular stomach with absence of early differentiation markers, including the GATA family of transcription factors and H⁺/K⁺-adenosine triphosphate (ATP4b), and abnormal expression of the hedgehog family of signalling molecules (Spencer-Dene et al., 2006). In the adult rat stomach, *Fgfr2b* mRNA is expressed at a level of two to three times higher than that in the liver and intestine (Housley et al., 1994). FGFR2b is also expressed in the normal human gastric tissue by parietal cells and surface mucous cells, and is upregulated in gastric carcinoma tissues (Matsunobu et al., 2006, Shin et al., 2000), suggesting a role for *Fgfr2b* in normal gastric homeostasis and carcinogenesis. However, the requirement of *Fgfr2b* in gastric homeostasis and injury has not been demonstrated, and therefore, this study broadly aims to understand how targeted ablation of *Fgfr2b* in adult stomach can affect epithelial proliferation, survival, differentiation, and response to injury.

A previous study has shown that parietal cell ablation leads to accumulation of pre-parietal, pre-neck cells and depletion of zymogenic chief cells, suggesting that parietal cells are required for normal coordination of migration-associated terminal differentiation of pit, neck and chief cells (Li et al., 1996). Therefore, using an animal model to study *Fgfr2b* ablation in the parietal cells will allow us to understand the requirement of *Fgfr2b* in gastric homeostasis. Additionally, the first line of defence against injury and *Helicobacter* species infiltration is the mucus produced by the gastric surface cells, and targeted ablation of

Fgfr2b in the gastric surface mucosa will allow us to understand the requirement of *Fgfr2b* during injury. We hypothesise that ablation of *Fgfr2b* in the gastric parietal and surface mucous cells leads to disrupted epithelial proliferation and apoptosis, aberrant differentiation, and increased susceptibility to injury.

As loss of *Fgfr2b* is perinatal lethal, this study will require the use of a stomach-specific inducible Cre-expressing line crossed with our conditional *Fgfr2b* line. Since there is currently no inducible stomach-specific Cre line, the initial aim of this study is to generate two novel inducible stomach-specific Cre lines that target Cre expression into the two sites of *Fgfr2b* expression independently: parietal cells and surface mucous cells. The minimal promoters used were from *Atp4b* and trefoil factor 1 (*Tff1*) respectively. The *Atp4b* minimal promoter has been well characterised and used successfully by several studies to direct transgene expression into the parietal cells (Syder et al., 2004, Oh et al., 2005). In contrast, the *Tff1* minimal promoter has been partially characterised (Terada et al., 2001), and has not been used in any study to date to drive transgene expression, therefore the *Tff1* promoter region will require further characterisation to find the optimal region to drive stomach-specific Cre expression. Generation of these inducible stomach-specific Cre-expressing lines would represent important new tools for the future to study loss-of-function of *Fgfr2b* and other conditional alleles in normal development and disease models, such as the *Helicobacter felis* infection model of gastric adenocarcinoma (Wang et al., 1998).

6.2 Materials and Methods

6.2.1 *In situ* hybridisation

Adult stomach tissue from three six-week-old male C57BL/6 mice weighing approximately 23 g to 26 g was dissected, fixed, and embedded as previously described (Section 2.5.2). To visualise the localisation and expression of *Fgfr2b* mRNA, ISH was performed on tissue sections using DIG-labelled antisense *Fgfr2* probe as previously described (Section 2.3.3).

6.2.2 DNA cloning

Genomic DNA was extracted from 0.5 cm³ of liver tissue from a six-week-old male C57BL/6 mouse according to Section 2.10.1. *Atp4b* minimal promoter and two *Tff1* promoter fragments (*Tff1*_Fragment 3 and *Tff1*_Fragment 5) were amplified by PCR from the genomic DNA using *PfuUltra* High-Fidelity Taq polymerase (600382; Stratagene) to obtain minimal nucleotide errors. Only two *Tff1* promoter fragments were generated by PCR because the remaining *Tff1*_Fragment 1, 2 and 4 could be easily generated by restriction digest from *Tff1*_Fragment 5. *Atp4b* and *Tff1* promoter fragments were generated with additional restriction sites on either end to facilitate cloning into a vector (Figure 6.1 and Table 6.1). The 20 µl PCR reaction contained 1X *PfuUltra* HF reaction buffer, 0.2 mM of each dNTP, 2.0 or 2.5 mM of MgCl₂, 0.3 µM of each primer, 1 U of *PfuUltra* HF DNA polymerase, and 50 ng of genomic DNA. The PCR cycling parameters were similar to Section 2.4.4, except the annealing temperature of the primers was according to Table 6.1. PCR products were analysed by agarose gel electrophoresis to confirm correct amplicon size (Section 2.4.5), and gel-purified to obtain a pure amplicon (Section 2.10.2).

PCR products were digested with the appropriate restriction enzymes corresponding to their additional restriction sites, and column-purified as previously described (Section 2.10.2). An appropriate amount (see formulae below) of luciferase cloning vector pGL3-Basic (E1751, Promega) was digested and column-purified as previously. The amount of luciferase cloning vector required was calculated according to the following formulae:

$$\frac{\text{amount of vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \frac{3}{1} = \text{amount of insert (ng)}$$

The purified PCR fragments were ligated into the purified cloning vectors, and the recombinant plasmids were transformed into *E. coli* DH5 α cells (Section 2.10.4 and 2.10.6). Twelve transformed colonies for each recombinant plasmid were grown in 5 ml mini LB cultures supplemented with 50 μ g/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7). DNA sequencing was performed on 150 ng of the recombinant plasmids to validate the nucleotide sequence accuracy of the PCR fragments (Section 2.10.8). Tff1_Fragment 5 was sequenced with primers SEQ1 to SEQ10, Tff1_Fragment 3 was sequenced with a combination of SEQ1, and SEQ6 to SEQ10, and Atp4b minimal promoter was sequenced with a combination of SEQ1, SEQ11 and SEQ12 (Table 6.2). Once the nucleotide sequences were verified to be 100% accurate, the mini-cultures were used to inoculate 250 ml maxi LB cultures supplemented with 50 μ g/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7).

The recombinant plasmid of Tff1_Fragment 5 was separately double digested with appropriate restriction enzymes corresponding to Tff1_Fragment 1, Tff1_Fragment 2 and Tff1_Fragment 4, and column-purified (Section 2.10.2, 2.10.3 and Table 6.1). An appropriate amount of the pGL3-Basic vector was digested and column-purified similarly. The purified PCR fragments were ligated into the purified cloning vectors, and the recombinant plasmids were transformed into *E. coli* DH5 α cells as

previously. Five transformed colonies for each recombinant plasmid were grown in 5 ml mini LB cultures supplemented with 50 µg/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7). Following a test restriction digest on a small amount of the recombinant plasmids and subsequent verification of DNA fragment size by agarose gel electrophoresis, one mini culture from each recombinant plasmid was used to inoculate 250 ml maxi LB cultures supplemented with 50 µg/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7).

6.2.3 Promoter analyses

All five *Tff1* luciferase constructs were transiently transfected into three cell lines: GSM10 (a mouse gastric surface mucous cell line), AGS and HGT101 (two human gastric cancer lines), whereas the *Atp4b* luciferase construct was transiently transfected into MGE507 (a mouse parietal cell line) (Section 2.11.1). Transient transfection and luciferase assay were performed according to Section 2.11.4 and 2.11.5. Following verification that *Tff1*_Fragment 1 (thereafter known as ‘*Tff1* minimal promoter’) and *Atp4b* minimal promoter gave the best luciferase activity, both luciferase constructs were transiently transfected into 3T3 (mouse fibroblast), HC11 (mouse mammary epithelium) and nTERT (human keratinocyte) cell lines to determine the specificity of these minimal promoters. Transient transfection and luciferase assay were performed as previously.

To determine the positive regulatory elements on the *Tff1* minimal promoter, the minimal promoter was transiently transfected into two separate GSM10 cell lines that were previously grown for 24 hrs in either serum-free media or serum-free media supplemented with 10 ng/ml epidermal growth factor (EGF). Transient transfection and luciferase assay were performed as previously.

6.2.4 Generation of transgenic constructs

Both luciferase constructs carrying the *Tff1* and *Atp4b* minimal promoters were double digested with their respective restriction enzymes to obtain the minimal promoters, made blunt-ended by Klenow polymerase (M0210L; NEB), and gel-purified (Section 2.10.2, 2.10.2 and Fig. 6.1). A CreER^{T2} plasmid (a kind gift from Prof. Chambon, Strasbourg, France) was double digested with NdeI and BspEI, made blunt-ended and gel-purified as previously. Both minimal promoters were ligated into the CreER^{T2} plasmid, and the recombinant plasmids were transformed into *E. coli* DH5 α cells (Section 2.10.4 and 2.10.6). Twelve transformed colonies for each recombinant plasmid were grown in 5 ml mini LB cultures supplemented with 50 μ g/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7). DNA sequencing was performed on 150 ng of the recombinant plasmids to verify the orientation of the cloned minimal promoters (Section 2.10.8 and Table 6.2). Once the correct colony was identified, the mini-cultures were used to inoculate 250 ml maxi LB cultures supplemented with 50 μ g/ml ampicillin, and recombinant plasmids were purified from these cultures (Section 2.10.7). 40 μ g of Tff1-CreER^{T2} and Atp4b-CreER^{T2} were double digested with SalI/AatII and SalI/BssSI and gel-purified (Section 2.10.2 and 2.10.3). DNA sequencing was performed on these purified constructs to confirm their nucleotide sequences (Section 2.10.8 and Table 6.2). Final transgenic constructs were then submitted to the Transgenic Service (Cancer Research UK) for pronuclear microinjection into fertilised CBA/B6 zygotes.

6.2.5 Validation of transgenic founders

CBA/B6 founders were crossed with ROSA26 reporter strain (R2R). The R2R line originated from Dr. Philippe Soriano, Fred Hutchinson Cancer Research Center, USA, and has been previously characterised (Soriano,

1999). F1 progenies were assessed by PCR genotyping for Cre (Section 2.1.3). Founders that had ten or more consecutive littermates that were negative for Cre genotype were culled. From founders that had progenies with positive a Cre genotype, two CreER^{T2+}/R2R and CreER^{T2wt}/R2R mice were culled, and reverse transcription PCR was performed on RNA extracted from their glandular stomachs to verify presence of *Cre* mRNA (Section 2.4.4). Following the confirmation of Cre mRNA, another two CreER^{T2+}/R2R and CreER^{T2wt}/R2R were given tamoxifen (1 mg per day) for five days consecutively and culled on the following day (Section 2.2.2). Glandular stomach tissues from these mice were dissected for wholemount X-gal staining (Section 2.6.3).

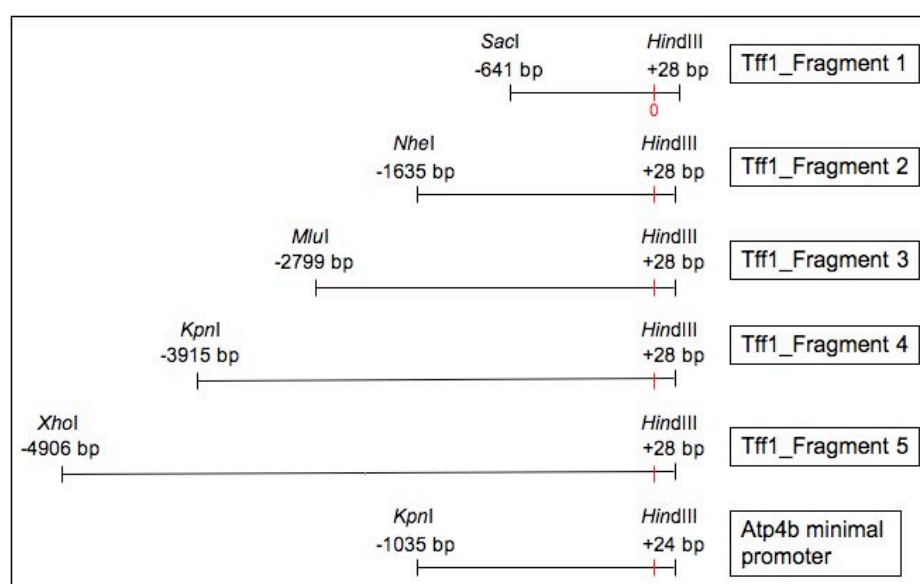


Figure 6.1 List of promoter fragments generated by PCR.

Each promoter fragment has restriction sites added on either end, which are denoted by the restriction enzyme names. The number below each enzyme denotes the position of the nucleotide relative to the transcriptional start site of the gene denoted as '0' in red. Nucleotide direction is shown as 5' to 3'.

Table 6.1 Primer sequences for amplifying *Tff1* promoter fragments and *Atp4b* minimal promoter.

Promoter fragment		Sequence (5' to 3')	Annealing temp (°C)	Amplicon size	Extension time
Tff1_Fragment 3	Forward	CCGACGCGTCTGAGTCC	69.0	2827 bp	3 min
	Reverse	TTTCATGGAGCACGA CCGAAGCTTAGCTTCAC GGGAAGACCACGA			
Tff1_Fragment 5	Forward	AAGGTGTGCGGAAATGG	62.4	4934 bp	5 min
	Reverse	ATTCAG CCGAAGCTTAGCTTCAC GGGAAGACCACGA			
Atp4b minimal promoter	Forward	ATTGGTACCTCTAGAGC	59.0	1071 bp	1 min 30 s
	Reverse	TCTTTCCTCTGG ATTAAGCTTGTCTCTCC TGCTTGCCTGGA			

Table 6.2 Primer sequences for DNA sequencing.

Primer label	Sequence (5' to 3')	Annealing temp (°C)
SEQ1	ACTAACATACGCTCTCCATC	53.0
SEQ2	GCATCACCTGTAGTCAGC	52.0
SEQ3	TGTGCACAGCTAGACCTG	55.0
SEQ4	GTTATTAACTACACCTATTCCCCG	55.0
SEQ5	ACAGATATGCGAGGTGGT	55.0
SEQ6	AAGTAGGAAACTCAACCC	52.0
SEQ7	TTCCCATCTGTGTTTGCATGC	60.0
SEQ8	TTCCATGTTAGCACTGTG	52.0
SEQ9	CTCAGAGACTATGACCCAG	52.0
SEQ10	CCAAGTCGATGACATTATAC	52.0
SEQ11	GCCCTGAATACCTAGATCC	54.0
SEQ12	TCCAGGTAGTTGTAGGCAC	54.0

6.3 Results

6.3.1 *Fgfr2b* mRNA is expressed in stomach epithelium

ISH was used to determine the localisation on *Fgfr2b* mRNA in an adult mouse stomach. Results show abundance of *Fgfr2b* mRNA in the forestomach squamous epithelium, pepsinogen-secreting chief cells, mucous-producing pit and neck cells, a minor portion of parietal cells, and basal and surface of antral glands (Figure 6.2).

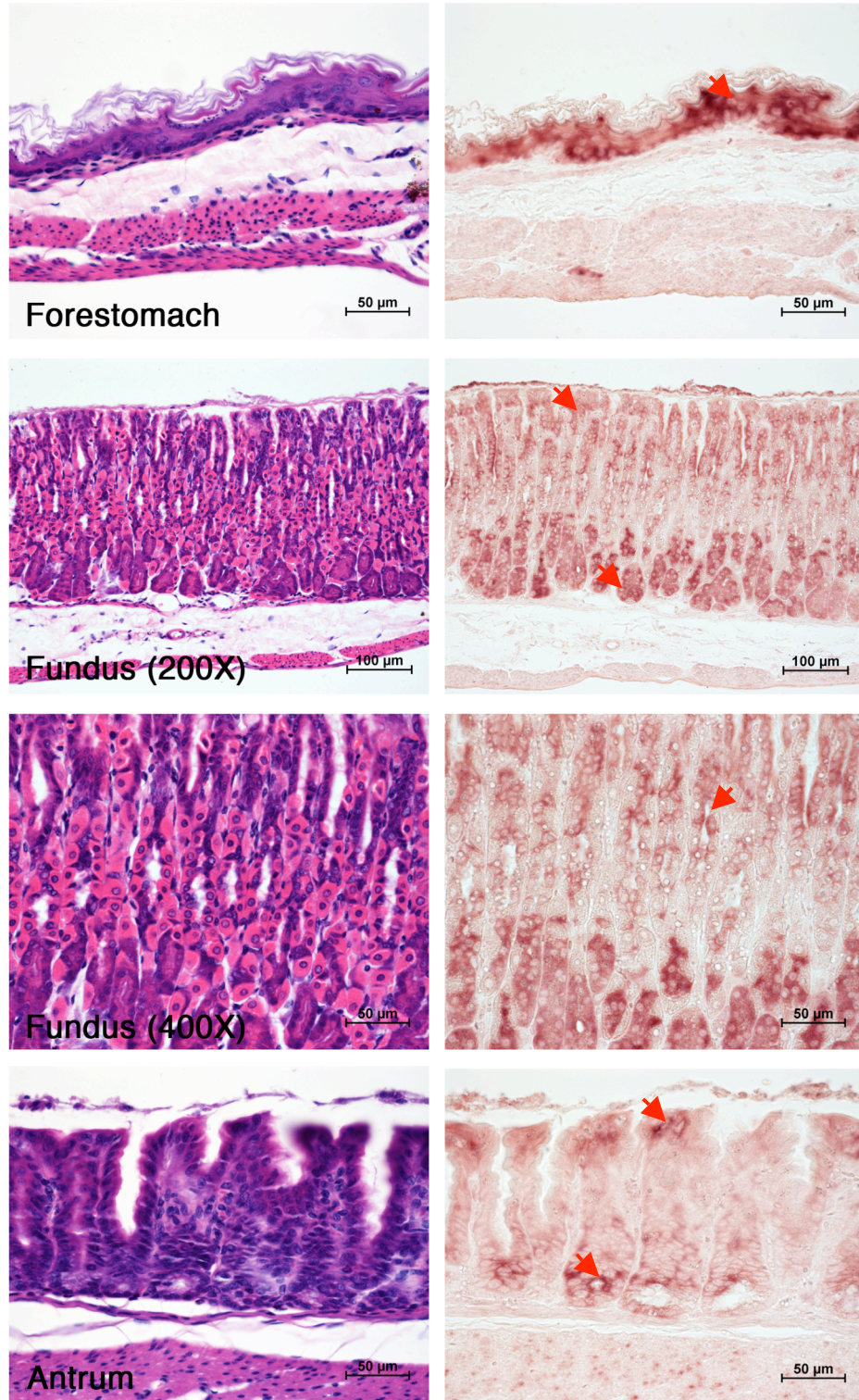


Figure 6.2 Expression sites of *Fgfr2b* mRNA in an adult C57/BL6 mouse gastric epithelium.

Images on the left panel were from H&E staining, and images on the right panel were from *Fgfr2b* ISH. Red arrows mark the expression site of *Fgfr2b* mRNA. Positive control from adult duodenum of C57/BL6 show

positive *Fgfr2b* staining in the crypts and villi, whereas negative control using DIG-labelled sense *Fgfr2* probe show no staining in the gastric epithelium (data not shown).

6.3.2 Stomach-specific promoters were successfully cloned and validated

All five *Tff1* promoter fragments and *Atp4b* minimal promoter were successfully generated and cloned into the pGL-3 Basic luciferase vector (Figure 6.3). Following the confirmation of nucleotide sequences by DNA sequencing, all five *Tff1* promoter fragments were subjected to luciferase assay in gastric-specific cell lines to determine the optimal minimal promoter. *Tff1*_Fragment 1 (-641 bp to +27 bp) gives the highest luciferase readout in GSM10 (approximately nine times more than the promoter-less luciferase vector), AGS and HGT101 cell lines compared to the other four promoter fragments (Figure 6.4). The *Atp4b* minimal promoter (-1,025 bp to +24 bp) also gives approximately four times more luciferase readout than the promoter-less luciferase vector in MGE507 cell line (Figure 6.4). Evaluation of minimal promoter specificity by luciferase assay in non-gastric cell lines (3T3, HC11, nTERT) show no significant difference in luciferase readout between the *Tff1*, *Atp4b* and promoter-less luciferase vector (Figure 6.4). Additionally, the *Tff1* minimal promoter yields three times more luciferase activity in the GSM10 cell line grown in serum-free media supplemented with EGF 10 ng/ml than in a plain serum-free media (Figure 6.5).

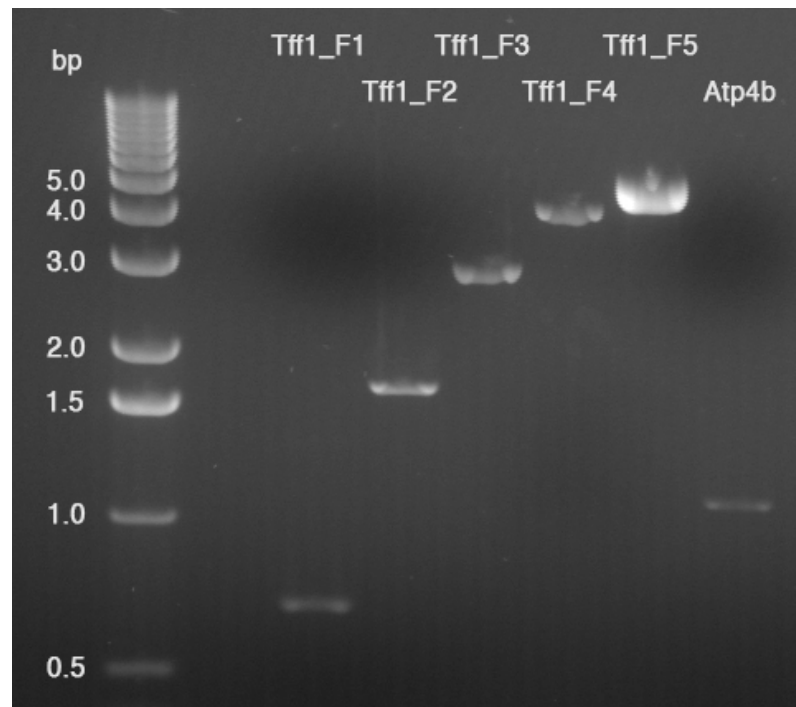


Figure 6.3 PCR amplification of all five *Tff1* promoter fragments and *Atp4b* minimal promoter.

Tff1_F3, Tff1_F5 and Atp4b fragments were generated by PCR, whereas Tff1_F1, Tff1_F2 and Tff1_F4 fragments were generated from restriction digest of Tff1_F5 fragment.

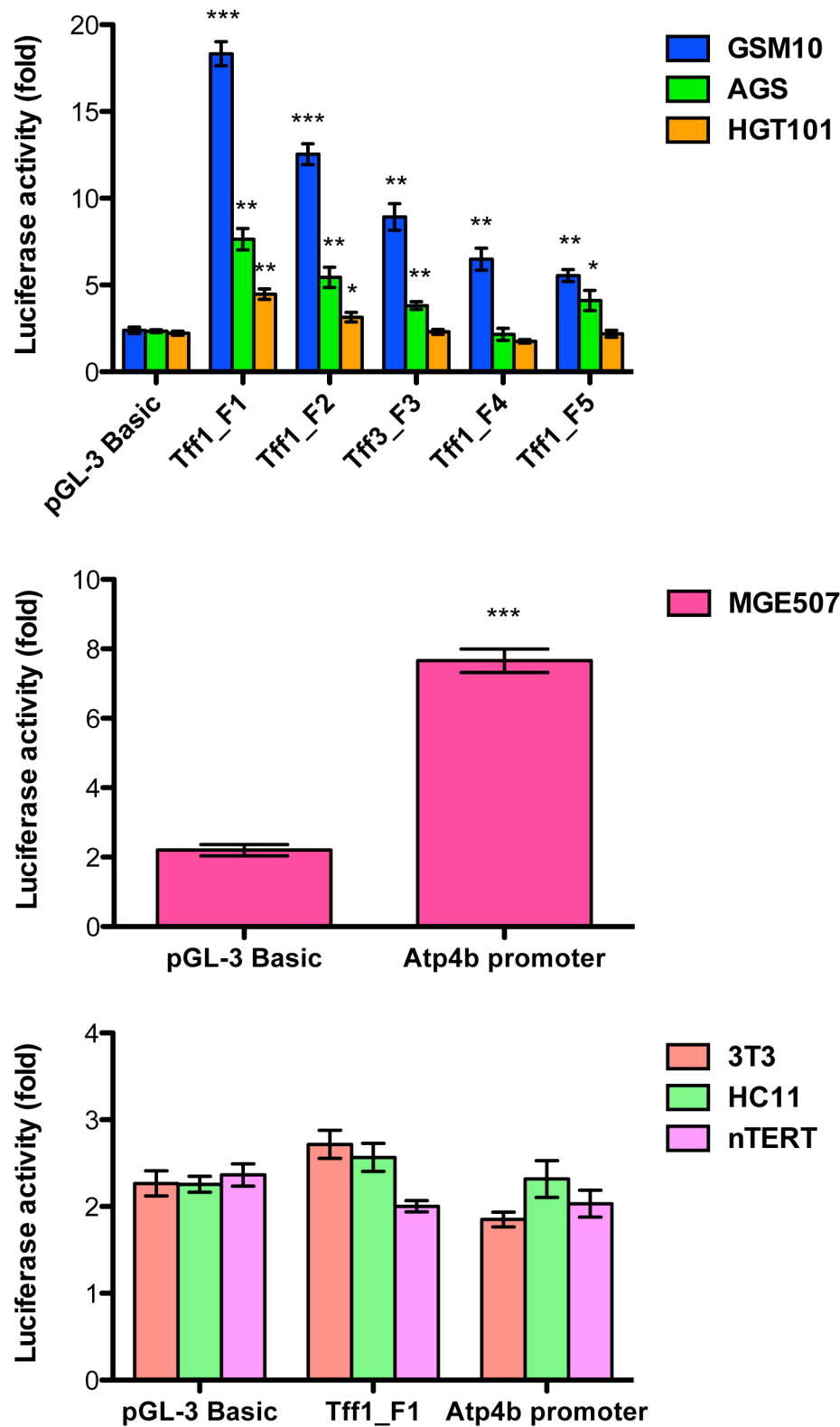


Figure 6.4 Promoter analyses by luciferase assays.

All *Tff1* promoter fragments and *Atp4b* minimal promoter were cloned upstream of the luciferase gene (Luc) of pGL-3 Basic vector. Luciferase vector bearing the promoter fragments were transfected into various cell

lines together with a constant amount of phRL-CMV (Renilla). Luciferase activity was measured and normalised to Renilla luciferase activity. Tff1_F1 gives the most optimal luciferase expression in GSM10 cells, and the cloned Atp4b minimal promoter activates luciferase expression in MGE507 cells. These minimal promoters do not activate luciferase expression in non-gastric cell lines. The values (relative to the promoter-less pGL-3 Basic vector) ($\pm$ SEM) indicated were the average of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001; $n = 3$ samples/group.

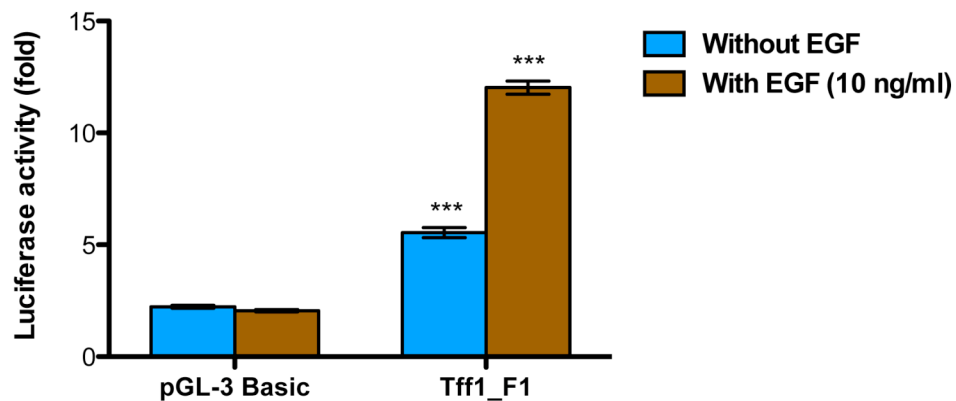


Figure 6.5 Effects of EGF on *Tff1* minimal promoter activity.

Luciferase vector bearing the *Tff1* minimal promoter was transfected into GSM10 cells together with a constant amount of phRL-CMV (Renilla) in media with or without EGF 10 ng/ml. Luciferase activity was measured and normalised to Renilla luciferase activity. The Tff1_F1 minimal promoter contains EGF response element, which upregulates luciferase expression in the presence of recombinant EGF. The values (relative to the promoter-less pGL-3 Basic vector) ($\pm$ SEM) indicated were the average of three independent experiments. *** P <0.001; $n = 3$ samples/group.

6.3.3 Identification of *Tff1-CreER^{T2}* and *Atp4b-CreER^{T2}* founders

Of the total number of zygotes injected with the *Tff1-CreER^{T2}* (175 zygotes) and *Atp4b-CreER^{T2}* (182 zygotes), approximately 23% of these were born as founders, and approximately 35% of these founders were positive for the *Cre* allele. These positive founders were crossed with R2R mice, and approximately 58% of these founders transmitted the *Cre* allele. A further 30% of these founders expressed *Cre* mRNA in the glandular stomach, and further X-gal staining showed that none of these founders produced active *Cre* protein (Table 6.3 and Figure 6.6).

Table 6.3 Identification of transgenic founders.

Construct	No. of zygotes injected	Number of founders				
		Born	+ve <i>Cre</i> genotype	Transmitted <i>Cre</i> genotype	+ve <i>Cre</i> mRNA	+ve for X-gal staining
<i>Tff1-CreER^{T2}</i>	175	45	13	7	2	0
<i>Atp4b-CreER^{T2}</i>	182	39	15	9	3	0

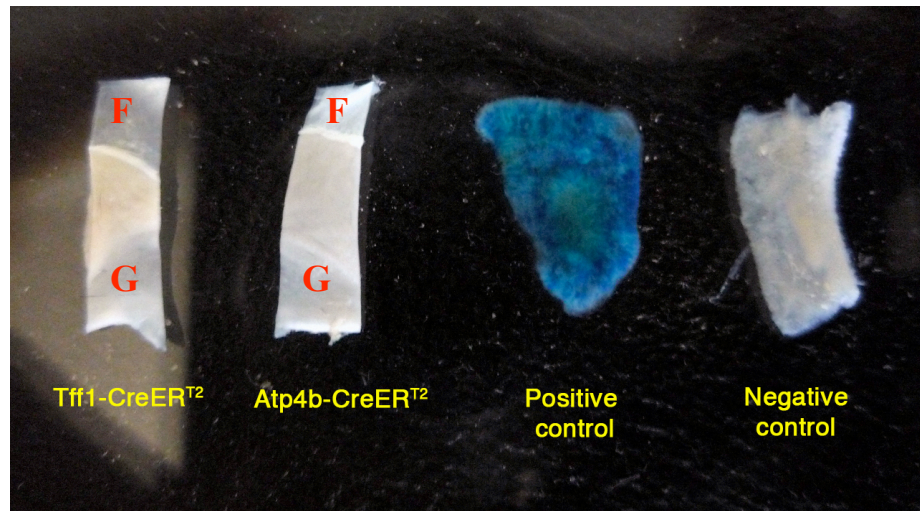


Figure 6.6 Validation of transgenic founders by wholemount X-gal staining.

Stomach tissues were isolated from the Tff1-CreER^{T2+}/R2R and Atp4b-CreER^{T2+}/R2R mice induced with tamoxifen and stained wholemount with X-Gal. 'F' and 'G' mark the forestomach and glandular regions respectively. Positive and negative controls came from duodenal tissue of adult Villin-CreER^{T2+}/R2R and Villin-CreER^{T2wt}/R2R mice respectively that were subjected to similar induction and X-gal staining protocols. Both stomach tissues, including negative control, showed absence of X-Gal staining, while positive control showed presence of staining, validating the induction and X-Gal staining protocols.

6.4 Discussion

In order to determine the requirement of *Fgfr2b* in gastric homeostasis and injury, this study initially aims to look at the expression site of *Fgfr2b* mRNA in a normal adult mouse. Our novel data using ISH shows that *Fgfr2b* mRNA is expressed by most gastric lineages, including the forestomach squamous epithelium, fundus chief cells, parietal cells, surface mucous cells, mucous neck cells, and antral surface and bottom glands. There are surprisingly few parietal cells that express *Fgfr2b* mRNA, which contrasts with a previous study reporting the restricted expression of *Fgfr2b* in parietal and surface mucous cells in the normal human gastric epithelium (Matsunobu et al., 2006), suggesting that expression site of *Fgfr2b* mRNA differs between human and murine. Additionally, these rare-stained parietal cells could be the immature pre-parietal cells that give rise to mature parietal cells during terminal differentiation (Karam et al., 2003), or represent a particular subtype of mature parietal cells. To date, no study has reported a marker for differentiating pre-parietal and parietal cell population, or reported different subtypes of mature parietal cells.

To generate two inducible Cre lines under the stomach-specific *Atp4b* and *Tff1* minimal promoters respectively, *Atp4b* minimal promoter (-1,035 bp to +24 bp) was initially cloned into the pGL-3 luciferase vector and successfully validated in a mouse gastric pre-parietal (MGE507) cell line. In order to thoroughly characterise the *Tff1* promoter, five *Tff1* promoter fragments with staggered lengths were cloned and compared by luciferase assays in three gastric cell lines (GSM10, AGS and HGT101), and promoter nucleotide -641 bp to +28 bp is determined to be the best *Tff1* minimal promoter. Preliminary testing on the specificity of these minimal promoters by luciferase assays in non-gastric cell lines suggests that both minimal promoters are specific for directing transgene expression in the gastric epithelium.

The efficiency of the *Tff1* minimal promoter can be attributed to the presence of an AP-1 site (-ATGAATCA-) (Terada et al., 2001), which is involved in the signalling pathway Src-homology tyrosine phosphatase 2 (SHP2)/Ras/ERK/AP-1 originating from gp130, a common interleukin (IL)-6 co-receptor (Taga and Kishimoto, 1997). IL-6 family cytokines are required for maintaining gastric homeostasis by regulating TFF1 via co-receptor gp130 (Giraud et al., 2007), and gp130 mutants with abrogated SHP2 signalling pathway develop gastric adenomas by three months of age, accompanied by significant downregulation of TFF1 expression (Tebbutt et al., 2002).

Another possible positive regulatory element on the *Tff1* minimal promoter is the EGF response element. TFF1 expression in human gastric epithelium has been closely associated with EGF secretion by the subjacent mucous neck cells (Wright et al., 1990). The -428 bp to -332 bp flanking region of the human *Tff1* gene contains a 10-fold enhancer element that responds to EGF (Nunez et al., 1989), even though the exact sequence of this enhancer element is still under investigation. Our investigation on the mouse *Tff1* minimal promoter by luciferase assays using media with and without EGF 10 ng/ml shows a 2.5-fold increase in promoter activity, confirming the presence of an EGF response element in our *Tff1* minimal promoter.

Comparison of the promoter activity between GSM10 and HC11 cell lines suggests the presence of a stomach-specific regulatory element in the *Tff1* minimal promoter. Both GSM10 and HC11 are epithelial cell lines, and have been maintained in growth medium containing EGF 10 ng/ml. Even though HC11 cells have been shown to express EGFRs (Taverna et al., 1991), the *Tff1* minimal promoter is only active in the GSM10 cell line and not in the HC11 cells, confirming the presence of stomach-specific element in this minimal promoter, and activation of this stomach-specific element is a pre-requisite for transgene expression under the *Tff1* minimal promoter. However, the exact sequence of this stomach-specific element is still under investigation.

After screening 175 founders of Tff1-CreER^{T2} and 182 founders of Atp4b-CreER^{T2}, none of the founders was positive for Cre protein activity in the mouse stomach. This observation is in common with a previous study generating a transgenic line expressing human growth hormone (hGH) under the similar *Atp4b* minimal promoter which showed an approximately 4% success rate (from the number of liveborn mice screened) of finding a transgenic founder that expressed hGH, suggesting an extremely low success rate of using this minimal promoter to create a transgenic line (Lorenz and Gordon, 1993). Observations from *in vitro* validation experiments do not always correspond to that in *in vivo* studies due to differences between both biological systems. An alternative explanation is that the transgene may have been integrated into transcriptionally inactive/repressive sites, thus reducing the transcription efficiency of the transgene. One possible solution is to inject the transgenic constructs into twice the number of zygotes that was originally used in this study, thereby increasing the number of live-born mice for screening and giving a higher success rate of identifying the transgenic founders. Alternatively, the stomach-specific Cre inducible constructs can be targeted more specifically into the X-linked hypoxanthine guanine phosphoribosyl transferase (*Hprt*) locus as this region has a high consistency for expression, consistent location of transgene integration, and provides the ability to control the copy number of transgene (Hatada et al., 1999, Guillot et al., 2000, Evans et al., 2000, Cvetkovic et al., 2000, Bronson et al., 1996).

6.5 Conclusion

In conclusion, we have shown that *Fgfr2b* is expressed in various cell lineages of the adult mouse stomach epithelium, including mucus-producing pit and neck cells, acid-producing parietal cells and pepsinogen-secreting zymogenic chief cells. We have also generated two tamoxifen-inducible Cre constructs under the stomach specific promoters: *Tff1* (-641 bp to +28 bp) and *Atp4b* (-1,035 bp to +24 bp) minimal promoters.

From our *in vitro* experiments, we have validated that these minimal promoters are efficient and specific for driving transgene expression in mouse stomach. Further characterisation on the *Tff1* minimal promoter reveals the presence of EGF response element and a stomach-specific element. While random integration of these transgenic constructs gave no reliable founder, this study has nevertheless increased our understanding of the regulatory elements of the *Tff1* promoter.

Chapter 7: Final conclusions

Fgfs and *Fgfrs* mediate epithelial-mesenchymal crosstalk that is essential in tissue proliferation, survival, differentiation and motility across multiple organs, including skin and the GI tract. We have used several established mouse models, complemented by *in vitro* studies, to investigate loss-of-function of *Fgf10* and *Fgfr2b* in GI development, homeostasis and injury. In brief, our experiments have shown that:

1. *Fgf10* and *Fgfr2b* are required for normal duodenal development, as loss of either *Fgfr2b* or *Fgf10* results in disproportionately short small intestine (due to reduced epithelial proliferation), duodenal atresia (due to increased tissue apoptosis at a focal point on the duodenum), and gastric heterotopia in the rostral duodenum (due to defective anterior-posterior patterning at the stomach-duodenum boundary prior to intestinal differentiation). Aberrant expression of Wnt is associated with anterior-posterior patterning defect, with *Fgf10-Fgfr2b* signalling regulating *Tcf4* expression via the Grb2/Sos/Ras/MAPK pathway.
2. *Fgfr2b* is not required for maintaining adult intestinal homeostasis, as substantial downregulation of *Fgfr2b* does not significantly affect body weight, and intestinal proliferation, survival and differentiation. Possible functional redundancy of *Fgfr2b* in adult GI tract could have contributed to these observations.
3. *Fgfr2b* is required to fully protect against intestinal injury and to promote wound healing. Downregulation of *Fgfr2b* in our DSS-induced ulcerative colitis model shows an increase in severity and delays wound healing, a consequence of reduced epithelial proliferation.
4. *Fgfr2b* is expressed by various lineages of the adult gastric epithelium, including the mucus-secreting pit and neck cells, acid-secreting

parietal cells, and pepsinogen-secreting chief cells. The *Tff1* (-641 bp to +28 bp) and *Atp4b* (-1,035 bp to +24 bp) minimal promoters are optimal for constructing inducible stomach-specific Cre lines to ablate *Fgfr2b* in the adult stomach epithelium.

Publications

Abstracts

Ding SS, Spencer-Dene B and Stamp GW, “FGF10-FGFR2b signalling in gut development, homeostasis and injury”, LRI Symposium, 2009.

Ding SS, Spencer-Dene B and Stamp GW, “FGF10-FGFR2b signalling in intestinal development and homeostasis”, International Graduate Student Conference, 2009.

Ding SS, Spencer-Dene B and Stamp GW, “The requirement of FGFR2b in intestinal development and homeostasis”, FGF Gordon Conference, 2008.

Ding SS, Spencer-Dene B and Stamp GW, “FGF10-FGFR2b in GI development”, International Graduate Student Conference, 2007.

Peer review article

Ding SS, Spencer-Dene B and Stamp GW, Dependence on FGF10/FGFR2b signalling in duodenal morphogenesis and differentiation in the mouse, *Developmental Dynamics*, 2009 (under review).

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Appendix

Buffers and solutions

Unless otherwise stated, distilled water refers to 'Mili-Q Plus' water produced by the reverse osmosis ultra pure water system (Milipore).

Carnoy's fixative

Absolute ethanol (101077Y; VWR)	60 ml
Chloroform (22711.324; VWR)	30 ml
Glacial acetic acid (100015N; VWR)	10 ml

10% neutral buffered formalin pH 7.8 (NBF)

Formalin, full strength (37-40% formaldehyde) (361387P, VWR)	100 ml
Na ₂ HPO ₄ (S7907; Sigma-Aldrich)	6.5 g
NaH ₂ PO ₄ (S8282; Sigma-Aldrich)	4 g
Distilled water	900 ml

4% paraformaldehyde (PFA)

Paraformaldehyde (16005; Sigma-Aldrich)	16 g
Phosphate buffered saline (PBS)	400 ml

Paraformaldehyde was dissolved in PBS at 70°C, and cooled to RT before use.

Phosphate buffered saline (PBS)

NaCl (S7653; Sigma-Aldrich)	8 g
KCl (P9333; Sigma-Aldrich)	0.35 g
Na ₂ HPO ₄ (Sigma-Aldrich)	1.43 g
NaH ₂ PO ₄ (Sigma-Aldrich)	0.25 g
Distilled water	1 L

pH adjusted to 7.2, and solution was autoclaved before use.

PBST solution was made by adding 1 ml of Tween-20 (63158; Sigma-Aldrich) into 1 L of PBS.

Base solution for RU486 mix

Carboxymethylcellulose (21901; Sigma-Aldrich)	25 mg
Tween-80 (P1754; Sigma-Aldrich)	50 µl
Distilled water	10 ml
Solution was autoclaved before use.	

20X SSC

NaCl (Sigma-Aldrich)	17.53 g
Sodium citrate (S1804; Sigma-Aldrich)	8.82 g
Distilled water	100 ml
pH adjusted to 5.0, and solution was autoclaved before use.	

Tris buffered saline (TBS)

NaCl (Sigma-Aldrich)	8.76 g
Tris (t1378; Sigma-Aldrich)	6.05 g
Distilled water	1 L
pH adjusted to 7.5.	

PBST solution was made by adding 1 ml of Tween-20 (63158; Sigma-Aldrich) into 1 L of PBS.

10X maleic buffer

Maleic acid (63180; Sigma-Aldrich)	116 g
NaCl (Sigma-Aldrich)	88 g
Distilled water	1 L
pH adjusted to 7.5.	

0.01 M sodium citrate buffer

Tri-sodium citrate (25114; Sigma-Aldrich)	2.94 g
0.2 M HCl	22 ml
Distilled water	1 L
pH adjusted to 8.0.	

Luria-Bertani broth (LB)

Tryptone (T7293; Sigma-Aldrich)	10 g
Yeast (51475; Sigma-Aldrich)	5 g
NaCl (Sigma-Aldrich)	5 g
Distilled water	1 L

Solution is autoclaved before used.

LB agar was made by dissolving 15 g of agarose (A4718; Sigma-Aldrich) into 1 L of LB broth.

Suppliers and distributors

Supplier	Address
Ambion	Austin, TX, USA
Applied Biosystems	Foster City, CA, USA
BD Biosciences	New Jersey, USA
BDH Laboratory Supplies Inc.	Hertfordshire, UK
BioGenex Laboratories Inc.	San Ramon, CA, USA
Calbiochem	Nottingham, UK
DAKOCytomation A/S	Cambridge, UK
Fisher Scientific	Leicestershire, UK
GE Healthcare	Buckinghamshire, UK
Gibco BRL Ltd.	Renfrewshire, UK
Graphpad Software	San Diego, CA, USA
Hollingsworth & Vose Co. Ltd.	Gloucestershire, UK
Invitrogen Ltd.	Paisley, UK
Launch Diagnostics Ltd.	Kent, UK
Mayne Pharma Plc	Leamington Spa, Warwickshire
Microzone Ltd.	West Sussex, UK
Milipore	Watford, UK
Nalgene	Rochester, NY, USA
NanoDrop Technologies	Wilmington, DE, USA
New England Biolabs (NEB)	New York, USA
Nunc	Rochester, NY, USA
Pierce Biotechnology	Cramlington, Northumberland
Promega	Hampshire, UK
Qiagen	West Sussex, UK
R&D Systems Europe Ltd.	Abingdon, UK
Roche Diagnostics Ltd.	Burgess Hill, UK

Supplier	Address
Sigma-Aldrich Co. Ltd.	Gillingham, UK
Stratagene	La Jolla, Ca, USA
Tesco	London, UK
The Binding Site	San Diego, CA, USA
Thermo Scientific	Rochester, NY, USA
Vector Laboratories	Burlingame, SA, USA
VWR International Ltd.	Leicestershire, UK