



THE UNIVERSITY OF SYDNEY

Faculty of Medicine and Health

FGF10 AND THE MYSTERY OF DUODENAL ATRESIA

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Preface

It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat.

It is war-worn Hotspur, spent with hard fighting, he of the many errors and valiant end, over whose memory we love to linger, not over the memory of the young lord who "but for the vile guns would have been a valiant soldier."

Theodore Roosevelt

Excerpts from "Citizenship in a Republic" speech

Delivered at the Sorbonne, in Paris, France on the 23rd of April 1910

Abstract

The cause of duodenal atresia (DA) is not known. Tandler's "solid cord" hypothesis conflicts with current biological evidence. In humans, a genetic aetiology is supported by the association with Trisomy 21. Interruption of *Fgf10* is the strongest genetic link to DA in mice, but the lethality of associated defects means it is an unlikely cause of DA in humans. **It is hypothesised that DA is caused by a genetic change downstream of the FGF10/FGFR2b signalling pathway.** This thesis aims to identify gene signalling changes that may cause DA.

Aim 1 was to validate the spectrum of DA present in CRISPR-derived *Fgf10*^{-/-} embryos. These embryos demonstrated a higher penetrance of DA than previously reported, with morphology distribution closely reiterating that observed in humans. DA penetrance and phenotype severity increased with gestational age, suggesting an evolutionary nature.

Aim 2 was to evaluate gene expression associated with DA, through the use of RNA-seq and RT-qPCR. Whilst multiple genes were up- and downregulated in the absence of *Fgf10*, a cluster, otherwise associated with pyloric muscle specification, was of particular interest. This possible interplay between pyloric development and DA unveils a new hypothesis, that: **in the duodenum of *Fgf10*^{-/-} mice, anterior-posterior mis-patterning with ectopic pyloric specification may be the basis of DA formation, and loss luminal continuity.**

Aim 3 was to develop an organoid model to facilitate future *ex vivo* DA research. Normal organoids were shown to grow from wild-type duodenum and growth patterns differed between *Fgf10* genotypes.

This thesis forms a foundation for future research into DA aetiology. Further animal research must combine gene analysis of human patients born with DA, to identify putative causative genes/pathways. Determining the cause of DA in humans is a clinical and scientific imperative, with immediate therapeutic relevance to antenatal counselling and future clinical research to ameliorate or prevent DA.

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Declaration

I certify that, to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

I understand that if my candidature is successful, this thesis will be lodged with the University Librarian and made available for immediate use.

Matthew Lachlan Markley Jones

13/12/2022

I certify that, as lead supervisor, it is my opinion that this thesis is sufficiently well presented to be examined and does not exceed the prescribed word limit.

Professor Jonathan Karpelowsky

13/12/2022

Authorship attribution statement

This thesis contains material published in (Jones et al., 2020) which is included (with minor modifications) as sections 1.1, 1.4, 1.5; and figures 1.1, 1.3. This publication is attached as appendix 1. My role in this published work was to conceive the project, conduct the review of literature, analyse the evidence, and write and finalise the manuscript.

This thesis contains material published in (Teague et al., 2018) which is included (with modifications, including updated experimental data) as sections 4.2.1, 4.2.2; and figures 4.1, 4.2. This publication is attached as appendix 2. My role in this published work was to assist in study design, perform molecular biology techniques, microsurgical tissue dissection, image capture, results analysis and interpretation, preparation, and finalising the manuscript.

Due to its relevance to the content of this thesis (Vinycomb et al., 2020) is attached as appendix 3. My role in this published work was to assist in results analysis and interpretation, preparation, and finalising the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Matthew Lachlan Markley Jones

13/12/2022

As a supervisor for the candidature upon which this thesis is based, and the corresponding author for the above published items, I can confirm that the authorship attribution statements above are correct.

Associate Professor Warwick Teague

13/12/2022

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2. Teague, W. J., **Jones, M. L. M.**, Hawkey, L., Smyth, I. M., Catubig, A., King, S. K., Sarila, G., Li, R. & Hutson, J. M. 2018. FGF10 and the Mystery of Duodenal Atresia in Humans. *Front Genet*, 9, 530. [attached as Appendix 2]
3. Vinycomb, T., Browning, A., **Jones, M. L. M.**, Hutson, J. M., King, S. K. & Teague, W. J. 2020. Quality of life outcomes in children born with duodenal atresia. *J Pediatr Surg*, 55, 2111-2114. [attached as Appendix 3]
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Abstract "FGF10 and the mystery of Duodenal Atresia"

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Abstract "Fgf10 expression during murine gut development and its role in intestinal atresia"

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Abstract "Novel use of embryonic gut organoid culture to investigate duodenal and colonic atresia"

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List of abbreviations

A	<i>Abat</i>	4-aminobutyrate aminotransferase
	<i>Actb</i>	Actin Beta
	ANOVA	Analysis of variance
B	BAIB	L- beta-aminoisobutyrate
	<i>Barx1</i>	BarH-Like Homeobox 1
	BLAST	Basic Local Alignment Search Tool
	BLAT	BLAST-Like Alignment Tool
	BMM	Basement Membrane Matrix
	BMP	Bone morphogenetic protein
	bp	Base pair
	BSA	Bovine serum albumin
C	Cas9	CRISPR-associated (Cas) endonuclease 9
	CCND1	Cyclin D1
	cDNA	Complementary DNA
	CPAM	Congenital pulmonary airway malformation
	CRISPR	Clustered regularly interspaced short palindromic repeats
	Ct	Threshold cycle
	<i>Cubn</i>	Cubilin
D	DAPI	Diamidino-2-phenylindole
	DMEM	Dulbecco's Modified Eagle Medium
	DNA	Deoxyribonucleic acid
E	EDTA	Ethylenediaminetetraacetic acid
	EEG	electroencephalogram
	<i>en bloc</i>	As a whole; all at once
	ERK	Extracellular signal-regulated protein kinase
	ETS	Erythroblast Transformation Specific
	ETV4	ETS Variant Transcription Factor 4
	ETV5	ETS Variant Transcription Factor 5
	<i>ex vivo</i>	Outside of the living body
F	FDR	False discovery rate
	FGF	Fibroblast Growth Factor
	<i>Fgf10</i>	Fibroblast Growth Factor 10 (mouse gene)
	FGF10	Fibroblast Growth Factor 10 (protein / human gene)
	<i>Fgf22</i>	Fibroblast Growth Factor 22 (mouse gene)
	FGF22	Fibroblast Growth Factor 22 (protein / human gene)
	<i>Fgf3</i>	Fibroblast Growth Factor 3 (mouse gene)
	FGF3	Fibroblast Growth Factor 3 (protein / human gene)

	<i>Fgf7</i>	Fibroblast Growth Factor 7 (mouse gene)
	FGF7	Fibroblast Growth Factor 7 (protein / human gene)
	FGFR	Fibroblast growth factor receptor
	FGFR1B	Fibroblast growth factor receptor 1 (protein / human gene)
	<i>Fgfr2b</i>	Fibroblast growth factor receptor 2 (mouse gene)
	FGFR2B	Fibroblast growth factor receptor 2 (protein / human gene)
	<i>Foxa2</i>	Forkhead Box A2
G	GABA	gamma aminobutyrate
	<i>Gata3</i>	GATA Binding Protein 3
	GFR	Growth factor reduced
	GSEA	Gene set enrichment analysis
H	HHEX	Hematopoietically Expressed Homeobox
I	IHH	Indian Hedgehog Signalling Molecule
	<i>in situ</i>	In position; locally
	<i>in vitro</i>	In the glass
	<i>in vivo</i>	Within the living body
	<i>Isl1</i>	ISL LIM Homeobox 1
K	<i>Kirrel2</i>	Kirre Like Nephtrin Family Adhesion Molecule 2
	<i>Kynu</i>	Kynureninase
L	LogFC	Log2 of the fold change
M	MAPK	Mitogen-activated protein kinase
	MCRI	Murdoch Children's Research Institute
	mins	Minutes
	mRNA	Messenger RNA
	mRNA-seq	mRNA sequencing
N	NCBI	National Centre for Biotechnical Information
	NCS	NovaSeq Control Software
	<i>Nepn</i>	Nephrocan
	<i>Nkx2-5</i>	NK2 Homeobox 5
	<i>Nmur2</i>	Neuromedin U Receptor 2
O	<i>Oxct1</i>	3-Oxoacid CoA-Transferase 1
P	PCR	Polymerase chain reaction
	PDX1	pancreatic and duodenal homeobox 1 (protein / human gene)
	<i>Pdx1</i>	pancreatic and duodenal homeobox 1 (mouse gene)
	<i>Penk</i>	Proenkephalin
	PFA	Perfluoroalkoxy alkanes

	PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
	PITX2	Paired-like homeodomain transcription factor 2
	PME	PIPES, MgCl ₂ , EGTA
R	RA	Retinoic acid
	<i>Raldh2</i>	Retinaldehyde dehydrogenase 2
	<i>Rbp4</i>	Retinol Binding Protein 4
	RNA	Ribonucleic acid
	<i>Rpl32</i>	Ribosomal Protein L32
	rpm	Revolutions per minute
	RT-qPCR	reverse transcription quantitative real-time PCR
	RTA	Real time analysis
S	s	Seconds
	SDS	Sequence Detection Software
	SEM	Standard error of the mean
	<i>Sfrp5</i>	Secreted Frizzled Related Protein 5
	<i>Sgk3</i>	Serum/Glucocorticoid Regulated Kinase Family Member 3
	SHH	Sonic hedgehog protein
	<i>Six2</i>	Sine Oculis Homeobox 2
	<i>Sox2</i>	(Sex determining region Y)-box 2
	<i>Sox9</i>	(Sex determining region Y)-box 9
	<i>Spp1</i>	Secreted Phosphoprotein 1
	SRY	Sex determining region Y
	SSA	succinate semialdehyde
	STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
T	TBX	T-box protein
	TBX4	T-box protein 4
	TBX5	T-box protein 5
	TE	Tris and EDTA
	TESI	Tissue-engineered small intestine
	<i>Tifa</i>	TRAF Interacting Protein with Forkhead Associated Domain
	tm	Targeted mutation
	TRAF	Tumour Necrosis Factor, Receptor Associated Factor 1
	Tris	tris(hydroxymethyl)aminomethane
U	UCSC	University of California Santa Cruz
	UV	Ultraviolet
W	WEHI	Walter and Eliza Hall Institute of Medical Research
Z	<i>Zfc3h1</i>	Zinc Finger C3H1-Type Containing

1. Introduction

1.1. Defining duodenal atresia

Duodenal atresia (DA) is a congenital foregut malformation that affects 1 in 5,000–10,000 live births (Escobar et al., 2004). There are three morphological types of DA described which reflect an increasing degree of obstruction or intestinal discontinuity: Type 1 involves bowel continuity with luminal obstruction or stenosis; type 2 comprises bowel discontinuity with a connecting "bridge" of tissue; and type 3 refers to bowel discontinuity with complete separation (Skandalakis and Gray, 1994) (**Figure 1.1, A-D**). Although Calder first described 'duodenal atresia' in humans in 1733 (Calder, 1733), it was not until 1905 that Vidal and Ernst successfully performed the first documented surgical repair of DA (Vidal, 1905, Ernst, 1916).

The cause of DA is not known. In 1900, Julius Tandler published a hypothesis on the origins of DA based on his studies of normal duodenal development (Tandler, 1900). Tandler meticulously dissected 11 human embryos and theorised, based on his macroscopic observations, that normal duodenal development includes a 'solid cord' phase due to exuberant endodermal growth. He proposed that the failure to re-canalise this solid cord may lead to in DA in humans. Even then, Tandler cautioned readers, stating: "It is clear to me that the opinion represented here does not exceed the status of a new hypothesis, and it is not meant to exceed this." (Nichol et al., 2011). Notwithstanding, most paediatric surgeons accept Tandler's 'solid cord' hypothesis worldwide, despite conflicting developmental biology findings (Cheng and Tam, 1998, Botham et al., 2012) and the failure of Tandler's theory to account for the morphological variations of DA (Merrot et al., 2006).

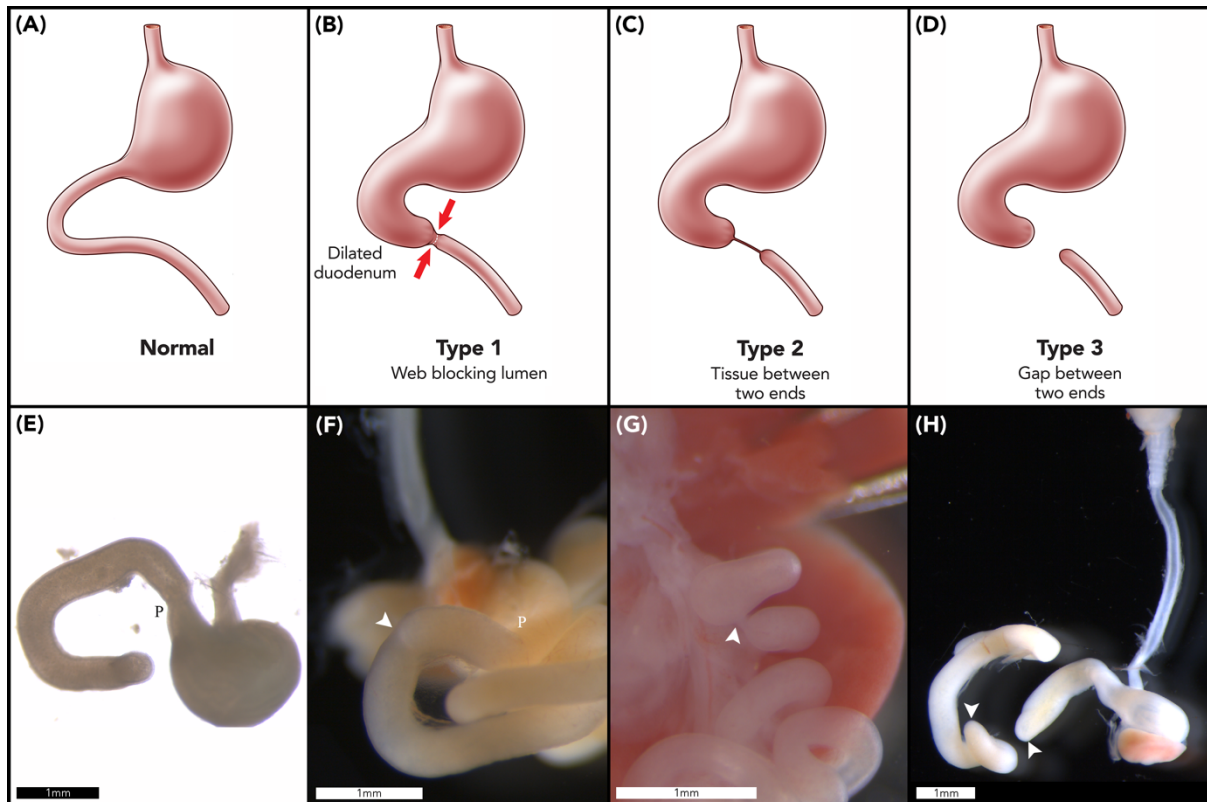


Figure 1.1: Duodenal atresia phenotype characterisation. Normal gastric, pyloric, and duodenal morphology demonstrated pictorially (A) and as seen in wild-type murine embryos (E). Type 1 DA is characterised by bowel continuity with luminal obstruction or stenosis (B). Type 2 DA demonstrates bowel discontinuity with a connecting "bridge" of tissue (C). Type 3 DA shows bowel discontinuity with complete separation (D). CRISPR-derived *Fgf10* knockout embryos provided examples of type 1 DA (F); type 2 DA (G); and type 3 DA (H), including a demonstration of an intact oesophagus, in the presence of tracheal atresia (H). Annotations denote scale bars and the location of the pylorus, P. Arrows indicate the location of atresia.

Murine studies have shown that interruption of the *Fgf10-Fgfr2b* signalling axis results in DA in 35-75% of knockout mice (Fairbanks et al., 2004b, Botham et al., 2012, Kanard et al., 2005, Reeder et al., 2012b, Teague et al., 2018). This supports a genetic aetiology for DA in humans, although no specific causative mutation has yet been demonstrated in humans. The association between DA and Trisomy 21 in humans is well recognised, with DA occurring in 20% of cases (Khan et al., 2017).

Nevertheless, animal models of Trisomy 21 fail to demonstrate DA or other associated gastrointestinal malformations (Delabar et al., 2006). Again, in humans, an autosomal recessive inheritance pattern for familial cases has been proposed, albeit based on a limited number of isolated and historical case reports (Berant and Kahana, 1970, Gross et al., 1996, Lambrecht and Kluth, 1998, Fonkalsrud et al., 1969, Best et al., 1989). Finally, although associations between DA and specific chromosomal anomalies have been reported (Zamfir et al., 2016, Mora et al., 2014), none relate to Chromosome 21 or Chromosome 5, which houses the *FGF10* gene in humans.

1.2. Management of duodenal atresia

Most cases of DA are diagnosed antenatally, with antenatal ultrasound showing polyhydramnios and a 'double bubble' (dilated stomach + dilated proximal duodenum) for 60% of patients. Postnatal diagnosis may be made via abdominal radiography, which demonstrates a gas-filled 'double bubble'.

Neonates present with feed intolerance, and typically a green (bile-stained) coloured vomit. This is due to the obstruction being distal to the ampulla of Vater in 80% of cases. Where diagnostic uncertainty persists, an upper gastrointestinal contrast study is recommended, however this is rarely required. Late presentations are uncommon, however an incomplete obstruction secondary to a widely perforated membrane (i.e. type 1 atresia) may present in infancy or childhood.

Many cases of DA have clinically important associated conditions (i.e. Down Syndrome (20%), oesophageal atresia (5%) and major congenital cardiac lesions). One third of patients with DA have congenital heart lesions. However, only 16% have 'major' lesions with expected concern to pre- and intra-operative care (Khan et al., 2017).

Current practice for DA management implies an attempt at surgical correction typically in the first week of life, with the re-establishment of duodenal continuity and minimal postoperative morbidity or mortality (Zani et al., 2017, Wright et al., 2021). Vinycomb et al. (attached as appendix 3) showed that children undergoing DA surgery in the neonatal period typically grow up to have a quality of life comparable to a healthy population (Vinycomb et al., 2020). The surgical techniques and technologies used to repair DA and achieve these favourable postoperative outcomes have since evolved from what was described by Vidal and Ernst a century ago (Vidal, 1905, Ernst, 1916), including both open and laparoscopic approaches.

Ladd was the first to publish a series of DA patients undergoing surgical repair (LADD, 1932). He reports on the outcome of 11 patients with duodenal obstruction, five patients with 'intrinsic' duodenal obstruction (i.e. DA) and six patients with 'extrinsic' duodenal obstruction (i.e. malrotation volvulus). Those with DA were repaired by a posterior gastroenterostomy, a posterior duodenojejunostomy (**Figure 1.2, A**) or a jejunostomy with a mortality rate of 40%.

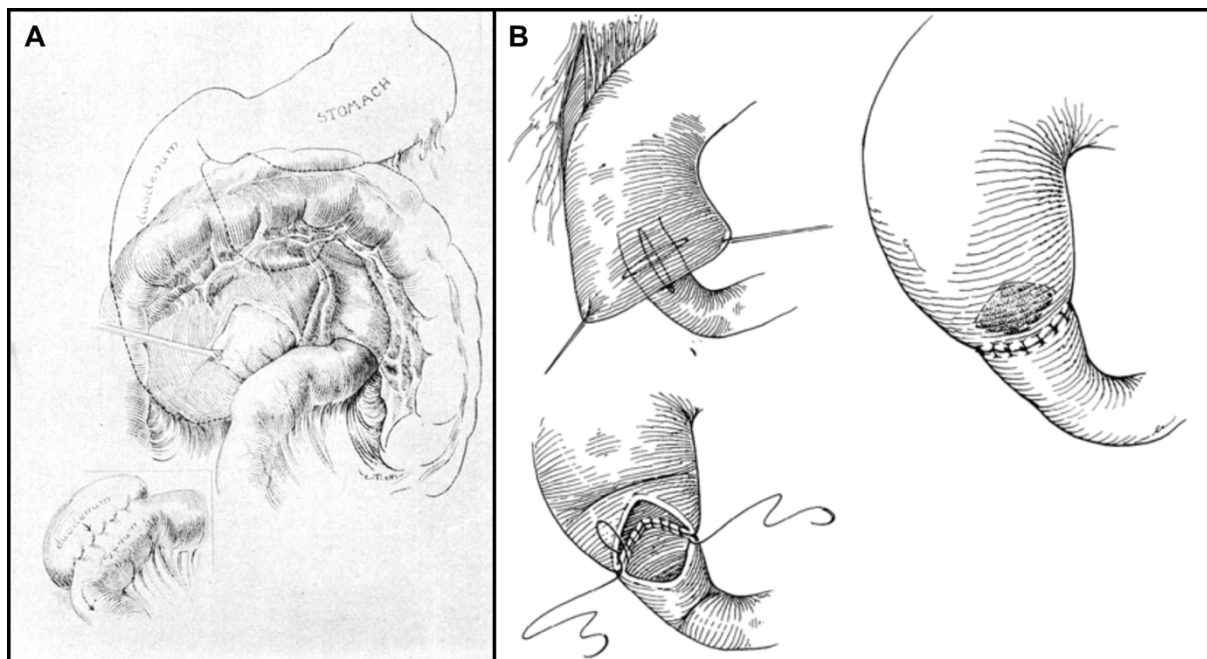


Figure 1.2: Surgical techniques for DA repair. (A) Ladd's drawing shows the method of performing a posterior duodenojejunostomy for Case No. 143386 (LADD, 1932). (B) The 'Diamond-shaped anastomosis' was described in 1990 by Kimura et al. as part of a Duodenoduodenostomy (Kimura et al., 1990).

It was not until 1990 that Kimura et al. proposed a duodenoduodenostomy (**Figure 1.2, B**). This technique sees a transverse incision in the distal end of the proximal duodenum and a longitudinal incision in the distal duodenum, forming a diamond-shaped anastomosis.

The advent of minimally invasive surgical equipment and techniques has resulted in the development of a laparoscopically performed duodenoduodenostomy, first reported by Bax et al. (Bax et al., 2001) and then Rothenberg (Rothenberg, 2002). However, a high complication rate led to this approach being abandoned until advances in intra-corporeal sutures and other technical aspects were perfected. With these advances, centres with considerable minimal access surgical expertise have successfully resumed laparoscopic duodenal atresia surgery in the last decade. Subsequently, multiple studies have compared the laparoscopic to open techniques without finding any significant difference between laparoscopic and open surgical options (Chung et al., 2017, Chiarenza et al., 2017, Jensen et al., 2013). Only one larger, single-centre, retrospective study has found any difference (Son and Kien, 2017), identifying a reduction in the mean time to initial oral feeding, reduction in mean postoperative length of stay, and improved cosmesis in patients undergoing laparoscopic DA repair. Both open and laparoscopic approaches are considered acceptable and safe options for repair when performed by an appropriately trained surgeon.

1.3. Duodenal embryology

In the third week of fetal development, the primitive gastrointestinal tract is formed by a hollow cylinder of endodermal cells surrounded by mesoderm (Williams and Bhatia, 2014). This endoderm cylinder then lengthens, folding ventrally at the cranial and caudal ends, meeting near the yolk sac to form a closed tube. Two other portions of the endoderm-lined cavity, the yolk sac and the allantois, remain outside the embryo (Sadler, 2019). In the fourth week, the cranial end of the digestive tube is closed by the resorption of the buccopharyngeal membrane (Huisman and Kellenberger, 2008).

From here, the gastrointestinal tract develops into three distinct regions, giving rise to specific anatomical portions (Sinnatamby and Last, 2011). The foregut forms the pharynx, oesophagus, stomach, liver, gallbladder, pancreas and the cranial portion of the duodenum. The midgut (which spends weeks six to ten herniated through the umbilical opening, where it develops almost entirely outside the peritoneal cavity before rotating and returning to the abdomen) forms the remainder of the duodenum, along with the jejunum, ileum, the ascending and cranial two-thirds of the transverse colon. The hindgut forms the caudal transverse and descending colon, the rectum and the urogenital sinus.

Regional specification of the gut tube into different components is initiated by transcription factors expressed in the different regions. SOX2 'specifies' the oesophagus and stomach, PDX1 the duodenum (Chen et al., 2009), CDXC the small intestine, and CDXA the large intestine and rectum (Sadler, 2019, Roberts, 2000). Sonic hedgehog (SHH) stabilises the epithelial-mesenchymal interactions throughout the gut, upregulating the aforementioned 'specifying' transcription factors (Nielsen et al., 2004).

During maturation, the mucosal surface of the intestine rapidly proliferates, out-pacing the outer layers, causing it to fold and form villi. Within the duodenum, it has been widely accepted that the epithelium proliferates so rapidly that during this period it occludes the lumen (Grand et al., 1976, Montgomery et al., 1999). This 'overcrowding' of mucosal tissue is suggested to have been what Tandler observed, leading him to propose the 'solid cord' hypothesis (Meio et al., 2008), and its apoptosis was the proposed process through which recanalisation 'failed' leading to DA (Moore and Persaud, 1998). Several studies in human embryos found inconsistent duodenum obstruction during this phase, leading the authors to question the validity of a normal physiological obstructed phase in duodenal development (O'Rahilly and Müller, 1984, Kluth et al., 1987, Boyden et al., 1967).

Notwithstanding, the most significant human embryonic study on duodenal development was performed by Matsumoto et al. in 2002 (Matsumoto et al., 2002). Some 133 embryos were analysed; 111 with light microscopy, six with scanning electron microscopy and 16 with transmission electron microscopy. These authors found that the middle and lower duodenum underwent a decrease in the calibre and area of the lumen, period of 'occlusion', before the secondarily re-establishing of the lumen with apparent coalescence of vacuoles (termed by these authors 'recanalisation'), all occurring in a craniocaudal timeline. This occlusion was observed at Carnegie Stage 15-18 (middle) and 17-20 (lower). Notably, Matsumoto et al. identified that occlusion was not associated with increased mitotic activity, and the so-called recanalisation demonstrated no increases in apoptosis within the lumen. Hence, they concluded that while, in humans, a 'solid cord' phase of duodenal development did occur, it was likely a disturbance in the epithelial-mesenchymal interaction, namely the budding morphogenesis occurring based on complex differential expressions of genes such as Shh and Pdx1 (Roberts, 2000), which was the cause of DA.

Importantly, these observations and interpretations of human duodenal development to include solid core and recanalization stages is at odds with the more detailed and rigorous parsing of murine duodenal development, in which there is explicitly no solid core stage, i.e. no loss of duodenal lumen (Cheng and Tam, 1998, Botham et al., 2012).

1.4. Fibroblast Growth Factor 10 and associated genes and proteins

The mammalian fibroblast growth factors (FGF) family contains 22 genes, 18 of which bind and activate tyrosine kinase FGF receptors (FGFR) using heparin-like molecules or klotho co-factors receptor binding (Ornitz and Itoh, 2015). These 18 FGFs are grouped into six sub-families based on biochemical function, sequence homology and evolutionary traits (Ornitz and Itoh, 2001). The FGF7 sub-family comprises FGF3, FGF7, FGF10 and FGF22 and preferentially binds and activates FGFR2b (the IIIb splice variant of the FGFR2 receptor).

FGFR2b is expressed in the epithelial layer and can be activated by FGF7 and FGF10 ligands (Ibrahimi et al., 2001). These ligands show no binding activity with the mesenchyme-expressed FGFR2c variant (Igarashi et al., 1998, Zhang et al., 2006). Conversely, the FGF8 ligand preferentially binds and activates FGFR2c, with no binding activity towards FGFR2b (Ornitz et al., 1996, MacArthur et al., 1995).

The activation of FGFR2b by FGF10 is associated with mesenchymal/epithelial interactions, which instruct branching and budding morphogenesis. This is a key signalling pathway for the normal development of the gastrointestinal tract (e.g. stomach (Spencer-Dene et al., 2006), duodenum (Kanard et al., 2005) and large intestine (Burns et al., 2004)) and its derivatives [e.g. lung (Park et al., 1998)]. Indeed, gastrointestinal development involves multiple signalling pathways and their associated transcription factors, including FGF10-FGFR2b, are summarised in **Figure 1.3A** and reviewed elsewhere (Kim and Shivdasani, 2016, Danopoulos et al., 2017).

FGF10-FGFR2b binding activates multiple intracellular signalling pathways, including the ERK1/2, MAP kinase pathway, to induce downstream activation of proliferation and cell survival genes (Luo et al., 2008, Yang et al., 2011) (**Figure 1.3B**). TBX4/TBX5 may induce *Fgf10* expression to promote region-specific stimulation of the mesenchymal proliferation at the initial sites of foregut growth. This growth depends on the regulation of the HOX and TBX genes, which are mediated by retinoic acid (RA) (Wang et al., 2006). Potentially, RA acts on TBX genes, such as TBX4 and TBX5, as a co-factor to activate FGF10 transcription and, as a result, mediates the FGF10-FGF8 positive feedback loop, which is required for outgrowth (Nishimoto et al., 2015). RA-deficient Embryos fail to outgrow due to defective FGF10 signalling, resulting in a lack of FGF-target genes in the epithelium. Of some interest, the development of the foregut may be partially restored in the defective embryos by introducing RA supplementation early on in development (between E7.5 to E8.5), suggesting that RA regulation is upstream of FGF10 (**Figure 1.3B**) (Wang et al., 2006).

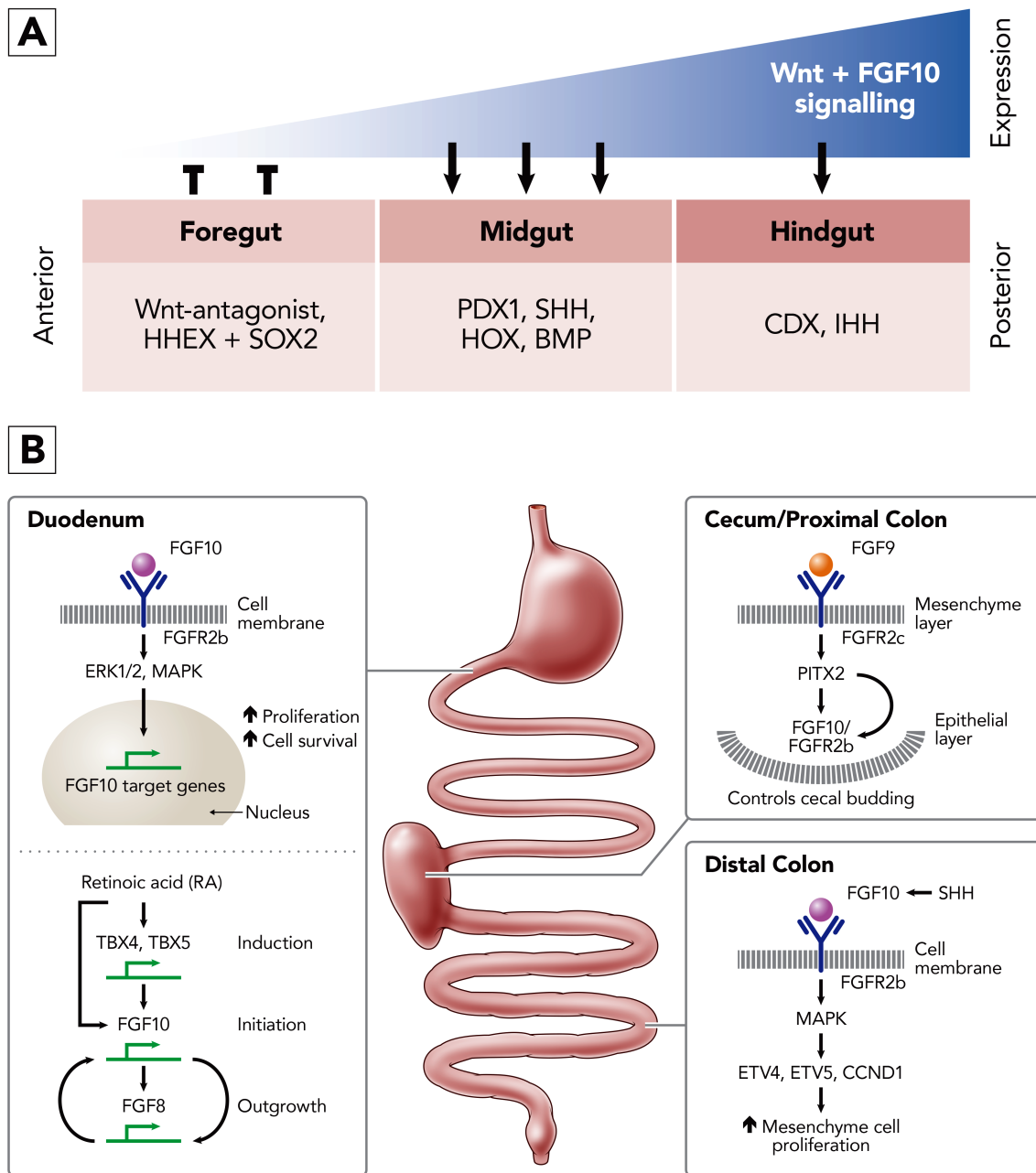


Figure 1.3: Schematic illustrations of the role of FGF10 in the developing and developed murine intestine. (A) The expression-dependent changes of key transcription factors in the foregut, midgut and hindgut mediated by differing levels of FGF10 and Wnt-signalling expression; Hematopoietically-expressed homeobox protein (HHEX), SRY (sex determining region Y)-box 2 (SOX2) in the foregut; pancreatic and duodenal homeobox 1 (PDX1), Sonic Hedgehog (SHH), homeobox genes (HOX), Bone morphogenetic proteins (BMP) in the midgut; Caudal-Type Homeobox Protein (CDX), Indian hedgehog (IHH) in the hindgut. **(B)** Key FGF10-pathways involved the development of the duodenum, cecum/proximal colon and distal colon; Fibroblast growth factor (FGF) 8, 9 and 10; Tyrosine kinase fibroblast growth factor receptors (FGFR) 2b, 2c; Extracellular signal-regulated kinases (ERK1/2); mitogen-activated protein kinase (MAPK); Thromboxanes B4 (TXB4); Thromboxanes B5 (TBX5); ETS translocation variant 4 (ETV4); ETS translocation variant 5 (ETV5); Cyclin D1 (CCND1); Sonic Hedgehog (SHH).

Within the caecum and proximal colon, FGF9 binds to the mesenchyme-expressed FGFR2c receptor to direct caecal mesenchyme expansion via the expression of PITX2. In conjunction with this, FGF10 triggers the epithelium's budding into the caecal mesenchyme, again through the mediation of PITX2 expression. Thus, the interaction between FGF10 and PITX2 is critical for the colon's maintenance and proliferation of the epithelial progenitor cells (**Figure 1.2B**). During embryonic development, FGF10 is also expressed in the mesenchyme of the distal colon, where it binds and activates FGFR1b and FGFR2b to promote proliferation, differentiation and survival of the epithelium. This activation maintains progenitor cells within the embryonic small intestine by targeting E26 transformation-specific (ETS)-Family genes such as ETV4, ETV5 and CCND1 through the MAP kinase (MAPK) pathway (Nyeng et al., 2011) (**Figure 1.3B**).

The homozygous deletion of the *Fgf10* or *Fgfr2b* gene results in mice with multiple organ defects, including lung, limb and mammary glands (with failure to form buds 1, 2, 3 and 5). The resultant mice are non-viable after birth due to lung agenesis (De Moerlooze et al., 2000, Mailleux et al., 2002).

1.5. The role of Fibroblast Growth Factor 10 in duodenal atresia

In 2004, Fairbanks et al. (Fairbanks et al., 2004b) were the first to identify a link between the *Fgf10/Fgfr2b* signalling pathway and intestinal atresia in mice. Using *Fgfr2b* knockout mice, they observed DA in 35% of null embryos when examined at gestational stage E18.5. These authors reported examples of both type 1 DA (57%) and type 3 DA (43%) but no type 2 DA (Fairbanks et al., 2004b). The same group then turned their attention to the interruption of the ligand instead of the receptor, knocking out *Fgf10* (Kanard et al., 2005). It is noteworthy that *Fgf10* knockout embryos were found to have a similar penetrance of DA (38%) compared to *Fgfr2b* knockouts, but with a notably different relative frequency of DA types; type 1 DA (8%), type 3 DA (92%), and still no type 2 DA (Kanard et al., 2005).

Further emphasising the importance of the *Fgf10/Fgfr2b* pathway in intestinal development, deletion of either *Fgf10* or *Fgfr2b* also resulted in a more distally located intestinal atresia, i.e. colonic atresia (Fairbanks et al., 2004c). In contrast with the incomplete penetrance of DA, colonic atresia was present in 100% of *Fgf10* and *Fgfr2b* knockout mice embryos (Fairbanks et al., 2004c)

Notably, the morphology of the duodenum is normal in *Fgf10* hypomorphic mice, with no examples of DA in this setting of *Fgf10* under-expression (Teague et al., 2018). Thus, even reduced *Fgf10* signalling is sufficient for morphologically normal duodenal development. Similarly, Sala et al. (Sala et al., 2006) found normal colonic development in *Fgf10* hypomorphic mice, whereas complete loss of *Fgf10* gene function resulted in colonic atresia. In the colon, *Fgf10* expression has been shown to be expressed first distally before extending proximally and found to be critical for the maintenance and proliferation of the epithelial progenitor cells in the colon. Furthermore, crypt formation seen within the proximal colon in the absence of *Fgf10* has suggested that other signalling pathways were active in the development of this region, reinforcing the early anatomical and functional regionalisation of the gastrointestinal tract early during development. Another important finding of Sala et al. (Sala et al., 2006) was that atresia occurred without an associated loss of mesenchyme. This finding makes vascular compromise an unlikely mechanism for colonic atresia in *Fgf10* knockouts, as a vascular event would be expected to cause loss of both mesenchyme and epithelium.

The impact of *Fgf10* over-expression has also been investigated in *ex vivo* and *in vivo* models. Torashima et al. (Torashima et al., 2016) showed that adding *Fgf10* to tissue-engineered small intestine (TESI) resulted in increased size and weight of the sample and increased villi height and crypt depth. In this setting, epithelial differentiation was not inhibited by over-expression of *Fgf10*, a finding attributed to the lack of typical feedback inhibitory mechanisms in the *ex vivo* TESI samples.

Nyeng et al. (Nyeng et al., 2011) studied *in vivo* over-expression of *Fgf10* within the developing duodenum. In these embryos, *Fgf10* over-expression caused attenuation of cell differentiation and expansion of the progenitor niche along with upregulation of FGFR signalling targets known to exert feedback inhibition on the FGF pathway (e.g. Sprouty2, Etv4, Etv5) (Nyeng et al., 2011, Minowada et al., 1999). Conversely, the complete loss of *Fgf10* led to the premature differentiation of all enteric cell lineages in the duodenum (Nyeng et al., 2011). These findings support the inference that *Fgf10* is necessary to prevent premature differentiation of enteric cell lineages and maintain the epithelial progenitor cell population during development. In an extension of this conclusion, these authors hypothesised that premature differentiation and the resultant depletion of the progenitor cell population are responsible for the epithelial deficiencies of DA in *Fgf10* knockout mice (Nyeng et al., 2011).

An alternative to the premature differentiation hypothesis is that proposed by Botham et al. (Botham et al., 2012). These investigators concluded that exaggerated endodermal apoptosis, rather than terminal differentiation of progenitor cells, was the root cause for DA in *Fgfr2b* knockout mice. In the atretic region, endodermal apoptosis was seen at E10.5, with complete endodermal disappearance by E11.5 and involution of the mesoderm at E13.5. This timeline corresponded with the first recognition of DA in these *Fgfr2b* knockout mice, also at E13.5. Comparisons were also made between the duodenum of *Fgfr2b* knockout mice and otherwise normal human embryo samples. In mice, loss of endoderm is complete at an earlier stage of development (E11.5, Carnegie Stage 16). In humans, however, 'epithelial crowding' with a corresponding apparent loss of duodenal lumen is not seen until Carnegie Stage 17, with 'recanalisation' only at Carnegie Stage 18. Thus, there is a temporal dislocation between luminal occlusion in atretic murine and normal human duodenal development (Botham et al., 2012, Matsumoto et al., 2002). The timeline for murine DA is supported by the findings of Nichol (Nichol et al., 2012), who showed murine duodenal narrowing evident from E11.5 and DA from E13.5.

Another hypothesis is that of Merei (Merei, 2004) who proposed that intestinal atresia may result from aberrant notochord signalling. Reeder et al. (Reeder et al., 2012a) used *Fgfr2b* knockout mice and reported 42% penetrance of DA and 100% penetrance of colonic atresia. These investigators refuted the role of aberrant notochord signalling as the basis for murine DA, showing neither disruption of sonic hedgehog (*Shh*) expression nor notochordal discontinuity in *Fgfr2b* knockouts. Furthermore, organ culture of explanted wild-type murine gut in the presence of supra-physiological concentrations of *Shh* failed to induce atresia in the duodenum or the colon.

One striking feature of the DA reported in the aforementioned seminal studies is the morphological types of DA reported in *Fgf10* and *Fgfr2b* knockout mice (Fairbanks et al., 2004b, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012b). In humans, the distribution of DA types shows similar proportions of type 1 and type 3 DA, and few cases of type 2 DA (e.g. 54% type 1, 4% type 2, and 42% type 3 DA (Khan et al., 2017)). In contrast to this, when collated together, 93% of all murine DA cases reported in these studies were examples of type 3 DA, i.e. the most severe type of DA with complete discontinuity of the duodenum (**Figure 1.1D**) (Fairbanks et al., 2004b, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012b). None of these studies reported any examples of type 2 DA in mice.

Of relevance to DA type and the severity of malformation is the work by Reeder et al. (Reeder et al., 2012b). Reeder et al. investigated a role for retinaldehyde dehydrogenase 2 (*Raldh2*), a gene that encodes the enzyme responsible for the final step of vitamin A conversion to retinoic acid. *Raldh2* is critical for normal posterior foregut development (Wang et al., 2006) and is under-expressed in the duodenum of *Fgfr2b* knockout mice (Botham et al., 2012). Therefore, Reeder et al. combined heterozygous *Raldh2* deletion with homozygous deletion of *Fgfr2b* with the hypothesis of generating a more severe DA phenotype than with homozygous deletion of *Fgfr2b* alone.

Contrary to this hypothesis, the addition of *Raldh2* haploinsufficiency resulted in a reduced rather than increased penetrance of atresia (21% vs 45%) and a shift toward less severe atresia types: *Fgfr2IIIb*^{-/-}; *Raldh2* ^{+/-} (80% type 1 DA, 20% type 2 DA, and no type 3) versus *Fgfr2IIIb*^{-/-}; *Raldh2* ^{+/+} (7% type 1 DA, 0% type 2 DA, and 93% type 3 DA). These authors suggested that signalling downstream of *Fgf10/Fgfr2b* (i.e. *Raldh2*) was responsible for the phenotypic variations in DA-type morphology.

Teague et al. have recently reported (see appendix 2) a more human-like distribution of DA types, including type 2 DA, in two strains of CRISPR-derived *Fgf10* knockout mice (Teague et al., 2018). Of note, these newly reported strains, tm1 (B6-*Fgf10*<c.[464_470dup;506_645 del]APNMu>), and tm2 (B6-*Fgf10*<c.495_507delAPNMu>) (Eppig et al., 2006), each demonstrate all three DA types and a significantly higher penetrance of DA than previous animal models (74% (Teague et al., 2018) versus 35-45% (Fairbanks et al., 2004b, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012b)). The basis for differences in type distribution and penetrance compared with previously reported *Fgf10* knockout mouse strains remains unclear. The various strains are of consistent background, namely C57/Bl6 (Fairbanks et al., 2004b, Kanard et al., 2005), and whilst the genetics differ, each represents a “nonsense mutation” (Eppig et al., 2006). It is therefore proposed that signalling molecules and pathways downstream of *Fgf10-Fgfr2b* are moderating the morphology and penetrance of the DA phenotype in these various strains.

The findings of Al Alam et al. (Al Alam et al., 2012) support this. While an FGF9/Pitx2/FGF10 signalling axis had been proposed within the lung (De Langhe et al., 2008), Al Alam et al. demonstrated that the same signalling axis is active in the developing gut (Al Alam et al., 2012). Using tissue-specific *Fgf9* and *Pitx2* conditional knockout, they demonstrated that, within the cecum, FGF9 controls mesenchymal *Pitx2* expression and, in turn, *Fgf10* expression. They also found that *Pitx2* induced the expression of *Fgf10*, even in the absence of *Fgf9*, suggesting that *Pitx2* is downstream of *Fgf9*.

In further attempts to define pathways upstream of *Fgf10* involved in atresia, Reeder et al. investigated the role that Sonic hedgehog (*Shh*) plays with *Fgfr2b* (Reeder et al., 2014). Using a Matrigel culture system and focusing on the colon of *Fgfr2b* knockout mice, they found downregulation of *Shh* at E11.5 throughout the entire colon and then loss of *Foxf1* expression 12 hours later in the mid-to-distal colon prior to its involution. They also found that the exogenous addition of *Shh* to the *Fgfr2b* deficient colon failed to prevent the formation of atresia, suggesting that the loss of *Shh* is not a critical event in atresia formation.

2. Hypotheses and aims

2.1. General hypothesis

Interruption of FGF10-FGFR2b signalling is the best demonstrated genetic link to DA in mice. This corresponds in importance, and hypothesis-formulating relevance, to the association between DA and Trisomy 21 in humans. *FGF10* haploinsufficiency and *FGF10* mutations have been linked to human disease, principally to craniofacial syndromes (Milunsky et al., 2006, Entesarian et al., 2005, Rohmann et al., 2006), chronic respiratory disease (Klar et al., 2011), but no human gastrointestinal tract atresia cases to date (Tatekawa et al., 2007). In contrast to this narrative of haploinsufficiency, homozygous *Fgf10* invalidation appears necessary for DA in animal models (Teague et al., 2018), albeit accompanied by non-survivable anomalies such as lung agenesis. Therefore, based upon these findings and the currently available literature regarding *Fgf10-Fgfr2b* signalling in duodenal atresia, the principal hypothesis for this research is that:

The aetiology of DA in humans is genetically driven, and the causative genetic changes are downstream of the *FGF10/FGFR2b* signalling pathway.

This downstream locus may account for both the incomplete penetrance of DA in the murine model and the different morphological types (i.e. severity) of DA. It may also explain why human DA patients lack the non-survivable associations of *Fgf10* deletion. Therefore, this hypothesis was explored by investigating the up- and downregulation of genes and pathways in atretic and morphologically normal duodenum. Gene expression analysis focused on the duodenum of CRISPR-derived *Fgf10* knockout mice with and without duodenal atresia and additional wild-type controls. The assumption was that all three of these mice populations are necessary to discern candidate genes and pathways responsible for DA within the murine model.

2.2. PhD thesis hypotheses and specific aims

This PhD thesis consists of three components which together support the principal hypothesis:

Hypothesis 1: *Fgf10* deletion results in an incomplete penetrance of DA in mice, with a human-like morphology distribution. (Chapter 4)

Aim 1: To characterise the general and gastrointestinal morphology of CRISP-derived *Fgf10* knockout mice by performing microdissection on *Fgf10*^{-/-} embryos, documenting the presence and type of DA.

Hypothesis 2: Signalling pathways related to the *Fgf10-Fgfr2b* axis moderate the morphology and penetrance of the DA phenotype seen in *Fgf10*^{-/-} embryos. (Chapters 5, 6 and 7)

Aim 2: To identify the novel *Fgf10*-mediated gene expression pathways in the duodenum using genome-wide mRNA sequencing at the embryonic age demonstrating the most significant *Fgf10* activity. Key genes shown as up- and downregulated in mRNA sequencing will be profiled using RT-qPCR across differing embryonic ages to assess activity before and after the occurrence of DA.

Hypothesis 3: Organoids grown from the duodenal tissue of *Fgf10*^{-/-} embryos will demonstrate differences in growth pattern compared to wild-type duodenal tissue. (Chapter 8)

Aim 3: Develop an *ex vivo* model to demonstrate embryonic duodenal growth. To assess the growth pattern of duodenal organoids, including the ability to form crypts. To perform staining to compare cell activity, including proliferation and differentiation.

3. Material and methods

3.1. General chemicals

Table 3.1 List of general chemicals

Reagent	Manufacturer	Catalogue no.
Agarose	Astral	AM0710
Bovine serum albumin (BSA)	Sigma-Aldrich	A9418
Diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542
DNA ladder (1kb)	New England Biolabs	N3200L
DNaseI	Qiagen	79254
dNTP	Promega	U1511
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	11965084
EDTA	Sigma-Aldrich	3609
Ethanol	Sigma-Aldrich	7023
Fast Start Universal SYBR Green Master	Roche	4913914001
Gel Red	Gene Target Solutions	41003
Glycerol	Sigma-Aldrich	G5516
GoTaq	Promega	M3001
GoTaq Buffer (5x)	Promega	M7911
Growth Factor Reduced Matrigel	Corning	354230
Isopropanol	Crown Scientific	425
Lysis buffer	Qiagen	74104
Magnesium chloride (MgCl ₂)	Promega	A3511
Mini RNasey Kit	Qiagen	74104
Nsi1	Promega	R5351
Nuclease free water (dH ₂ O)	Astral	E476
Phosphate-buffered saline	Sigma-Aldrich	P5493
PIPES	Sigma-Aldrich	RDD004
Potassium chloride (KCl)	Sigma-Aldrich	60135
Tris-base ultra-pure,	Astral	AM0497
Tris-borate-EDTA (TBE) buffer	Sigma-Aldrich	T4415
Tris-Hydrogen Chloride (HCl)	Roche	10812846001
Triton X100	Sigma-Aldrich	93426
Tween-20	Sigma-Aldrich	P1379

3.2. *Fgf10* knockout mice (*Fgf10*^{-/-})

3.2.1. Generation of *Fgf10* knockout mice

Mutant animals were produced via CRISPR/Cas9 injection of C57/Bl6 oocytes by Monash University as a node of the Australian Phenomics Network (APN). In contrast to Yasue *et al.* (Yasue *et al.*, 2014), who used CRISPR to target exon 1 of the *Fgf10* gene, these strains were derived using RNA guides targeting exon 3, designed to minimise 'off-target' events.

Thus, two novel murine strains were developed, the first with a 7bp duplication and 140bp deletion (B6-*Fgf10*<c.[464_470dup; 506_645del]APNMu>), and the second with a 13bp deletion (B6-*Fgf10*<c.495_507delAPNMu>) (Eppig *et al.*, 2006). These strains are named 'tm1' (464_470dup; 506_645del) and 'tm2' (495_507del), respectively.

3.2.2. General husbandry and breeding

Mice were maintained in a temperature- (24°C) and lighting- (14:10 h light-dark cycle) controlled room with free access to food and water within the accredited, institutional animal facility and cared for strictly with animal welfare and ethical requirements.

To establish breeding colonies for each strain, wild-type mice were mated with heterozygous mice, and liveborn pups were genotyped to inform successive matings (see genotyping protocols below). *Fgf10* mutation is fatal in its homozygous (null) form due to lung agenesis (Sekine *et al.*, 1999). Therefore, heterozygous plug-mating was reserved for experimental use only to generate null, heterozygote and wild-type embryos. For experimental matings, pregnant dams were humanely culled at timed gestations to provide timed embryos ranging from E9.5 to E19.5.

3.2.3. Genotyping

3.2.3.1. DNA extraction

For genotyping, tail or ear clippings were placed in 600µl of extraction buffer (50mM of NaOH, pH 8.5), centrifuged at 14,000rpm for 1min and placed on a heat block for 15mins at 100°C. Each sample was vortexed for 1min, then returned to the heat block for a further 5mins at 100°C. Samples were removed from the heat and allowed to cool for 20mins at room temperature. Genomic DNA was precipitated with 50µl of 1M Tris-HCl (pH 8.0), mixed well in readiness for PCR. DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientifics, 2000) at 260nm wavelength.

3.2.3.2. Polymerase chain reaction (PCR)

The primer sequences used to genotype tm1 mice are shown in **Table 3.2**. These primers were then used for the PCR program, which was conducted on DNA as per **Table 3.3**.

Table 3.2 The primer sequences used to genotype tm1 mice

Primer ID	Primer sequence 5' – 3'	PCR target and size	
		Wild Type	Mutant
Forward	GGAGTGTAGATCATTACATGGC	348bp	215bp
Reverse	GTGAGGATACCATCTCTTTCTGTCC		

Primer set for establishing the PCR for the detection of tm1 mutation

Table 3.3 Optimised PCR conditions for identifying tm1 deletion on exon 1 of *Fgf10*

Reaction Mix		Reaction Conditions	
Genomic DNA	1 μ L	<i>Initial denaturation</i>	95 °C for 3 mins
Primers	0.05mcM each	<i>Denaturation</i>	95 °C for 30 s
GoTaq (Promega Cat:M3001)	0.025U	<i>Annealing</i>	60 °C for 30 s
5x GoTaq Buffer	1.5mM		
dNTP	0.2mM	<i>Extension</i>	72 °C for 1 min
MgCl	1.5mM		(return to denaturation for 36 cycles)
H2O to make a reaction volume	20 μ l	<i>Completion</i>	72°C for 5 mins

Polymerase chain reaction (PCR), Minutes (mins), Seconds (s)

The primer sequences used to genotype tm2 mice are shown in **Table 3.4**. These primers were then used for the PCR program, which was conducted on genomic DNA as per **Table 3.5**. On completion of the PCR, a Nsi1 digestion protocol was conducted as per **Table 3.6** to split the wild-type allele to 242bp and 148bp.

Table 3.4 The primer sequences used to genotype tm2 mice

Primer ID	Primer sequence 5' – 3'	PCR target and size	
		Wild Type	Mutant
Forward	GGAGTGTAGATCATTACATGGC	390bp	377bp
Reverse	GAATTCAGGGCTATGTCTTTGC	(242bp and 148 bp after Nsi1 digestion)	

Primer set for establishing the PCR for the detection of tm1 mutation

Table 3.5 Optimised PCR conditions for identifying tm2 deletion on exon 1 of *Fgf10*

Reaction Mix		Reaction Conditions	
Genomic DNA	1 μ L	<i>Initial denaturation</i>	95 °C for 3 mins
Primers	0.1mcM each	<i>Denaturation</i>	95 °C for 30 s
GoTaq (Promega Cat:M3001)	0.05U	<i>Annealing</i>	60 °C for 1 min
5x GoTaq Buffer	3mM	<i>Extension</i>	72 °C for 1 min
dNTP	0.4mM	<i>(return to denaturation for 30 cycles)</i>	
MgCl	3mM		
H2O to make a reaction volume of	10 μ l	<i>Completion</i>	72°C for 5 mins

Polymerase chain reaction (PCR), Minutes (mins), Seconds (s)

Table 3.6 Nsi1 digestion protocol for the tm2 Wild-type allele

Reaction Mix		Reaction Conditions	
PCR products	10 μ L	<i>Digestion</i> 37 °C for 90mins	
Nsi1 (Promega, Cat:R5351)	0.05U		
10x Nsi buffer	1 μ L		
10x BSA	1 μ L		
H2O to make a reaction volume of	20 μ l		

Polymerase chain reaction (PCR), Minutes (mins), Seconds (s)

3.2.3.3. Electrophoresis

After PCR amplification, 1.5% agarose gel electrophoresis was performed in 1x Tris-Boric acid-EDTA (TBE) buffer (90mM Tris-boric, 2mM EDTA) at 100V for 35mins, using 1kb DNA ladder (New England Biolabs, Cat: N3200L). PCR products were stained with Gel Red (Gene Target Solutions, Cat:41003) and visualised with Bio-Rad Gel Doc at standard UV transilluminator 302nm (Life Sciences, 2001).

3.2.4. Animal ethics

All procedures were performed at the Murdoch Children's Research Institute (MCRI) and Walter and Eliza Hall Institute of Medical Research (WEHI) with the approval of the MCRI Animal Ethics Committee (A792 and A950).

3.3. Tissue collection

3.3.1. Microdissection for DA profiling

Tm1 and tm2 embryos of gestation E9.5-E19.5 were humanely collected, culled, and micro-dissected under a bright field dissecting microscope (Leica, M165 FC). Key phenotypic features were noted, and still images were recorded using the Leica Application Suite image capture. Particular attention was shown to the morphology of the foregut and the determination of the presence and type of DA. Where ambiguous, duodenal morphology and patency were further investigated using wholemount immunostaining or paint-filling as adapted from techniques to delineate embryonic murine inner ear morphology (Morsli et al., 1998). The entirety of the duodenum from the pylorus to the duodenojejunal flexure was preserved intact, with back-light microscopy to confirm patency. The duodenal type was determined by consensus of two investigators at the time of dissection, and then representative images were secondarily re-assessed independently by two or more authors blinded to the original type determination to provide internal validation.

3.3.2. Tissue processing for mRNA extraction

Tm1 embryos of gestation E9.5, E11.5, E13.5, E15.5, and E19.5 were humanely collected, culled, and micro-dissected fresh in Phosphate-buffered saline (PBS) under a bright-field microscope (Leica, M165 FC). For *Fgf10* ^{-/-} embryos, particular attention was paid to the presence and type of DA. Emphasis was placed on the careful but prompt identification and removal of the duodenum from the embryo before being immediately snap-frozen in dry ice to preserve mRNA quality.

The oesophagus, stomach, small intestine and large intestine were also harvested via this same method for use in parallel or future experiments. This additional tissue was collected to ensure maximal scientific benefit was extracted from each experimentally bred embryo. Extracted tissue was stored at -80°C in preparation for mRNA extraction (see section 3.4.1).

3.3.3. Tissue processing for organoid studies

Tm1 embryos of gestation E12.5 were humanely collected, culled, and micro-dissected fresh in Phosphate-buffered saline (PBS) under a bright-field microscope (Leica, M165 FC). For *Fgf10* ^{-/-} embryos, particular attention was paid to the presence and type of DA. During the dissection process, the ‘organ block’ of the embryo was regularly transferred to fresh PBS-filled wells to ensure no cell contamination of the duodenal tissue from surrounding organs. Once isolated, the duodenum was placed on a fresh well before being minced with dissection scissors. The minced duodenal tissue with a small volume of PBS was then transferred into a 1mL tube (Eppendorf) in preparation for organoid seeding (see section 3.5.1).

3.4. Molecular biology

3.4.1. mRNA extraction

Embryonic tissue samples isolated were pooled (i.e. three duodena per group sample), then minced and rapidly homogenised in 500µl of lysis buffer (Qiagen, Cat:74104) using a homogeniser. Samples were then centrifuged at max speed for 3mins, and RNA solution was transferred to RNasey® Mini Kit spin columns (Qiagen, Cat:74104), spun down at 12000rpm for 30 seconds discarding the flow through each time. Columns were washed with RW1 buffer (from Qiagen mini RNasey Kit) and centrifuged once again for 30 seconds at 12000rpm facilitating binding to the spin columns. A mixture of DNaseI (Qiagen, Cat:79254) and RDD buffer was made (1:7 dilution, RDD buffer provided in Kit) and placed onto the spin columns containing the bound mRNA for 15 minutes at room temperature.

RW1 buffer was, again, added to the spin columns and spun down at 12000rpm for 30 seconds, followed by two washes of RPE buffer with the same conditions. Spin columns were dry span at max speed for 2 minutes with a new collection tube to remove any residue of wash buffer on the column membrane. The RNasey spin column was air-dried for 10 minutes at room temperature. Twenty to 30 microliters of elution buffer (nuclear-free H₂O) were added to the spin columns, incubated for 1 minute and spun down into a new 1.5ml tube at 12000rpm for 1min. The extracted mRNA from the spin column was transferred into a new tube and stored at -80°C until required for analysis.

mRNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientifics, 2000) at 260nm wavelength. Two microliters of sample were placed on the spectrophotometer reader and measured. Concentration was determined, and nuclease-free water was used as a blank. In order to evaluate the quality of the mRNA sample, the absorbance at 260nm was compared to the wavelengths of 280nm and 230nm, which determined the organic and protein contamination within the RNA samples. The samples that read 260/290 and 260/230 ratios close to 2 were accepted as good-quality mRNA.

This process was mirrored for experiments utilising tissue from alternate gut regions to allow comparison between regions.

3.4.2. mRNA sequencing (mRNA-seq)

Duodenal and colonic tissue harvested underwent mRNA-Sequencing (mRNA-seq). The library preparation, data processing and Illumina sequencing was performed at the Walter and Eliza Hall Institute of Medical Research (WEHI) Melbourne. Sequencing libraries were prepared and then sequenced at a concentration on the NovaSeq platform (Illumina Inc). Differential gene expression analysis was performed using EdgeR; differential expression was defined as fold change (Log₂FC) ≥0.2.5 with a false discovery rate (FDR) and adjusted P-value ≤0.05. Gene set enrichment analysis (GSEA) was used to identify pathways and functionally-grouped gene sets.

3.4.3. Complementary DNA formation

Complementary DNA (cDNA) was synthesised from the isolated RNA using the BioRad iScript Advanced cDNA kit (BioRad, Cat: 170-8842). The synthesis was performed in a thermal cycler with the following conditions; 25°C for 10 minutes, followed by 37°C incubation for two hours, then at 85°C for 5 minutes to stop the enzymatic reaction and then 4°C indefinitely. The total volume of cDNA with nuclease-free water was adjusted to 10ng/μl and stored at -20°C. 2μl of the cDNA was used as a template for real-time quantitative polymerase chain reaction (RT-qPCR).

3.4.4. Real-time quantitative polymerase chain reaction (RT-qPCR)

Nucleotide sequences for the target mRNA were extracted using the National Centre for Biotechnical Information (NCBI) browser (<http://www.ncbi.nlm.nih.gov/nucleotides>). Primers were designed using NCBI Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). To ensure that the primers target the gene of interest, they were confirmed using University of California Santa Cruz (UCSC) In-Silico PCR (<https://genome.ucsc.edu/cgi-bin/hgPcr>) and UCSC BLAT (<https://genome.ucsc.edu/cgi-bin/hgBlat>). Primers were ordered from Sigma-Aldrich and resuspended in TE buffer (100mM Tris, 1mM EDTA) to make at 100pmol/μl. For qPCR reactions, the primer stock was diluted 1:10 for 10pmol/μl in reaction mixture. 2μl sample was added onto each well of a 96-well optical plate (Applied Biosystems, Cat: 4346906). 6.5μl of Fast Start Universal SYBR® Green Master (Roche, Cat: 04913914001), 0.5μl of each primer (forward and reverse) and 3.5μl TE buffer were added to each well and samples were amplified in duplicate using the Sequence Detector (Applied Biosystems, Prism 7500). Reactions were first incubated at 95°C for 10 minutes, followed by 30-45 cycles of 95°C for 10 seconds and 60°C for 30 seconds per cycle. Threshold cycles (Ct) were measured in the exponential phase for all samples.

3.4.5. Analysis of cDNA expression in RT-qPCR

Expression of *Rpl32* and *Actb* housekeeping genes were used to normalise the expression levels in the cDNA samples (Kriegova et al., 2008). The relative expression was calculated as (Gene Ct – *Actb/Rpl32* Ct = $C\Delta t$). To determine the fold change, the formula was first calculated ($\Delta\Delta Ct = \text{Untreated } C\Delta t - \text{Treated } C\Delta t$), then as $\text{Fold Change} = 2^{-(\Delta\Delta Ct)}$. All primer sequences used for detecting RNA transcripts are in **Table 3.7**. The results were analysed using the 7500 SDS software (Applied Biosystems). Statistical analysis was performed in Prism 7.0 (GraphPad Software). Error bars on the results are presented as a standard error of the mean (SEM). Statistical significance was evaluated using Student's unpaired t-test; a p-value of <0.05 was considered significant.

Table 3.7: List of RT-qPCR primers for cDNA analysis

Gene ID	Forward (5'-3')	Reverse (3'-5')
<i>Abat</i>	CCATTGGTTAAAGCCCAGAA	GGTCAGAGACACACCCACCT
<i>Actb</i>	CATTGCTGACAGGATGCAGAAAGG	TGCTGGAAGGTGGACAGTGAGG
<i>Barx1</i>	GGACAATTAAGGGCCAGACA	GGATAAAAGTGTGGGCAAGG
<i>Cubn</i>	TCCGCTTCACATCAGATGGCAG	GGAGCAGTTGAGATTGGGAAGG
<i>Fgf10</i>	CACCTATGCATCTTTTAACTGGC	TCTATGTTTGGATCGTCATGGGG
<i>Foxa2</i>	CGAGCACCATTACGCCTTCAAC	AGTGCATGACCTGTTTCGTAGGC
<i>Gata3</i>	CAAGAGCAGCTCCTTCAACC	AGGGCTCTGCCTCTCTAACC
<i>Isl1</i>	AACAGCATGGTAGCCAGTCC	ACAGACTTCATGCGCTTCCT
<i>Kirrel2</i>	AATAGGATTCTCCCGCCTA	TGATAGCACGCCTCTCACAC
<i>Kynu</i>	CTGCGCTGAGAAGAAAATCC	TTGGGAATGGAAAAGGTGAG
<i>Nepn</i>	GTTTCCCAAACCAGCTTCA	CCTCGAACAAGCTGACAACA
<i>Nkx2-5</i>	CTACAAGTGCAAGCGACAGC	GGGTAGGCGTTGTAGCCATA
<i>Nmur2</i>	ATCCCTTCCAGTGTCTGTGG	ACCTAAGAGCAGGACCAGCA
<i>Oxct1</i>	TCCCCTTTAGCCTCTCCATT	TGAGCCTTTCCCATCATTTTC
<i>Pdx1</i>	TTCCCGAATGGAACCGAGCCTG	TTTTCCTCGGGTTCGCTGTGT
<i>Penk</i>	TACTGGGAACGGGAGACAAC	GCTTCTGCAGCTCTTTTGCT
<i>Rbp4</i>	CTGTGGACGAGAAGGGTCAT	AAAGGAGGCTACACCCCAGT
<i>Rpl32</i>	GAGGACCAAGAAGTTCATCAGG	CATTGTGGACCAGGAACTTGC
<i>Sfrp5</i>	TGGAGCCCAGAAGAAGAAGA	AGCAGCAGCTGTCCCTCTAC
<i>Sgk3</i>	CGCCGGATCTTCTTACCATA	ATAGCCCCGGAGGAAACTAA
<i>Six2</i>	GCCTGCAGGACTCCATACTC	TGAAAATGAATCCCCAAAGG
<i>Spp1</i>	GCTTGGCTTATGGACTGAGGTC	CCTTAGACTCACCGCTCTTCATG
<i>Tifa</i>	CAGGTGTCCCGAATTCAGTT	CTCCGTCTTCCTTCTGCAAC
<i>Zfc3h1</i>	GTTCCACAGAGGACCTCGTCTT	CTTCTCCGTCAGACTCACTGTC

3.5. Organoid model

3.5.1. Organoid seeding

Organoids were cultured using sterile techniques under a tissue culture hood, with the air-liquid interface method proposed by Ootani et al. (Ootani et al., 2009) to allow epithelial and mesenchymal structures to develop and interact. This culture system utilised a 12mm polycarbonate cell culture insert (Millipore, Millicell Cell culture insert, Cat: PIHP01250) with a permeable pore size of 0.4µm, which sit in a 24-well culture plate (Greiner).

300µL of Growth Factor Reduced Matrigel (GFR Matrigel) Basement Membrane Matrix (Corning, Cat: 354230), which was kept on ice to prevent gel formation, was pipetted into each cell culture insert. These cell inserts then sat for at least 30 minutes to allow the Matrigel to solidify. While the Matrigel was setting, the duodenum was extracted as per section 3.3.3. This experiment was performed in that order to ensure the duodenal tissue suffers the shortest period between being *in vivo* to *in vitro* in the 3D matrix.

Once prepared, the tubes containing minced duodenum (see section 3.3.3) were centrifuged at 800 rpm for 3 minutes. The supernatant was removed, and the duodenal tissue was resuspended in 300µL of GFR Matrigel while kept on ice. The duodenal-tissue-containing GFR Matrigel is then transferred into the cell culture insert to sit as an even layer above the first layer (now solid). The cell culture inserts are left for 30 minutes, sitting within the empty 24-well culture plate, allowing the top layer of GFR Matrigel to set with the duodenal tissue.

Once the GFR Matrigel had solidified, 600µL of Dulbecco's Modified Eagle Medium (DMEM) was added to the 24-well culture plate containing the cell culture inserts. Attention was paid to ensuring the level of DMEM surrounding the cell insert sits below the level of the suspended duodenal tissue (see **Figure 3.1**) and that no DMEM was placed on the surface of the GFR Matrigel. This was to ensure the presence of the air-liquid interface required for epithelial-mesenchymal interaction.

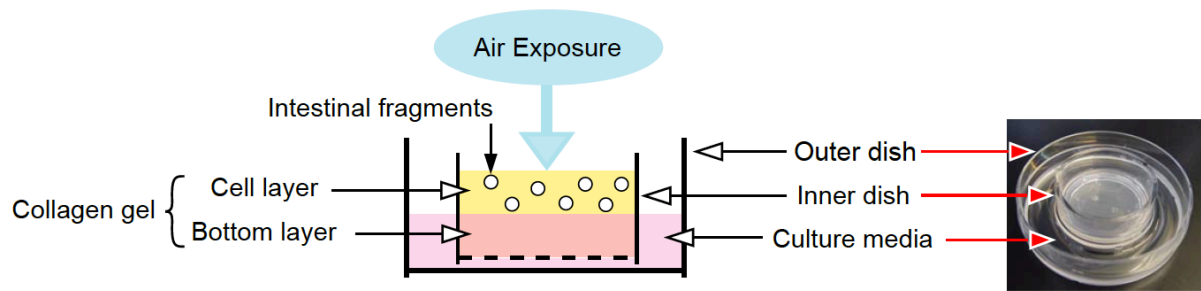


Figure 3.1: Air–Liquid Interface Culture System for 3D Organoid Culture of intestinal tissue. (Li et al., 2016a, Ootani et al., 2009)

3.5.2. Culture and growth of organoids

The 24-well plates containing the duodenal tissue in GFR Matrigel were kept covered and stored in a 37°C incubator with 5% CO₂ to facilitate organoid culturing. Plates were only removed from the incubator to have DMEM removed and replaced every 48 hours, during which light microscopy and photography (Leica, M165 FC) of the organoids within the wells was performed. This protocol was completed under sterile conditions within the tissue culture hood. Organoids were harvested for fixation and staining after 14 days or kept for 21 days to compare growth patterns.

3.5.3. Comparison of growth patterns

Every 48 hours, after the removal of DMEM from the surrounding well, the cell culture inserts were photographed. The timeframe for organoid formation, the number and size of spheroids and organoids formed, the presence of crypts, and the occurrence of peristaltic movements were documented. Organoids were grouped by their *Fgf10* genotype and by the pre-harvest duodenal morphology.

3.5.4. Organoid isolation and fixation

Organoids grown for wholemount staining were isolated and fixed after 14 days of culturing. The protocol was modelled off O'Rourke et al. with minor modifications made to account for the volume of GFR Matrigel surrounding the organoids (O'Rourke et al., 2016).

DMEM was removed from the outer well. The GFR Matrigel containing organoids, was carefully removed *en bloc* from within the cell culture insert and placed inverted (organoid containing half of the Matrigel block, facing down) into 8-well chamber slides (Thermo Fisher Scientific, Lab-Tek™, cat: 154532). In order to dissolve the Matrigel and fix the organoids, 300µL of freshly prepared 4% PFA in PME buffer (10x PME Buffer: 500 mM PIPES; 25 mM MgCl₂; 50 mM EDTA) were added to each well. After being left to sit for two hours (to fully dissolve the Matrigel), the fixative was removed by carefully pipetting on the sidewall of each chamber. This technique was used from here on out to remove solutions while ensuring the organoids were not lost from the chambers.

The organoids were washed in 300µl IF buffer (PBS containing 0.2% Triton X-10 and 0.05% Tween) on a benchtop orbital shaker (Ratek OM6) at 60 rpm. After the IF buffer was removed, 300µL of Permeabilising solution (PBS containing 0.5% Triton X-100) was added to the well and left at room temperature for 20 minutes. This solution was removed, and organoids were again washed with IF buffer as above, before adding 300µl of Blocking solution (IF buffer containing 1% bovine serum albumin (BSA)) and sitting at room temperature for 30 minutes.

3.5.5. Organoid wholemount staining

Primary antibodies used for staining were rabbit anti-KI-67 (1:200) and goat anti-Cytokeratin (1:200). Secondary antibodies were anti-rabbit Red (1:500) and anti-goat Green (1:500).

Primary antibodies were prepared in the Blocking solution for staining, and 200µL was added to each chamber. The chamber slide was left overnight, sitting in a larger humidified chamber, uncovered and protected from UV light, at 4°C. After overnight incubation, each chamber was washed three times for five minutes with 300µl IF buffer. This was performed on a benchtop orbital shaker (Ratek OM6) at 60 rpm. 200µl of Blocking solution containing the secondary antibodies was added to each chamber and left to sit for one hour at room temperature, protected from UV light.

This blocking solution was removed, 200µl of Diamidino-2-phenylindole (DAPI) in IF buffer (1µg/ml) was added to each chamber, and allowed to sit for five minutes. Each chamber was again washed three times with IF buffer, as above.

The remaining IF buffer was removed, and the chambers were detached from the base slide using the removal kit which comes with the slides. 20µl of ProLong Gold antifade was added to cover each organoid before the coverslip (Cover Glass, Rectangular #1 (24 × 50 mm, 0.12–0.16 mm) (Corning, Cat: 2975-245)) was carefully placed on top, and clear nail polish used to seal it to the slide. The slide was allowed to sit overnight, protected from UV light, at 4°C prior to being imaged.

3.5.6. Wholemout stained organoid image capture

Slides containing wholemount, stained organoids underwent image capture using a Dragonfly Spinning Disk Confocal microscope (Andor). Standard filter sets for red, green and blue spectra were used to capture staining for Cytokeratin, KI-67 and DAPI, respectively. Image acquisition was performed with Fusion software. Z-series acquisition was used to capture images at varying depths and surface locations over the organoid and combine these into a single 3D image.

3.6. Sample analysis and statistics

Statistical analysis was performed in SPSS Statistics (IBM). Error bars on the results are presented as a standard error of the mean (SEM), and a p-value of <0.05 was considered significant. Statistical significance was evaluated using student's unpaired t-test or two-way ANOVA, followed by Bonferroni test.

4. Generation and gastrointestinal characterisation of the *Fgf10* knockout mouse

To clarify the role and nature of genetic pathways in DA occurrence, a murine model was established, employing *Fgf10* knockout via the CRISPR method and utilising heterozygous cross-breeding to establish *Fgf10*^{-/-} embryos for investigation. These embryos were studied for phenotypical changes through microdissection and image capture. Their gastrointestinal tissue (including duodenum) was analysed for up- and down-regulation of alternate genetic pathways and harvested for *in vitro* analysis of growth patterns. In this chapter, it will be demonstrated that this CRISPR-derived *Fgf10* knockout mouse model is both valid and advantageous in the study of DA, warranting its inclusion in order to test hypotheses related to the role of *Fgf10* in DA development.

4.1. Introduction

The currently limited understanding of DA aetiology presents the most significant barrier to any research designed to identify potential strategies for preventing its occurrence in humans or delivering non-surgical management options to families. Investigation of the pathogenesis of DA is not possible in human embryos; therefore, a suitable animal model is required to advance the scientific knowledge regarding DA. Although Down syndrome is the best-understood genetic link with DA in humans, the animal models for Down syndrome fail to demonstrate DA or any other Trisomy 21-associated gastrointestinal abnormalities (Delabar et al., 2006).

Like Trisomy 21 in humans, and as previously detailed in section 1.5, interruption of FGF10/FGFR2b signalling is the best demonstrated genetic link to DA in mice (Fairbanks et al., 2004a, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012a).

Historically, the most substantial limitation in using these animal models in the study of DA is the relatively poor penetrance of the DA phenotype. DA is only present in 35-45% of *Fgf10*^{-/-} embryos, and these strains have failed to reiterate the full spectrum of DA phenotypes seen in humans (Fairbanks et al., 2004a, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012a).

Therefore, a more phenotypically robust *Fgf10* knockout model was required to provide an efficient, ethically sound, and scientifically valid basis for DA research. Details of how this mouse model was derived can be found in section 3.2. As will be later elaborated upon, only embryos of E13.5 or older were used to profile the penetrance and phenotypical spectrum of DA in this mouse model.

4.2. Results

4.2.1. Genotype

The product from classic PCR genotyping confirmed the expected genetics according to the tm1 and tm2 mutations (**Figure 4.1**). The PCR product for each strain was formally sequenced to confirm the correct action of the primers in each protocol. Semiquantitative PCR showed RNA expression for heterozygote and *Fgf10*^{-/-} embryos in both tm1 and tm2 strains (**Figure 4.1 A**). With wild-type litter mates providing controls for *Fgf10* gene expression in both strains, the heterozygous embryos expressed approximately half the *Fgf10* (tm1 = 42.29% and tm2 = 51.16%), and no expression was seen in *Fgf10*^{-/-} embryos.

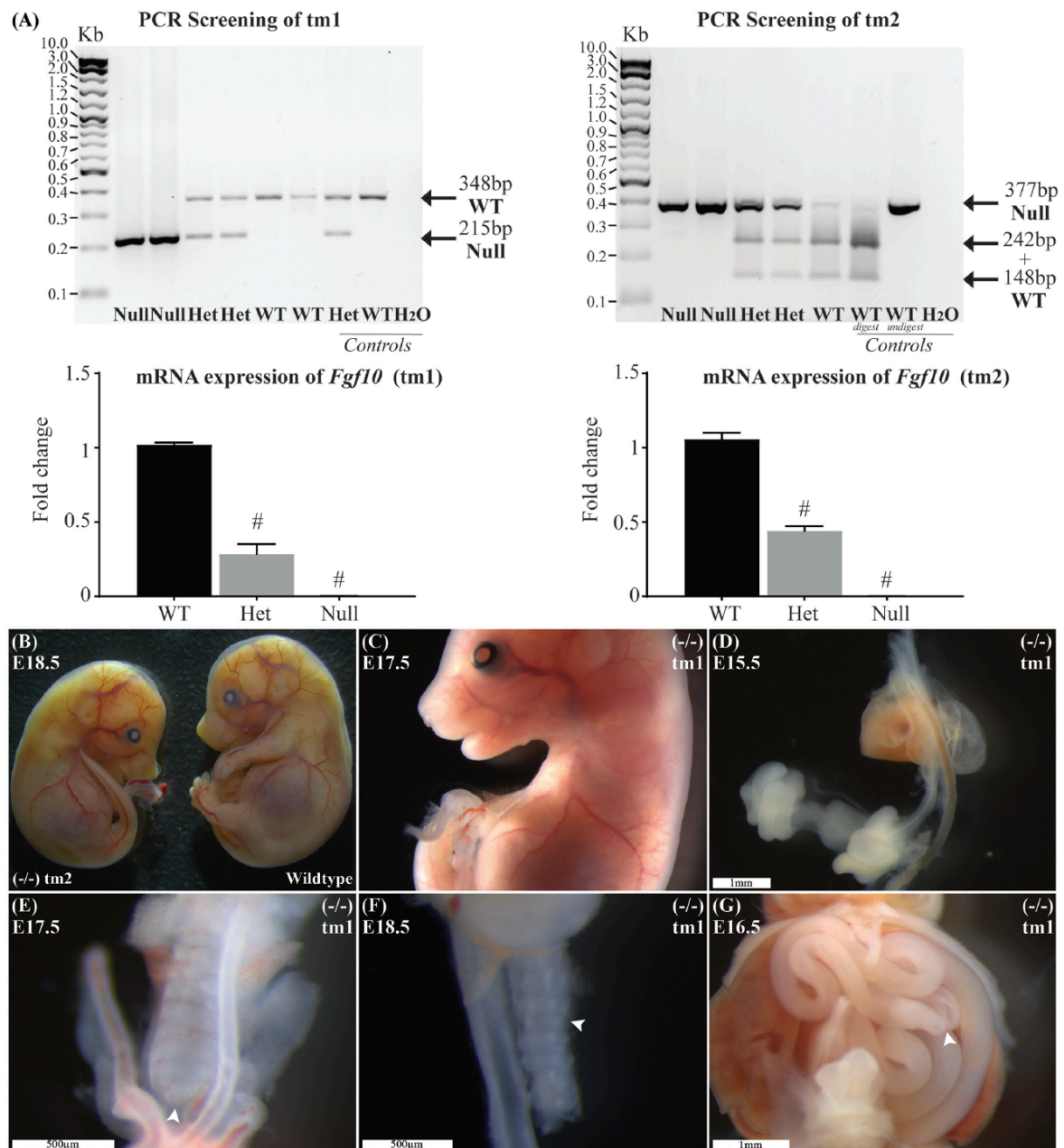


Figure 4.1: Genotype and general phenotype characterization. (A) Product from standard and RT-qPCR shows genotyping and RNA expression for both *tm1* and *tm2* strains. (B) General phenotype of a representative *Fgf10*^{-/-} embryo (left embryo) with the expected lack of limbs and smaller body size compared to wild-type litter mate (right embryo). *Fgf10*^{-/-} embryos also demonstrated: abnormal facies (C), lung agenesis (D–F), anomalous tracheal rings (F), and caecal atresia (G). Arrows highlight features of interest. Gestational ages for panels B–G are as stated. #*p*-value < 0.001, comparing either *Fgf10*^{+/-} or *Fgf10*^{-/-} mice to their wild-type littermates using Student's *t*-test.

4.2.2. General phenotype

The general phenotype of both tm1 and tm2 strain *Fgf10*^{-/-} embryos was consistent with previously reported *Fgf10* knockout mouse strains (Sekine et al., 1999, Teshima et al., 2016, Sala et al., 2011). These *Fgf10*^{-/-} embryos demonstrated a characteristic lack of limbs and smaller than average body size (**Figure 4.1, B-C**), abnormal facies (**Figure 4.1C**), lung agenesis (**Figure 4.1, D-F**), anomalous tracheal rings (**Figure 4.1F**), and caecal atresia (**Figure 4.1G**). Expected (i.e. normal) changes in organ morphology during development notwithstanding, these abnormalities in general phenotype were represented at all gestational ages of *Fgf10*^{-/-} embryos assessed. Further, as the experimental question and aim focused primarily on abdominal foregut morphology, detailed comparisons of these general phenotypic features during *Fgf10*^{-/-} embryo development were not performed, e.g. between tm1 and tm2.

The general phenotype of tm1 and tm2 *Fgf10* heterozygotes (+/-) mirrored that of wild-type (i.e. normal) litter mates, including morphologically normal limb buds, bilateral and branched lungs, normal appearance of tracheal rings and no caecal atresia. Finally, whilst the morphology and ultrastructure of some organs in *Fgf10*^{+/-} mice are known to be abnormal (Jaskoll et al., 2005, El Agha et al., 2012), these were not formally assessed for the tm1 and tm2 strains, except for foregut morphology as detailed below.

Wild-type littermates provided controls for normal gastric, pyloric and duodenal morphology (**Figure 4.2A**). Without exception, *Fgf10*^{+/-} embryos also demonstrated normal gastroduodenal morphology (**Figure 4.2, B-C**), distinct from *Fgf10*^{-/-} embryos.

4.2.3. DA phenotype

Of 132 *Fgf10*^{-/-} embryos E13.5 or older, 82 (62%) demonstrated DA. DA was observed in 61 (60%) tm1 and 21 (70%) tm2 *Fgf10*^{-/-} embryos. *Fgf10*^{-/-} embryos without DA did, however, demonstrate microgastria, which was a universal phenotypic feature for the *Fgf10*^{-/-} cohort (**Figure 4.2D, 4.2E**).

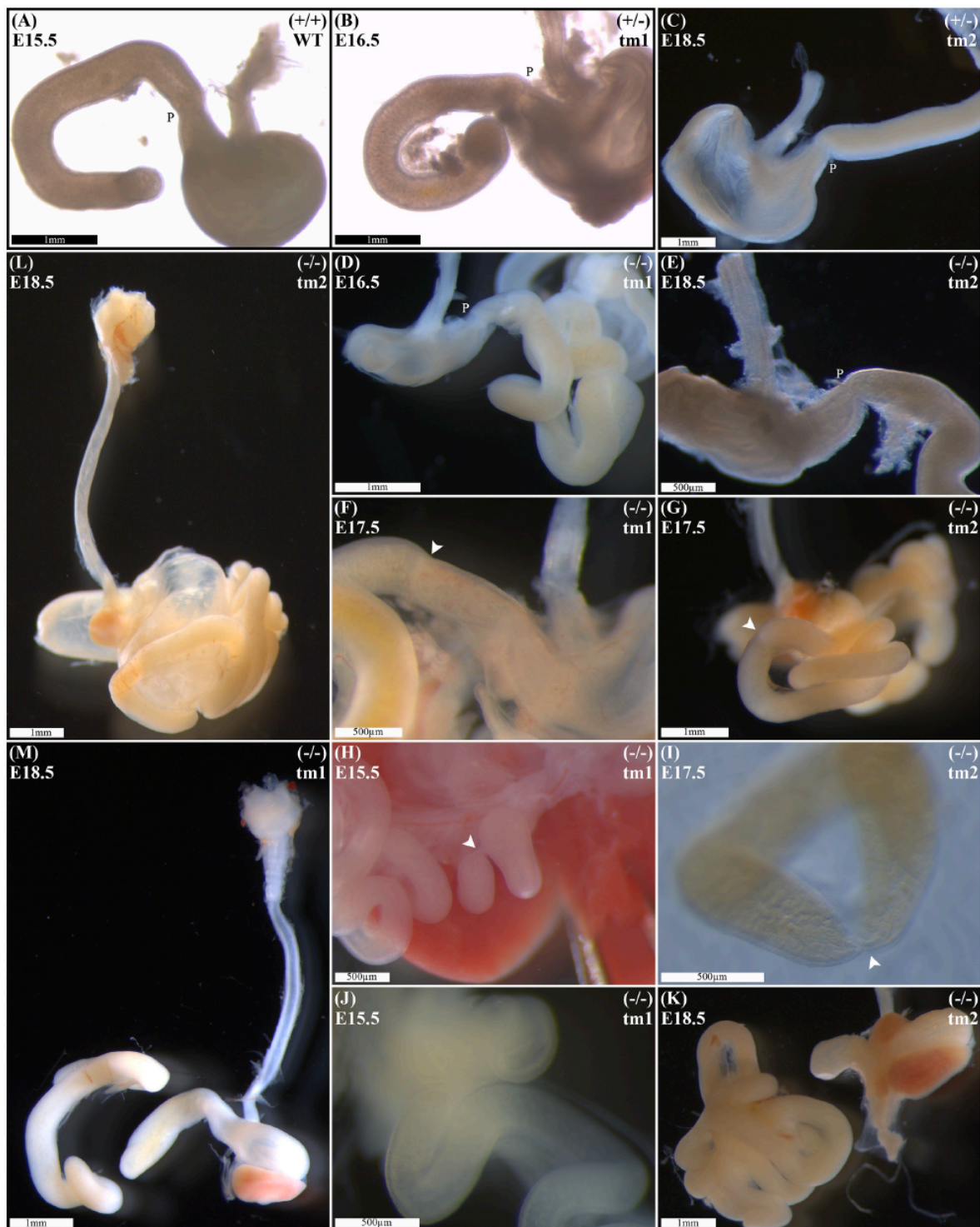


Figure 4.2: Duodenal phenotype characterisation. Normal gastric, pyloric, and duodenal morphology was demonstrated by wild-type embryos (A), as well as *Fgf10* heterozygous embryos for *tm1* (B) and *tm2* (C) strains. *Fgf10*^{-/-} embryos universally demonstrated microgastría but varied according to the presence and type of DA. *Fgf10*^{-/-} embryos provided examples of: normal continuity and morphology of the duodenum for *tm1* (D) and *tm2* (E); type 1 DA, *tm1* (F) and *tm2* (G); type 2 DA, *tm1* (H) and *tm2* (I); and type 3 DA, *tm1* (J) and *tm2* (K). (L) Type 2 DA demonstrating a “double bubble” with significantly dilated proximal duodenum; *tm2*. (M) Type 3 DA demonstrating an intact oesophagus in the presence of tracheal atresia; *tm1*. Annotations denote genotype, scale bars, and location of the pylorus, P. Arrows indicate location atresia in type 1 DA and type 2 DA examples. Gestational ages for each panel are as stated.

Both tm1 and tm2 strains provided examples for all three DA types: tm1 with 33 (32%) type 1, 10 (10%) type 2, and 18 (18%) type 3 DA; tm2 with 10 (48%) type 1, 4 (19%) type 2, and 7 (33%) type 3 DA (**Figures 4.2, F-K**).

DA in *Fgf10*^{-/-} embryos reiterated the type morphology of DA in humans. In each case, the morphology of DA was recognisably distinct from the physiological luminal narrowing at the junction between the stomach and duodenum, i.e. pylorus (**Figure 4.2, A-E**). Type 1 DA is characterised in these examples (**Figure 4.2F, 4.2G**) by continuity of the outer (serosal) aspect of the fetal gut wall, whilst a web obstructs the lumen at the site of atresia. Other examples of type 1 DA (not shown in **Figure 4.2**) demonstrate stenosis of the gut lumen, such that the lumen is notably and abnormally narrowed, whilst epithelial continuity persists. Type 2 DA (**Figure 4.2H, 4.2I, 4.2M**) represents a more severe duodenal anomaly in which the gut wall and lumen are discontinuous, albeit a bridge or span of tissue maintains a physical connection between the two ends. Finally, type 3 DA (**Figure 4.2 J-L**) is the most severe duodenal phenotype, recognised here by complete disconnection of the atretic gut ends.

Given the recognised association between DA and oesophageal atresia in humans, DA-affected *Fgf10*^{-/-} embryos were analysed for oesophageal continuity. **Figure 4.2L** (tm1) and **Figure 4.2M** (tm2) provide representative images of the universally intact oesophagus for these embryos, i.e. no oesophageal atresia. 100% of tm1 and tm2 *Fgf10*^{-/-} embryos demonstrated caecal atresia.

4.2.4. Distribution by embryonic gestational age

A significant variation of DA occurrence and penetrance is seen between the gestational ages of both tm1 and tm2 *Fgf10*^{-/-} embryos. Of 145 *Fgf10*^{-/-} embryos dissected (either for phenotypical assessment, mRNA extraction, or organoid seeding), 115 were tm1 (see **table 4.1**), and 30 were tm2 (see **table 4.2**).

Table 4.1 Occurrence of DA by gestational age in tm1 *Fgf10*^{-/-} embryos

	No DA	%	Type 1	%	Type 2	%	Type 3	%	Total
E11.5	13	100%							13
E13.5	17	52%	11	33%	4	12%	1	3%	33
E15.5	14	37%	10	26%	4	11%	10	26%	38
E17.5	10	32%	12	39%	2	6%	7	23%	31
Total	54	47%	33	29%	10	9%	18	16%	115

Table 4.2 Occurrence of DA by gestational age in tm2 *Fgf10*^{-/-} embryos

	No DA	%	Type 1	%	Type 2	%	Type 3	%	Total
E11.5									
E13.5									
E15.5	6	35%	5	29%	1	6%	5	29%	17
E17.5	3	23%	5	38%	3	23%	2	15%	13
Total	9	30%	10	33%	4	13%	7	23%	30

When stratified for gestational age, there was no statistically significant difference between DA occurrence between tm1 and tm2. At E15.5, DA was observed in 24 (63%) tm1 and 11 (65%) tm2 *Fgf10*^{-/-} embryos. At E17.5, DA was observed in 21 (68%) tm1 and 10 (77%) tm2 *Fgf10*^{-/-} embryos. The small number of tm2 embryos precluded any meaningful comparison in DA morphology between groups. No tm2 E11.5 or E13.5 embryos were harvested, meaning there was no ability to compare to tm1 at an earlier gestational age.

The tm1 and tm2 datasets were combined, providing a total of 145 *Fgf10*^{-/-} embryos (see **table 4.3**). DA frequency was seen to increase across gestational age. At E11.5 no cases of DA were observed; at E13.5, DA was observed in 16 (48%) *Fgf10*^{-/-} embryos; at E15.5, DA was observed in 35 (64%) *Fgf10*^{-/-} embryos; and, at E17.5, DA was observed in 31 (70%) *Fgf10*^{-/-} embryos.

Table 4.3 Occurrence of DA by gestational age (tm1 and tm2 combined)

	No DA	%	Type 1	%	Type 2	%	Type 3	%	Total
E11.5	13	100%	0	0%	0	0%	0	0%	13
E13.5	17	52%	11	33%	4	12%	1	3%	33
E15.5	20	36%	15	27%	5	9%	15	27%	55
E17.5	13	30%	17	39%	5	11%	9	20%	44
Total	63	43%	43	30%	14	10%	25	17%	145

This increase in frequency coincided with a relative increase in the more severe duodenal anomalies (i.e. type 2 and type 3 DA). **Figure 4.3** pictorially demonstrates this increase in penetrance of DA with increasing gestational age. All groups were statistically significant in their DA distribution compared to E11.5 ($p < 0.001$). There was also a statistically significant difference between groups E13.5 to E15.5 ($p = 0.007$) and E13.5 to E17.5 ($p = 0.012$). No significance existed ($p = 0.846$) between groups E15.5 and E17.5.

Caecal atresia was present in 100% of tm1 and tm2 *Fgf10*^{-/-} embryos, regardless of age.

Upon completing the phenotypical profiling of both groups, as presented in Teague et al. (Teague et al., 2018), the decision was made to proceed with only one *Fgf10* knockout model for genetic pathway analysis in order to simplify the data analysis for genetic testing. When stratified for gestational age, tm1 and tm2 demonstrated no significant difference in the presence or morphology of DA. Therefore, tm1 was chosen as the preferred strain for ongoing experimental work and analyses due to its more straightforward genotyping protocol.

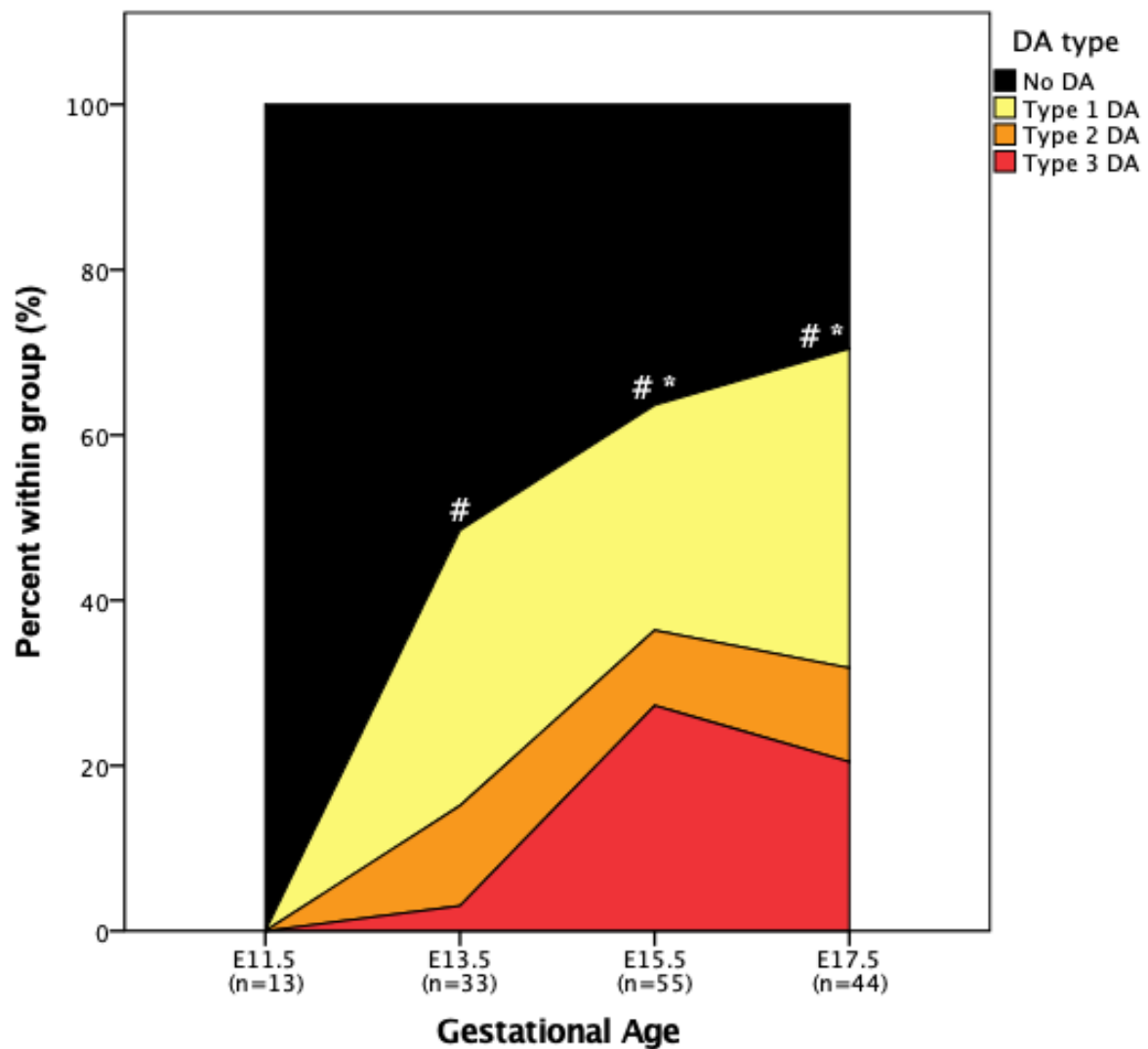


Figure 4.3: Distribution of DA in *Fgf10*^{-/-} embryos (tm1 and tm2; n=145) grouped by gestational age. # demonstrates statistical significance between groups when compared to E11.5. * demonstrates statistical significance between groups when compared to E13.5. No significant difference existed between E15.5 and E17.5. * p-value <0.05 were considered significant.

4.3. Discussion

The DA penetrance and types evident in these novel, CRISPR-derived, *Fgf10* knockout mouse strains, reported here as tm1 and tm2, represent an important and positive development in the field. In distinction to previous reports, these novel *Fgf10*^{-/-} embryo strains demonstrate a significantly higher penetrance (62%) and examples for all three morphological types of DA.

The basis for the differences in DA penetrance and DA type distribution between these and previously reported *Fgf10* knockout mouse strains remains unclear. The strains are of consistent background, namely C57/Bl6 (Fairbanks et al., 2004a, Kanard et al., 2005), and whilst the genetics differ, each represents a nonsense mutation (Eppig et al., 2006). The exact genetics, and its interplay on downstream targets, may well play a role in the penetration of the DA phenotype. Interestingly, Reeder et al. observed adding haploinsufficiency of retinaldehyde dehydrogenase 2 (*Raldh2*^{+/-}) to *Fgfr2IIIb* homozygous null embryos resulted in a reduced penetrance of DA, accompanied by a less severe DA phenotype (Reeder et al., 2012b). Of note, type 2 DA was evident in *Fgfr2IIIb*^{-/-} *Raldh2*^{+/-} but not in *Fgfr2IIIb*^{-/-} mouse embryos. Their results could be interpreted as demonstrating a relationship between DA penetrance and type distribution in the murine model. If this were so, a model demonstrating all DA types might be expectedly undermined by weak penetrance. However, the results presented here demonstrate improved penetrance and increased expression of all three DA types over gestational age, affirming the applicability of the tm1 and tm2 strains as promising models of DA.

The relation between DA frequency, morphology and gestational age is a critical revelation. The presence of a normal duodenum in all *Fgf10*^{-/-} embryos before E13.5 suggests this is a key time point related to the onset of DA. Furthermore, the increase in DA frequency and severity of the phenotype suggests this condition continues to progress over this period, with stable morphology observed beyond E15.5.

These findings earmark the key gestational ages in *Fgf10*^{-/-} embryos to bracket the study of genetic expression. They also indicate E13.5 and earlier as the significant age group to harvest *Fgf10*^{-/-} duodenum in best hope to witness, *ex vivo*, the growth pattern of the duodenum destined to become atretic. The argument could be made that duodenal tissue persisting in *Fgf10*^{-/-} embryos beyond E15.5 is subject to survivorship bias, with that set to become atretic already gone.

Another point of interest is that of caecal atresia. Previous studies have demonstrated this same 100% occurrence of caecal atresia in *Fgf10*^{-/-} and *Fgfr2b*^{-/-} embryos (Fairbanks et al., 2004c). Although not the focus of this thesis, the presence of a different type of gastrointestinal atresia with complete penetrance in all *Fgf10*^{-/-} embryos, regardless of gestational age, creates an additional area of interest. The epithelial-mesenchymal interactions moderating development of the caecum are distinct from those responsible for development of the duodenum, triggered by epithelial expression of *CdxA* in caecum versus *Pdx1* in the duodenum. *Fgf10* is strongly expressed in the mesenchyme of the caecum, and an FGF9/Pitx2/FGF10 signalling pathway has been proposed to control bud formation (Al Alam et al., 2012). Therefore, it makes sense that the knockout of *Fgf10* will immediately affect the caecum, with atresia occurring as soon as the caecal bud fails to form at E10.5.

The incomplete penetrance of the duodenal phenotype in *Fgf10*^{-/-} embryos is distinct from the complete penetrance of caecal atresia and other phenotypic features (e.g. absent limbs, absent lungs) in these nulls. Presently undefined developmental differences notwithstanding, it is considered that the incomplete penetrance of DA indicates redundancy in the requirements for “normal” duodenal morphogenesis. As such, tm1 and tm2 *Fgf10*^{-/-} embryos without DA provide an essential population for future study to discern signature signalling differences when compared with wild-type, heterozygote and DA-affected *Fgf10*^{-/-} embryos.

4.4. Conclusion

This the findings of this chapter have confirmed **hypothesis 1**, that *Fgf10* deletion results in an incomplete penetrance of DA in mice, with a human-like morphology distribution. These results have characterised the general and gastrointestinal morphology of CRISP-derived *Fgf10* knockout mice by performing microdissection on *Fgf10*^{-/-} embryos, documenting the presence and type of DA, subsequently achieving **aim 1**. Furthermore, the temporal variation in DA in *Fgf10*^{-/-} embryo suggests an onset of DA at a timepoint of approximately E13.5.

However, before proceeding with experimental work focussing on E13.5 as a possible onset of DA, this concept must be validated by studying the expression of FGF10 throughout the intestine of genetically normal murine embryos.

5. FGF10 expression during embryogenesis throughout the gastrointestinal tract

5.1. Introduction

Understanding the temporo-spatial expression patterns relevant to the *Fgf10/Fgfr2b* signalling pathway in the murine gastrointestinal tract is a fundamental precursor to identifying changes in up- and downstream pathways. Existing research into *Fgf10* expression patterns has focused on the foregut (Nyeng et al., 2007, Nyeng et al., 2008), gastrointestinal boundaries and junctions (San Roman and Shivdasani, 2011) or lacked the detail required to inform further investigation within the CRISPR derived *Fgf10*^{-/-} DA model (Fon Tacer et al., 2010). It was aimed to characterise the expression of *Fgf10* along the gastrointestinal tract during different stages of embryogenesis to understand better where and when this gene is most active.

The results presented in chapter 4 indicate that, in *Fgf10*^{-/-} embryos, the onset and evolution of DA penetrance and morphology occurs between gestational ages E11.5 and E15.5. Therefore, it was hypothesised that the principal *Fgf10* activity would occur within the duodenum between these gestational ages.

Wild-type C57 embryos were harvested at gestational ages E8.5, E10.5, E11.5, E12.5, E13.5, E14.5, E16.5 and E18.5. The gut was micro-dissected (see section 3.3.2), and RT-qPCR was used (see section 3.4) to quantify the presence of *Fgf10* mRNA. mRNA expression was standardised against the housekeeping gene *Actb*. Where anatomically possible, separate analysis of the oesophagus, stomach, duodenum, small intestine and colon was performed. Gastrointestinal tissue from each region and gestational age was pooled into three groups to provide technical replicates (n=3) for data analysis of variation and significance.

5.2. Results

The gastrointestinal tissue from 24 embryos was harvested, with seven from each gestational age studied ($n=7$). No gastrointestinal anomalies (e.g. duodenal or caecal atresia) were identified in any of these wild-type embryos. Within the E8.5 cohort, the gastrointestinal tract was still developing, so in place of discrete gastrointestinal regions, the foregut diverticulum, gastrointestinal plate, and the remainder of the whole embryo were harvested. At E10.5, the duodenum is still under formation (Botham et al., 2012); therefore, discrete isolation of this region was not possible, so the whole of the small intestine was grouped for analysis. The profile of embryos E8.5 to E16.5 are shown in **Figure 5.1**, demonstrating the significant variation in size for these early gestational ages.

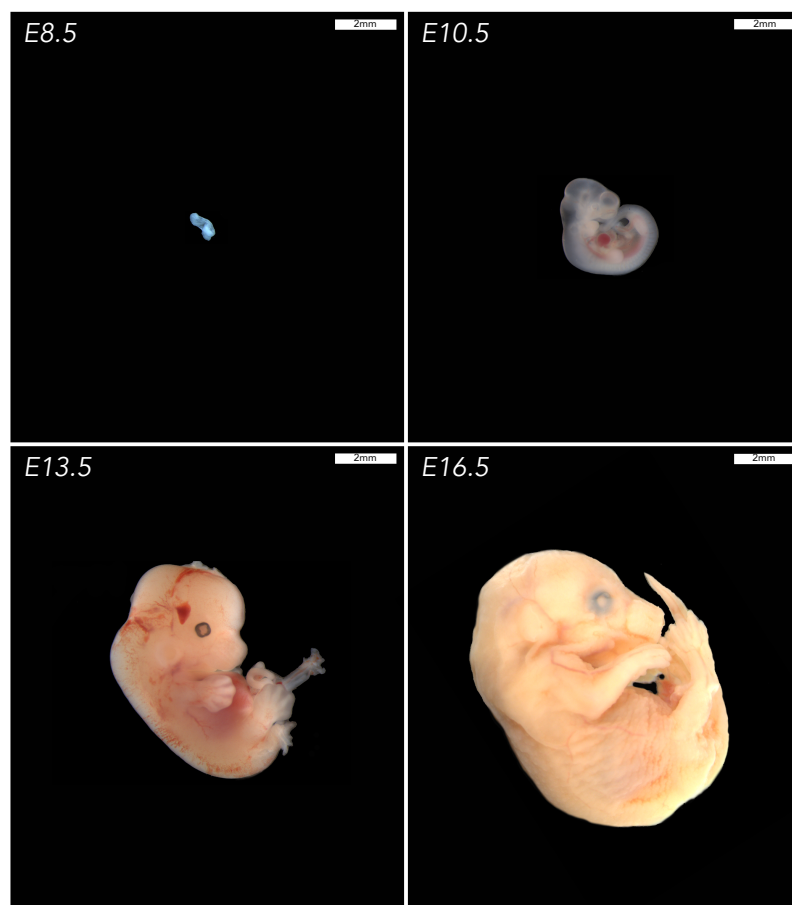


Figure 5.1. Wild-type embryos at E8.5, E10.5, E13.5 and E16.5. *The side profiles of still-whole C57 wild-type embryos demonstrating a significant variance in the overall size.*

5.2.1. *Fgf10* expression throughout the gastrointestinal tract

Fgf10 was expressed in all sites of the gastrointestinal tract, at all gestational ages studied.

Figure 5.2 demonstrates *Fgf10* expression grouped by gestational age. The most prominent expression of *Fgf10* was seen at E13.5 (**Figure 5.2E**), with the bulk of this increase due to its expression within the duodenum, colon and stomach, respectively.

Figure 5.3 compares *Fgf10* expression within the individual gut regions at different stages of development (excluding E8.5 and E10.5, where not all regions could be isolated). A statistically significant increase can be seen at E13.5, greatest for the duodenum (**Figure 5.3C**) and colon (**Figure 5.3E**), with a less significant increase seen in the stomach (**Figure 5.3B**).

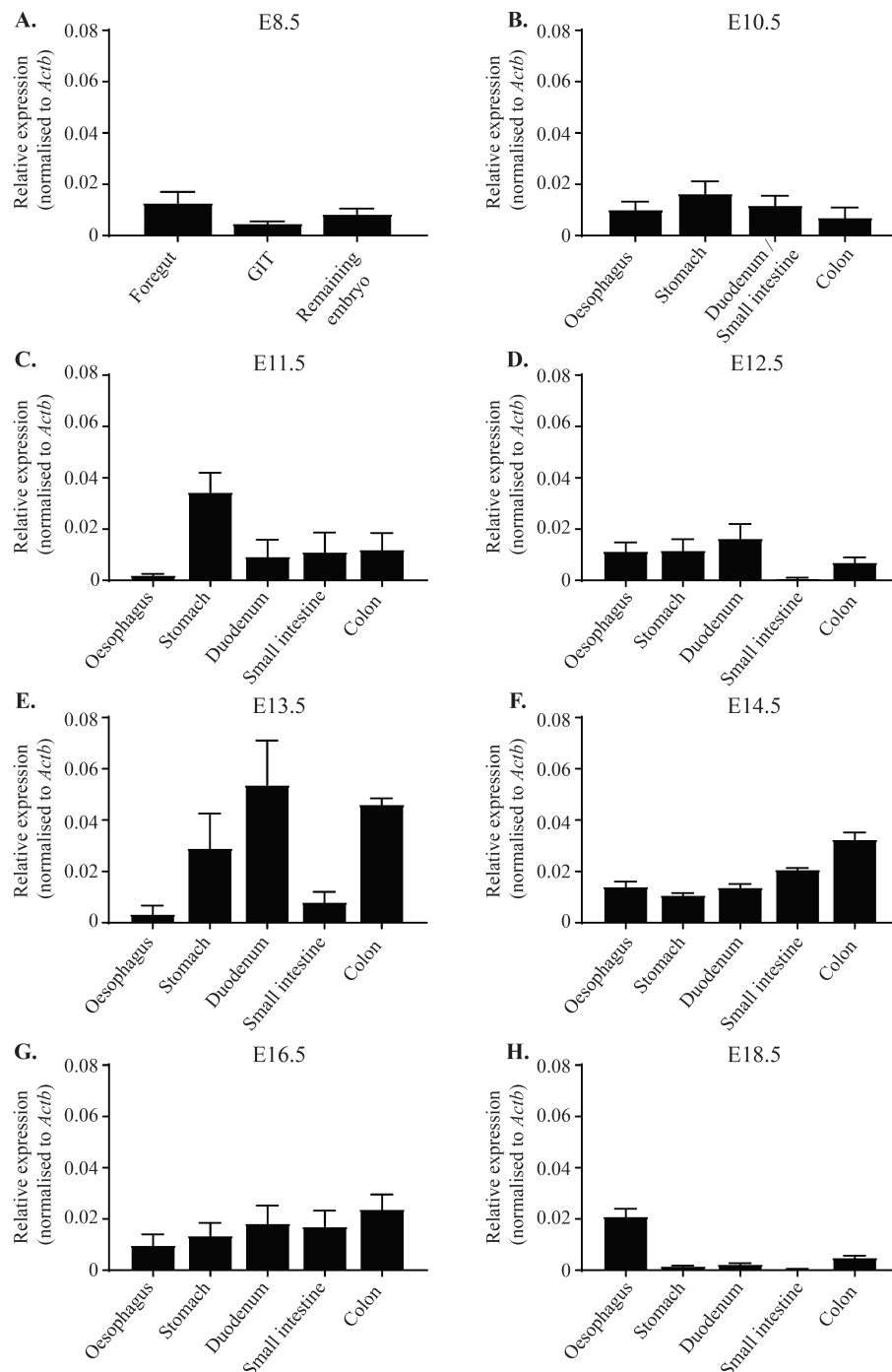


Figure 5.2 Expression profile of *Fgf10* within the gastrointestinal tract of C57Bl/6 mice during embryogenesis. mRNA expression analysis of *Fgf10* in (A) E8.5 foregut, gastrointestinal tract (GIT) and the remaining embryo (B) E10.5 oesophagus, stomach, duodenum combined with the small intestines, and colon (C-H) E11.5 - E18.5 oesophagus, stomach, duodenum, small intestines, and colon separately, were measured by RT-qPCR. All expression changes were normalised to *Actb*. $n=7$ per group per age, $n=3$ technical replicates with error bars represent $\pm$ SEM

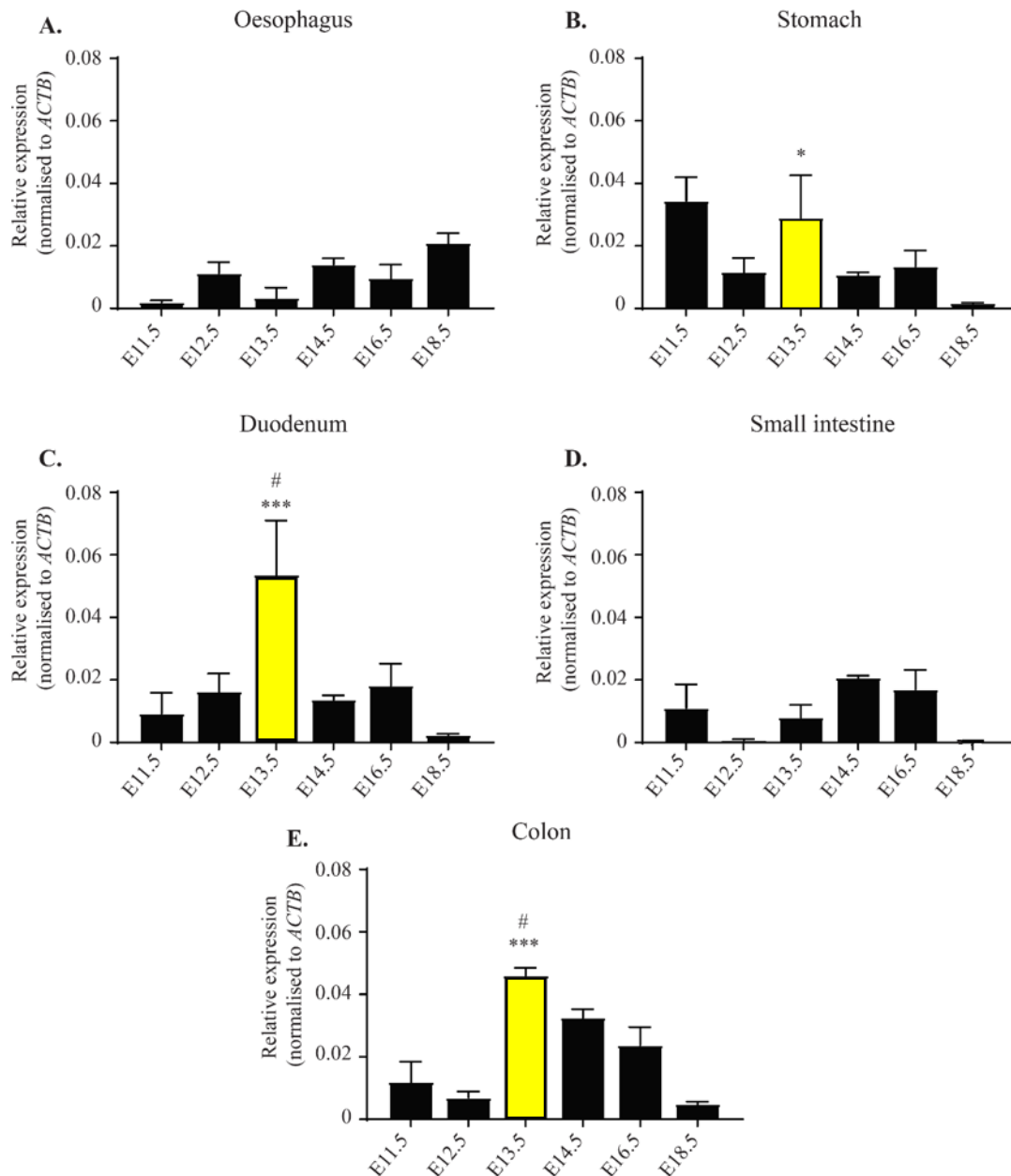


Figure 5.3 *Fgf10* is significantly increased within the duodenum stomach and colon in C57Bl/6 mice at E13.5. mRNA expression analysis of *Fgf10* in (A) oesophagus, (B) stomach, (C) duodenum, (D) small intestines and (E) colon during embryogenesis, respectively, were measured by RT-qPCR. All expression changes were normalised to *Actb*. Two-way ANOVA (Bonferroni) test was performed comparing *Fgf10* gene expression from all embryonic ages to E13.5, * p -value < 0.05 , # p -value < 0.01 and *** p -value < 0.001 were considered significant. $n=7$ per group per age, $n=3$ technical replicates. Error bars represent $\pm$ SEM

5.3. Discussion

These results demonstrate a key increase in *Fgf10* expression in the duodenum, colon and stomach at E13.5. Of note, these three regions have all demonstrated phenotypical changes in these *Fgf10*^{-/-} embryos, in the form of duodenal and caecal atresia, along with microgastria. These results provoke further thought when weighed against the aforementioned finding that DA is not observed in *Fgf10*^{-/-} embryos before the gestational age of E13.5. Thus, the deduction can be made that E13.5 represents a key gestational age for DA development. E13.5, therefore, provides a key point of focus for further investigation. It is proposed that E13.5 represents the most suitable gestational age to delineate the up- and downregulation of genes and pathways in atretic and morphologically normal duodenum in these *Fgf10*^{-/-} embryos. This further research would continue to build towards the primary hypothesis that the aetiology of DA in humans is genetically driven and the causative genetic changes are downstream of FGF10/FGFR2b signalling pathway.

The increased expression of *Fgf10* in the colon at E13.5, is an interesting finding, given the complete penetrance of colonic atresia, regardless of gestational age, in the setting of *Fgf10-Fgfr2b* signalling interruption. It is proposed that genome-wide analysis of the colon in *Fgf10*^{-/-} embryos, while not the focus of this thesis, may aid in the discernment of the genetic changes which hold actual relevance for atresia formation amongst the high volume of genetic changes expected to be observed in this model.

5.4. Conclusion

The findings within this chapter have confirmed the embryonic age demonstrating greatest *Fgf10* activity, forming part of **aim 2**. This knowledge will be used to scrutinise gene expression differences between the three morphological types of DA present in *Fgf10* knockout mice, through the use of genome-wide mRNA sequencing. This work continues to build towards **hypothesis 2**, that signalling pathways related to the *Fgf10-Fgfr2b* axis are moderating the morphology and penetrance of the DA phenotype seen in *Fgf10*^{-/-} mice.

6. Genome-wide expression profile in the duodenum of the *Fgf10*^{-/-} mouse

6.1. Introduction

Interruption of the FGF10/FGFR2b pathway is the only known genetic link to DA in mice. However, even in the CRISPR-derived *Fgf10*^{-/-} embryos described in chapter 2, which demonstrate the most significant proportion of DA occurrence to date, DA is not universally present (Fairbanks et al., 2004b, Kanard et al., 2005, Botham et al., 2012, Reeder et al., 2012b). Conversely, caecal atresia is present in all *Fgf10*^{-/-} embryos. This makes logical sense, given the known link between FGF10 and caecal bud formation at E10.5 (Al Alam et al., 2012). Known pathways for duodenal formation do not present this same link to FGF10 (Chen et al., 2009). Therefore, it is hypothesised that the epigenetic effects of FGF10/FGFR2b interruption result in other genetic changes leading to DA, which would account for incomplete DA penetrance.

Furthermore, a greater proportion of DA in E15.5 and E17.5 embryos is represented by type 2 and type 3 DA compared to what is seen at the onset of DA presentation in E13.5. It is also hypothesised that DA morphology represents an evolving spectrum of disease, and the epigenetic changes triggering this transformation may be of most considerable significance in the more severe phenotypes. Thus, fundamental interest sits in comparing the genetic changes between the duodenal tissue of wild-type embryos to that of *Fgf10*^{-/-} without DA, *Fgf10*^{-/-} with type 1 DA, and *Fgf10*^{-/-} with type 2 or 3 DA.

The previous chapter explored *Fgf10* expression in the gastrointestinal regions of wild-type embryos throughout embryogenesis. *Fgf10* was shown to be most active in the duodenum at the gestational age of E13.5. This finding coincides with the first observation of DA in *Fgf10*^{-/-} embryos at this gestational age.

Therefore, E13.5 was the chosen gestational age to undergo genome-wide expression analysis, where the most significant disparity exists between the genetic activity of wild-type and *Fgf10*^{-/-} tissue and where DA is expected to be present in all forms but still evolving.

Characterisation of various signalling pathways and genes up- or downregulated in DA is a critical next step to finding those which are causative. The current investigation endeavoured to identify these key genes through parallel mRNA sequencing in the duodenum of E13.5 embryos, which were *Fgf10*^{+/+} (wild-type), *Fgf10*^{+/-} (heterozygous), *Fgf10*^{-/-} without DA, *Fgf10*^{-/-} with type 1 DA, and *Fgf10*^{-/-} with type 2 or 3 DA. By analysing both genome-wide expression changes and gene set enrichment analysis (GSEA) and comparing the discrete *Fgf10* and DA-related groupings, this chapter describes widespread changes in the duodenal transcriptome seen in *Fgf10* deletion and DA formation.

In addition to the duodenum, genome-wide analysis was performed on the *Fgf10*^{-/-} embryos colon. This was to aid in the discernment of the genetic changes which hold actual relevance for atresia formation amongst the high volume of genetic changes expected to be observed in this model. Despite the complete penetrance of colonic atresia in *Fgf10*^{-/-} mice, these colons were grouped by the DA phenotype of the embryos from which they were harvested. This was to validate that any genetic variance seen in the duodenum of these phenotypes was not replicated in the colon, where no phenotypical variance exists.

This chapter aims to investigate global gene expression and gene set pathways using next-generation sequencing (mRNA-seq) in the duodenum of *Fgf10*^{-/-} mice to understand the role of *Fgf10* in various signalling or disease pathways and characterise the signalling pathways and genes up- or downregulated in DA.

6.2. Results

6.2.1. mRNA sequence and quality control

63 tissue samples were analysed from 36 embryos (36 duodenum and 27 colons). In order to meet the mRNA quantity required for analysis, tissue was pooled into samples of 3 (see table 6.1). Biological replicates were formed, where possible, to analyse the variance within groups. However, this was not feasible for the 'type 2 and 3 DA' grouping due to the low penetrance of severe DA morphology at E13.5 in *Fgf10*^{-/-} embryos (12% and 3%, respectively). Technical replicates were formed, through the use of two run lanes, to quantify the variance within the experiment.

No features of atresia or other gastrointestinal anomalies were present in wild-type or heterozygous embryos. Caecal atresia was present in all *Fgf10*^{-/-} embryos.

Image analysis was performed in real time by the NovaSeq Control Software (NCS) v1.6.0 and Real Time Analysis (RTA) v3.4.4, running on the instrument computer. RTA performs real-time base calling on the NovaSeq instrument computer. Then the Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. mRNA-seq was performed in dual lanes, and data yield for all samples (shown in table 6.2) were deemed acceptable for progression to mRNA-seq.

Table 6.1 sample groupings for mRNA sequencing

Sample	Tissue type	Phenotype	Animal ID
1WTDuon1	Duodenum	No duodenal atresia and no caecal atresia	181, 182, 183
2WTDuon2	Duodenum	No duodenal atresia and no caecal atresia	184, 185, 203
3WTDuon_3	Duodenum	No duodenal atresia and no caecal atresia	187, 206, 232
4HetDuon_1	Duodenum	No duodenal atresia and no caecal atresia	180, 188, 189
5HetDuon_2	Duodenum	No duodenal atresia and no caecal atresia	192, 191, 193
6HetDuon_3	Duodenum	No duodenal atresia and no caecal atresia	208, 210, 211
7NoDADuon_1	Duodenum	No duodenal atresia but has caecal atresia	190, 200, 201
8NoDADuon_2	Duodenum	No duodenal atresia but has caecal atresia	207, 215, 221
9NoDADuon_3	Duodenum	No duodenal atresia but has caecal atresia	259, 262, 267
10TypeoneDuon_1	Duodenum	Has type 1 duodenal atresia and has caecal atresia	230, 233, 235
11TypeoneDuon_2	Duodenum	Has type 1 duodenal atresia and has caecal atresia	247, 251, 265
12TypetwoDuon_1	Duodenum	Has type 2 or type 3 duodenal atresia and has caecal atresia	186, 280, 281
13ColonWTn_1	Colon	No duodenal atresia and no caecal atresia	181, 182, 183
14ColonWTn_2	Colon	No duodenal atresia and no caecal atresia	184, 185, 203
15ColonHetn_1	Colon	No duodenal atresia and no caecal atresia	180, 188, 189
16ColonHetn_2	Colon	No duodenal atresia and no caecal atresia	192, 191, 193
17ColonNoDan_1	Colon	No duodenal atresia but has caecal atresia	207, 215, 221
18ColonNoDan_2	Colon	No duodenal atresia but has caecal atresia	259, 262, 267
19Colontypeonen_1	Colon	Has type 1 duodenal atresia and has caecal atresia	230, 233, 235
20Colontypeonen_2	Colon	Has type 1 duodenal atresia and has caecal atresia	247, 251, 265
21Colontype2n_1	Colon	Has type 2 or type 3 duodenal atresia and has caecal atresia	186, 280, 281

Table 6.2: Data yield in mRNA-seq (100bp Single End)

Lane	Sample Name	Single Reads	Data Yield (bp)
1	1WTDuon1	14,179,611	1.43 Gb
	2WTDuon2	11,357,278	1.15 Gb
	3WTDuon_3	11,687,847	1.18 Gb
	4HetDuon_1	12,104,096	1.22 Gb
	5HetDuon_2	12,806,357	1.29 Gb
	6HetDuon_3	11,450,848	1.16 Gb
	7NoDADuon_1	12,553,875	1.27 Gb
	8NoDADuon_2	10,697,107	1.08 Gb
	9NoDADuon_3	11,469,713	1.16 Gb
	10TypeoneDuon_1	12,030,483	1.22 Gb
	11TypeoneDuon_2	10,934,008	1.10 Gb
	12TypetwoDuon_1	11,981,359	1.21 Gb
	13ColonWTn_1	13,175,178	1.33 Gb
	14ColonWTn_2	10,623,872	1.07 Gb
	15ColonHetn_1	3,392,369	0.34 Gb
	16ColonHetn_2	10,693,314	1.08 Gb
	17ColonNoDan_1	12,022,803	1.21 Gb
	18ColonNoDan_2	11,568,989	1.17 Gb
	19Colontypeonen_1	11,447,792	1.16 Gb
	20Colontypeonen_2	10,696,664	1.08 Gb
	21Colontype2n_1	13,425,217	1.36 Gb
2	1WTDuon1	14,344,637	1.45 Gb
	2WTDuon2	11,544,044	1.17 Gb
	3WTDuon_3	11,822,566	1.19 Gb
	4HetDuon_1	12,301,678	1.24 Gb
	5HetDuon_2	12,945,654	1.31 Gb
	6HetDuon_3	11,616,779	1.17 Gb
	7NoDADuon_1	12,716,701	1.28 Gb
	8NoDADuon_2	10,792,013	1.09 Gb
	9NoDADuon_3	11,659,902	1.18 Gb
	10TypeoneDuon_1	12,179,787	1.23 Gb
	11TypeoneDuon_2	11,095,557	1.12 Gb
	12TypetwoDuon_1	12,149,597	1.23 Gb
	13ColonWTn_1	13,390,353	1.35 Gb
	14ColonWTn_2	10,770,677	1.09 Gb
	15ColonHetn_1	3,427,073	0.35 Gb
	16ColonHetn_2	10,832,575	1.09 Gb
	17ColonNoDan_1	12,206,769	1.23 Gb
	18ColonNoDan_2	11,678,418	1.18 Gb
	19Colontypeonen_1	11,630,723	1.17 Gb
	20Colontypeonen_2	10,797,781	1.09 Gb
	21Colontype2n_1	13,674,703	1.38 Gb
Total		528,294,451	53.36 Gb

6.2.2. Differential gene expression in the duodenum of *Fgf10*^{-/-} embryos

Following confirmation of mRNA quality and mRNA-seq read analysis, statistical analysis of differential gene expression profiles was performed using the edgeR package from Bioconductor. The data set was treated as an ordinal data set, and the expression is tested as a progressive increase or decrease in expression from the wild-type compared to *Fgf10*^{+/-}; *Fgf10*^{-/-} without DA; *Fgf10*^{-/-} with type 1 DA; and *Fgf10*^{-/-} with type 2/3 DA.

A heat map (**Figure 6.1**) shows the clustering of differentially expressed genes in the duodena of *Fgf10*^{-/-} embryos. When all groups were compared to wild-type duodenum, mRNA sequencing identified upregulated 2677 genes, and 3337 downregulated. Venn diagrams were formed using VENNY 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>) to identify overlapping genes between groups. These show the distribution across groups of upregulated (**Figure 6.2**) and downregulated (**Figure 6.3**) genes. A total of 93 differentially expressed genes were common across all *Fgf10*^{-/-} groups compared to wild-type. Of these 29 were upregulated (including *Cym*, *Six2*, *Barx1*, *Gata3*, *Nkx2-5*, *Isl1* and *Penk*) and 64 downregulated (including *Rbp4*, *Sfrp5*, *Kirrel2*, *Kynu*, *FoxA2* and *Pdx1*). Only one gene (*Nepn*) was found to have significantly altered expression in *Fgf10*^{+/-} duodenum, which was universally downregulated in all groups with *Fgf10* interruption.

RT-qPCR was used to validate the expression of a panel of genes identified by RNA-seq. Genes without human equivalent, or non-protein coding, were excluded from further analysis of the role of DA. Potentially important genes for duodenal formation were chosen for RT-qPCR analysis. Student's t-test was performed, and a p-value of <0.05 was considered significant. This experimental analysis validated 83% (5 out of 6) of the upregulated genes (**Figure 6.4**) and 100% (6 out of 6) of the downregulated profile (**Figure 6.5**).

Figure 6.1 mRNA-seq identified significant up- and downregulated differential gene expressions in the *Fgf10*^{-/-} mouse duodenum. Colour coded heat map of 80 significant genes. Genes were clustered using hierarchical clustering. Genes that are highly expressed, normal expression and low expression are represented as different shades of purple (high = dark purple; low = white). Negative logFC illustrates down-regulated gene expression (blue) and positive logFC demonstrates up-regulated genes (red). Key genes (*)

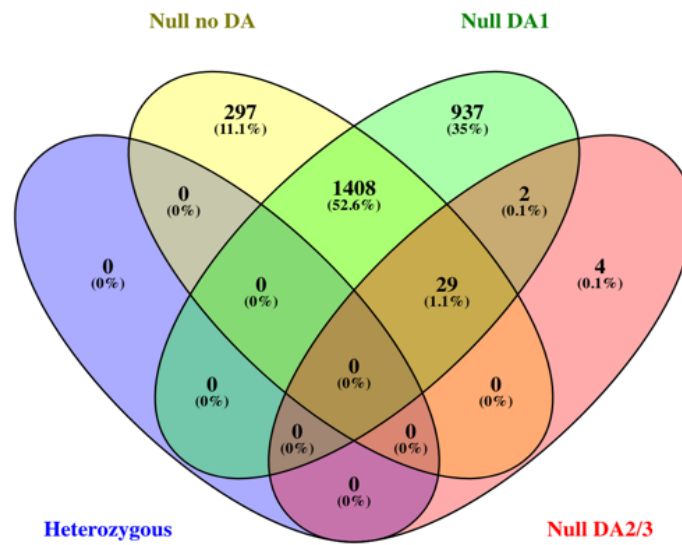


Figure 6.2: Distribution of upregulated genes identified across groups *Fgf10* genotype and DA phenotype. Of 2677 genes upregulated when compared to wild-type embryos, none were common between all groups. 29 were common between all *Fgf10*^{-/-} embryos, 2 common between all DA-affected embryos, and 4 upregulated only in DA type 2/3.

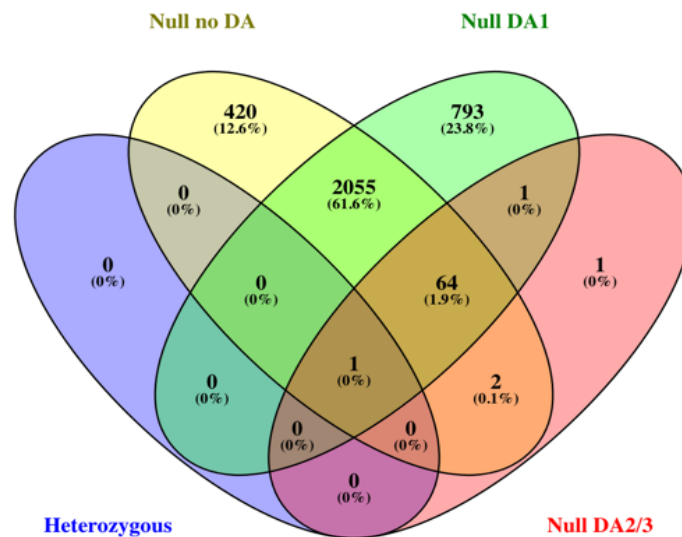


Figure 6.3: Distribution of downregulated genes identified across groups *Fgf10* genotype and DA phenotype. Of 3337 genes downregulated when compared to wild-type embryos, one was common between all groups. 64 were common between all *Fgf10*^{-/-} embryos, one common between all DA-affected embryos, and one downregulated only in DA type 2/3.

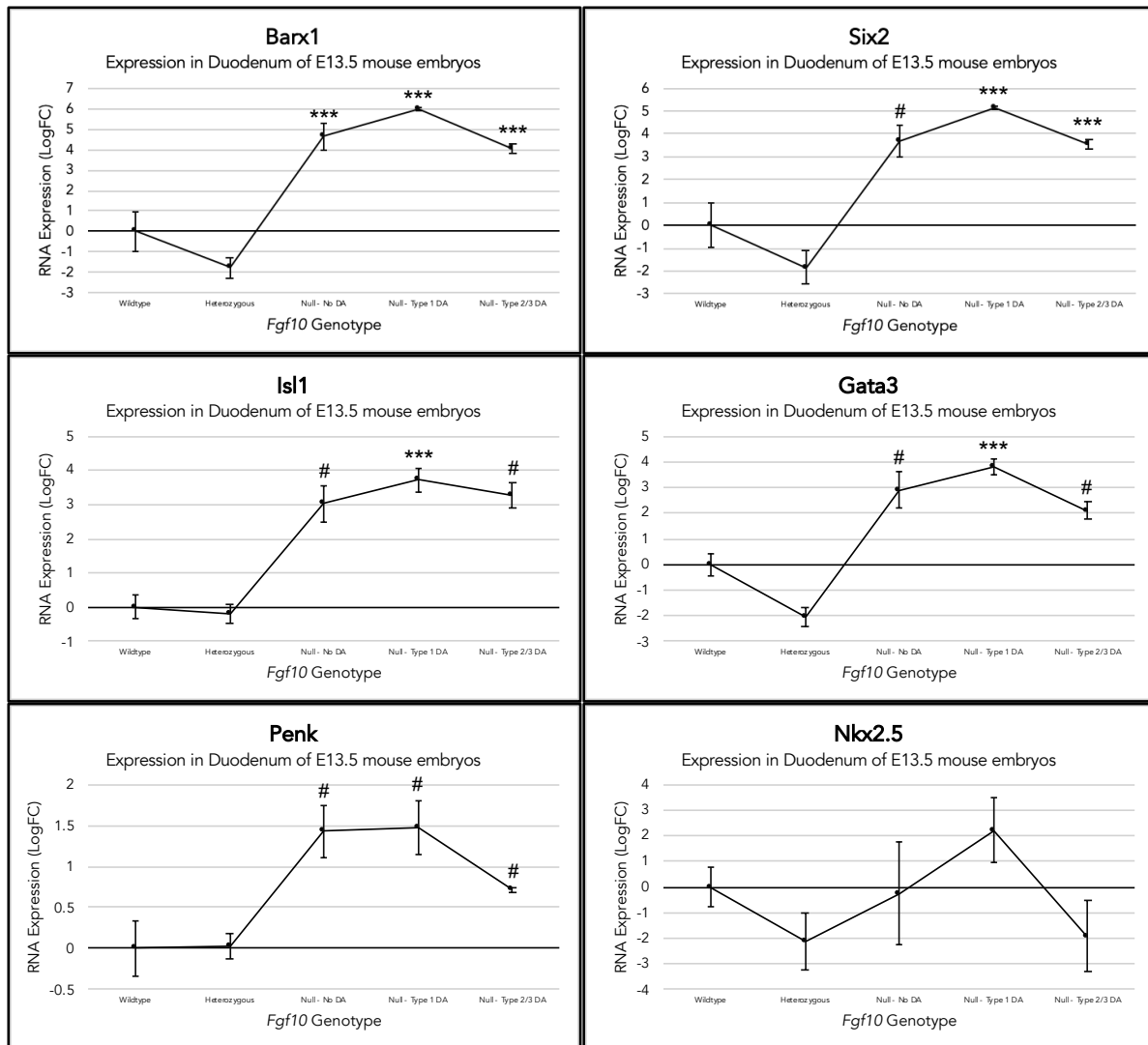


Figure 6.4 Validation of upregulated gene expression (wild-type vs *Fgf10*^{-/-}) identified in mRNA-seq analysis by RT-qPCR. RT-qPCR was performed and primers were designed to validate the gene expressed of the up and down-regulated target genes identified in the mRNA-seq within the Duodenum of the *Fgf10*^{-/-} mice compared to the wild type mice. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. Error bars represent $\pm$ SEM. * p-value < 0.05, # p-value < 0.01 and ***p-value < 0.001 were considered significant. All genes were validated as significant except *Nkx2-5*.

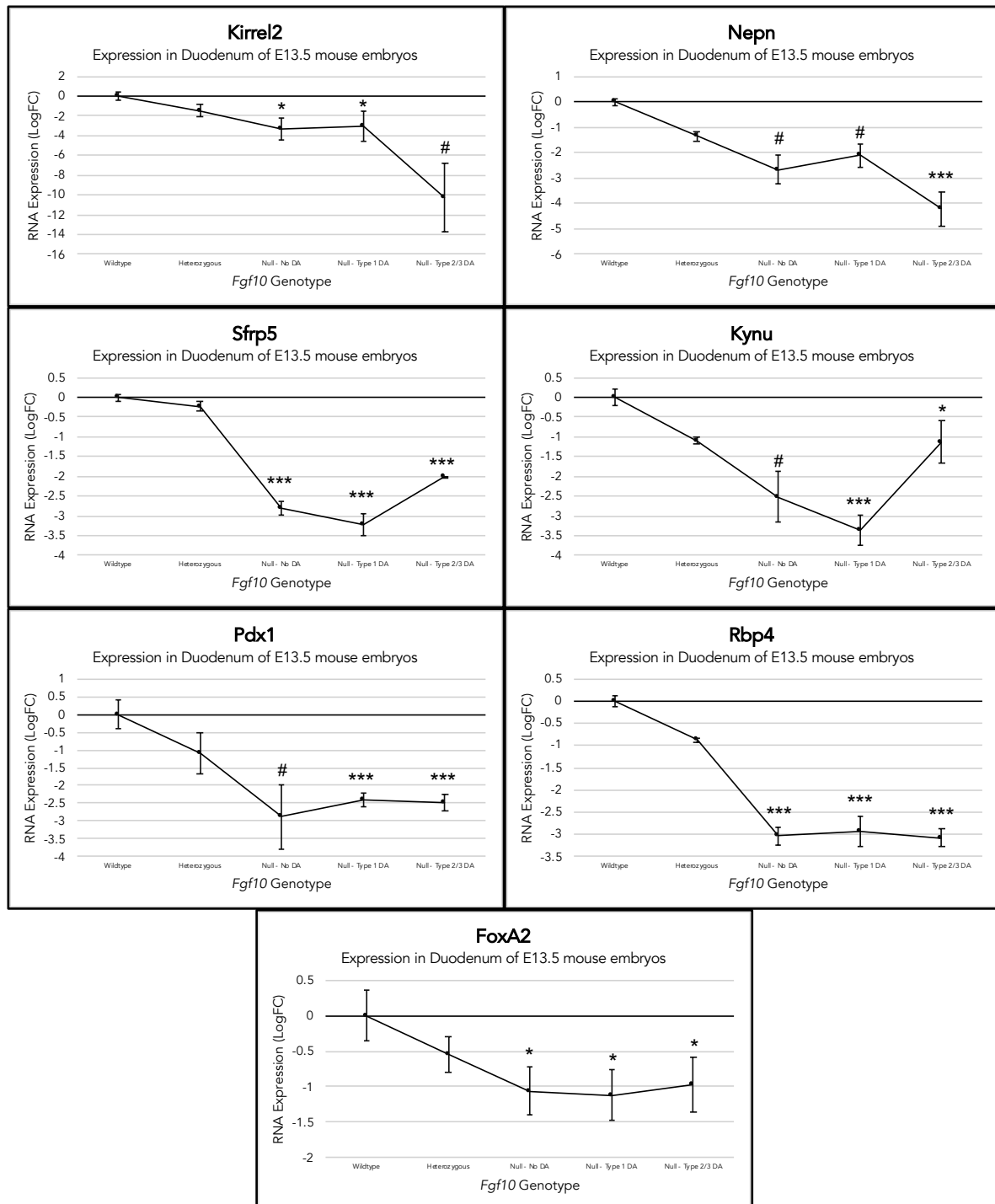


Figure 6.5 Validation of downregulated gene expression (wild-type vs *Fgf10*^{-/-}) identified in mRNA-seq analysis by RT-qPCR. RT-qPCR was performed and primers were designed to validate the gene expression of the up and down-regulated target genes identified in the mRNA-seq within the Duodenum of the *Fgf10*^{-/-} mice compared to the wild type mice. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. Error bars represent ± SEM. * p-value < 0.05, # p-value < 0.01 and ***p-value < 0.001 were considered significant.

6.2.3. Differential gene expression in the duodenum of DA-affected embryos

In order to identify any genetic changes associated with DA morphology, genes were compared between *Fgf10*^{-/-} groups (normalised against wild-type embryos) with varying DA phenotypes (no DA, type 1 DA and type 2/3 DA). Gene expression changes isolated to only DA-affected *Fgf10*^{-/-} embryos were identified.

Two genes were upregulated (*Nmur2* and *Oxct1*) and one downregulated (*Spp1*) in both Type 1 and Type 2/3 DA. Four genes were solely upregulated in the duodenum of embryos with type 2/3 DA (*Cubn*, *Tifa*, *Abat*, and *Sgk3*), and one was downregulated (*Zfc3h1*).

RT-qPCR was again used to validate the expression of these genes (**Figure 6.6**). Student's t-test was performed, and a p-value of <0.05 was considered significant. This experimental analysis validated 100% (6 out of 6) of the upregulated genes and 50% (1 out of 2) of the downregulated profile.

Zfc3h1 was unable to be validated, with RT-qPCR demonstrating upregulation in this gene, as opposed to the down-regulation seen in mRNA-seq. Of note, *Abat* and *Sgk3* demonstrated upregulation in all DA phenotypes, as opposed to only type 2 and 3 in mRNA-seq. Furthermore, an increase in *Oxct1* was seen in all *Fgf10*^{-/-} duodenum, as opposed to only DA-affected duodenum seen in mRNA-seq.

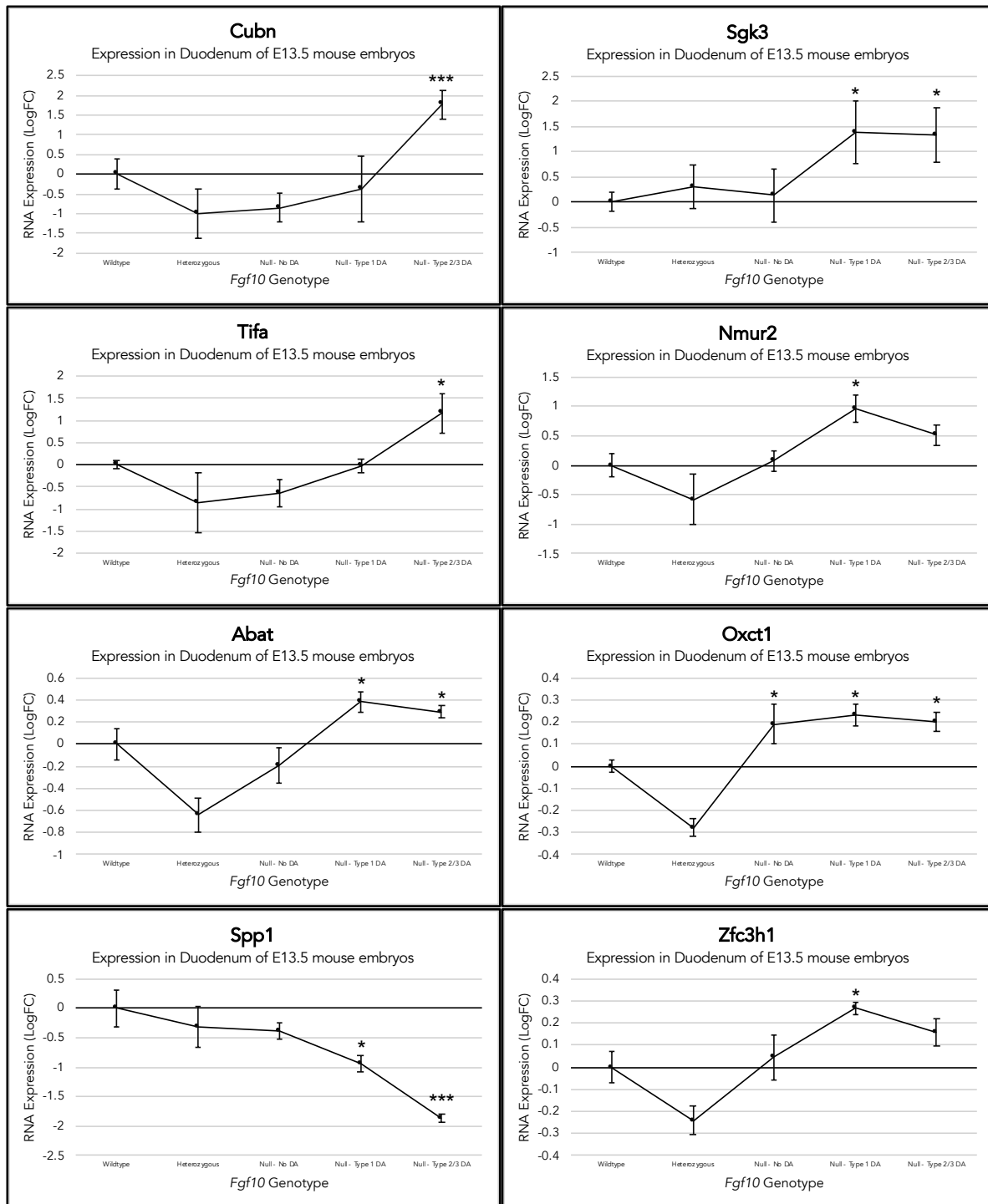


Figure 6.6 Validation of differential gene expression (*Fgf10* DA vs no-DA) identified in mRNA-seq analysis by RT-qPCR. RT-qPCR was performed and primers were designed to validate the gene expressed of the up and down-regulated target genes identified in the mRNA-seq within the Duodenum of the *Fgf10*^{-/-} mice compared to the wild type mice. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. Error bars represent $\pm$ SEM. * *p*-value < 0.05, # *p*-value < 0.01 and ****p*-value < 0.001 were considered significant.

6.2.4. Genetic pathway changes seen in *Fgf10*^{-/-} duodenum

The ordinal data set was examined for pathway differences using gene set enrichment analysis (GSEA). Reactome pathways were identified as being up- or downregulated in all *Fgf10*^{-/-} duodena (**Table 6.3**) or exclusively in *Fgf10*^{-/-} duodena featuring DA (**Table 6.4**). GSEA Molecular Signatures Database was used to provide context for the altered Reactome or biological pathways found (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>).

Fgf10^{-/-} duodenal tissue demonstrated upregulation of the same gene pathways downregulated in neural stem cells 36 hours after Cre-mediated knockout of TLX (Zhang et al., 2008a). Also upregulated was the RB1 TARGETS GROWING pathway of genes involved in growing fibroblast cells after the knockout of RB1 (Chicas et al., 2010). The MUSCLE CELL FATE COMMITMENT pathway was upregulated, suggesting an increase in the genes involved in acquiring and determining the cellular identity of muscle cells. Other common pathways upregulated included those related to chromosome assembly and maintenance (i.e. HAT1, SMC4L1, SMC1L1) and Oncogenesis (i.e. DEK, NASOPHARYNGEAL CARCINOMA, EWINGS SARCOMA, OVARIAN CANCER, BRCA1, BRCA2, ATM, and CHEK2).

Downregulated pathways in *Fgf10*^{-/-} duodenum included several associated with pancreatic development (PANCREATIC EXOCRINE PROGENITOR, ENDOCRINE PANCREAS DEVELOPMENT, MATURITY ONSET DIABETES YOUNG, and REGULATION OF BETA CELL DEVELOPMENT). These pathways are associated with: transcription factor expression in progenitors of the exocrine pancreas (Zhou et al., 2008); formation and maturation of the endocrine pancreas (including beta cells); and hyperglycaemia at a young age with no or minor insulin dependency. Other downregulated pathways include those associated with LCAT and CYP2B6, genes related to cholesterol transport and synthesis. The pathways of genes associated with cadherin cell-cell adhesion and tight junction formation were also reduced.

Table 6.3 List of pathways altered by Fgf10 knockout

Reactome pathway	Alteration	Genes	FDR
ZHANG TLX TARGETS 36HR DN	Upregulated	179	0.0006
GNF2 HAT1	Upregulated	47	0.0014
CHICAS RB1 TARGETS GROWING	Upregulated	216	0.0020
GO MUSCLE CELL FATE COMMITMENT	Upregulated	8	0.0022
CTRL VS ANTI IGM STIM BCELL 12H UP	Upregulated	166	0.0026
GNF2 SMC4L1	Upregulated	82	0.0026
ZHANG TLX TARGETS DN	Upregulated	87	0.0038
GNF2 DEK	Upregulated	56	0.0052
SENGUPTA NASOPHARYNGEAL CARCINOMA UP	Upregulated	248	0.0055
FERREIRA EWINGS SARCOMA UNSTABLE UP	Upregulated	148	0.0121
MORF SMC1L1	Upregulated	59	0.0142
RIZ ERYTHROID DIFFERENTIATION HEMGN	Upregulated	27	0.0195
PUJANA BRCA CENTERED NETWORK	Upregulated	115	0.0211
LIM MAMMARY LUMINAL MATURE DN	Upregulated	94	0.0225
WAMUNYOKOLI OVARIAN CANCER LMP DN	Upregulated	177	0.0235
PUJANA XPRSS INT NETWORK	Upregulated	155	0.0349
ZHOU PANCREATIC EXOCRINE PROGENITOR	Downregulated	10	<0.0001
GO ENDOCRINE PANCREAS DEVELOPMENT	Downregulated	35	0.0002
KEGG MATURITY ONSET DIABETES YOUNG	Downregulated	20	0.0003
GNF2 CEBPA	Downregulated	27	0.0014
GNF2 LCAT	Downregulated	54	0.0034
GNF2 HPX	Downregulated	61	0.0034
GNF2 GSTM1	Downregulated	50	0.0045
GNF2 TST	Downregulated	51	0.0055
GNF2 HPN	Downregulated	63	0.0079
GNF2 CYP2B6	Downregulated	18	0.0114
CADHERIN REGULATION OF CELL ADHESION	Downregulated	8	0.0259
TIGHT JUNCTION INTERACTIONS	Downregulated	22	0.0262
REGULATION OF BETA CELL DEVELOPMENT	Downregulated	25	0.0317

There were no significant downregulated pathways common to DA-affected embryos only. The most significantly upregulated Reactome pathway in DA-affected embryos was associated with pseudopodia of fibroblast cells in response to haptotactic, migratory stimulus by fibronectin (Mili et al., 2008). Multiple oncogenesis-associated Reactome pathways were also upregulated. This includes those associated with: genes upregulated in breast cancer stem cells (Zhang et al., 2008b); genes upregulated, alongside SMARCA2, in prostate cancer cells (Shen et al., 2008); genes upregulated in melanoma cells, sensitive to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Hamaï et al., 2006); and, genes upregulated in endometroid endometrial cancer cells (Bidus et al., 2006).

Table 6.4 List of pathways altered in DA-affected embryos

Reactome pathway	Alteration	Genes	FDR
MILI PSEUDOPODIA HAPTOTAXIS UP	Upregulated	463	0.0007
ZHANG BREAST CANCER PROGENITORS UP	Upregulated	382	0.0096
SHEN SMARCA2 TARGETS UP	Upregulated	399	0.0190
HAMAI APOPTOSIS VIA TRAIL UP	Upregulated	516	0.0223
BIDUS METASTASIS UP	Upregulated	193	0.0228
GCM DDX5	Upregulated	60	0.0235

6.2.5. Differential gene expression seen in the atretic colon of *Fgf10*^{-/-} mice

mRNA-seq analysis of the colon in *Fgf10*^{-/-} mice revealed >9,000 significantly downregulated genes. A heat-map (**Figure 6.7**) shows the clustering of differentially expressed genes in the colon of *Fgf10*^{-/-} embryos. Notably, the key genes identified as up- and downregulated in the duodenum of *Fgf10*^{-/-} mice (either with or without DA) do not appear to be altered in the colon. Key downregulated genes included the Fork-head box (FOX) and Homeobox (HOX) gene families. Conversely, the histone demethylase activity pathway was upregulated.

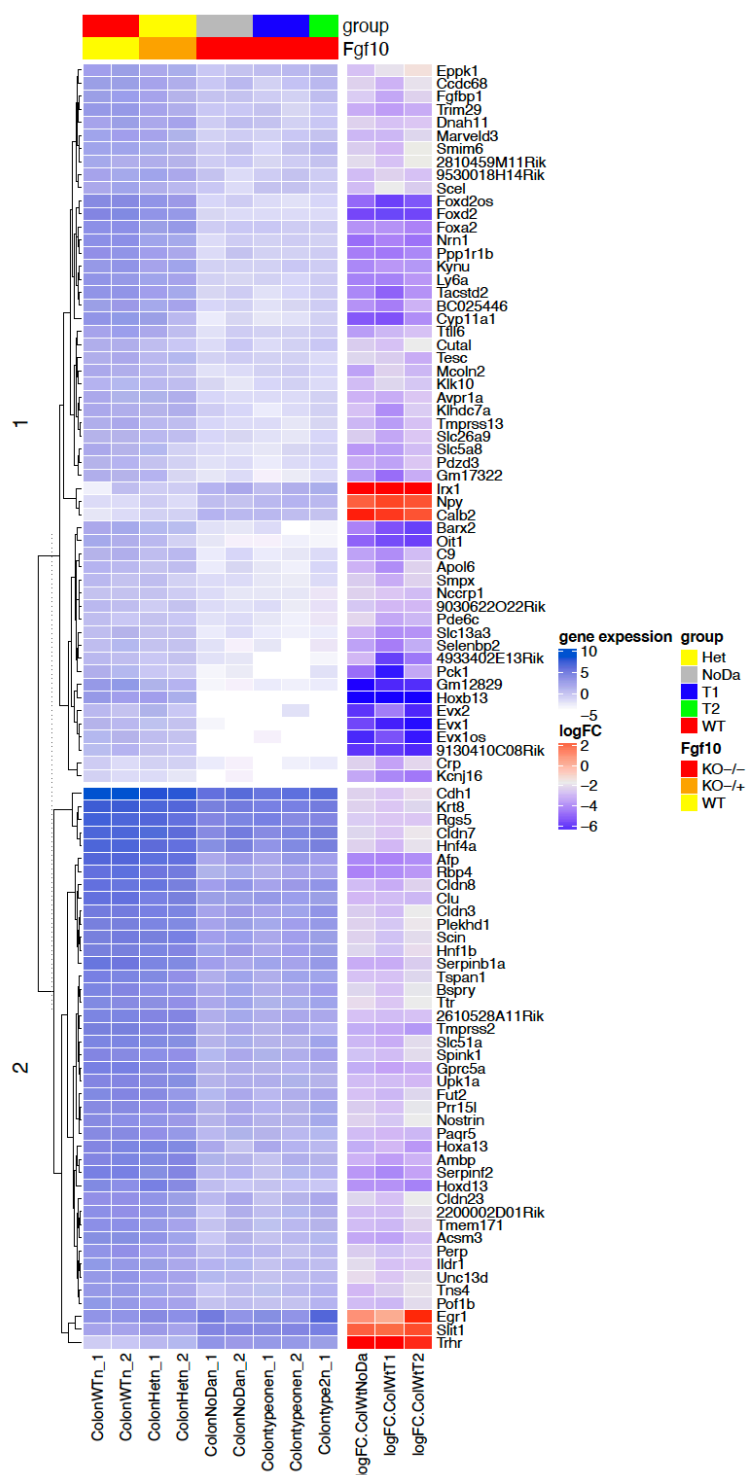


Figure 6.7 mRNA-seq identified significant up- and downregulated differential gene expressions in the *Fgf10*^{-/-} mouse colon. Colour coded heat map of 98 significant genes. Genes were clustered using hierarchical clustering. Genes that are highly expressed, normal expression and low expression are represented as different shades of purple (high = dark purple; low = white). Negative logFC illustrates down-regulated gene expression (blue) and positive logFC demonstrates up-regulated genes (red).

6.3. Discussion

These results list the key up- and downregulated genes in the duodenum of *Fgf10*^{-/-} embryos. Of particular interest are the genes where regulation changes are isolated to DA-affected embryos. Using differences at E13.5 to create a shortlist of key genes allows their study at other gestational ages. Nineteen of the 21 genes identified have been validated.

The expression of these genes will next be profiled in wild-type mice across earlier and later gestational ages before comparative analysis of *Fgf10*^{-/-} duodenum with and without DA. This will allow the analysis of whether regulatory changes identified at E13.5 persist or change in a meaningful pattern associated with DA.

Performance of pathway analysis provides insight into the broader role of genes across the whole genome, which are up- and downregulated within *Fgf10*^{-/-} duodenum. Predictably, genes linked to fibroblast activity and migration were upregulated to counterbalance *Fgf10*^{-/-} embryos. However, the upregulation of gene pathways involved in muscle cell fate, and downregulation of those involved in pancreatic development and function, provide particular interest. This finding demonstrates that pathways involved in *Fgf10*-led pancreatic development are also associated with duodenal development. A genetic link between pancreatic and duodenal development could help explain the association of annular pancreas, DA and trisomy 21 (Yigiter et al., 2010, Ali Almoamin et al., 2022).

There was no significant overlap between genetic changes in the atretic colon and up- or downregulated genes/pathways seen in the duodenum. This analysis offers new insights into the role of *Fgf10* and its downstream targets in colonic development. The decreased FOX and HOX gene expression, which correlated with *Fgf10* deletion in the atretic colon, is predicted to be modulated via enzymes within the histone demethylase activity pathway. Collectively, these results define a novel role for FGF10 in caecal atresia. However, they fail to support or rationalise findings within the duodenum. Therefore, the decision was made to cease further parallel genetic analysis of the colon in support of DA research.

6.4. Conclusion

The findings within this chapter have confirmed up- and downregulation within global gene expression and gene set pathways in the duodenum of *Fgf10*^{-/-} mice. The key pathways and genes identified propose a role of *Fgf10* in various signalling or disease pathways. These results help support **aim 2** of this thesis.

Key genes that have been characterised as up- or downregulated will now be profiled across varying gestational ages to identify key patterns between gestational ages or genes. This work continues to build towards **Hypothesis 2**, that signalling pathways related to the *Fgf10*-*Fgfr2b* axis moderate the morphology and penetrance of the DA phenotype seen in *Fgf10*^{-/-} mice.

7. Duodenal expression profile of key genes during DA development

7.1. Introduction

Using the E13.5 gestational age to identify 19 key genes up- or downregulated in *Fgf10*^{-/-} and DA-affected duodenum, it was essential to understand their role and interactions, along with their patterns of expression in the duodenum. Along with the generation of expression patterns, the literature was reviewed to profile these genes (**Figure 7.1**) in mice and humans to provide context for analysis. Using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database, their encoded proteins were analysed for interactions, co-expression, and association in common pathways (<https://string-db.org/>).

In order to elaborate on the expression pattern provided in chapter 6 (E13.5), duodenal tissue was harvested from embryos at gestational ages E11.5, E15.5, E17.5 and E19.5. These embryos were grouped by genotype and the presence and morphology of DA.

This tissue was pooled into two samples of three duodena per group to achieve the mRNA concentration required for analysis via RT-qPCR. A minimum of three technical replicates were run per sample to measure variability within the protocol. Gene expression of all samples was normalised against Rpl32.

Gene expression was represented as the LogFC of each group compared to wild-type expression. Student's t-test was performed and p-value <0.05 were considered significant. Gene expression was then compared for each gene across all gestational ages studied (including E13.5 presented previously) in order to identify patterns.

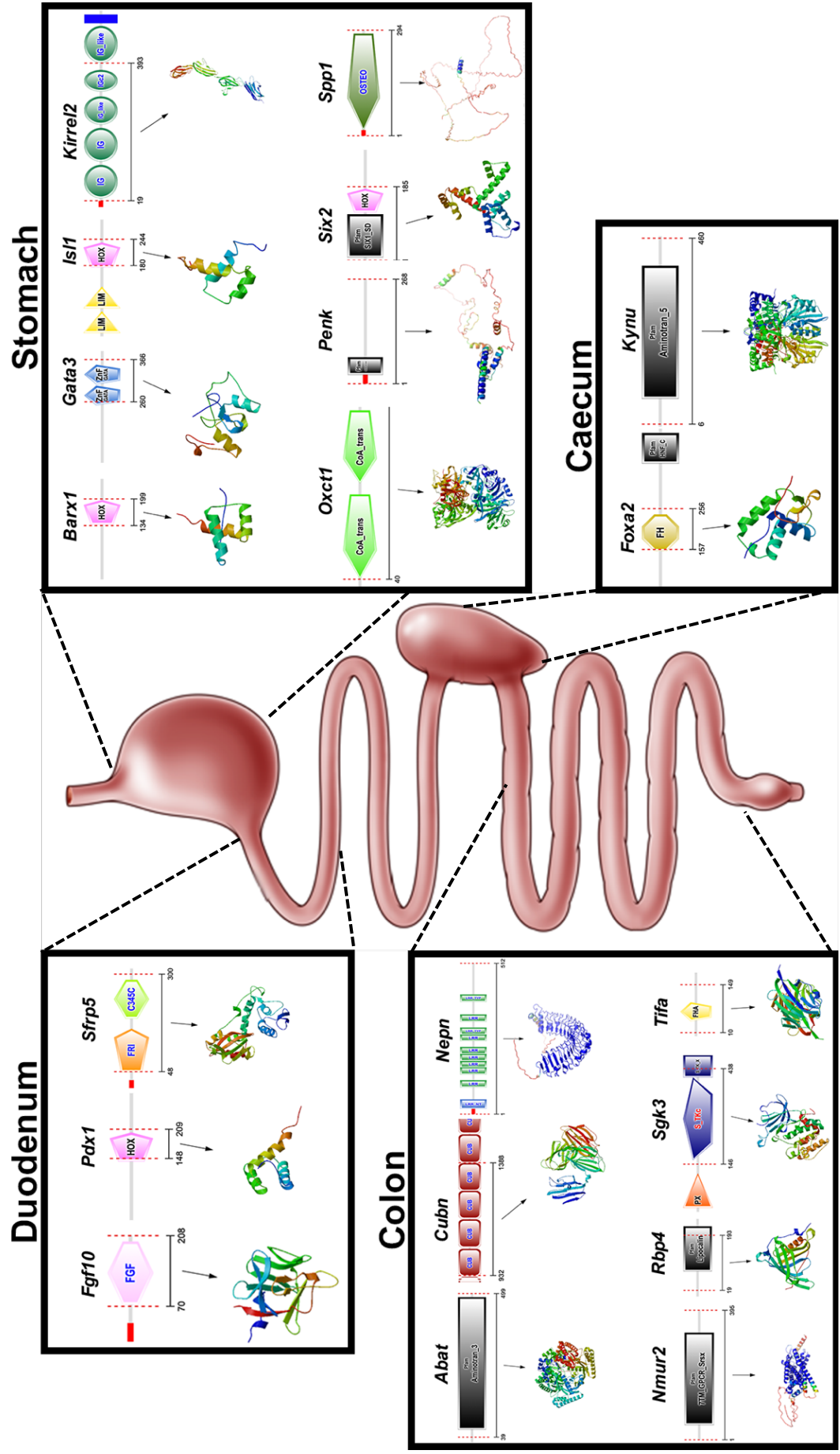


Figure 7.1 Gastrointestinal regions of greatest expression for key protein-coding genes. List of key genes, including transcription regions and coded protein, grouped based on regions maximum expression within the gastrointestinal tract. Expression regions based on (Yue et al., 2014). Transcription images sourced from (Letunic et al., 2020) and proteins sourced from (Waterhouse et al., 2018).

7.2. Results

No DA was observed in E11.5 embryos. 138 embryos were harvested (18 E11.5 embryos and 30 embryos from each other gestational age), with duodena from 6 embryos per group per age (n=6).

7.2.1. Expression pattern of key upregulated genes in *Fgf10*^{-/-} duodenum

7.2.1.1. *Barx1*

In mice, *Barx1* is a protein-coding gene located on chromosome 13. It encodes the BarH-like 1 subclass of homeobox transcription factors. It is orthologous to BARX1 in humans, located on chromosome 9. It is involved in epithelial-mesenchymal interactions during craniofacial development and stomach organogenesis (Tissier-Seta et al., 1995). BARX1 has been shown to support the differentiation of stomach epithelium (Miletich et al., 2005, Stringer et al., 2008), possess an inhibitory role in Wnt signalling in the control of tracheo-oesophageal septation (Woo et al., 2011), and direct spleen and pancreas laterality (Hecksher-Sørensen et al., 2004). In humans, BARX1 is associated with congenital hiatal hernia (Chang et al., 2020) and oesophageal squamous cell carcinoma (Yan et al., 2018).

FGF10 and BARX1 are both linked to early palate development, albeit in the separate anterior and posterior regions, respectively (Smith et al., 2012, Li et al., 2019).

Figure 7.2 demonstrates *Barx1* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows the greatest expression at E13.5 and E19.5.

RT-qPCR has shown that, in *Fgf10*^{-/-} duodenum, *Barx1* is upregulated in all DA phenotypes at E11.5, E13.5, E15.5 and E17.5 (**Figure 7.3**). The greatest upregulation was seen in DA-affected duodena at E17.5 with LogFC of 6.8 and 6.3 for embryos with type 1 and type 2/3 DA, respectively.

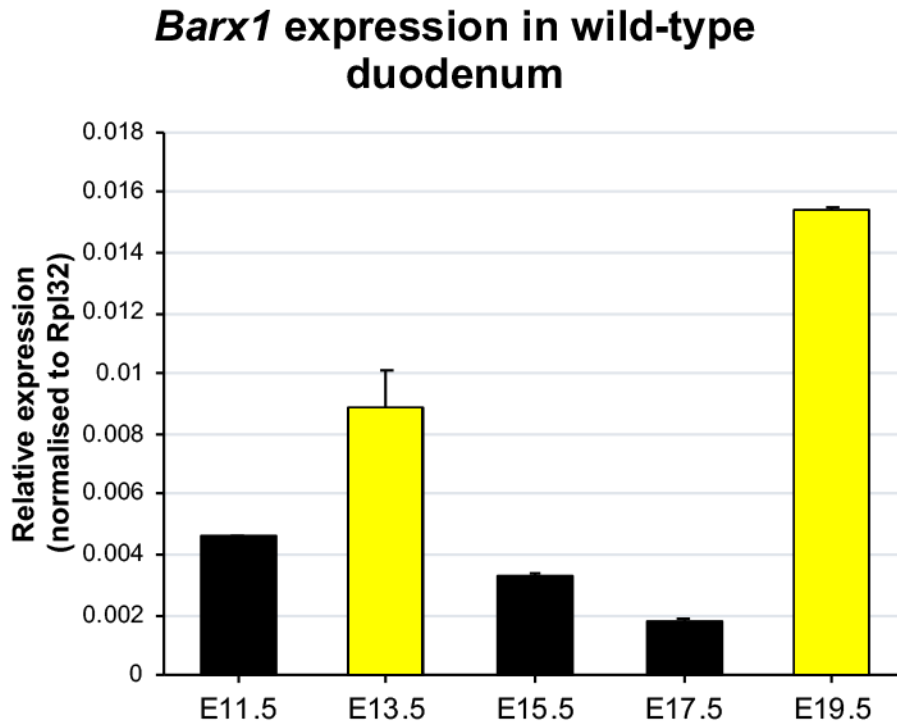


Figure 7.2 *Barx1* is significantly increased within the duodenum of embryos at E13.5 and E19.5. mRNA expression analysis of *Barx1* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E18.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

At E15.5, the upregulation of *Barx1* in all *Fgf10*^{-/-} groups was reduced compared to E13.5 and E17.5. Its expression almost returned to normal (LogFC= 0.3 $\pm$ 0.08) in those embryos without DA. Furthermore, at E17.5 *Barx1* expression from *Fgf10*^{-/-} embryos without DA remained significantly lower than DA -affected *Fgf10*^{-/-} phenotypes (LogFC= 3.9 $\pm$ 1.4).

At E19.5, *Barx1* was downregulated in DA-affected embryos compared to all other groups, demonstrating a small but statistically significant change, with a LogFC of -0.22 and -0.18 for DA type 1 and type 2/3, respectively.

This pattern demonstrates an initial (E11.5-E13.5) rise in *Barx1* expression in the duodenum of all *Fgf10*^{-/-} embryos, followed by a fall (E15.5), and then a secondary pulse (E17.5) much more significant in DA-affected duodena. These DA-affected duodena are subsequently reduced in their *Barx1* expression in the final stages of embryogenesis.

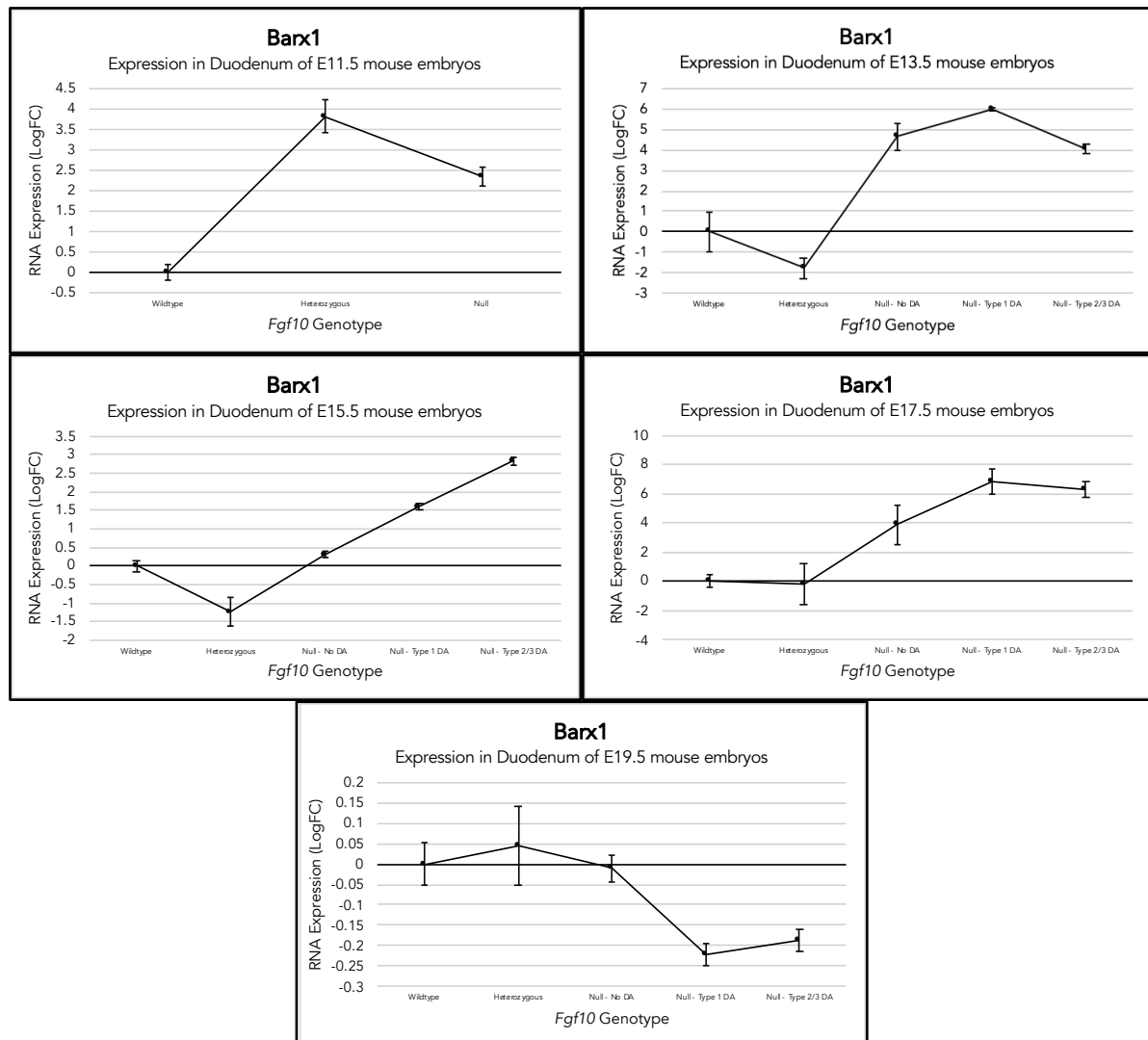


Figure 7.3 Expression of *Barx1* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Barx1* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.1.2. *Gata3*

Gata3, located on chromosome 2 in mice, encodes the Trans-acting T-cell-specific transcription factor GATA-3. It is a Transcriptional activator that binds to the enhancer of the T-cell receptor alpha and delta genes (Nakamura et al., 2020), forming a role in the innate and adaptive immune response (Kasal et al., 2021).

Gata3 has been shown as upstream of *Fgf10* in the regulation of mouse inner ear formation (Economou et al., 2013, Lillevall et al., 2006).

The human analogue, GATA3 (located on chromosome 10), has a similar role in T-cell development (Jenner et al., 2009), as well as epithelial tumour suppression (Zeng et al., 2019). Heterozygous mutations of the GATA3 gene results in hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome (Lemos and Thakker, 2020). It has been identified as a prognostic marker in breast cancer (Fararjeh et al., 2018), with patients with a high GATA3 demonstrating better clinical overall survival, with mutations promoting growth in malignant breast epithelium (Emmanuel et al., 2018).

Figure 7.4 demonstrates *Gata3* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows the greatest expression at E13.5 and E19.5.

RT-qPCR failed to demonstrate the upregulation of *Gata3* in all *Fgf10*^{-/-} embryos, earlier observed at E13.5, at other gestational ages (**Figure 7.5**). However, *Gata3* was upregulated in *Fgf10*^{-/-} embryos with DA at E15.5 (more so in type 2/3 DA, LogFC= 2.5, +/-0.25) and E17.5.

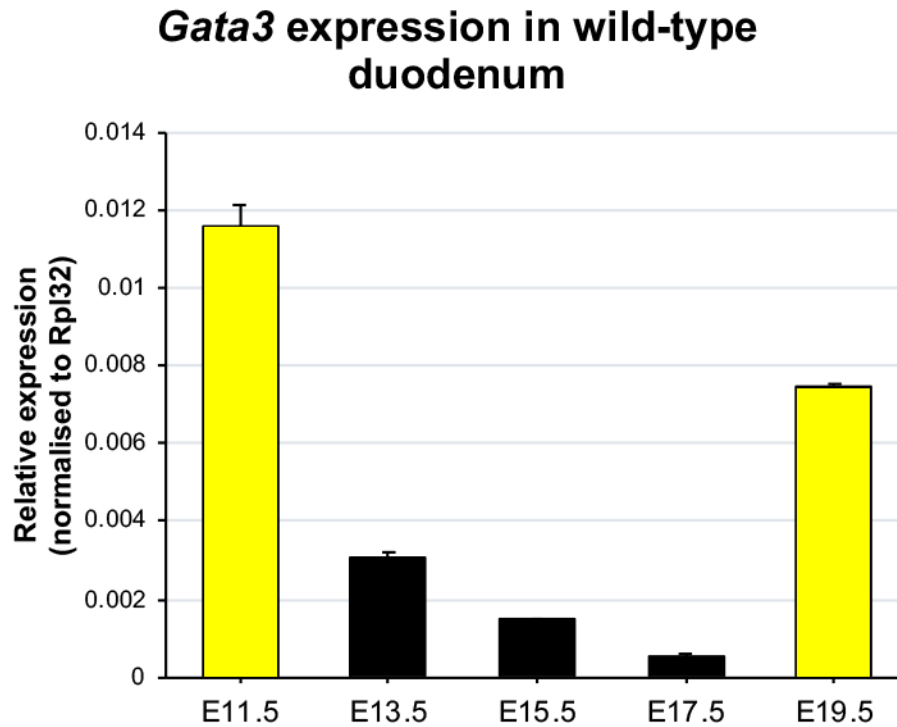


Figure 7.4 *Gata3* is significantly increased within the duodenum of embryos at E11.5 and E19.5. mRNA expression analysis of *Gata3* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

At E19.5, duodenum affected by DA type 2/3 was seen to reduce its expression of *Gata3* (LogFC= -0.4, $\pm$ 0.11).

This pattern demonstrates an initial (E13.5) upregulation of *Gata3* in the duodenum of all *Fgf10*^{-/-} embryos, which persists only for those with DA until E17.5. This is followed by a fall in *Gata3* expression in DA type 2/3 affected duodenum at E19.5.

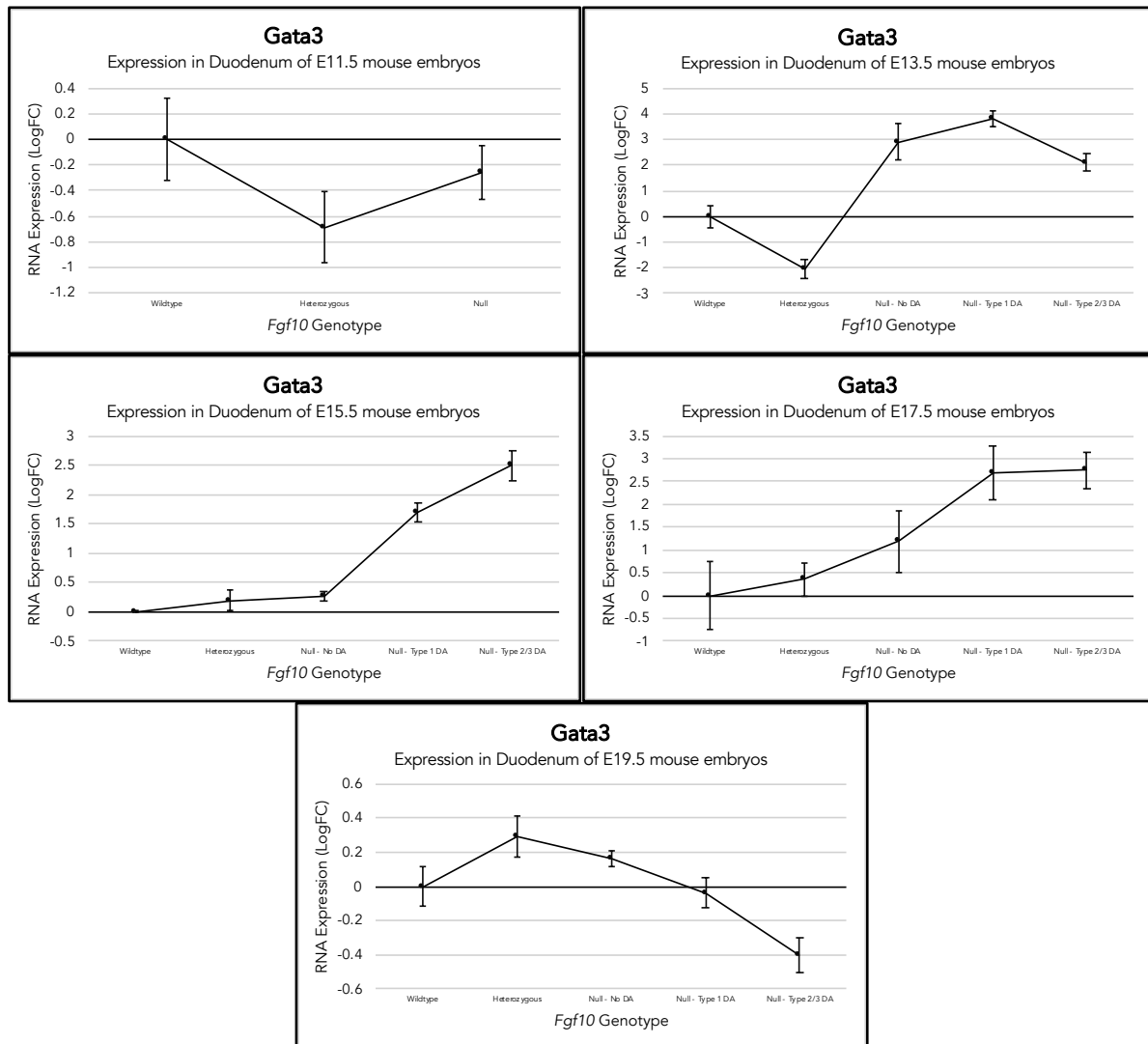


Figure 7.5 Expression of *Gata3* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Gata3* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.1.3. *Isl1*

Isl1 is a protein-coding gene found in mice on chromosome 13. It encodes the Insulin gene enhancer protein ISL-1. It is several processes, including heart, tongue and auditory neuron development (Jing et al., 2021, Zhang et al., 2022, Filova et al., 2022). It plays a fundamental role in the gene regulatory network essential for retinal ganglion cell differentiation (Elshatory et al., 2007). Is expressed in in multiple regions including the gastrointestinal tract (duodenum, stomach and colon); the central nervous system; genitourinary system; and heart and pericardium (Yue et al., 2014).

Fgf10 is associated upstream of *Isl1* in mediating mesenchymal expansion in developing external genitalia (Ching et al., 2018).

In humans, ISL1 (located on chromosome 5) encodes ISL LIM homeobox 1 a member of the LIM/homeodomain family of transcription factors. This protein plays an important role in regulating insulin gene expression (Yan et al., 2016). The encoded protein is central to the development of pancreatic mesenchyme and islet cells (Ahlgren et al., 1997) and may also be required for motor neuron generation (Pfaff et al., 1996, Solomon et al., 2021). Mutations in this gene have been associated with early onset type-2 diabetes (Yokoi et al., 2006). ISL1 is also associated with heart conditions (Dong et al., 2020, Ma et al., 2019), including atrial fibrillation (Wu et al., 2020) and ventral septal defects (Zheng et al., 2021b).

FGF10 is downstream of ISL1 in the regulation of human cardiac outflow formation (Golzio et al., 2012) and upstream in regulating pancreas development (Manfroid et al., 2007).

Figure 7.6 demonstrates *Isl1* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows greatest expression at E11.5 and E19.5, closely resembling that seen in *Gata3*.

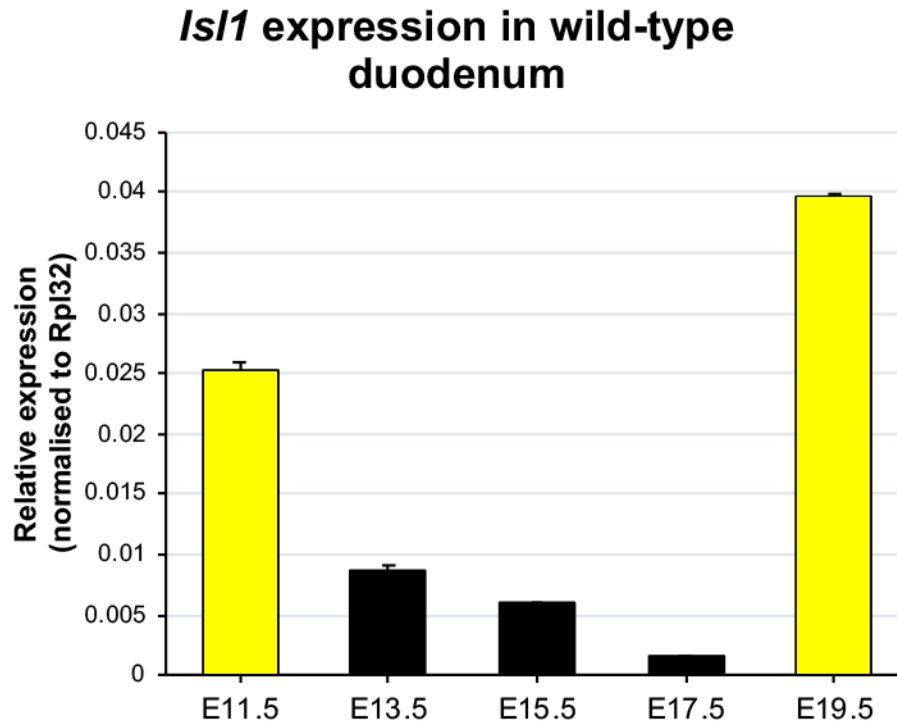


Figure 7.6 *Isl1* is significantly increased within the duodenum of embryos at E11.5 and E19.5. mRNA expression analysis of *Isl1* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

In the duodenum *Fgf10*^{-/-} embryos, *Isl1* is demonstrated by RT-qPCR (**Figure 7.7**) to be upregulated in all DA phenotypes at E11.5, E13.5 and E17.5. At E15.5 only DA-affected *Fgf10*^{-/-} duodenum demonstrate an increased *Isl1* expression. At E19.5 these DA-affected duodenum demonstrate a downregulation of *Isl1*, worse in type 2/3 DA than type 1.

The expression pattern of *Isl1* holds similarities to that of *Barx1* (section 7.2.1.1), with an initial (E11.5-E13.5) increase in the duodenum of all *Fgf10*^{-/-} embryos, followed by a fall (E15.5) of greater significance in *Fgf10*^{-/-} duodenum without DA. And then secondary pulse (E17.5), before DA-affected duodena are reduced in their *Isl1* expression at E19.5.

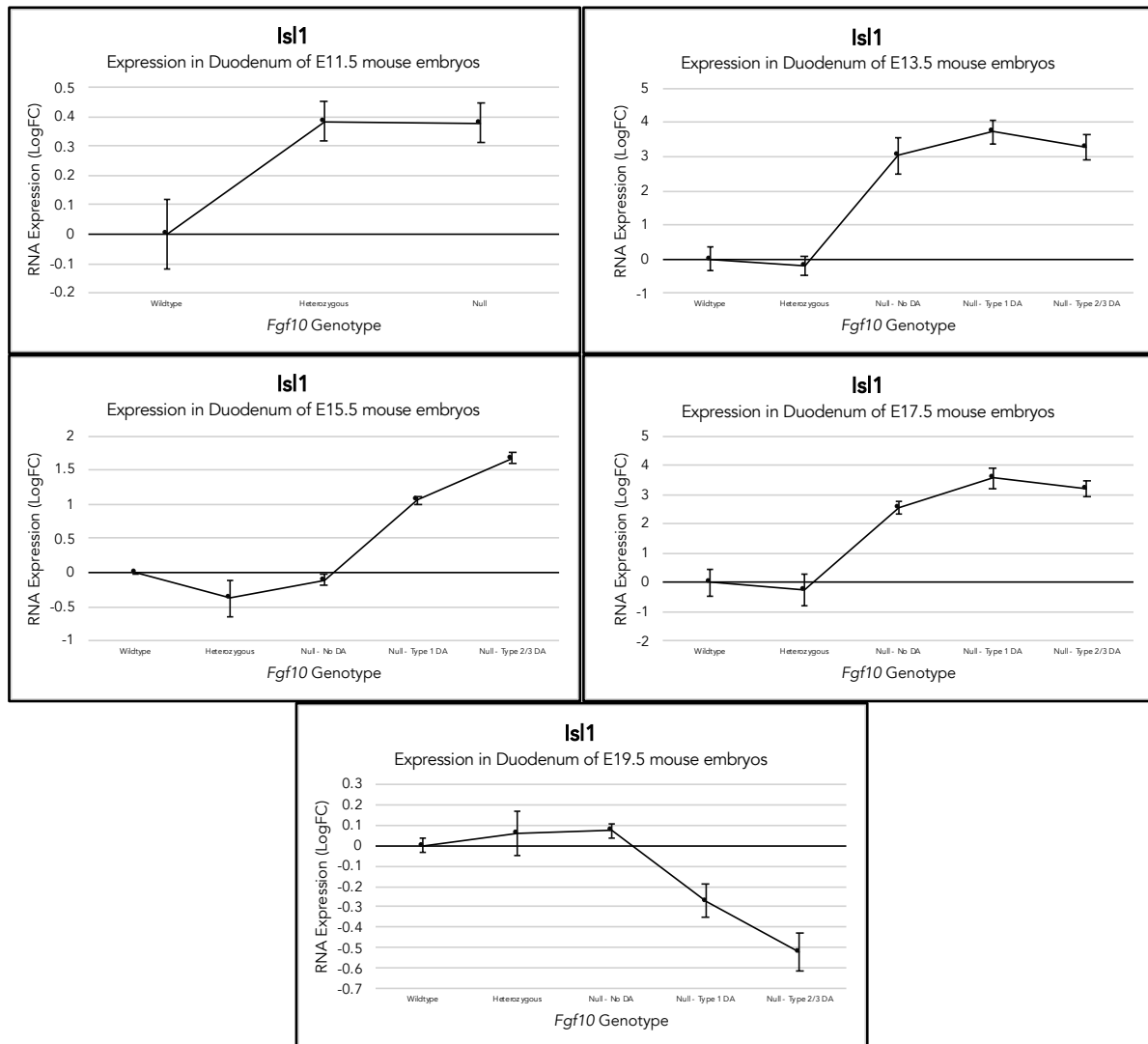


Figure 7.7 Expression of *Isl1* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Isl1* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. n=6 per group per age, n=2 samples per group, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.1.4. *Oxct1*

Oxct1 is found on chromosome 15 in mice and encodes 3-ketoacid coenzyme A transferase 1, a key enzyme in ketone body catabolism. Located in mitochondria and expressed in the foregut (Yue et al., 2014), it is required to oxidise ketone bodies, with knockout mice dying from hyperketonaemic hypoglycaemia (Cotter et al., 2011).

The human orthologue, OXCT1, is found on chromosome 5. Its mutation is associated with the metabolic disorder Succinyl-CoA:3-oxoacid coenzyme A transferase deficiency (SCOTD) (Grünert et al., 2021, Shafqat et al., 2013).

Figure 7.8 demonstrates *Oxct1* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows a significant increase in expression at E13.5 and E19.5, closely resembling that seen in *Barx1*.

In the duodenum of *Fgf10*^{-/-} embryos, *Oxct1* is demonstrated by RT-qPCR (**Figure 7.9**) to be upregulated in all DA phenotypes, from a minimal increase at E11.5 (LogFC= 0.16, +/- 0.02), to its greatest upregulation, seen in type 1 DA-affected embryos at E17.5 (LogFC= 2.0 +/- 0.2).

Some variance exists between *Fgf10*^{-/-} groups, with DA type 1 affected duodenum appearing to express *Oxct1* at a lower (E15.5) and then higher (E17.5) level when compared to other DA phenotypes.

No significant difference was seen between groups, in the expression of *Oxct1*, at E19.5.

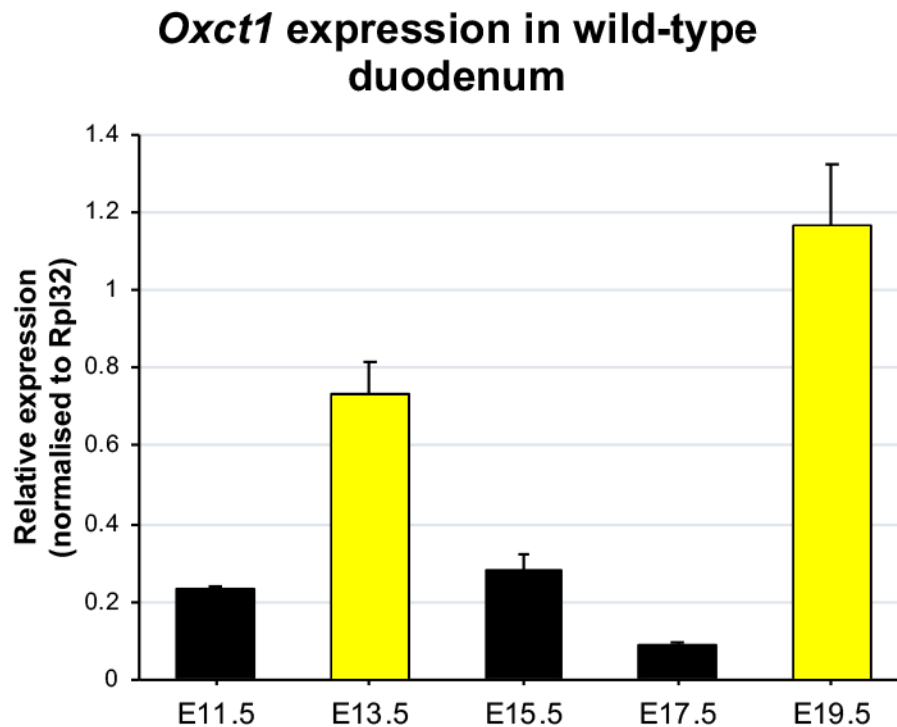


Figure 7.8 *Oxct1* is significantly increased within the duodenum of embryos at E13.5 and E19.5. *mRNA expression analysis of *Oxct1* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to Rpl32. n=6 per age, n=2 biological replicate samples, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.*

While it's difficult to interpret the meaning of this type 1 DA variation, the overarching pattern of *Oxct1* expression in *Fgf10*^{-/-} duodenum is that of a small and gradual increase from E11.5 to E17.5 before returning to normal levels.

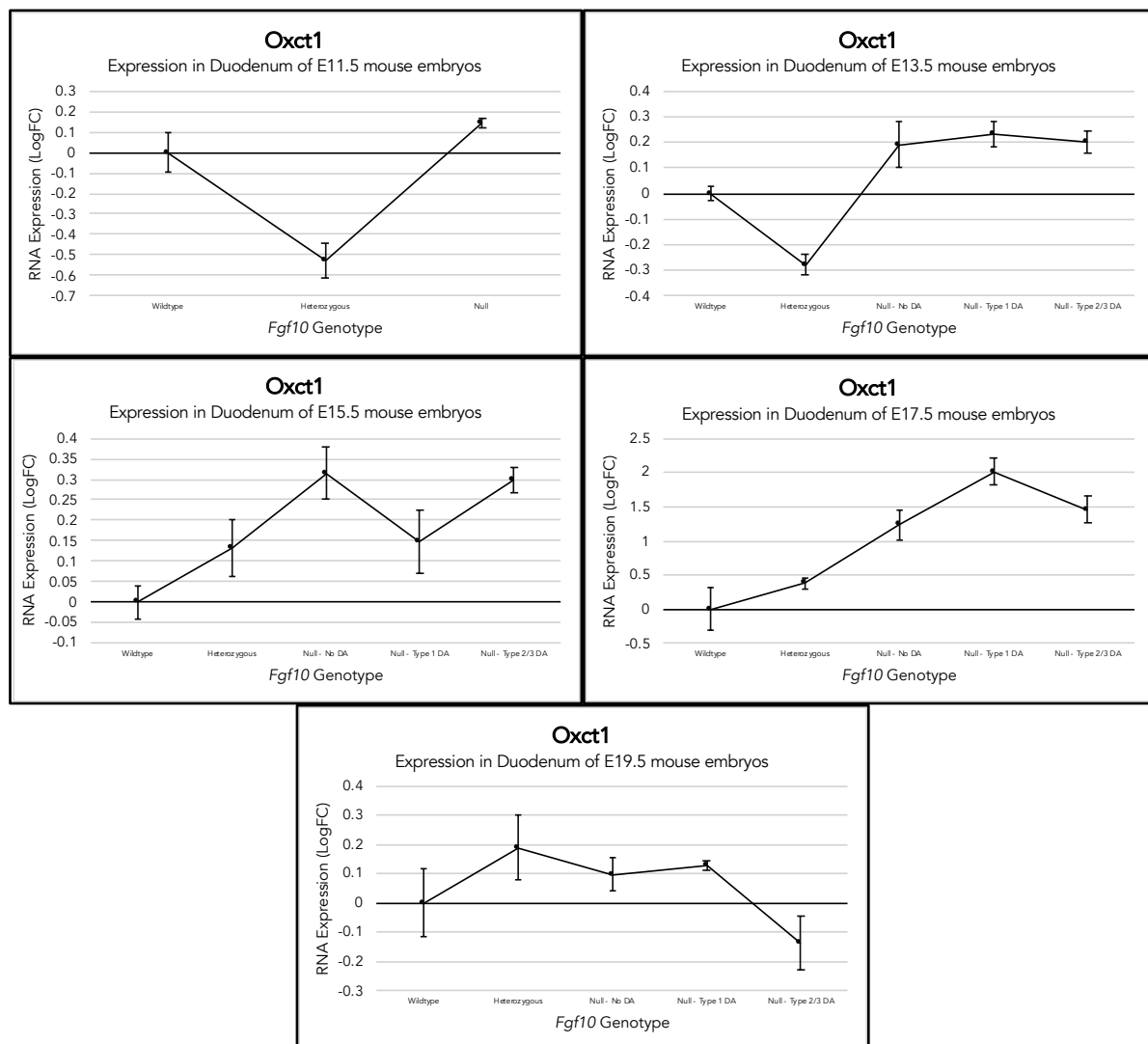


Figure 7.9 Expression of *Oxct1* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Oxct1* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.1.5. *Penk*

Penk is located on mouse chromosome 4 and encodes preproenkephalin, a protein involved in opioid receptor binding. Preproenkephalin has a role in the perception of pain (François et al., 2017), anxiety and aggression (König et al., 1996) and in the regulation of feeding and body weight (Mendez et al., 2015). It is expressed in GABAergic neurons in the spine (Huang et al., 2008), with over-expression associated with improved symptoms in Huntington's disease mouse models (Bissonnette et al., 2013).

In humans, *PENK* is located on chromosome 8, also expressing preproenkephalin. This endogenous opioid peptide has been shown to be associated with cannabis and cocaine use disorder (Moeller et al., 2015, Jutras-Aswad et al., 2012) and downregulated in alcoholics (Sarkisyan et al., 2015). It is associated with heart failure and chronic kidney disease (Emmens et al., 2019, Kieneker et al., 2018) and inhibits osteosarcoma migration (Zhang et al., 2020).

Figure 7.10 demonstrates *Penk* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows a significant increase in expression at E13.5 compared to all other embryonic ages. Expression is also relatively reduced at E15.5 and E17.5.

In the duodenum of *Fgf10*^{-/-} embryos, *Penk* is demonstrated by RT-qPCR (**Figure 7.11**) to initially (E11.5) be downregulated (LogFC= -0.14, +/-0.05), then (E13.5-E17.5) upregulated in all groups. The greatest upregulation of *Penk* is seen in the DA-affected type 2/3 *Fgf10*^{-/-} phenotype at E17.5 (LogFC= 3.13, +/-0.02). Downregulation of *Penk* is seen at E19.5 in Type 2/3 DA-affected embryos only (LogFC= -0.58, +/- 0.02).

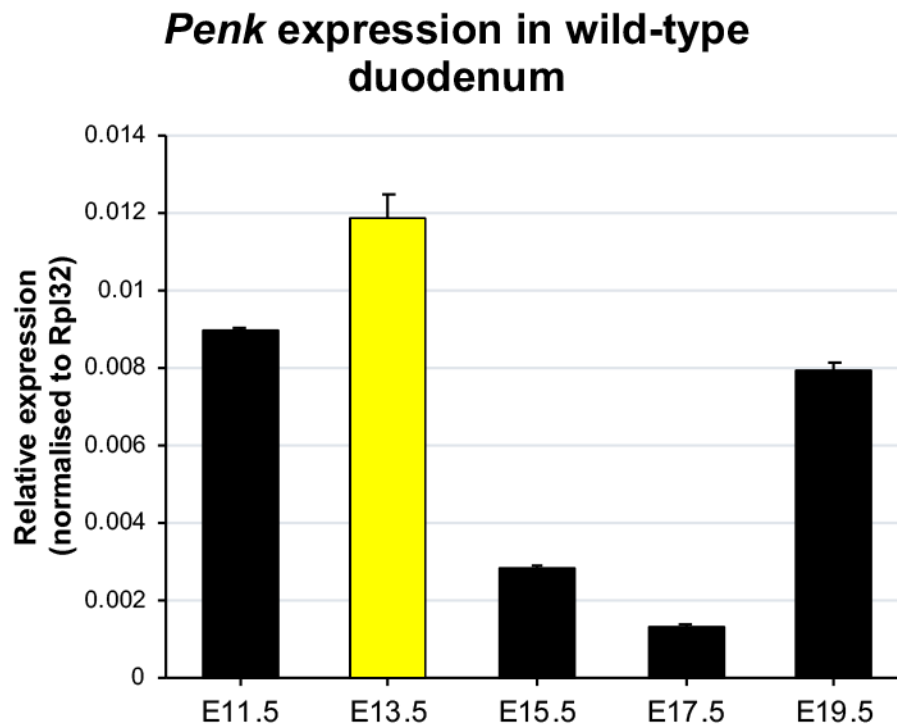


Figure 7.10 *Penk* is significantly increased within the duodenum of embryos at E13.5 and significantly reduced at E15.5 and E17.5. *mRNA expression analysis of *Penk* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

Between *Fgf10*^{-/-} groups, initially (E13.5) DA type 2/3 lags behind the upregulation of other phenotypes, before expression matches the severity of DA phenotype at E15.5 and E17.5.

The overall expression pattern of *Penk* in the *Fgf10*^{-/-} duodenum, is that of a rise (from initial downregulation at E11.5, to a peak of upregulation at E17.5) then a fall at E19.5 only for DA type 2/3.

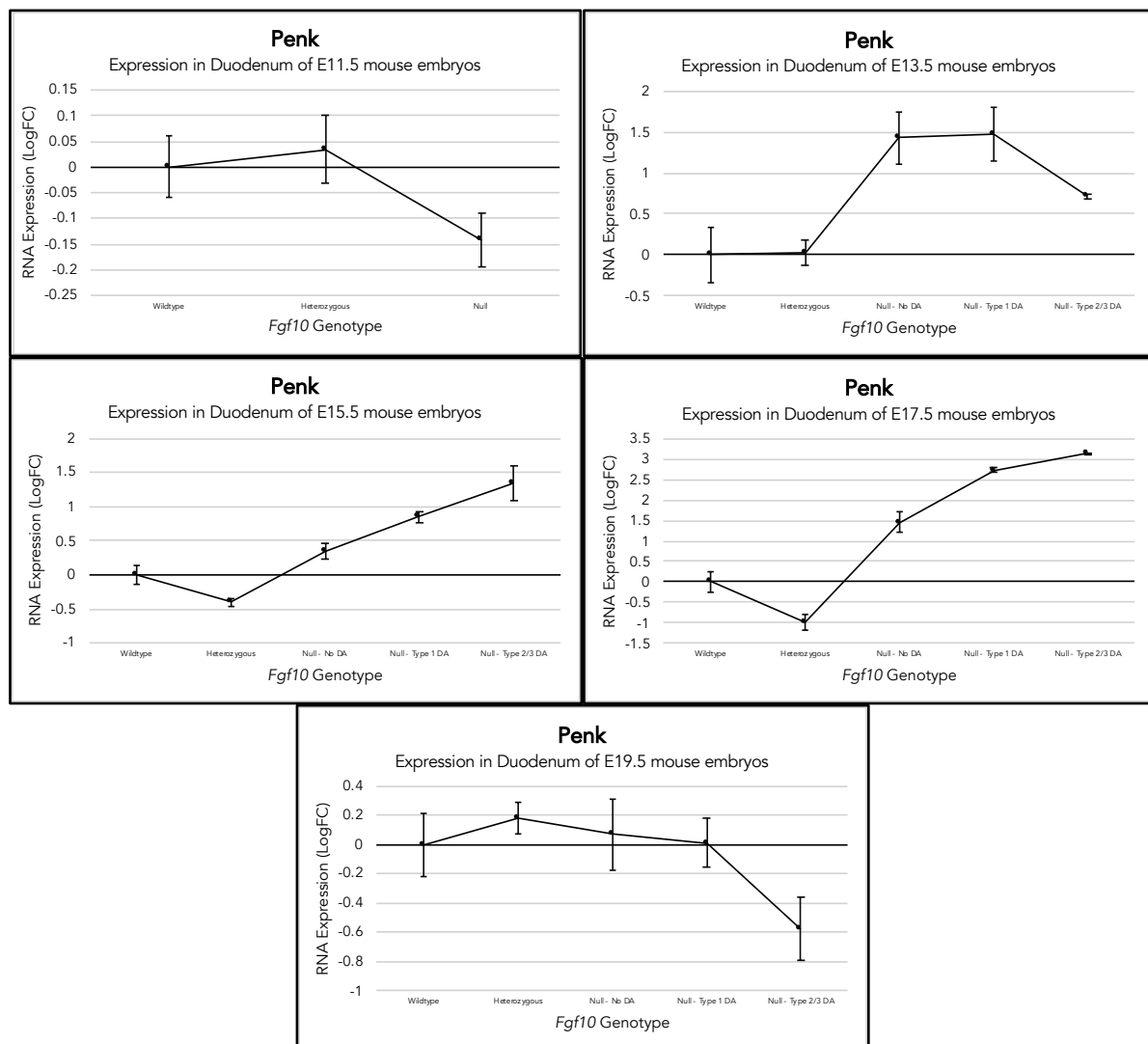


Figure 7.11. Expression of *Penk* in the duodenum of wild-type, *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5. RT-qPCR was performed using primers specific for *Penk* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.1.6. *Six2*

Six2 is located on mouse chromosome 17, encoding the protein sine oculis-related homeobox 2. It plays an important role in stomach, kidney and craniofacial development (Li et al., 2020, Kiefer et al., 2012). In the kidney it is known to maintain mesenchyme multipotent nephron progenitor cells in an undifferentiated state, and renew their proliferation through Wnt signalling (Li et al., 2016b). Thus, it is involved in kidney cell apoptosis (Xia et al., 2017) and mesenchyme to epithelial transformation (He et al., 2018).

In humans, *SIX2* is located on chromosome 2. In humans, *SIX2* influences the development of numerous organs, primarily through the regulation of cell proliferation, survival, migration, and invasion (Christensen et al., 2008). In the pancreas, *SIX2* has demonstrated a role in regulating beta-cell differentiation (Velazco-Cruz et al., 2020). It is involved in normal eye, craniofacial and ear development (Henn et al., 2018). Expression linked to poor survival of colon adenocarcinoma (Jiang et al., 2020) and hepatocellular carcinoma (Wan et al., 2019).

Figure 7.12 demonstrates *Six2* expression in the duodenum of wild-type embryos from E11.5 to E19.5. This pattern shows a significant increase in expression at E13.5 compared to all other embryonic ages.

RT-qPCR (**Figure 7.13**) has demonstrated that *Six2* is upregulated in the duodenum of all *Fgf10*^{-/-} embryos at E11.5, E13.5 and E17.5. Greatest relative upregulation is shown in DA-affected duodenum at E17.5 (type 1 DA LogFC= 7.50, +/-0.97; type 2/3 DA LogFC= 7.09, +/-0.84).

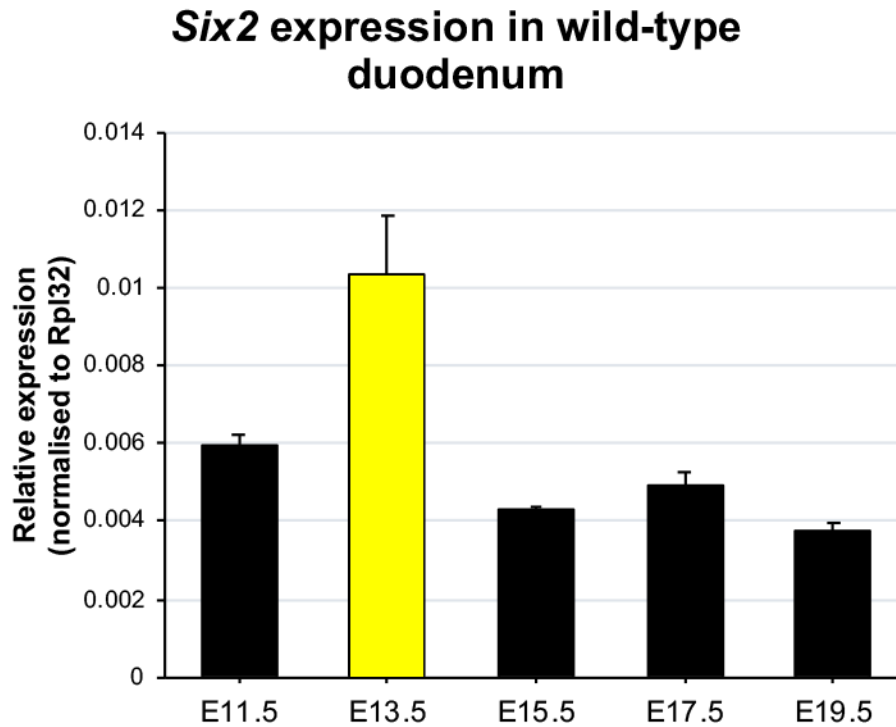


Figure 7.12 *Six2* is significantly increased within the duodenum of embryos at E13.5.

mRNA expression analysis of Six2 in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to Rpl32. n=6 per age, n=2 biological replicate samples, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

At E15.5 only DA-affected *Fgf10*^{-/-} duodena demonstrate upregulation of *Six2*. This relative increase compared to *Fgf10*^{-/-} embryos without DA, remains significant at the peak of *Six2* expression at E17.5.

Six2 is shown to be downregulated at E19.5 in DA-affected *Fgf10*^{-/-} duodenum, more significantly in type 2/3 than type 1. The expression pattern of *Six2* holds similarities to that of *Isl1* (section 7.2.1.3) and *Barx1* (section 7.2.1.1), with an initial (E11.5-E13.5) increase in the duodenum of all *Fgf10*^{-/-} embryos, followed by a fall (E15.5) of greater significance in *Fgf10*^{-/-} duodenum without DA. And then secondary more significant pulse (E17.5), before DA-affected duodena are reduced in their *Six2* expression at E19.5.

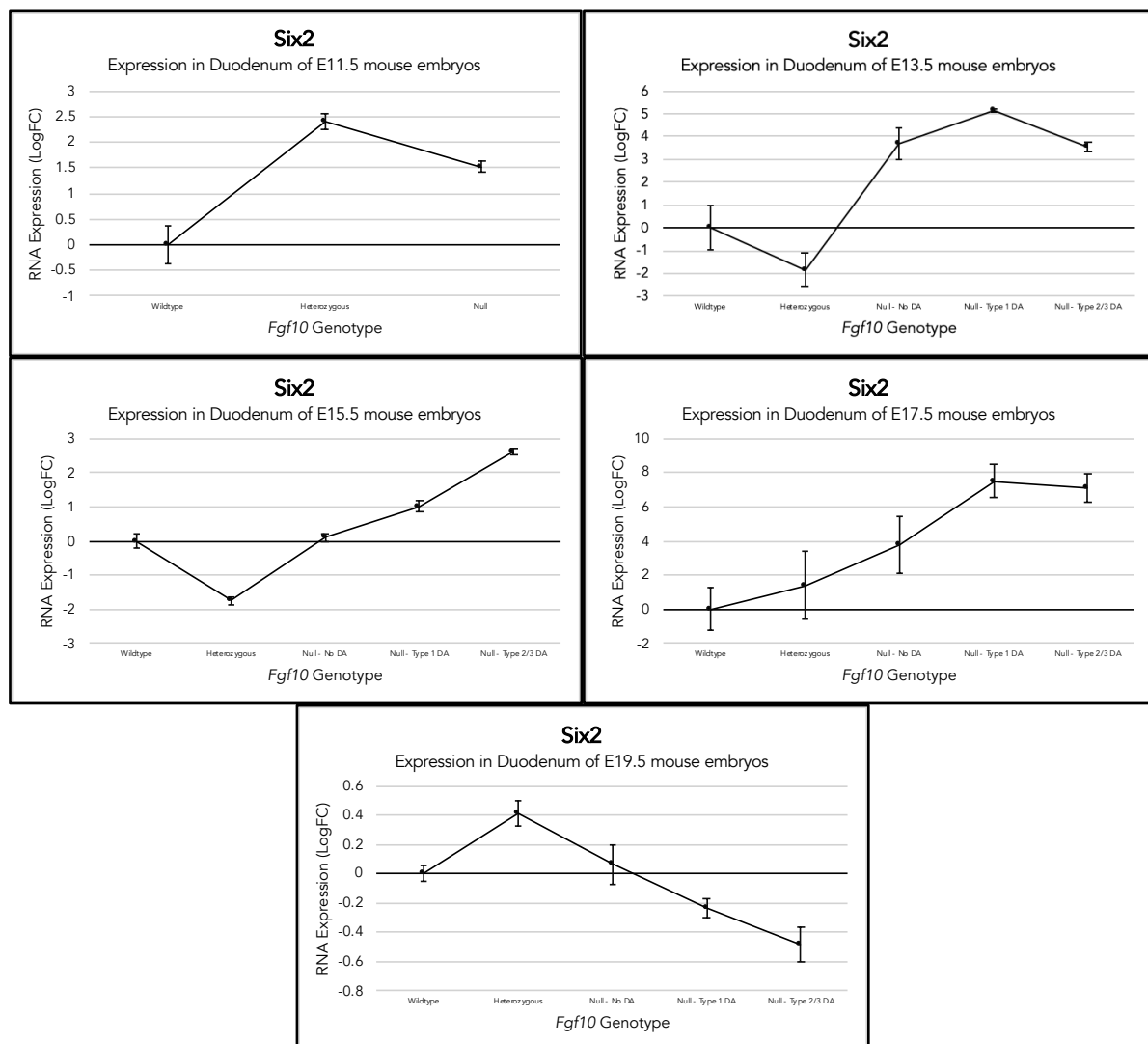


Figure 7.13 Expression of *Six2* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Six2* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2. Expression pattern of key downregulated genes in *Fgf10*^{-/-} duodenum

7.2.2.1. *Foxa2*

In mice, *Foxa2* encodes the Hepatocyte nuclear factor 3-beta transcription factor, and is located on chromosome 2. This transcription factor is a member of the forkhead class of DNA-binding proteins and opens the compacted chromatin for other proteins enabling DNA-binding activity. It holds a role in the embryonic pancreas and liver development and is associated with the establishment and regulation of gene expression related to metabolism (Wu et al., 2016). It has been demonstrated upstream of *Pdx1* (see section 7.2.12) in pancreatic beta-cells (Lee et al., 2002).

The human gene *FOXA2* (located on chromosome 20) is a hepatocyte nuclear factor and transcriptional activator for the liver-specific genes albumin and transthyretin. It is required for pancreatic differentiation, responsible for chromatin remodelling during enhancer priming (Lee et al., 2019). In humans, *FOXA2* is associated with type-2 diabetes (Fogarty et al., 2014, Tabassum et al., 2008), including early onset cases (Yamada et al., 2000). A tumour suppressor role has been proposed, with *FOXA2* mutation associated with oesophageal squamous cell carcinoma progression (Chen et al., 2022, Gao et al., 2021), hepatocellular carcinoma migration (Huang et al., 2021a), ovarian cancer (Shang et al., 2021), and uterine carcinosarcomas and carcinomas (Le Gallo et al., 2018).

FGF10 has been demonstrated to act upstream of *FOXA2* in the regulation of pancreatic fate for human embryonic stem cells (Mfopou et al., 2010) and they are co-expressed in Congenital pulmonary airway malformation (CPAM) epithelium (Fakler et al., 2020).

Figure 7.14 demonstrates *Foxa2* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A graded increase in duodenal expression is seen from E13.5 to E19.5.

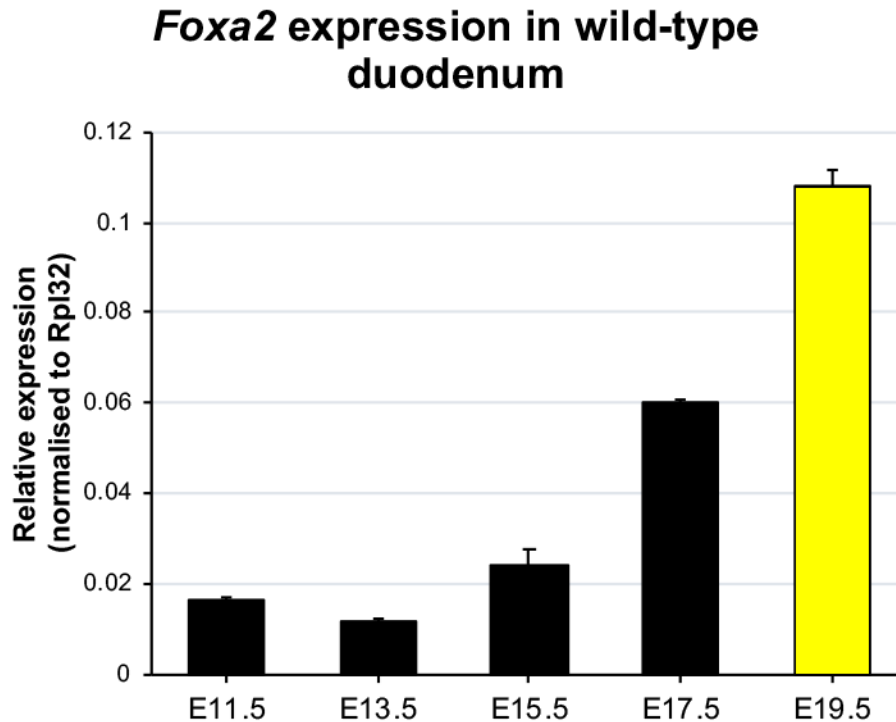


Figure 7.14 *Foxa2* undergoes a significant graded increase within the duodenum of embryos from E13.5 to E19.5. mRNA expression analysis of *Foxa2* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

RT-qPCR has shown (**Figure 7.15**) that *Foxa2* is downregulated in all *Fgf10*^{-/-} duodenal phenotypes from E13.5 (where expression is at its relative lowest) to E19.5. At E15.5 and E17.5 DA-affected duodenum are more significantly downregulated in their expression of *Foxa2* than non-DA-affected *Fgf10*^{-/-} embryos.

This pattern of *Foxa2* expression demonstrates a persisting, and relatively stable, downregulation of the gene in all *Fgf10*^{-/-} phenotypes, with greater significance in those affected by DA.

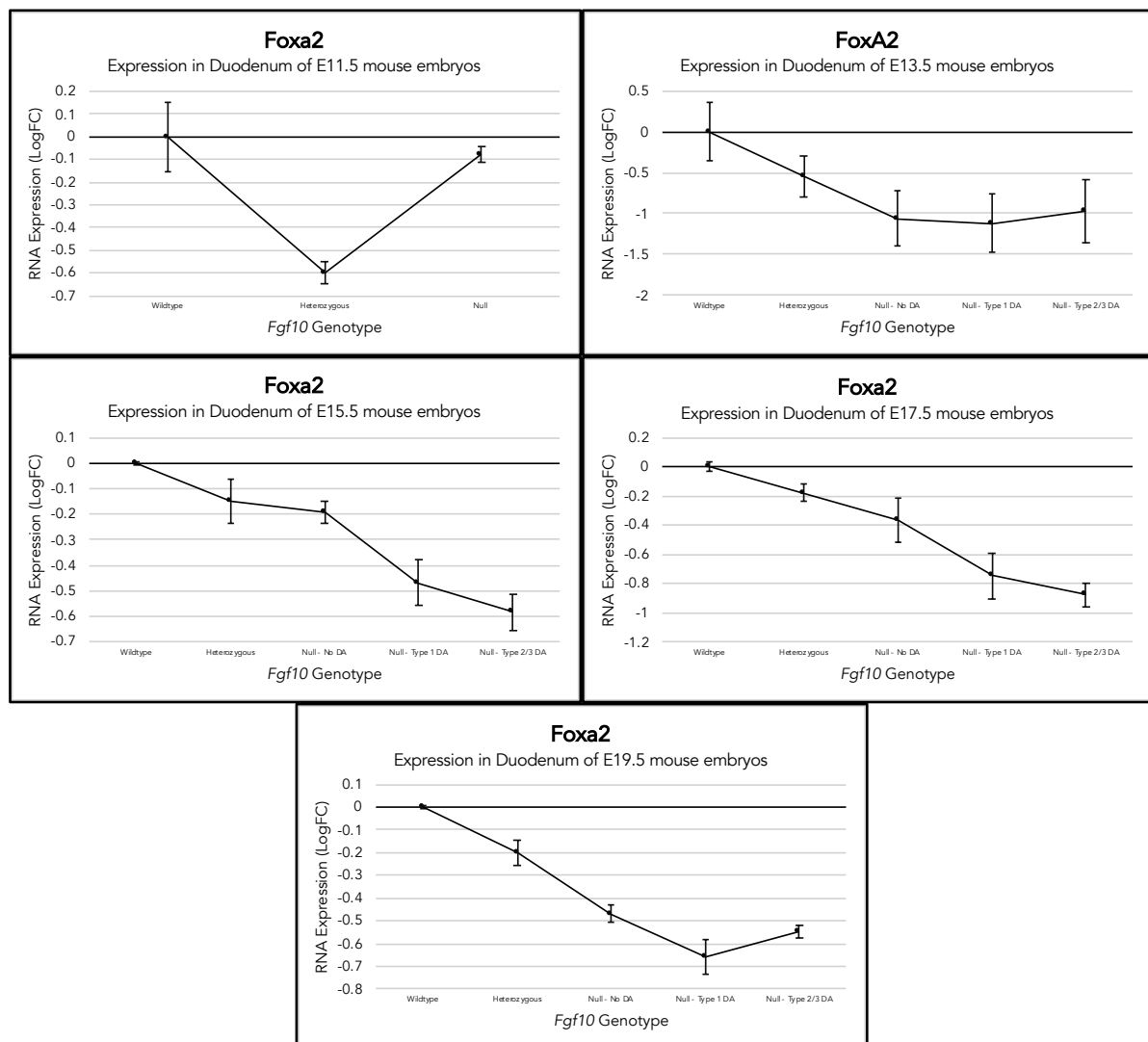


Figure 7.15. Expression of *Foxa2* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Foxa2* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

7.2.2.2. *Kirrel2*

In mice, *Kirrel2* is located on chromosome 7 and encodes kirre like nephrin family adhesion molecule 2. It belongs to the immunoglobulin superfamily and is expressed primarily in pancreatic beta-cells (Sun et al., 2003). It has been demonstrated to regulate basal insulin secretion (Yesildag et al., 2015) and is involved in olfactory neuron glomeruli function (Vaddadi et al., 2019, Brignall et al., 2018).

In humans, KIRREL2 is located on chromosome 19, and encodes the same named protein. It has been suggested to support the maintenance of the glomerular filtration barrier in the kidney, with its downregulation associated with protein-uric diseases (Ihalmo et al., 2007). KIRREL2 is also linked to diabetes, with auto-antibodies detected in people with type-1 diabetes (Rinta-Valkama et al., 2007).

Figure 7.16 demonstrates *Kirrel2* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A significant increase is seen at E19.5, compared to all other embryonic ages. A statistically significant increase is also seen at E13.5 compared to E11.5, E15.5 and E17.5.

RT-qPCR has demonstrated (**Figure 7.17**) that *Kirrel2* is downregulated in *Fgf10*^{-/-} duodenum at E11.5 and E13.5, with the most significant reduction of relative expression seen in DA type 2/3 affected embryos at E13.5 (LogFC= -10.2, +/-3.4).

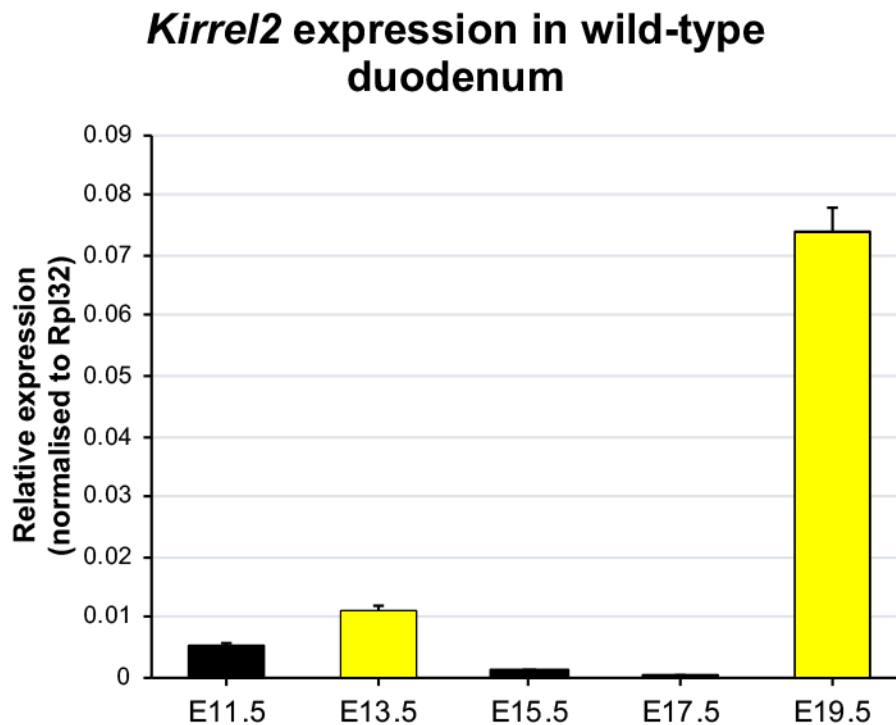


Figure 7.16 *Kirrel2* expression is significantly increased within the duodenum of embryos at E13.5 and E19.5. *mRNA expression analysis of *Kirrel2* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to Rpl32. n=6 per age, n=2 biological replicate samples, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.*

Kirrel2 is, however, upregulated at E15.5 in DA-affected duodenum, and then by all *Fgf10*^{-/-} duodenum at E17.5. The most significant increase in relative expression of *Kirrel2* is seen in DA type 2/3 affected embryos at E15.5 (LogFC= 1.63, +/-0.19), a significant swing from its E13.5 expression. In DA type 2/3 affected duodenum, *Kirrel2* is again downregulated at E19.5 (LogFC= -0.41, +/- 0.11).

The overarching pattern of *Kirrel2* expression in *Fgf10*^{-/-} duodenum is of downregulation at E11.5 to E13.5 (much more significant in DA type 2/3), before upregulation at E15.5 at E17.5 (again more significant in DA type 2/3), then downregulation only in DA type 2/3.

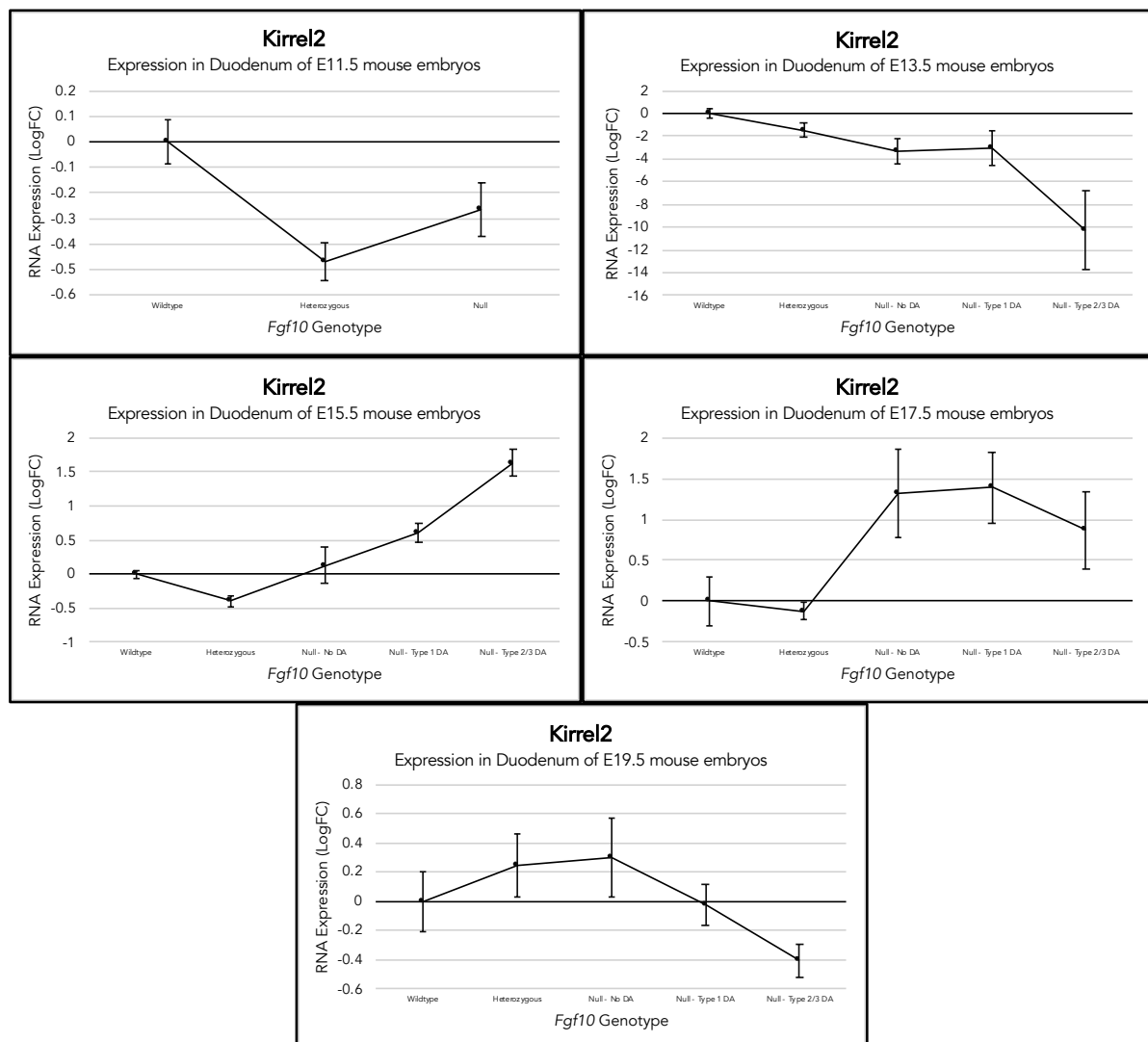


Figure 7.17. Expression of *Kirrel2* in the duodenum of *Fgf10*^{+/+} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Kirrel2* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2.3. *Kynu*

In mice, *Kynu* is located on chromosome 2 and encodes the protein kynureninase. This protein catalyses tryptophan metabolism (Phillips, 2014) and has been shown to have a pro-inflammatory role in the development of psoriasis (Wang et al., 2022).

The human orthologue of this gene, KYNU, is also located on chromosome 2. Kynureninase is also linked to psoriasis in humans (Harden et al., 2016) and has been shown to suppress breast cancer cell growth (Liu et al., 2019). KYNU mutation is associated with essential hypertension (Zhang et al., 2011), and xanthurenic aciduria (Christensen et al., 2007).

Figure 7.18 demonstrates *Kynu* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A significant expression increase is seen to be graded from E15.5 to E19.5.

RT-qPCR has demonstrated (**Figure 7.19**) that *Kynu* is downregulated in all *Fgf10*^{-/-} duodenum at E11.5 and E13.5, with greatest significance in type 1 DA-affected E13.5 embryos (LogFC= -3.36, +/-0.38).

Fgf10^{-/-} duodenum then demonstrates upregulation of *Kynu* at E15.5, before downregulation is seen only in type 2/3 affected DA at E17.5 and E19.5.

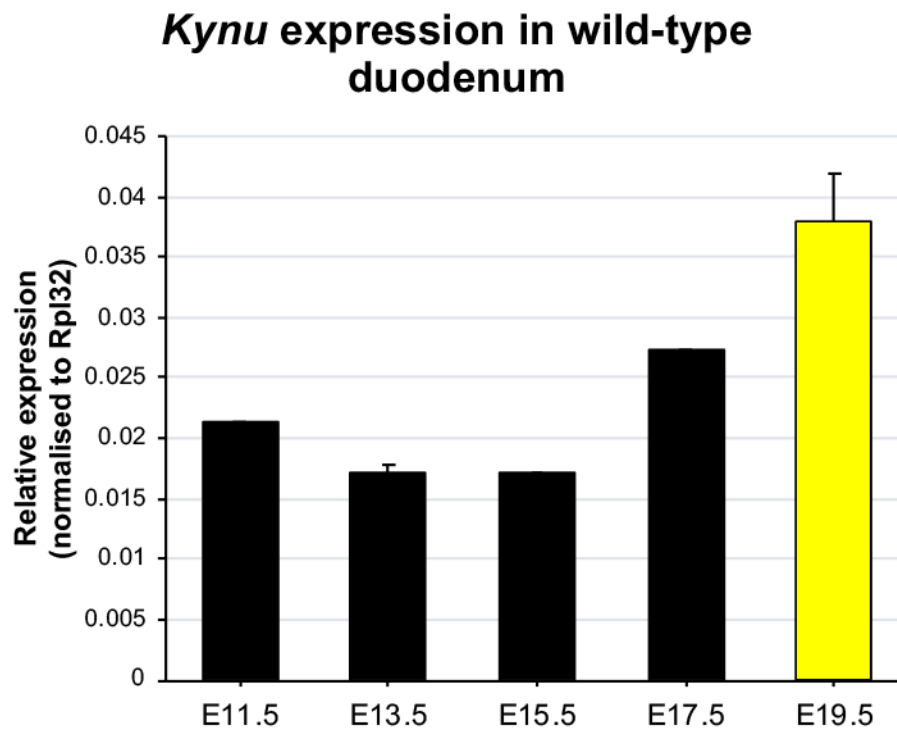


Figure 7.18 *Kynu* expression undergoes an increase within the duodenum of embryos from E15.5 to E19.5. *mRNA expression analysis of Kynu in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to Rpl32. n=6 per age, n=2 biological replicate samples, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.*

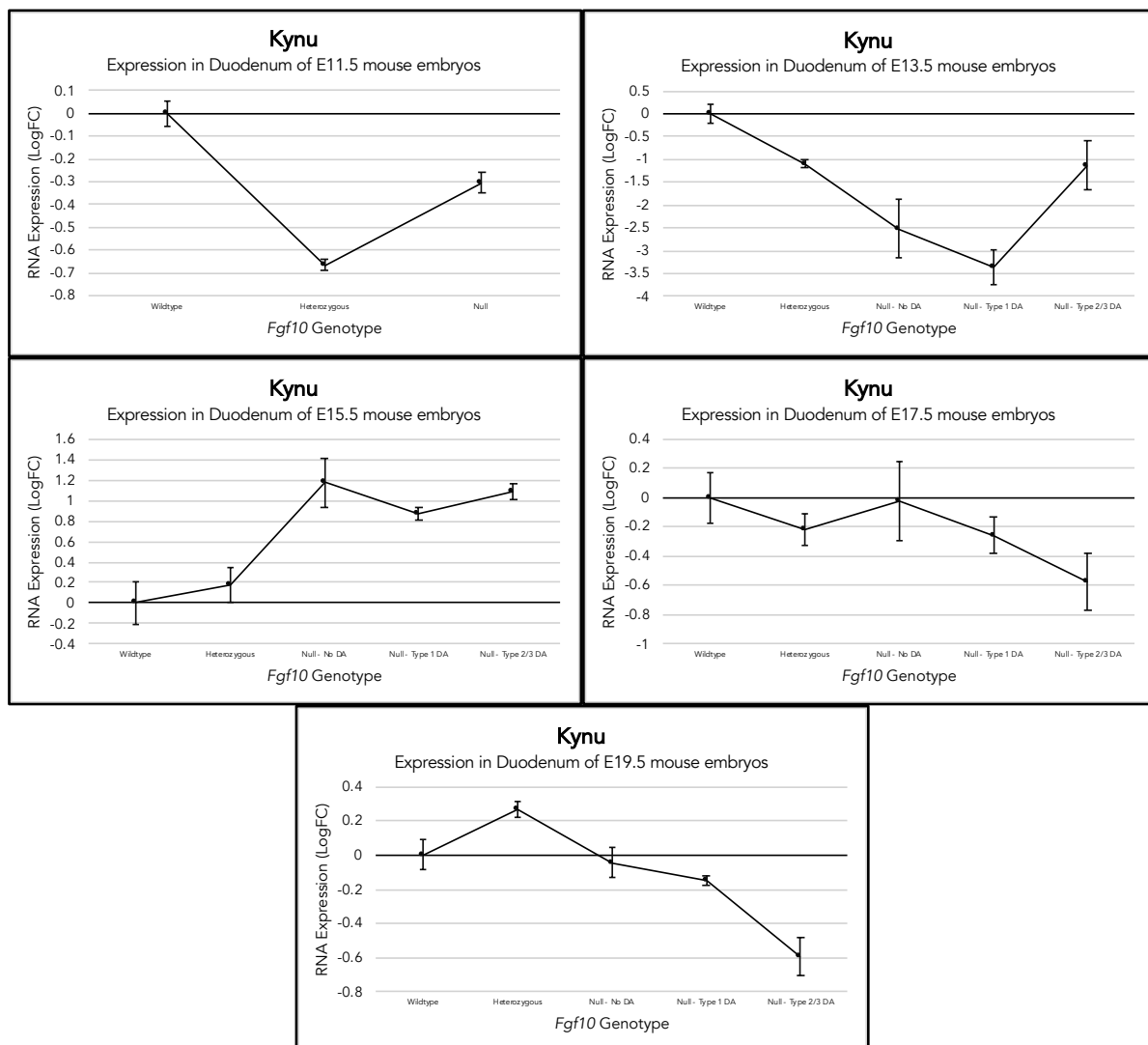


Figure 7.19 Expression of *Kynu* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Kynu* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2.4. *Nepn*

In mice, *Nepn* encodes nephrocan, and is located on chromosome 10. Nephrocan supports early stages of endoderm specification (Hou et al., 2014) with loss of *Nepn* impairing endoderm differentiation and the expression of posterior foregut-associated markers (Addeo et al., 2019). Specifically, it has been proposed to regulate boundary patterning events between the pylorus and stomach (Li et al., 2009). It is predicted to enable lipopolysaccharide binding activity and inhibit TGF-beta signalling (Mochida et al., 2006).

The human orthologue, NEPNP is located on chromosome 6. It is a pseudogene, inactivated by mutation, and is non-functional in humans (Zhang et al., 2010, Zhu et al., 2007).

Figure 7.20 demonstrates *Nepn* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A significant increase of expression is seen at E13.5.

RT-qPCR has demonstrated (**Figure 7.21**) that *Nepn* is downregulated in all *Fgf10*^{-/-} duodenum from E11.5 to E15.5, with greatest significance in type 2/3 DA-affected E13.5 embryos (LogFC= -4.22, +/-0.69).

At E17.5 there is a significant upregulation of *Nepn* in all *Fgf10*^{-/-} duodenum, greatest in those without DA (LogFC= 2.24, +/-0.77). Before a relative downregulation at E19.5 in DA-affected embryos.

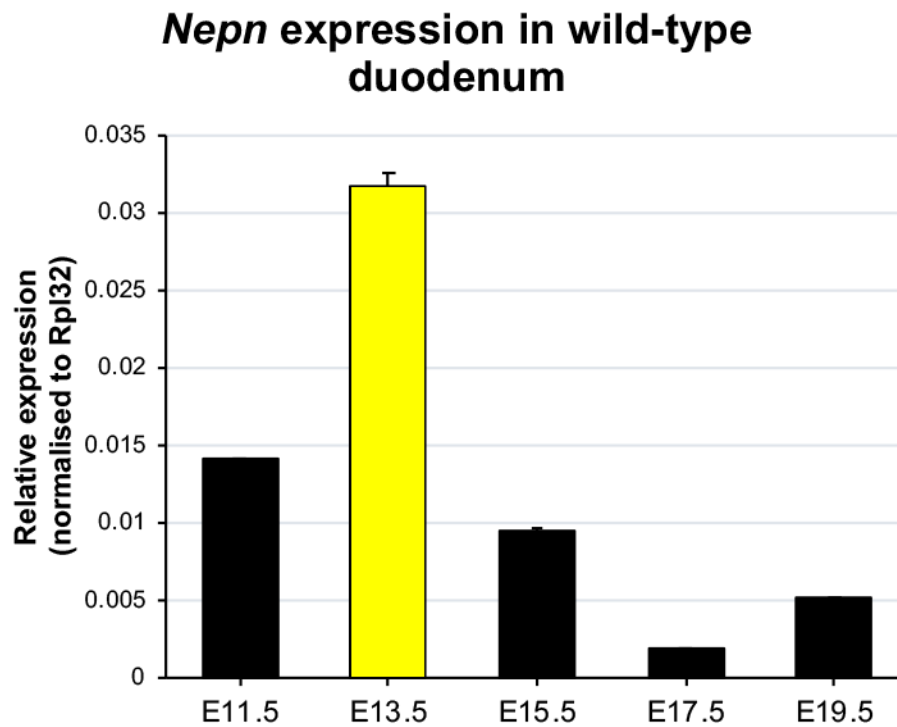


Figure 7.20 *Nepn* expression is increased within the duodenum of embryos at 13.5.

*mRNA expression analysis of *Nepn* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

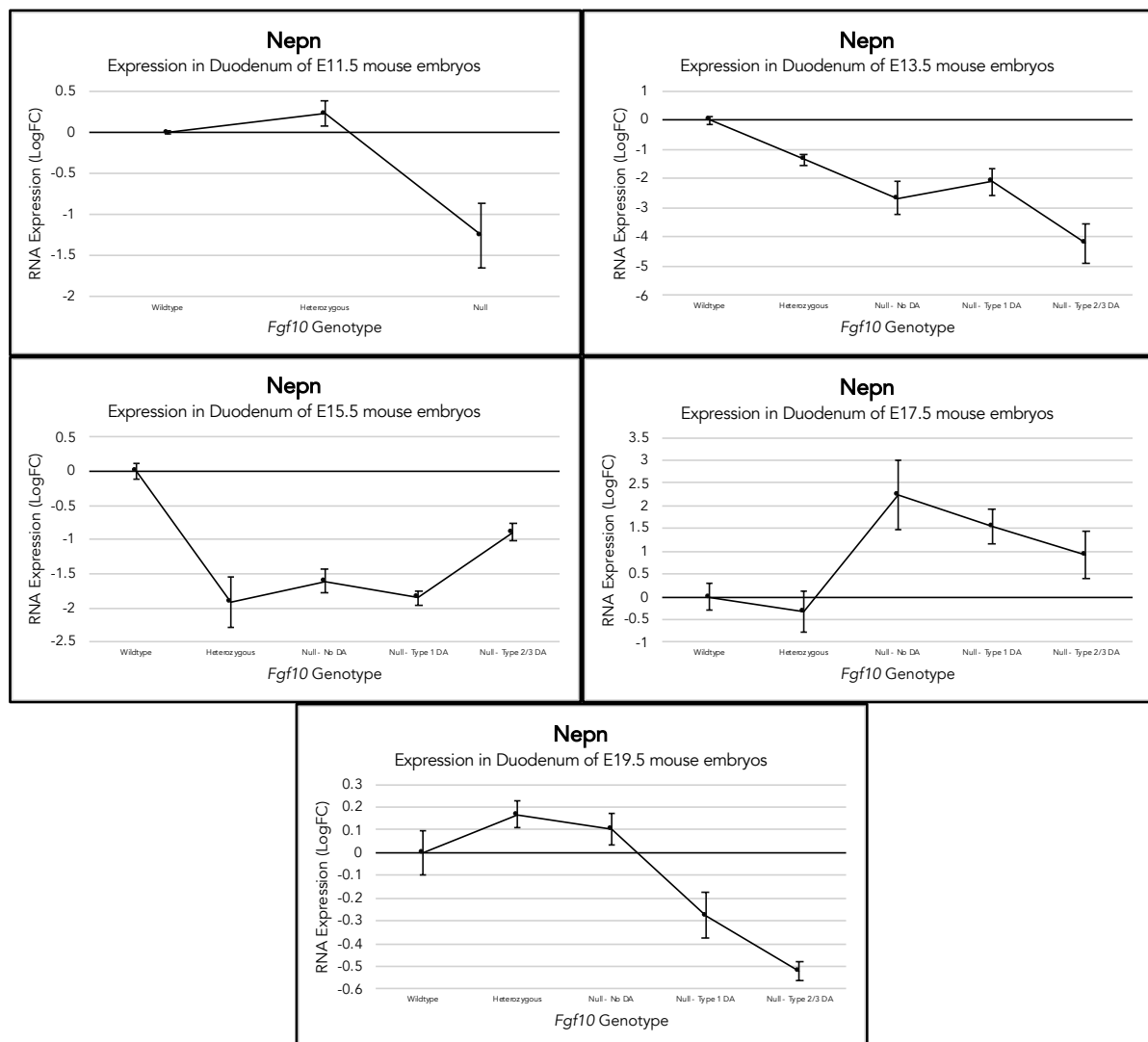


Figure 7.21 Expression of *Nepn* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Nepn* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2.5. *Pdx1*

In mice, *Pdx1* (located on chromosome 5) encodes the pancreas/duodenum homeobox protein 1. In the duodenum, *Pdx1* has been proposed to hold a role in the metabolism and absorption of lipids and iron (Chen and Sibley, 2012), as well as patterning appropriate gene expression during development (Chen et al., 2009). In pancreatic development, it supports the maturation and differentiation of pancreatic precursor cells (Zhong et al., 2022). PDX1 activates gene transcription of insulin and somatostatin (Zhou et al., 2012).

In the mouse pancreas, *Pdx1* has been demonstrated as downstream of *Fgf10*. *Fgf10* knockout is associated with reduced PDX1 production, and therefore failed pancreatic growth, differentiation and branching morphogenesis of the epithelium (Bhushan et al., 2001, Norgaard et al., 2003).

In humans, PDX1 is located on chromosome 13, and is a transcriptional activator of multiple genes, including insulin, somatostatin, glucokinase, islet amyloid polypeptide, and glucose transporter type 2. PDX1 mutations can cause pancreatic agenesis (Caetano et al., 2018) and impair beta-cell function leading to a predisposition for diabetes (Wang et al., 2019). It has been linked to a role in stomach (Ma et al., 2008, Ma et al., 2018), pancreatic (Liu et al., 2008, Kondratyeva et al., 2017), colorectal (Ballian et al., 2008, Lee et al., 2022), prostate (Jonmarker et al., 2008) and cervical (Chen et al., 2018) cancer.

As with mice, the human orthologue PDX1 is downstream of FGF10. FGF10 has been demonstrated to act upon PDX1 in the regulation of pancreatic fate for human embryonic stem cells, which also co-expressed FOXA2 (Mfopou et al., 2010).

Figure 7.22 demonstrates *Pdx1* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A graded increase of expression is seen from E13.5 to E19.5, in a similar pattern to *Foxa2*.

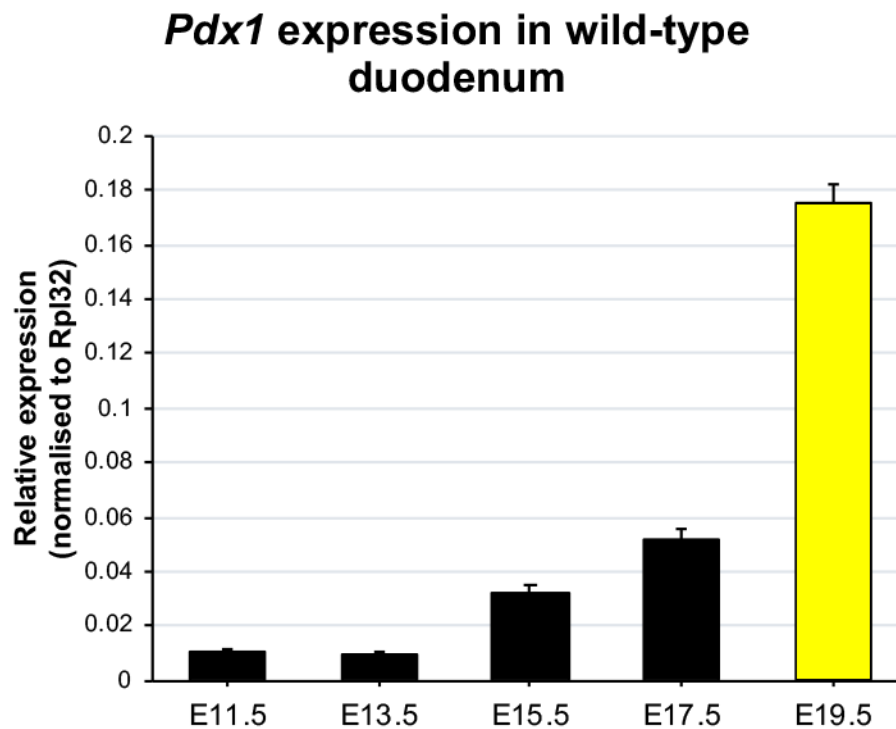


Figure 7.22 *Pdx1* expression undergoes a graded increase within the duodenum of embryos from E13.5 to E19.5. mRNA expression analysis of *Pdx1* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

RT-qPCR has demonstrated (**Figure 7.23**) that *Pdx1* is downregulated in all *Fgf10*^{-/-} duodenum from E11.5 to E17.5, with greatest significance in *Fgf10*^{-/-} E13.5 embryos without DA (LogFC= -2.89, $\pm$ 0.91).

Of note, at E15.5 DA-affected *Fgf10*^{-/-} embryos demonstrate significant less expression than their non-DA-affected counterparts. This difference is more prominent again at E17.5 where *Pdx1* expression and severity of DA phenotype correlate. There is no significant difference seen in *Pdx1* expression at E19.5.

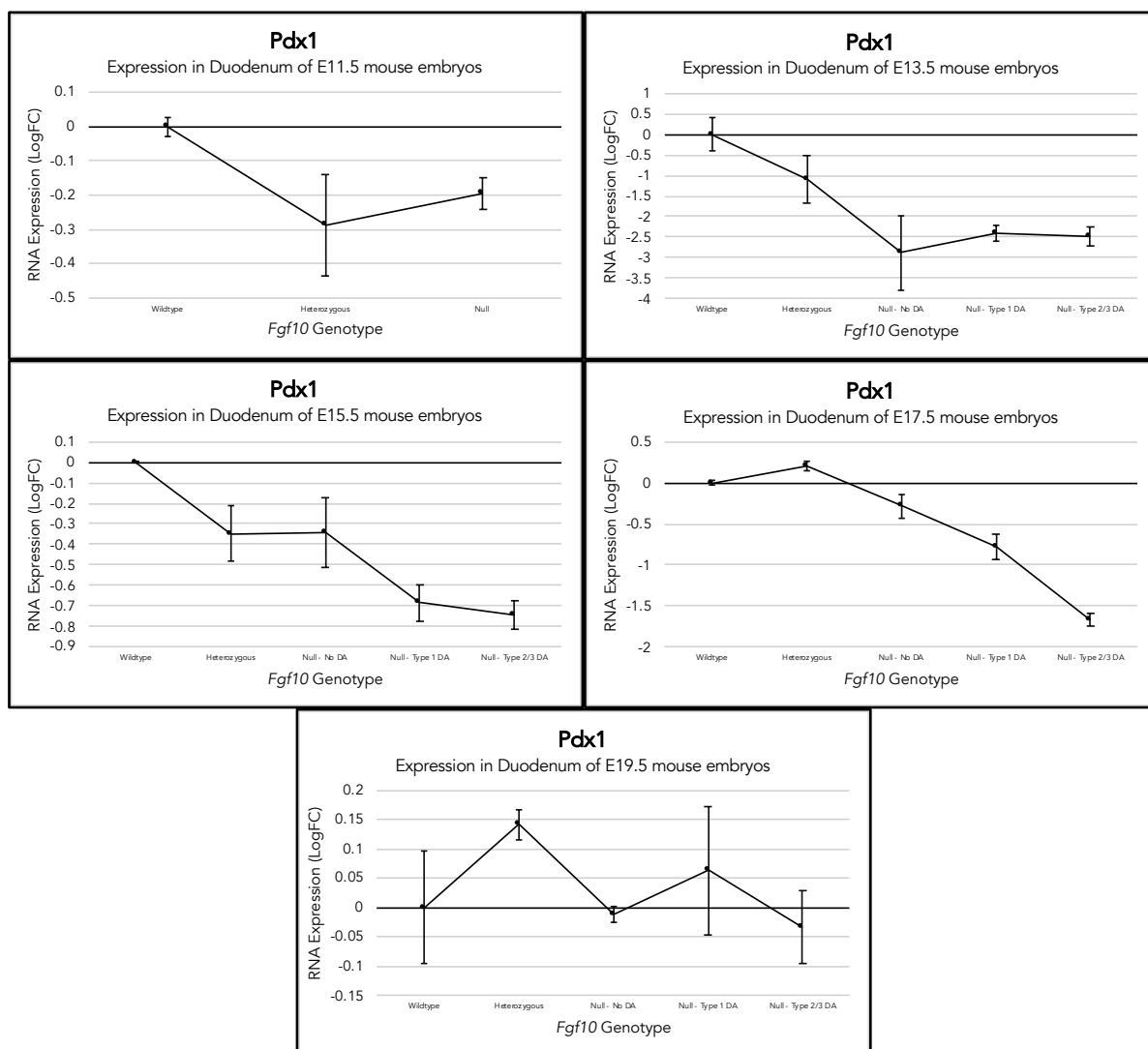


Figure 7.23 Expression of *Pdx1* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos

(with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Pdx1* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2.6. *Rbp4*

Rbp4 is located on chromosome 19, encoding retinol-binding protein 4. This protein mediates the systemic transport of retinol (vitamin A alcohol) from liver stores, which has been shown as necessary for ocular development and visual function (Shen et al., 2016). Its expression induces FGF21 expression within the liver (Steinhoff et al., 2022), increases the risk of non-alcoholic fatty liver disease (Tan et al., 2011), and promotes pancreatic beta-cell dysfunction in type-2 diabetes (Huang et al., 2021b)

In humans, the orthologue RBP4 is located on chromosome 10 and transports retinol similarly. It is associated with obesity and metabolic dysfunctions (Nono Nankam and Blüher, 2021), including gestational diabetes (Wu et al., 2022), insulin resistance (Chauhan et al., 2021), and may be involved in the dyslipidaemia associated with polycystic ovary syndrome (Sopher et al., 2012).

Through its transport of retinol, the precursor to retinoic acid (RA), *Rpb4* potentially has a role upstream of *Fgf10*. It has been proposed that RA acts on TBX genes as a co-factor to activate FGF10 transcription and, as a result, mediates the FGF10-FGF8 positive feedback loop required for outgrowth (Nishimoto et al., 2015). RA-deficient Embryos fail to outgrow due to defective FGF10 signalling, resulting in a lack of FGF-target genes in the epithelium.

Figure 7.24 demonstrates *Rpb4* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A significant increase in expression is seen at E13.5, with reduced expression at E15.5 and E17.5 compared to other ages.

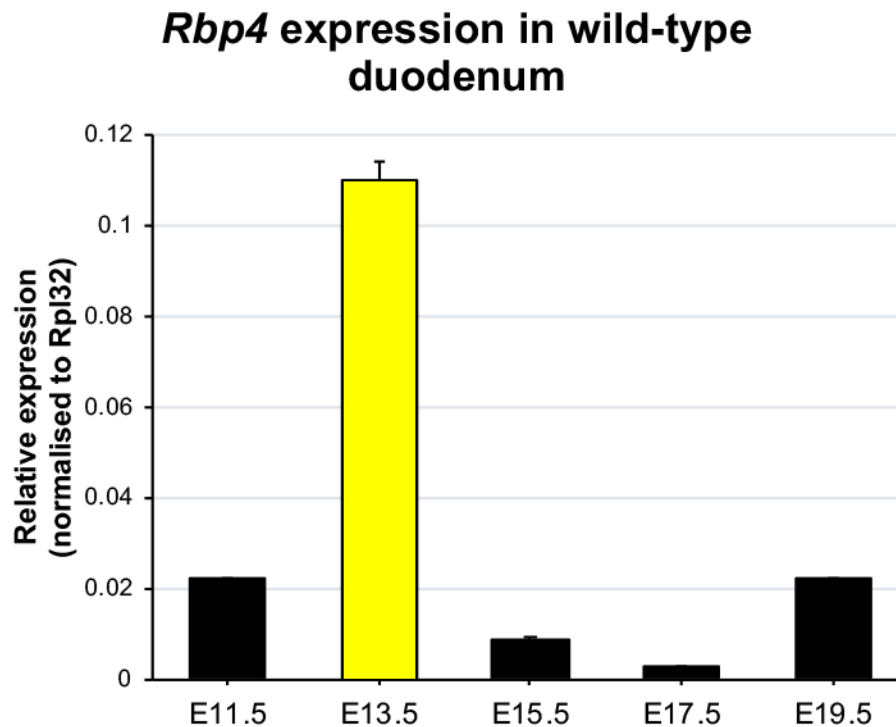


Figure 7.24 *Rbp4* expression is increased within the duodenum of embryos at E13.5.

*mRNA expression analysis of *Rbp4* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

RT-qPCR has demonstrated (**Figure 7.25**) that *Rbp4* is downregulated in all *Fgf10*^{-/-} duodenum from E11.5 to E15.5, with greatest significance in type 2/3 DA-affected E13.5 embryos (LogFC= -3.09, $\pm$ 0.20).

This is inversed at E17.5 and E19.5, where *Fgf10*^{-/-} embryos demonstrate upregulation of *Rbp4*.

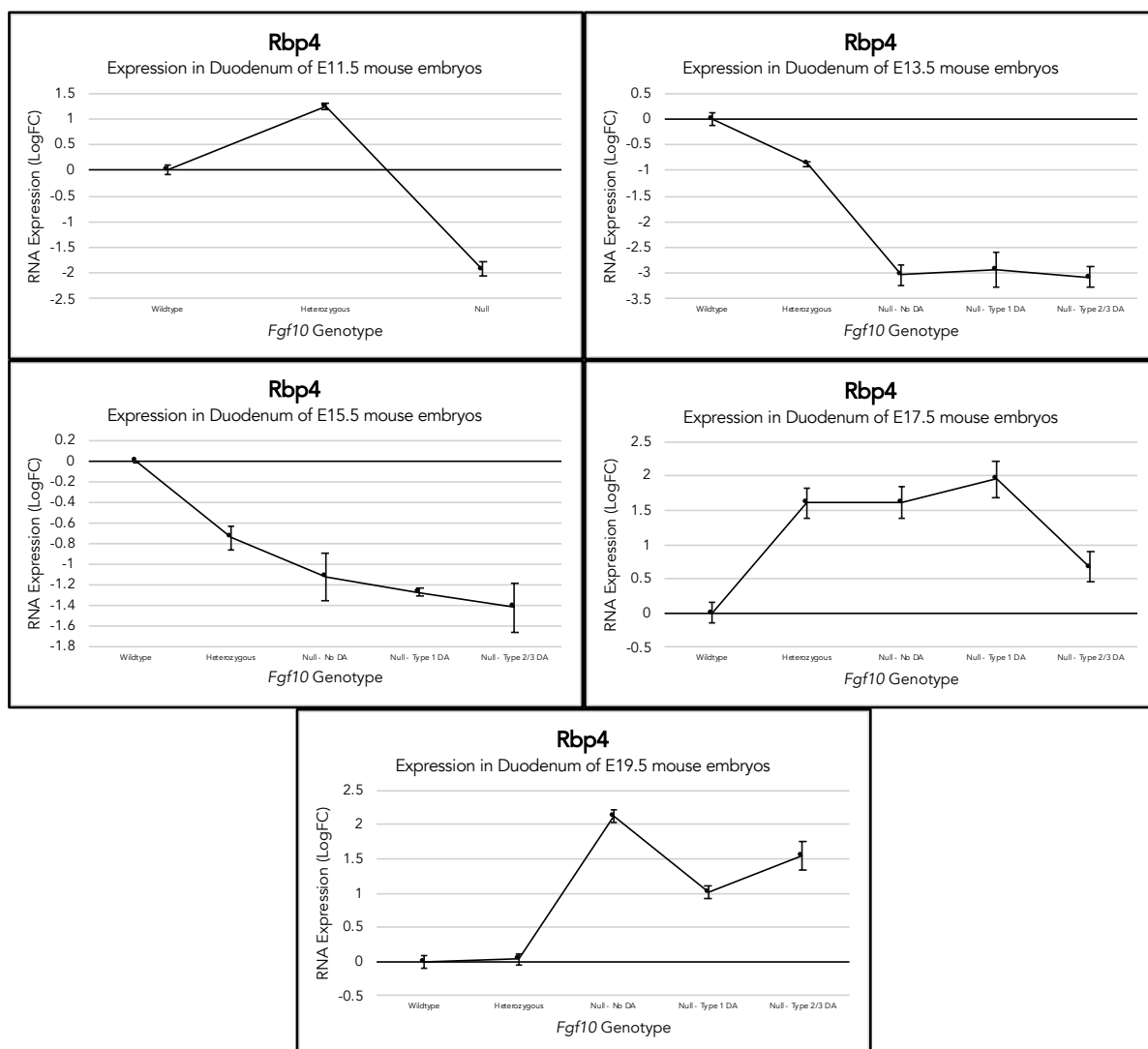


Figure 7.25 Expression of *Rbp4* in the duodenum of *Fgf10*^{+/+} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Rbp4* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.2.7. *Sfrp5*

Sfrp5 is located on mouse chromosome 19, encoding secreted frizzled-related sequence protein 5. This protein is a modulator of Wnt signalling through direct interaction enabling Wnt-protein binding activity (Mori et al., 2012). It is involved with identifying cardiac progenitors during myocardial development (Fujii et al., 2017) and has anti-inflammatory effects on injured cardiac tissue (Nakamura et al., 2016). It has been shown to modulate metabolic dysfunction in obesity (Ouchi et al., 2010).

The human orthologue, SFRP5, is located on chromosome 10 and its role with Wnt-protein signalling is thought to be similar. SFRP5 protein may be involved in determining the polarity of photoreceptor cells in the retina (Chang et al., 1999). Along with pigmented retina, SFRP5 is expressed in the pancreas (Bu et al., 2008), where an aberrant expression may be involved in pancreatic carcinogenesis.

Figure 7.26 demonstrates *Sfrp5* expression in the duodenum of wild-type embryos from E11.5 to E19.5. Significantly increased expression is seen at E13.5 and E17.5 compared to other embryonic ages.

RT-qPCR has demonstrated (**Figure 7.27**) that *Sfrp5* is downregulated in all *Fgf10*^{-/-} duodenum from E11.5 to E15.5, with greatest significance in type 1 DA-affected E13.5 embryos (LogFC= -3.23, +/-0.26).

Downregulation of *Sfrp5* persists, albeit to a lesser extent, in DA-affected *Fgf10*^{-/-} embryos at E17.5. Then only in type 2/3 affected embryos at E19.5.

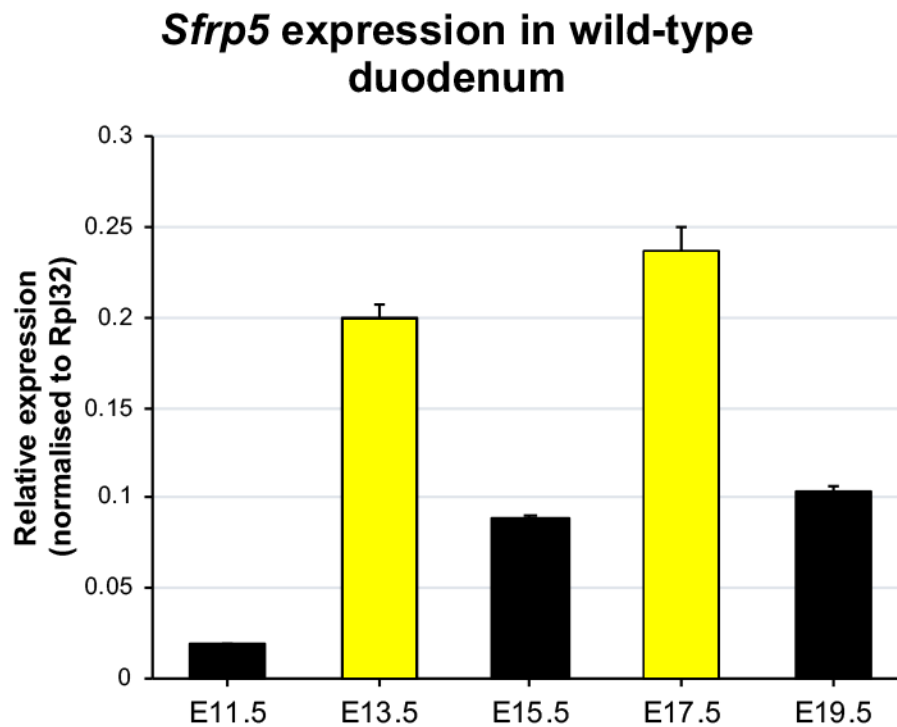


Figure 7.26 *Sfrp5* expression is increased within the duodenum of embryos at E13.5 and E17.5. mRNA expression analysis of *Sfrp5* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

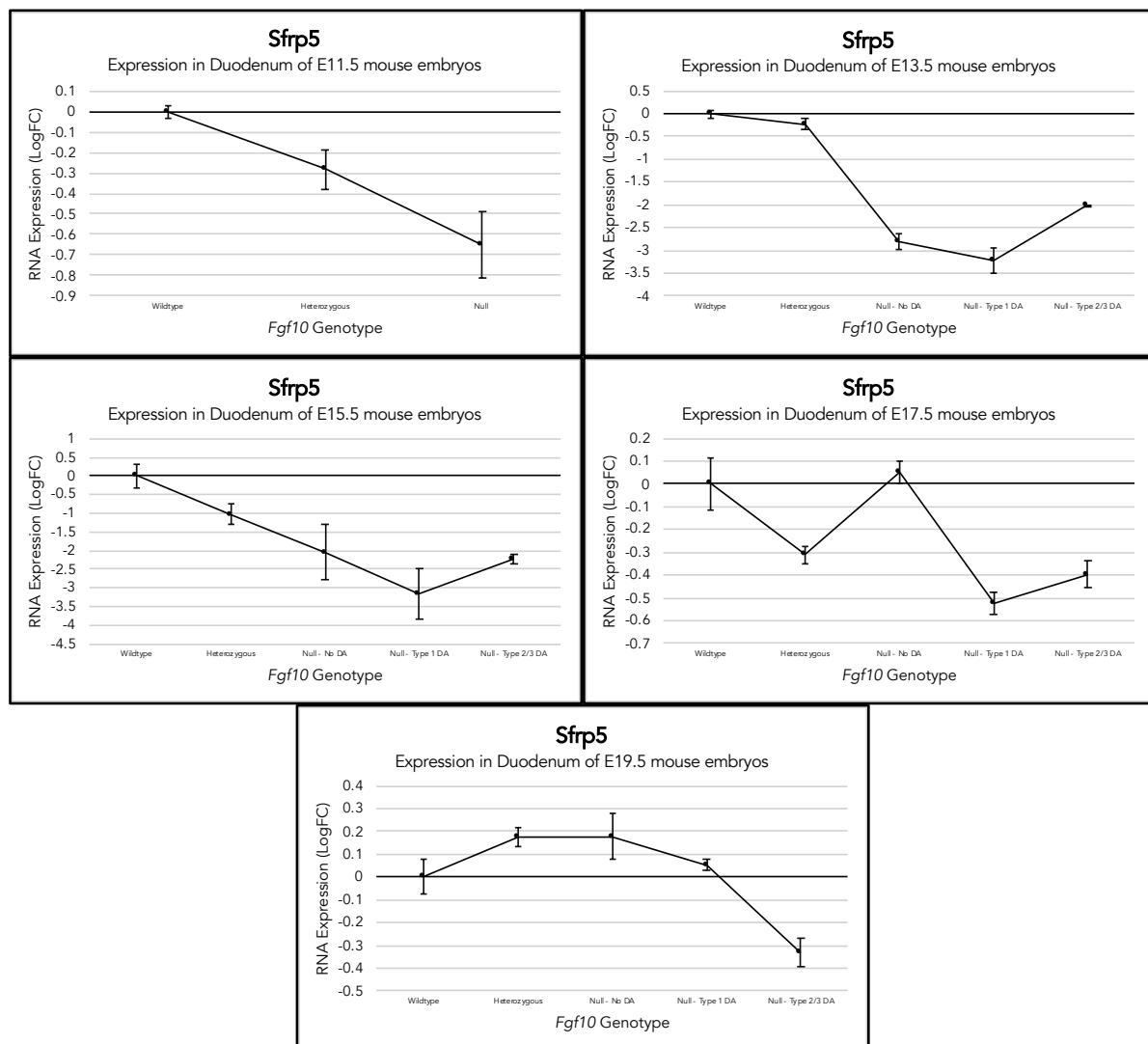


Figure 7.27 Expression of *Sfrp5* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Sfrp5* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.3. Expression pattern of key upregulated genes in DA-affected duodenum

7.2.3.1. *Abat*

In mice, *Abat* is a protein-coding gene located on chromosome 16. It encodes the protein 4-aminobutyrate aminotransferase. This protein catalyses the conversion of gamma-aminobutyrate (GABA, a predominantly inhibitory neurotransmitter in the central nervous system) and L- beta-aminoisobutyrate (BAIB) to succinate semialdehyde (SSA) and methylmalonate semialdehyde, respectively (Carninci et al., 2005). It can also convert delta-aminovalerate and beta-alanine.

ABAT is also located on chromosome 16 and performs the same role in humans. Deficiency of the encoded protein is associated with psychomotor retardation, hypotonia, hyperreflexia, lethargy, refractory seizures, and EEG abnormalities, with genetic variances associated with Alzheimer's Disease (Zheng et al., 2021a). GABA is located throughout the gastrointestinal tract (in both the enteric nervous system and endocrine-like cells) and has a significant role in motility and sensation of painful stimuli (Hyland and Cryan, 2010).

Figure 7.28 demonstrates *Abat* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A graded increase is seen from E11.5 to E17.5 before falling at E19.5.

RT-qPCR has demonstrated (**Figure 7.29**) that *Abat* is upregulated in DA-affected *Fgf10*^{-/-} duodenum at 13.5, and in all *Fgf10*^{-/-} duodenum at E15.5.

At E17.5 *Abat* is relatively downregulated in type 2/3 DA-affected duodenum, while remaining upregulated in type 1 DA. And at E19.5 only *Fgf10*^{-/-} with type 1 DA, or without DA demonstrate upregulation of *Abat*.

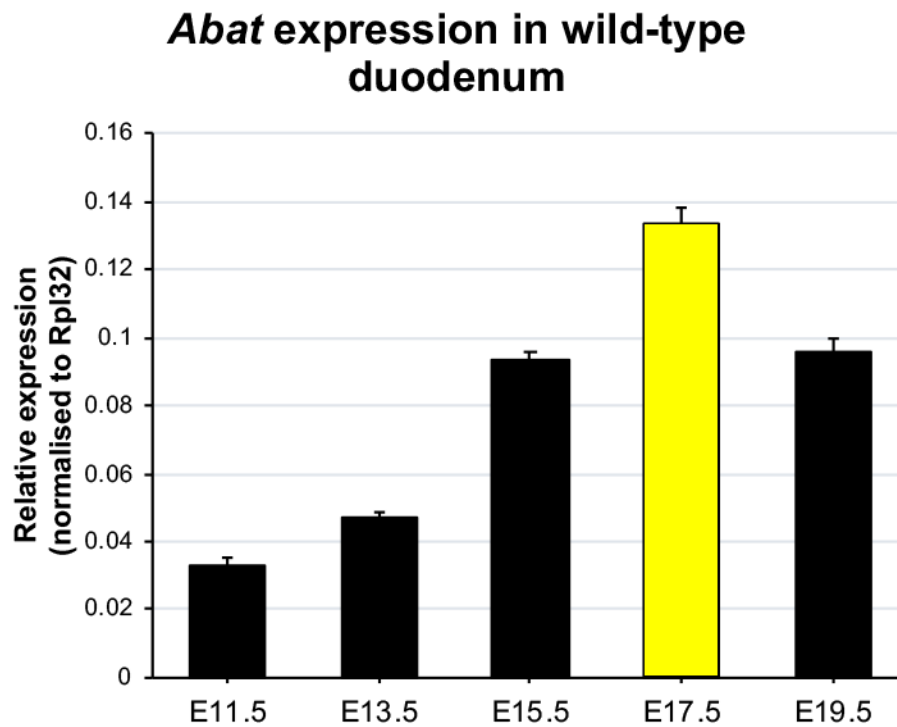


Figure 7.28 *Abat* expression undergoes a graded increase within the duodenum from E11.5 to E17.5. mRNA expression analysis of *Abat* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

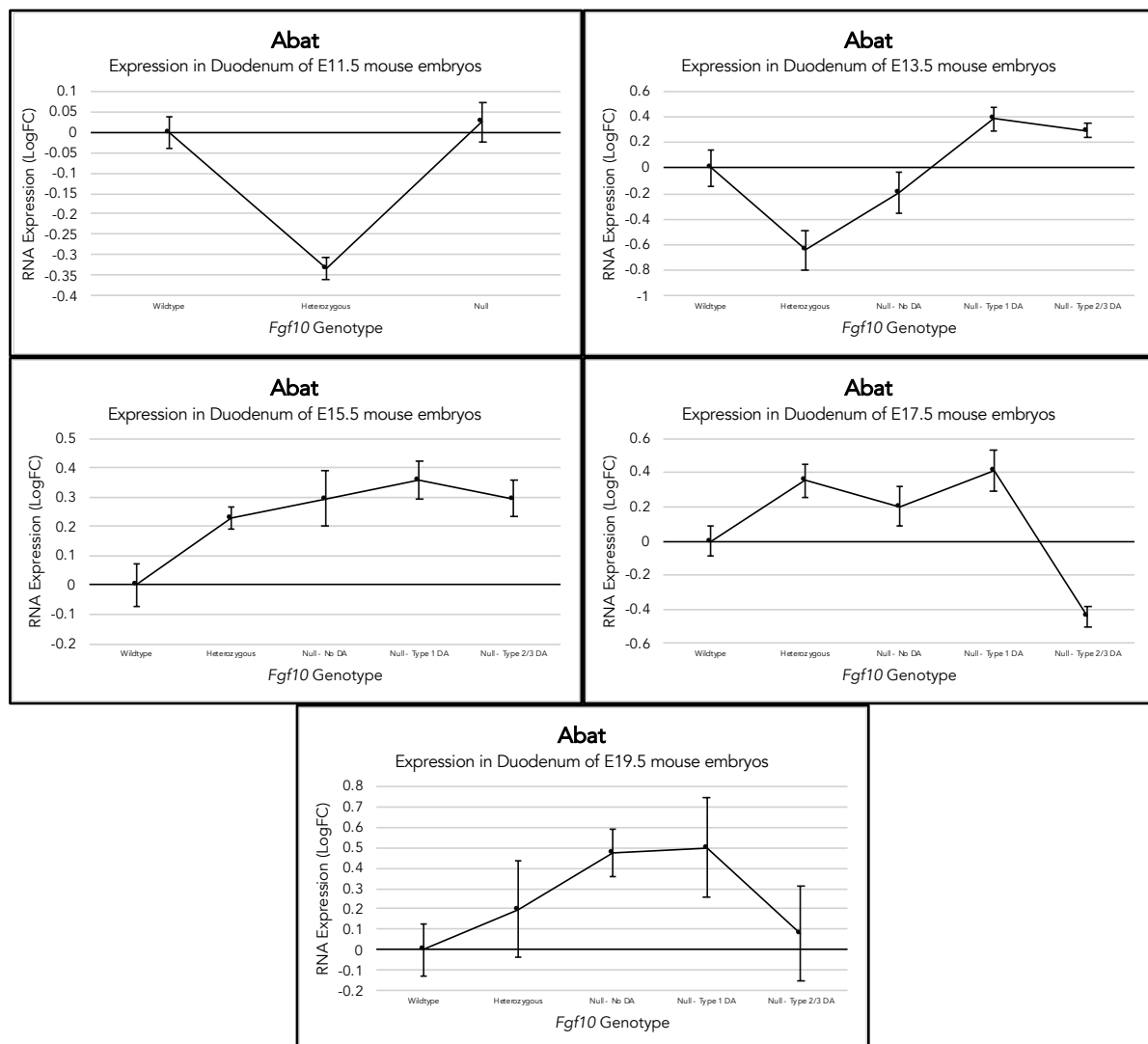


Figure 7.29 Expression of *Abat* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Abat* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.3.2. *Cubn*

In mice, *Cubn* is a protein-coding gene located on chromosome 2. It encodes the formation of the Cubilin protein, an intrinsic factor-cobalamin receptor. Cubilin is predicted to enable cargo receptor, haemoglobin binding, and protein homodimerization activity (Aseem et al., 2013). It has been shown to have a role in lipoprotein, receptor-mediated, and bacterial endocytosis (Hammad et al., 2000). *Cubn* is expressed in the embryonic endoderm, midgut, testes and renal proximal tubule (Drake et al., 2004, Oh et al., 2016)

Cubn is orthologous to the human gene CUBN, located on chromosome 10. Cubilin (a large endocytic protein) is the receptor for intrinsic factor-vitamin B12 complexes. It is primarily responsible for vitamin B12 uptake in the gut, and its mutation has been associated with recessive megaloblastic anaemia (Kristiansen et al., 2000). It is also responsible for the reabsorption of albumin and maturation of vitamin D in the kidney (Gan et al., 2022), and nutrient delivery during embryonic development (Kozyraki et al., 2022). It is associated with focal segmental glomerulosclerosis (Yang et al., 2022), colorectal cancer (Wu and Xu, 2020), type-2 diabetes (Tsekmekidou et al., 2020) and renal cell carcinoma (Gremel et al., 2017).

Figure 7.30 demonstrates *Cubn* expression in the duodenum of wild-type embryos from E11.5 to E19.5. A significant increase in expression is seen at E17.5 and E19.5 compared to other gestational ages.

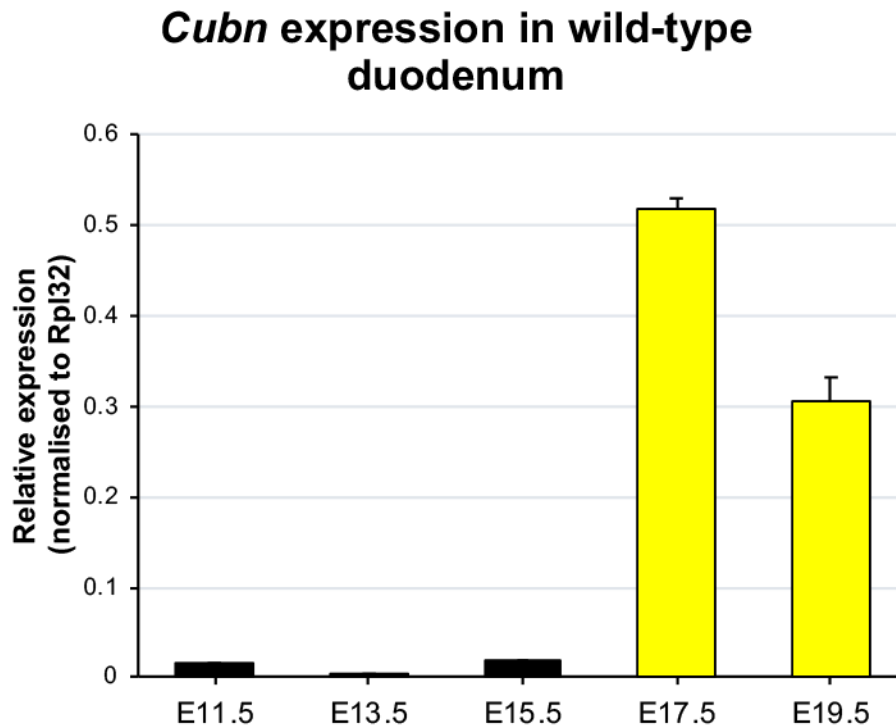


Figure 7.30 *Cubn* expression is increased within the duodenum at E17.5 and E19.5.

*mRNA expression analysis of *Cubn* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

RT-qPCR has demonstrated (**Figure 7.31**) that *Cubn* is downregulated in *Fgf10*^{-/-} duodenum at E11.5, before becoming relatively upregulated in type 2/3 DA-affected duodenum at E13.5 (LogFC= 1.78, $\pm$ 0.36).

At E15.5 all *Fgf10*^{-/-} phenotypes demonstrate *Cubn* upregulation. Before downregulation of only type 2/3 DA-affected duodenum at E17.5 (LogFC= -1.24, $\pm$ 0.35).

There is no significant expression difference seen at E19.5.

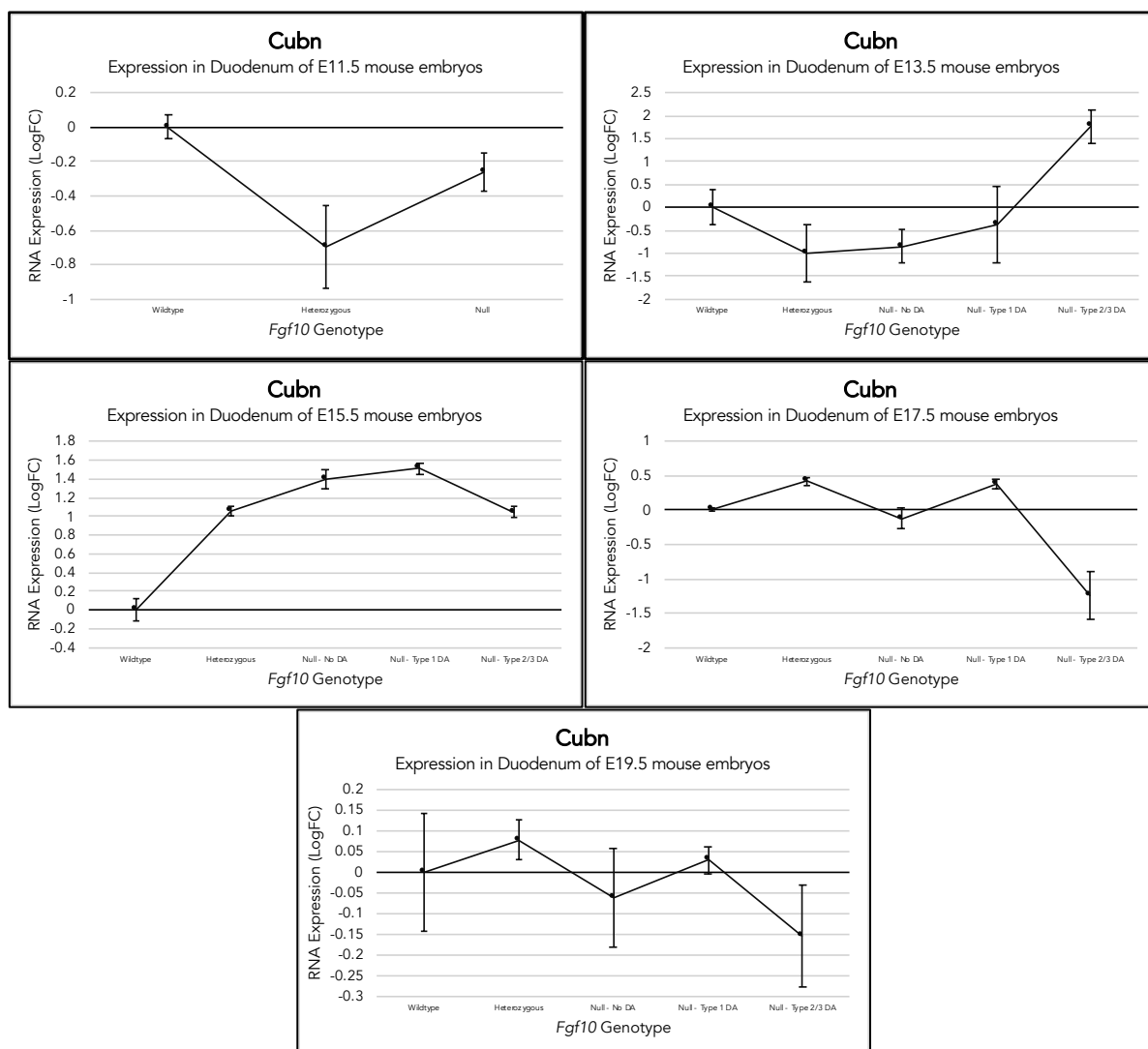


Figure 7.31 Expression of *Cubn* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Cubn* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.3.3. *Nmur2*

In mice, *Nmur2* (located on chromosome 11), encodes the neuromedin-U receptor 2, a receptor for the neuromedin-U and neuromedin-S neuropeptides. Through its binding with neuromedin-U, it holds a role in managing body weight, food intake and adiposity in female mice (Egecioglu et al., 2009). It is regulated by the circadian rhythm (Graham et al., 2005). It plays a significant role in central pain processing (Zeng et al., 2006), excluding neuropathic pain following nerve injury (Gilbert et al., 2013). It has been linked to autoantibody-mediated arthritis (Rao et al., 2012), and osteoblast differentiation and activity (Hsiao et al., 2020).

NMUR2 is located on chromosome 5 in humans, encoding the G-protein coupled, neuromedin U receptor 2. In humans, neuromedin U is widely expressed in the gastrointestinal tract and central nervous system (Fagerberg et al., 2014). Its mutation has not shown the same association with obesity in humans (Bhattacharyya et al., 2004).

Figure 7.32 demonstrates *Nmur2* expression in the duodenum of wild-type embryos from E11.5 to E19.5. Expression is seen to be increased at E11.5 and E19.5 compared to other gestational ages.

RT-qPCR has demonstrated (**Figure 7.31**) that *Nmur2* is upregulated in DA-affected *Fgf10*^{-/-} duodenum at E13.5 and E15.5, most significantly in type 2/3 DA-affected duodenum at E15.5 (LogFC= 2.75, +/-0.03).

At E17.5 all *Fgf10*^{-/-} duodenum demonstrate upregulation of *Nmur2*, albeit to a lesser extent. Then at E19.5 this falls again, with *Fgf10*^{-/-} without, or with type 1, DA remaining slightly upregulated, and type 2/3 DA demonstrating slight downregulation.

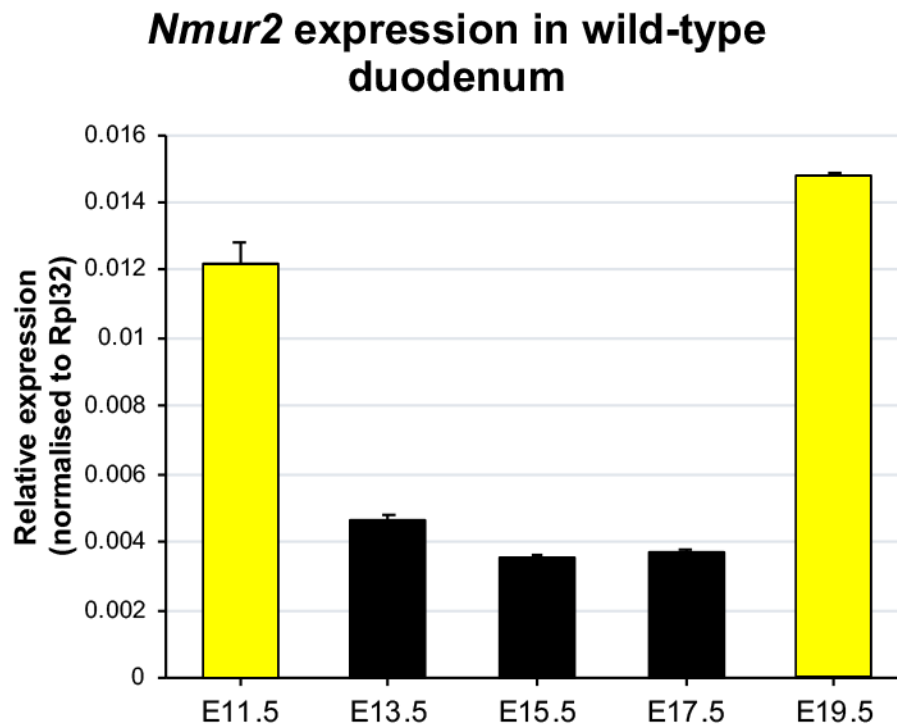


Figure 7.32 *Nmur2* expression is increased within the duodenum at E11.5 and E19.5.

*mRNA expression analysis of *Nmur2* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. n=6 per age, n=2 biological replicate samples, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.*

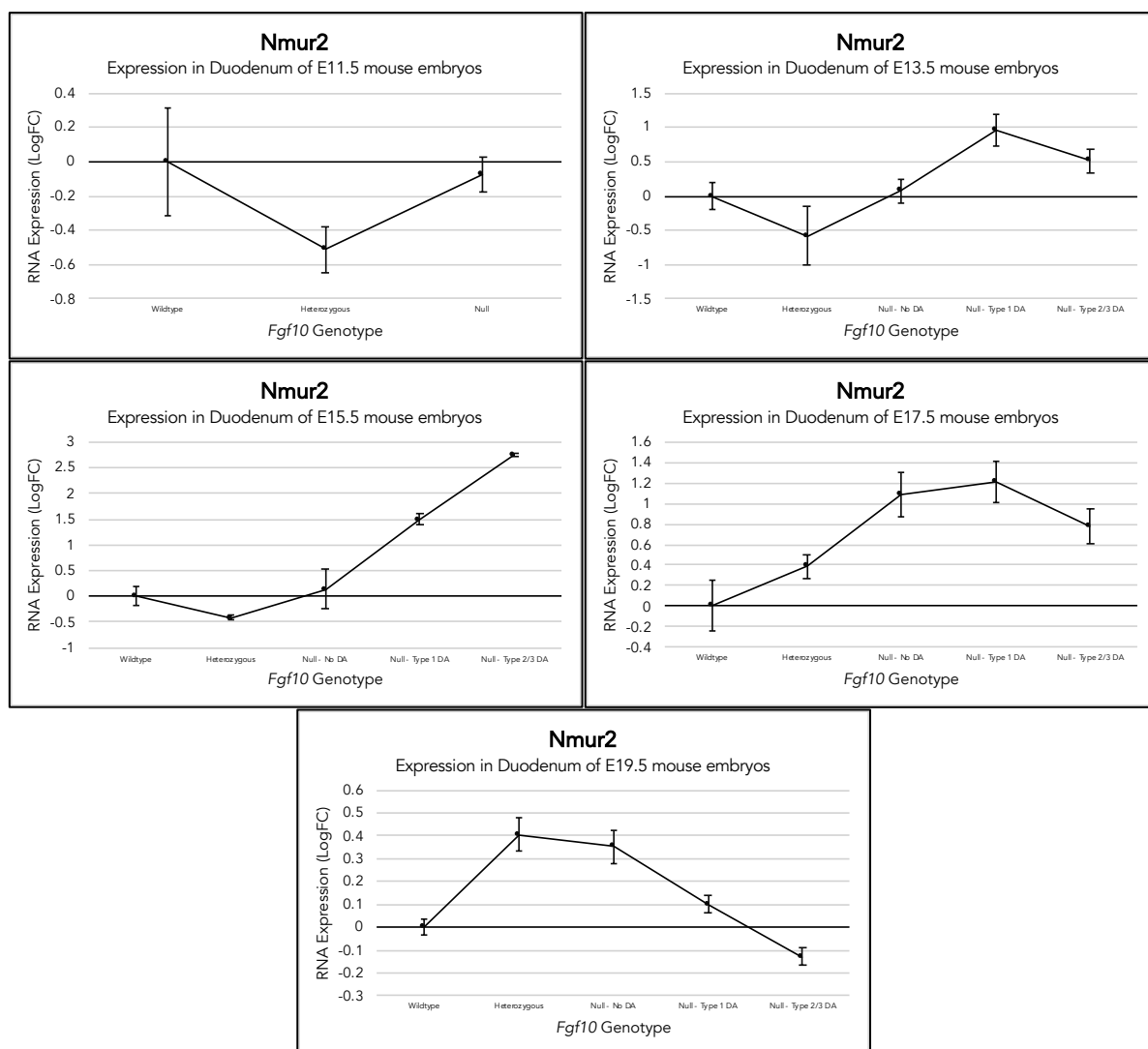


Figure 7.33 Expression of *Nmur2* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Nmur2* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.3.4. *Sgk3*

In mice, *Sgk3* is located on chromosome 8 and encodes serum/glucocorticoid-regulated kinase 3. This protein acts upstream of the extrinsic apoptotic signalling pathway in the absence of ligands, and is involved in the regulation of a wide variety of ion channels and membrane transporters (Loffing et al., 2006). It is thought to hold a significant role in regulating podocyte function, with reduced kidney podocin expression and elevated proteinuria seen in *Sgk3* knockout mice (Peng et al., 2018).

SGK3, in humans, is located on chromosome 8, its homonymous protein is also involved in ion transport, with its mutation causing hypophosphataemic rickets through misregulation of phosphate transport in the kidney (Cebeci et al., 2020). It is associated with oestrogen receptors in breast cancer (Xu et al., 2012), showing a positive association between expression and tumour prognosis. It is also associated with prostate cancer (Nalairndran et al., 2020).

Figure 7.34 demonstrates *Sgk3* expression in the duodenum of wild-type embryos from E11.5 to E19.5. Expression is seen to be increased at E11.5 and E19.5 compared to other gestational ages. This pattern appears similar to that presented in *Nmur2*.

RT-qPCR has demonstrated (**Figure 7.35**) that *Sgk3* is relatively upregulated in DA-affected duodenum at E13.5. This upregulation increases at E15.5, including non-DA-affected duodenum, with maximal relative increase in type 2/3 DA (LogFC= 2.74, +/- 0.06).

This begins to fall at E17.5, with relative downregulation of type 2/3 DA duodenum at E19.5, a pattern which is again similar to *Nmur2*.

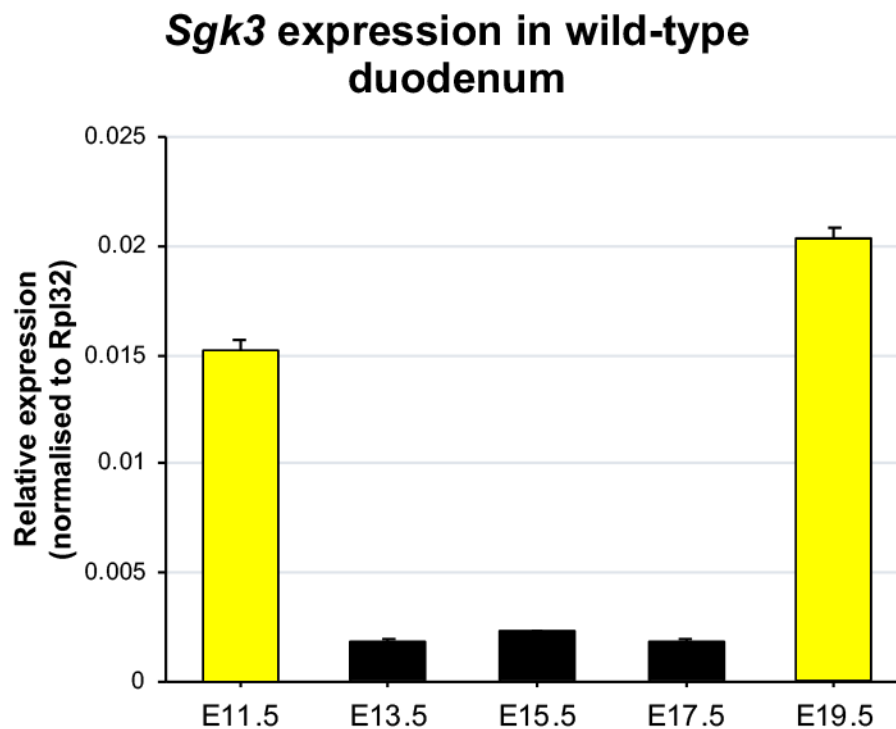


Figure 7.34 *Sgk3* expression is increased within the duodenum at E11.5 and E19.5.

*mRNA expression analysis of *Sgk3* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

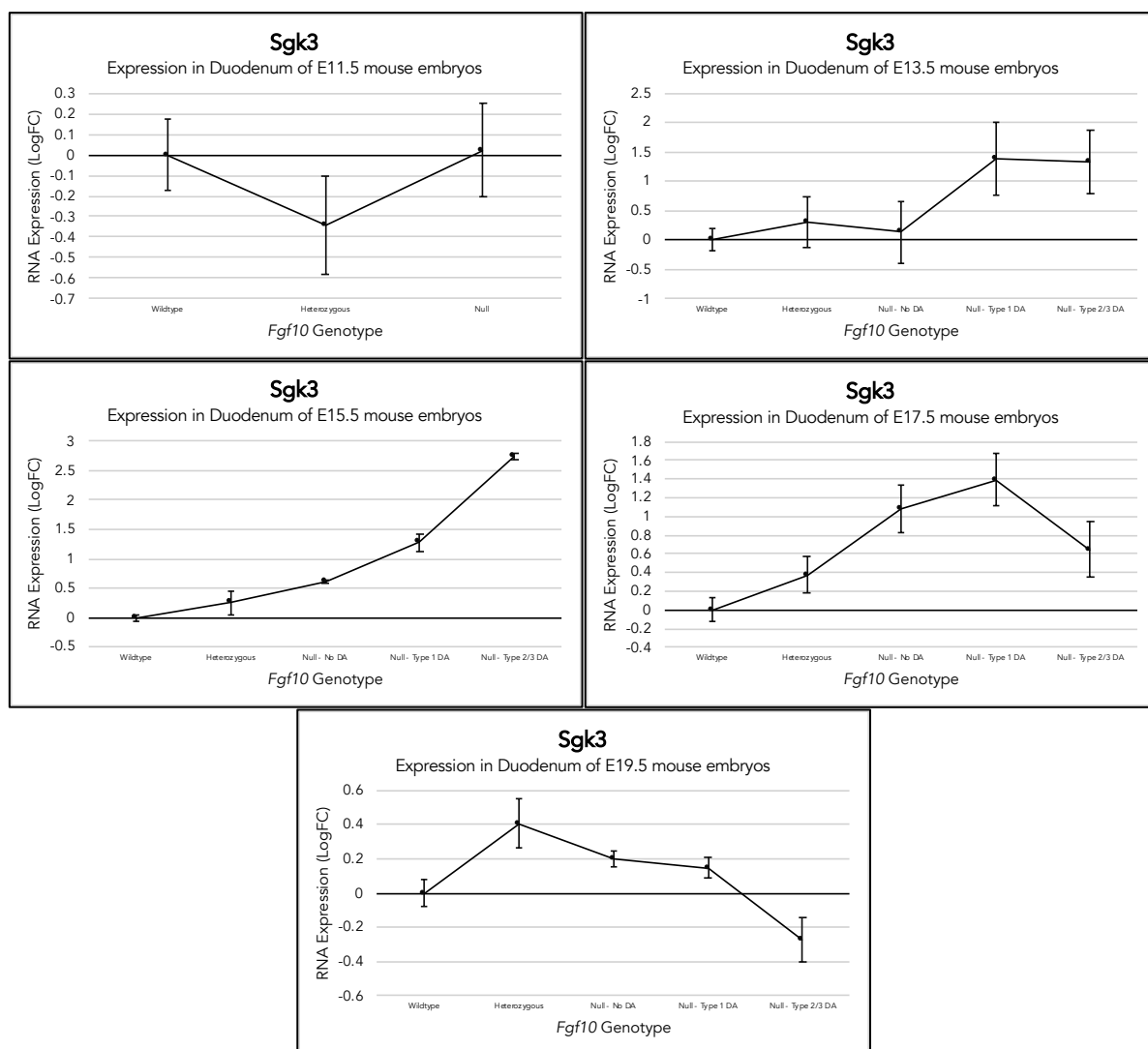


Figure 7.35 Expression of *Sgk3* in the duodenum of *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Sgk3* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.3.5. *Tifa*

Tifa is located on mouse chromosome 3 and encodes the TRAF-interacting protein with forkhead-associated domain. This is an adapter protein which mediates Interleukin-1 receptor-associated kinase 1 (IRAK1) and Tumour necrosis factor receptor (TNFR) associated factor 1 (TRAF6) interaction following IL-1 stimulation (Takatsuna et al., 2003).

TIFA is located on human chromosome 4, with TIFA protein holding a fundamental role in the recognition of, and immune response to, intracellular bacterial growth (Gaudet et al., 2017). TIFA has been shown to have a suppressive role in hepatocellular carcinoma progression through the promotion of apoptosis (Shen et al., 2015). Conversely, it promotes colorectal cancer cell proliferation (Shen et al., 2022), migration and survival of lung adenocarcinoma (Men et al., 2018)

Figure 7.36 demonstrates *Tifa* expression in the duodenum of wild-type embryos from E11.5 to E19.5. Expression is seen to increase in a graded manner from E13.5 to E19.5.

RT-qPCR has demonstrated (**Figure 7.37**) that *Tifa* is upregulated only in type 2/3 DA-affected duodenum at E13.5. This continues to increase in *Fgf10*^{-/-} duodenum at E15.5, with upregulation seen in all phenotypes, but greatest still in in type 2/3 DA-affected (LogFC= 1.62, +/-0.08).

This relative upregulation begins to reduce at E17.5, with type 2/3 DA-affected duodenum expression of *Tifa* downregulated at E19.5 (LogFC= -0.30, +/-0.12).

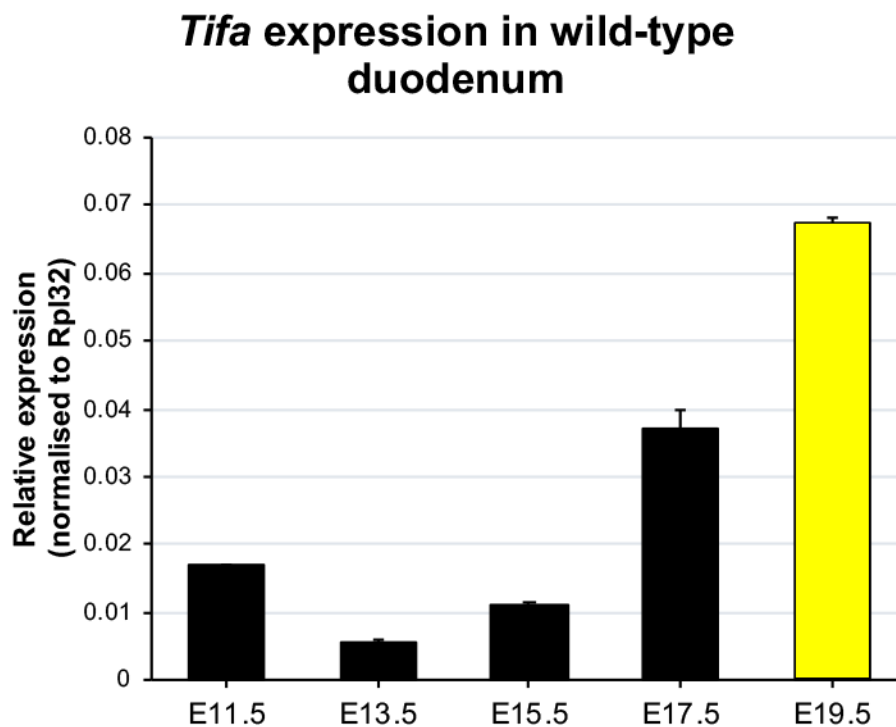


Figure 7.36 *Tifa* expression undergoes a graded increase within the duodenum from E13.5 to E19.5. mRNA expression analysis of *Tifa* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.

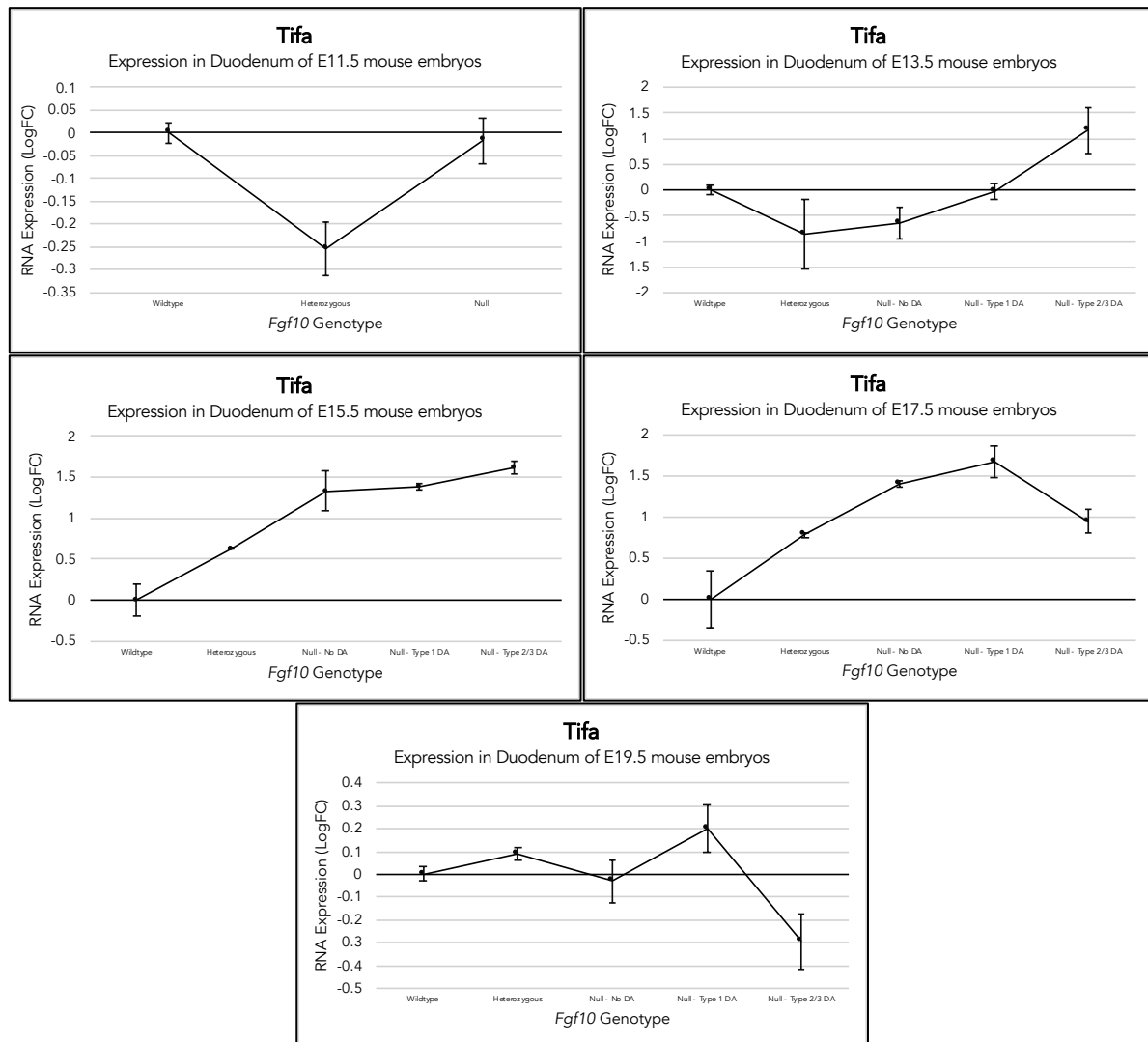


Figure 7.37 Expression of *Tifa* in the duodenum of *Fgf10*^{+/+} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Tifa* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. *n*=6 per group per age, *n*=2 samples per group, *n*=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.4. Expression pattern of key downregulated genes in DA-affected duodenum

7.2.4.1. *Spp1*

Spp1 located on mouse chromosome 5, encodes secreted phosphoprotein 1 (osteopontin). It forms an integral part of bone matrix mineralisation and is involved in osteoclast attachment (Rajachar et al., 2008, Franzén et al., 2008). It is involved with the development of the central nervous system (Jiang et al., 2019), and natural killer T-cells (Diao et al., 2008). It is associated with, but not necessary for, the formation of biliary atresia (Hertel et al., 2011).

A link has been demonstrated between *Spp1* and *Fgf10* in Apert syndrome mouse models, where increased osteopontin was seen in the limbs of *Fgfr2^{+/S252W}* mice (Yang et al., 2008).

The human orthologue, SPP1, is located on chromosome 4. Notably, in humans, osteopontin has been strongly linked to biliary atresia, with its expression correlating to proliferation and fibrosis (Whittington et al., 2005), and with the development of postoperative hepatic dysfunction and portal hypertension (Honsawek et al., 2011). It supports the repair of injured cardiac muscle (Rotem et al., 2022). It is a key regulator of both the adaptive and innate immune response., and is associated with multiple autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, type-1 diabetes, inflammatory bowel disease, Sjögren's, and myasthenia gravis (Xu et al., 2022).

Figure 7.38 demonstrates *Spp1* expression in the duodenum of wild-type embryos from E11.5 to E19.5. Expression is seen to be increased at E13.5 and E19.5 in a similar pattern to *Oxct1*.

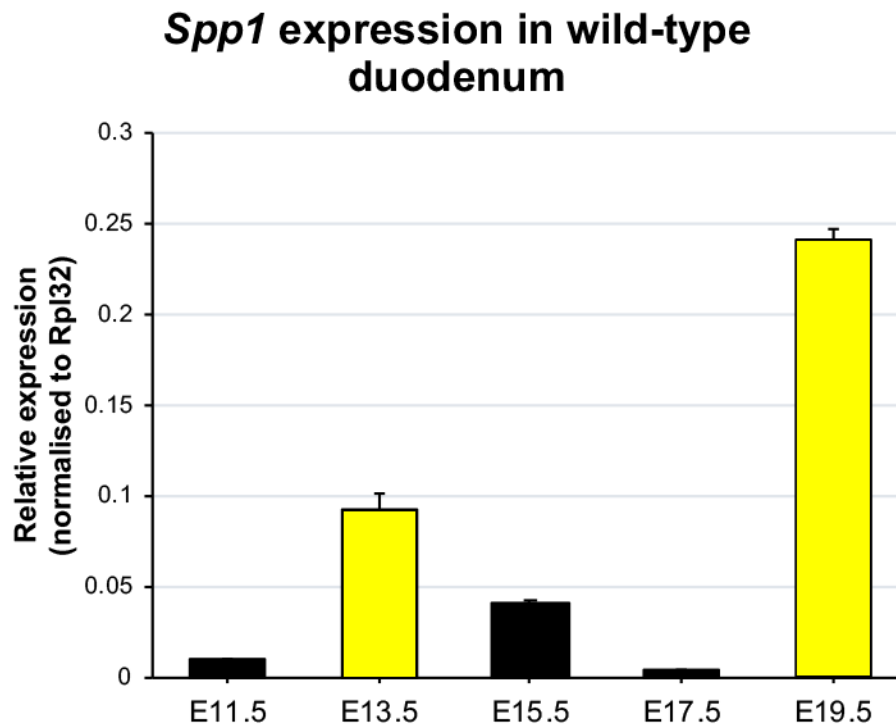


Figure 7.38 *Spp1* expression is increased within the duodenum at E13.5 and E19.5.

*mRNA expression analysis of *Spp1* in the duodenum of E11.5, E13.5, E15.5, E17.5 and E19.5 wild-type embryos, was measured by RT-qPCR. All expression changes were normalised to *Rpl32*. $n=6$ per age, $n=2$ biological replicate samples, $n=3$ technical replicates. Error bars represent the standard error of mean expression $\pm$ 95% confidence.*

RT-qPCR has demonstrated (**Figure 7.39**) that *Spp1* is downregulated in DA-affected duodenum at E13.5. This extends to all *Fgf10*^{-/-} duodenum at E15.5, before upregulation occurs in all phenotypes at E17.5 to E19.5.

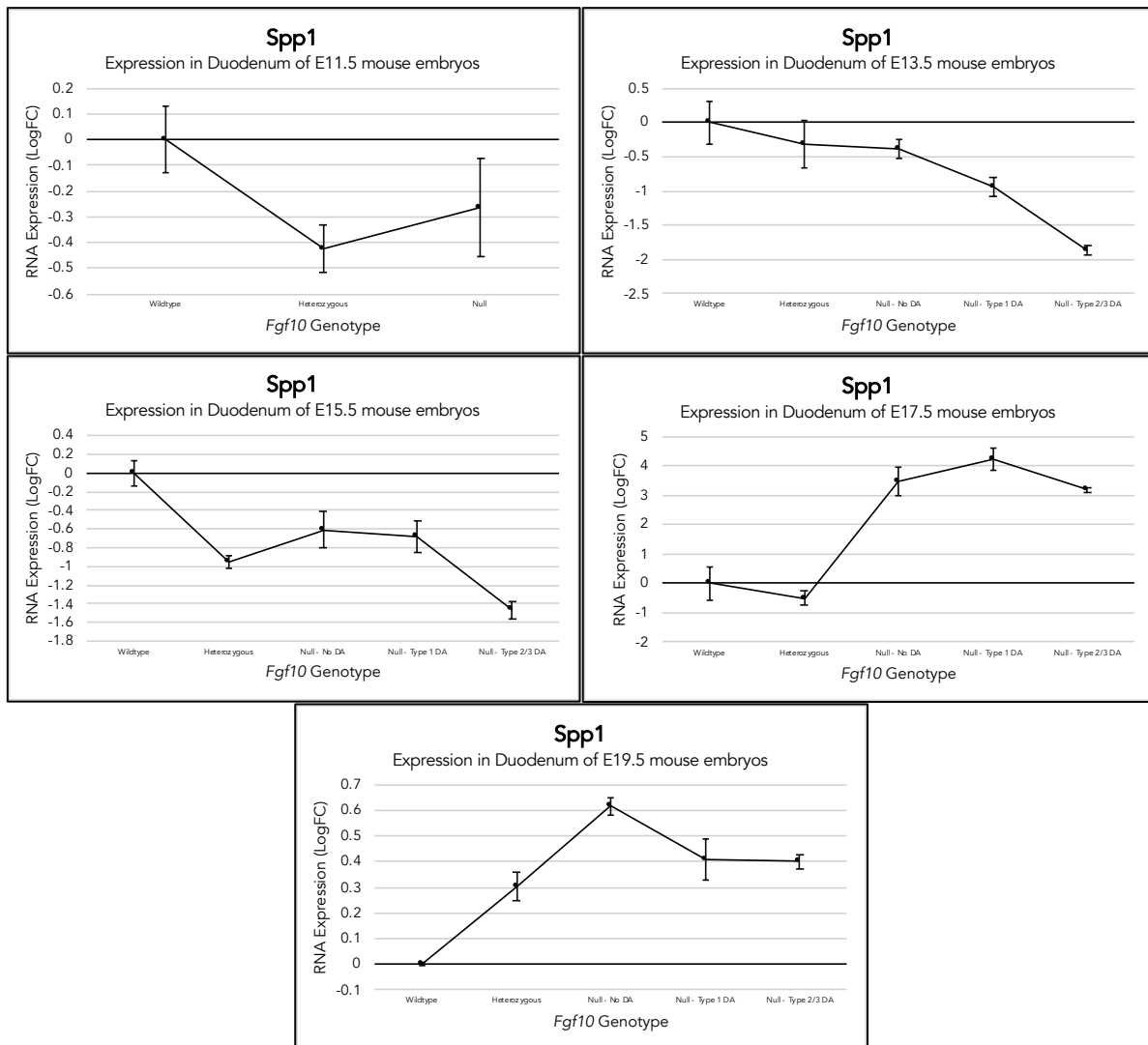


Figure 7.39 Expression of *Tifa* in the duodenum of *Fgf10*^{+/+} and *Fgf10*^{-/-} embryos (with and without DA), measured from E11.5 to E19.5, compared to wild-type. RT-qPCR was performed using primers specific for *Tifa* mRNA. Gene expression was normalised to housekeeping gene *Rpl32*. The mRNA expression of each genotype is represented as Log₂ fold change (LogFC) against the expression of wild-type duodenum. n=6 per group per age, n=2 samples per group, n=3 technical replicates. Error bars represent the standard error of mean expression +/- 95% confidence.

7.2.5. Interaction between key genes

The STRING database (<https://string-db.org/>) was used to assess for known and predicted protein-protein interactions (PPI) between these key genes (**Figure 7.1**) and *Fgf10* (Szkłarczyk *et al.*, 2019). A network was generated (**Figure 7.40**) to demonstrate nodes (i.e. all the proteins produced by a single, protein-coding gene locus) and edges (lines between nodes representing known and predicted PPIs or other associations). Of the 20 nodes formed from the input of key genes, ten edges were identified (PPI enrichment p-value: <0.001) between *Fgf10*, *Pdx1*, *Isl1*, *Gata3*, *Foxa2*, *Barx1*, and *Six2*. In order to validate cross-applicability between the genetics of this mouse model and humans, this process was repeated for the human analogue of each gene/protein, with the same PPIs found.

To delineate where these gene interactions/co-expression was occurring, they were then examined for functional enrichments in gene ontology, mammalian phenotype ontology, and Reactome pathways using STRING analysis (**Figure 7.41**). An FDR p-value <0.05 and functional enrichment score >1.5 were used to determine the significance of enrichments.

Pancreatic development Reactome pathways were identified, matching the earlier findings across the whole *Fgf10*^{-/-} genetic profile (section 6.2.4). Of key interest in the setting of DA was the correlation against mammalian phenotype ontology of abnormal pyloric sphincter morphology and gene ontology of embryonic digestive tract morphogenesis.

Building on these functionally enriched pathways, the list of key genes was simplified to the seven which were demonstrated as linked in **figure 7.40**, then genes associated with the pyloric sphincter morphology (Li *et al.*, 2014) (shown in **figure 7.43**) were added. The 'more' function within STRING was used to expand upon these key interrelated genes to form **figure 7.42**.

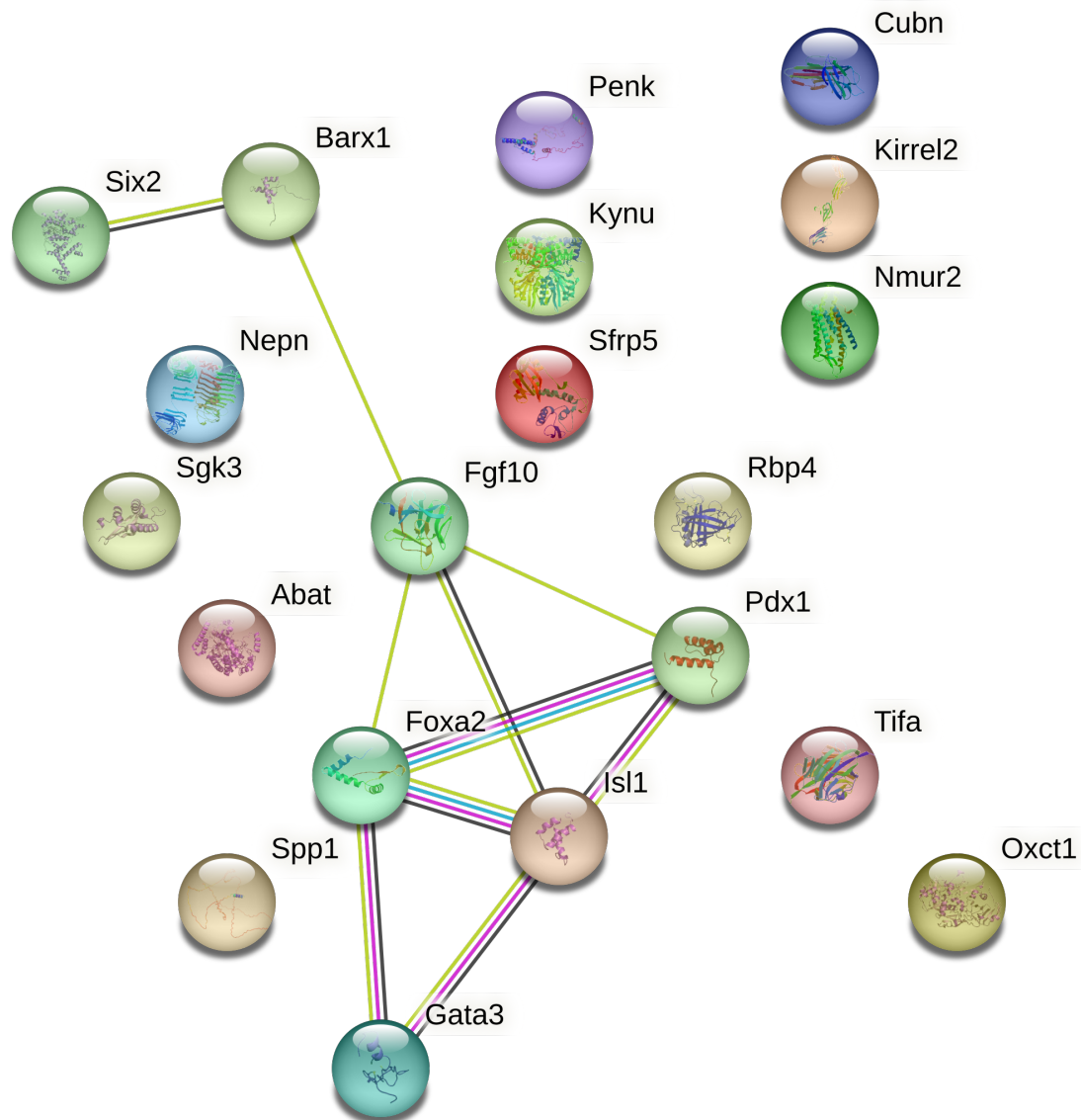


Figure 7.40: Network of known or predicted protein-protein interactions between key genes (mouse). *Known PPIs (both from curated databases, and experimentally determined) and evidence of co-expression existed between Pdx1, Foxa2 and Isl1. Experimentally determined PPIs and evidence of co-expression also existed between Gata3 and both Foxa2 and Isl1. Co-expression existed between Fgf10 and Isl1, as well as Six2 and Barx1. PPI links between Barx1, Fgf10, Pdx1 and Foxa2 PPI were identified through text-mining of PubMed data. Edges represent: known PPI (aqua [database curated] and pink [experimentally determined]); protein co-expression (black); text-mined PPI (green).* (Szklarczyk et al., 2019).

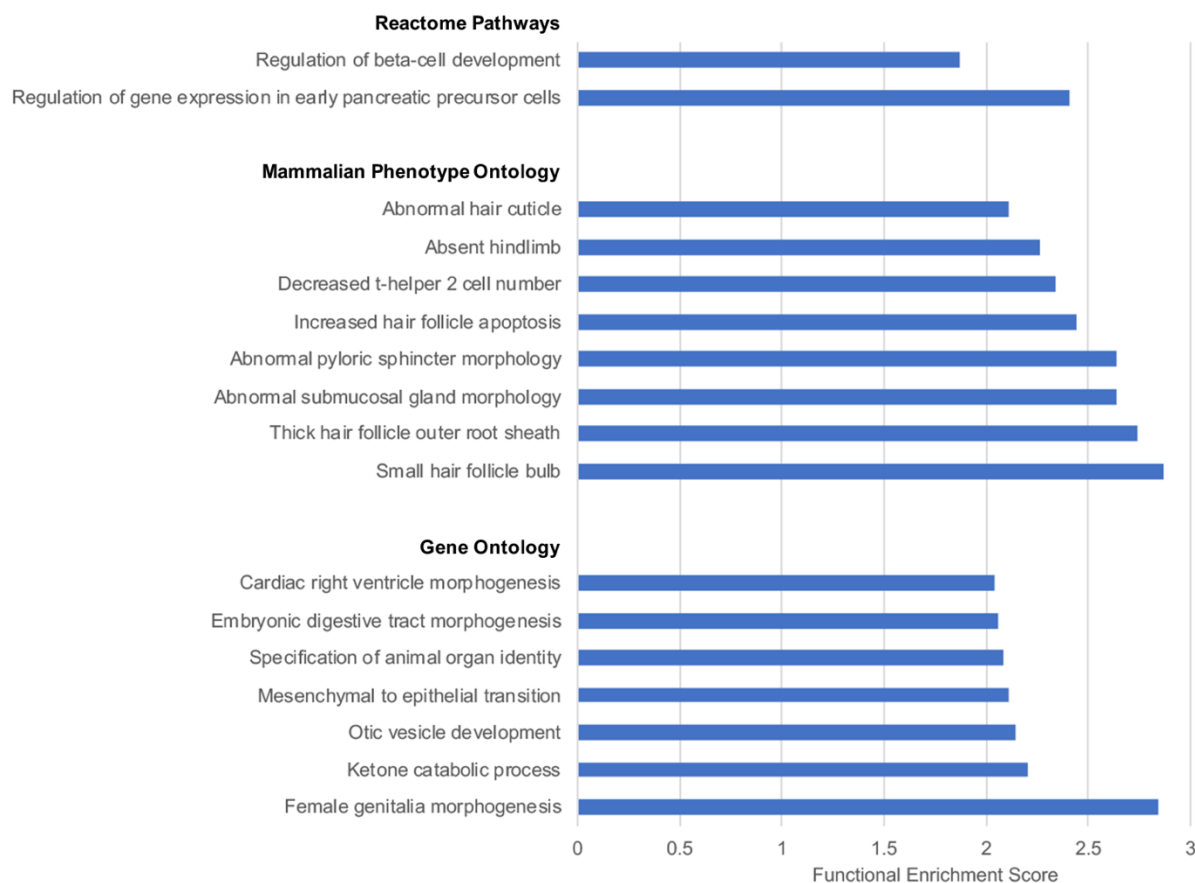


Figure 7.41: Functional enrichment scores of key genes and their associated Reactome Pathways, Mammalian Phenotype Ontology and Gene Ontology. *STRING* analysis was used to delineate the key gene functional enrichments in gene ontology, mammalian phenotype ontology, and Reactome pathways using. An FDR p -value <0.05 and functional enrichment score >1.5 were used to determine the significance of enrichments. Pancreatic development Reactome pathways matching the earlier findings, however phenotype ontology of abnormal pyloric sphincter morphology was of particular interest given the proximity to DA. (Szklarczyk et al., 2019).

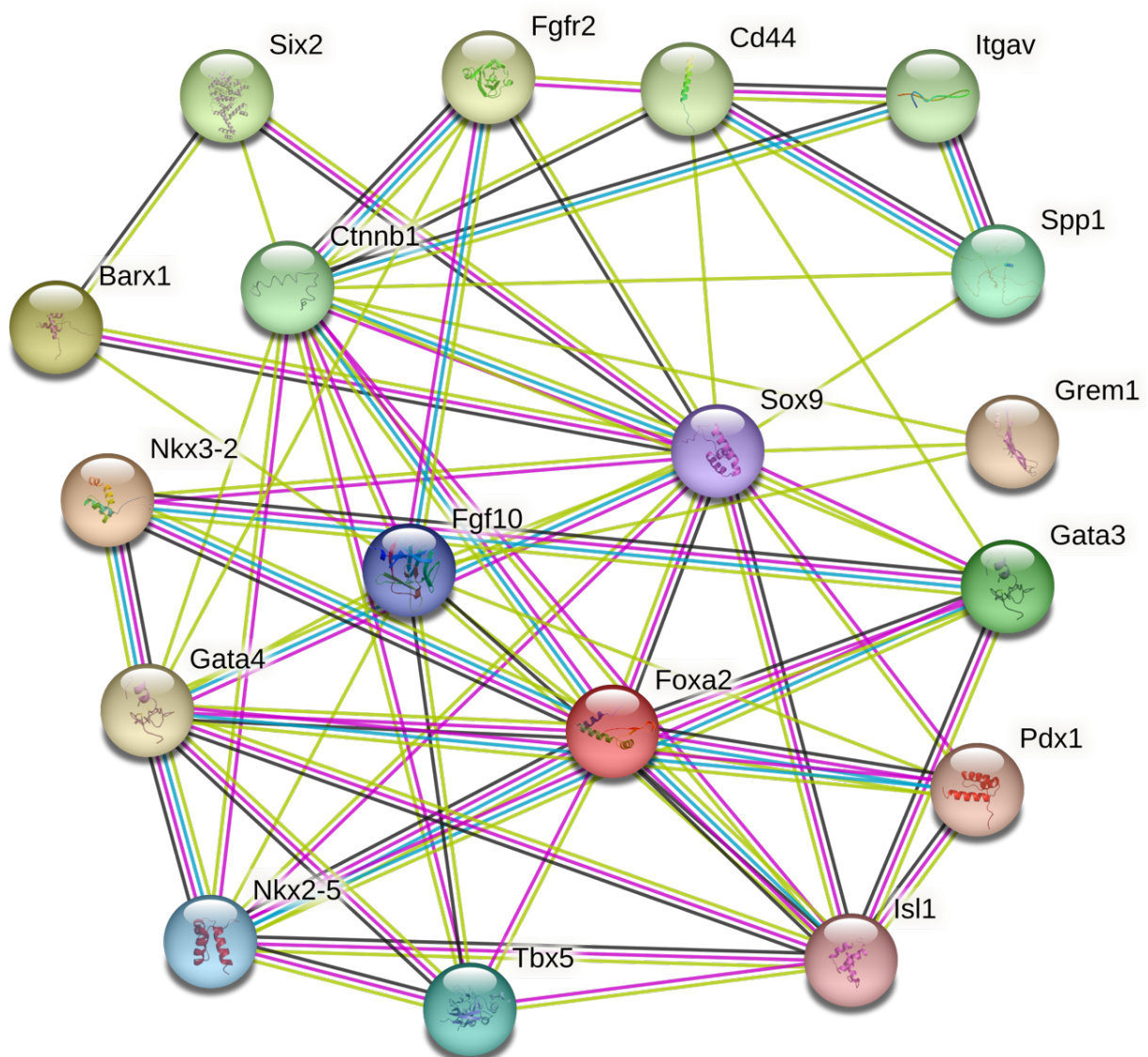


Figure 7.42. Network of known or predicted protein-protein interactions between key genes and those involved in pyloric sphincter specification. *Known PPIs (both from curated databases, and experimentally determined) and evidence of co-expression existed between multiple key genes, and those involved in Sox9 driven pyloric development. Edges represent: known PPI (aqua [database curated] and pink [experimentally determined]); protein co-expression (black); text-mined PPI (green). (Szkłarczyk et al., 2019).*

7.3. Discussion

These results demonstrate the action and interaction of key genes associated with *Fgf10*^{-/-} and DA throughout embryogenesis. Patterns between genes in normal expression and expression changes associated with DA and *Fgf10*^{-/-} can be seen.

In wild-type duodenum, a regular expression pattern demonstrating an initial rise at E13.5, followed by a more significant increase towards E19.5, was seen in *Barx1*, *Oxct1*, *Kirrel2* and *Spp1*. While there is no current evidence of interaction or co-expression between these genes or their proteins, *Oxct1* and *Kirrel2* hold key roles in regulating normal metabolism. A pattern of an initial fall and then rise was seen in *Gata3*, *Isl1*, *Kynu*, *Nmur2* and *Sgk3*. *Pdx1*, *Foxa2* and *Tifa* all demonstrate a similar rise in expression through embryogenesis from E13.5, with *Foxa2* and *Pdx1* associated (downstream of *Fgf10*) in pancreatic development.

Fgf10^{-/-} duodenum demonstrated a range of patterns in these key genes across gestational ages, with only *Pdx1* and *Foxa2* showing persistent downregulation (albeit at *Pdx1* E19.5, where no significant difference existed). Multiple genes (*Barx1*, *Isl1*, *Six2*, *Oxct1*, *Penk*, *Cubn*, *Nmur2*) shown initial relative upregulation in *Fgf10*^{-/-} duodenum, before demonstrating normal or downregulation at E19.5. *Kirrel2*, *Kynu* and *Nepn* all demonstrated initial downregulation in *Fgf10*^{-/-} before a period of upregulation and then returning to downregulation.

In DA-affected duodenum, a similar pattern emerged between *Barx1*, *Gata3*, *Isl1*, *Penk* and *Six2*. *Fgf10*^{-/-} with DA were seen to lead their DA-free counterparts in graded upregulation to E15.5, then downregulation at E19.5. Importantly, STRING analysis performed has demonstrated that *Barx1*, *Gata3*, *Isl1* and *Six2* are all associated through *Fgf10* (**Figure 7.40**). This correlation suggests these genes are of key interest, and their role in DA should be further elaborated. *Sfrp5* and *Barx1* are also linked through their role in Wnt signalling, which *Barx1* suppresses and *Sfrp5* enables the protein binding of.

Furthermore, the downregulation of *Pdx1* and *Sfrp5* in the DA-affected duodenum, and the upregulation of *Isl1*, *Gata3* and *Barx1*, known to be primarily expressed in the mesenchyme of more anterior regions (pylorus and stomach), suggests a disruption of normal gastro-duodenal regionalisation. *Gata3* and *Isl1* are known to be linked in their co-expression and association with failed pylorus muscle to development (Li et al., 2014). In addition, *Barx1* and *Six2* are both highly associated with these genes (**Figure 7.42**) including in *Sox9* mediated pyloric development (**Figure 7.43**). The upregulation of these pyloric smooth muscle development genes further supports mis-patterning of the duodenum in *Fgf10*^{-/-} embryos to be anteriorised and more gastric/pyloric. It is important to note, however, that while RNA-seq demonstrated that *Sox9* was downregulated, and *Grem1* and *Nkx3-2* were upregulated in all *Fgf10*^{-/-} duodenum, they failed to achieve statistical significance in the type 2/3 DA group. Therefore, these genes were not verified through RT-qPCR or investigated further within this chapter.

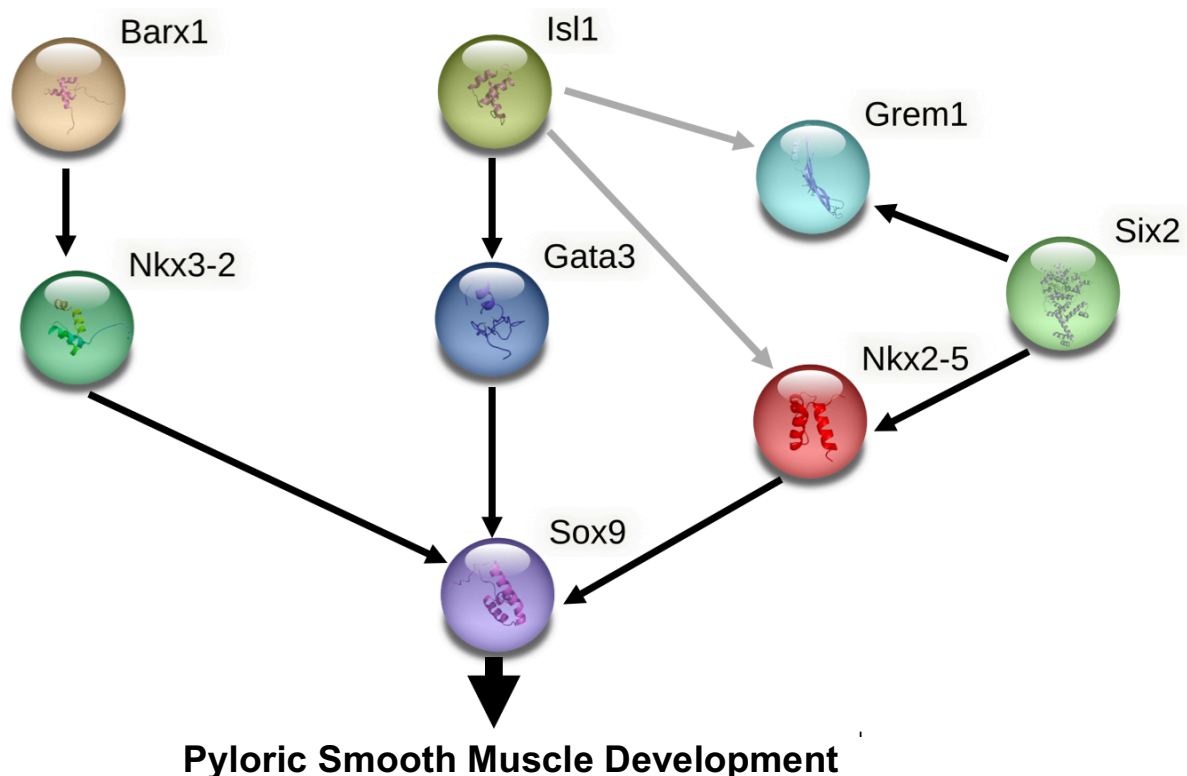


Figure 7.43 *Barx1*, *Isl1*, *Six2* and *Gata3* function in the *Sox9* driven model of pyloric muscle development. Currently hypothesised pathways (Li et al., 2014) involved in normal mouse pyloric development, demonstrate multiple key genes highlighted in this chapter (*Barx1*, *Isl1*, *Gata3*, *Six2*) acting through *Sox9*. Black lines indicate upregulation, grey lines indicate downregulation. Image generated through STRING (Szklarczyk et al., 2019).

Fgf10 has been shown to act upon *Sox9* in the pancreas in order to promote proliferation and pancreatic fate maintenance in progenitor cells (Ndlovu et al., 2018). It is therefore likely that *Fgf10* is also signalling through *Sox9* in the stomach and duodenum, which would explain the relative under-expression of *Sox9* in *Fgf10*^{-/-} mice (even in the setting of increased expression). This raises the hypothesis that disruption of the lumen in DA could result from anterior-posterior mis-patterning with ectopic pyloric specification, which will be discussed in greater detail in chapter 9.

It is complicated to ascribe meaning to these patterns in isolation. The multitude of effects that *Fgf10* knockout has on various gene pathways (including those which are auto-regulatory or able to interact with alternate ligands) creates significant complexity in understanding their actions and interaction. Therefore, these results must serve only as an indication of potential genes involved in the DA process. It is paramount that regionalisation patterns be expanded upon, and focus upon the expression of gastric and pyloric sphincter specification markers in *Fgf10*^{-/-} foregut (beyond the duodenum) could help answer this. Use of *in situ* hybridisation may provide increased spatial correlation between the genetic patterns observed in this chapter, and the physical expression in the stomach, pylorus, and duodenum both anterior and posterior to areas of atresia.

An *ex vivo* model should be established to observe this evolution of tissue growth and atresia. Such a model could provide tissue for RT-qPCR analysis itself, as well as a basis for the addition or removal of critical proteins to analyse changes in growth patterns.

The key genes profiled in this chapter serve as a basis for further analysis, not just for *in situ* hybridisation studies or in an *ex vivo* model, but in human cases of DA where the link to *Fgf10*^{-/-} associated DA in mice must be discovered.

7.4. Conclusion

This chapter has served the completion of **aim 2** to identify the novel Fgf10-mediated gene expression pathways in the duodenum using genome-wide mRNA sequencing at the embryonic age, demonstrating the most significant *Fgf10* activity. Secondly, key genes shown as up- and down-regulated in mRNA sequencing will be profiled across differing embryonic ages to assess activity before and after the occurrence of DA.

Key gene expression patterns presented in this chapter, unique to DA-affected *Fgf10*^{-/-} duodenum, provide the best evidence for **hypothesis 2**, that signalling pathways related to the *Fgf10-Fgfr2b* axis moderate the morphology and penetrance of the DA phenotype seen in *Fgf10* knockout mice. However, until a gene or pathway is linked to DA through a positively controlled model or human DA cases, these remain an association, not causation.

8. Organoids

8.1. Introduction

Organoids are three-dimensional tissue cultures grown from stem cells, which act as a simplified version of an organ produced *ex vivo* or *in vitro*. They are unique from more traditional cell-culture systems in that they are self-organising, are highly similar to the organs from which they are derived (Kim et al., 2020). Organoid systems allow for analysis in stem cell research, disease modelling (e.g., for colorectal cancer and cystic fibrosis) and regenerative medicine (Sato and Clevers, 2013). Therefore, they serve as the ideal system to test hypotheses for aforementioned genetic causes of DA.

The intestine is organised into crypts and villi and lined by simple columnar epithelium, which undergoes rapid renewal every five to seven days (Li and Xie, 2005, Barker et al., 2008). This rate of renewal, and the requirement for mesenchymal-epithelial interactions to sustain cell proliferation, created a significant barrier to the development of gastrointestinal organoids. Early stem-cell culture methods resulted in apoptosis of the epithelial cells within a few hours after removal from the basement membrane and underlying stroma (Frisch and Francis, 1994, Sträter et al., 1996).

It was in 2009 that Sato et al. published their work, building on the knowledge of endogenous intestinal stem cell niche components, to create a stable culture system capable of sustaining the long-term growth of near-physiological epithelia (Fatehullah et al., 2016). This system used Matrigel as an extracellular matrix substitute, supplemented with a mix of growth factors to allow for stem cell differentiation, maintain stem cell populations and promote cell proliferation (Sato et al., 2009). That same year Ootani et al. published their work with an air-liquid interface, where the culture of gastrointestinal epithelium, along with its underlying stromal elements, results in the growth of sphere-like organoids, capable of proliferation and differentiation (Ootani et al., 2009).

Since their publication, these two methods have served as the basis for intestinal organoid growth, providing a replenishable and novel experimental system to study both normal and abnormal gastrointestinal physiology (Mahe et al., 2013). Intestinal organoids are suitable for functional, cellular, and physiological studies of regenerating gastrointestinal epithelium (Mizutani et al., 2012, Anderson et al., 2021, Xu et al., 2017). However, no previous research has been conducted using organoids to support the study of gastrointestinal embryology and developmental defects.

In order to provide a basis for further analysis of aforementioned key genes and gene pathways, hypothesised to be involved in DA development, the aim then was to use organoid technology to develop an *ex vivo* model to demonstrate embryonic duodenal growth. Furthermore, this model could be used to assess the growth pattern of duodenal organoids, including the ability to form crypts and to perform staining to compare cell activity, including proliferation and differentiation.

This chapter addresses **hypothesis 3**, that organoids grown from the duodenal tissue of *Fgf10* knockout mouse embryos will demonstrate differences in growth pattern compared to wild-type duodenal tissue.

8.2. Results

8.2.1. Establishing an organoid model

The protocol used was modelled from that published by O'Rourke et al. with minor modifications made to account for the volume of GFR Matrigel surrounding the organoids (O'Rourke et al., 2016)

Growth Factor Reduced (GFR) Basement Membrane Matrix (BMM) (Corning, Matrigel, Cat: 354230) was chosen as the extracellular matrix. It was thought that this specific BMM would allow the best comparison of tissue in which *Fgf10* was reduced or absent compared to where the growth factor was present.

Traditional Matrigel, as used by Sato et al. (Sato et al., 2009), provides a rich environment of growth factors, including FGF10. It has been demonstrated in other 3D culture experiments on lung epithelium that where FGF10 was added or included in the Matrigel used, embedded epithelium still underwent branching even in *Fgf10*^{-/-} tissue (Bellusci et al., 1997, Park et al., 1998, Ohtsuka et al., 2001).

For these reasons, this methodology outlined in section 3.5 was used to generate organoids from embryonic duodenal tissue. In order to validate this organoid model, wild-type embryos at E12.5 were used for tissue harvesting of the duodenum.

Wild-type duodenum demonstrated growth in organoids for 21 days after tissue harvest (**Figure 8.1**), with the initial formation of spheroids from day 9, which then matured into organoids with crypts from day 13. Wild-type organoids were seen to migrate throughout the GFR Matrigel, with some demonstrating peristalsis under the microscope during DMEM change (video uploaded to YouTube at: <https://youtu.be/MhQQa28nUHo>). Peristalsis is considered an advanced sign of functional gastrointestinal epithelium (Lee et al., 2018), usually requiring pressure cycles and luminal flow to generate (Hofer and Lutolf, 2021).

Tissue debris from the epithelial layer is seen to shed within the organoid, and from the mesenchymal layer is deposited behind the organoid during migration. Even with regular DMEM changes, organoid growth did not progress significantly beyond day 21. By day 30, the GFR Matrigel in wells containing growing organoids appeared to dissolve, with only the debris of organoids remaining floating in DMEM within the Millipore insert. Attempts to sustain organoid growth beyond this phase, through the addition of further GFR Matrigel from day 21, failed to make any difference in this result.

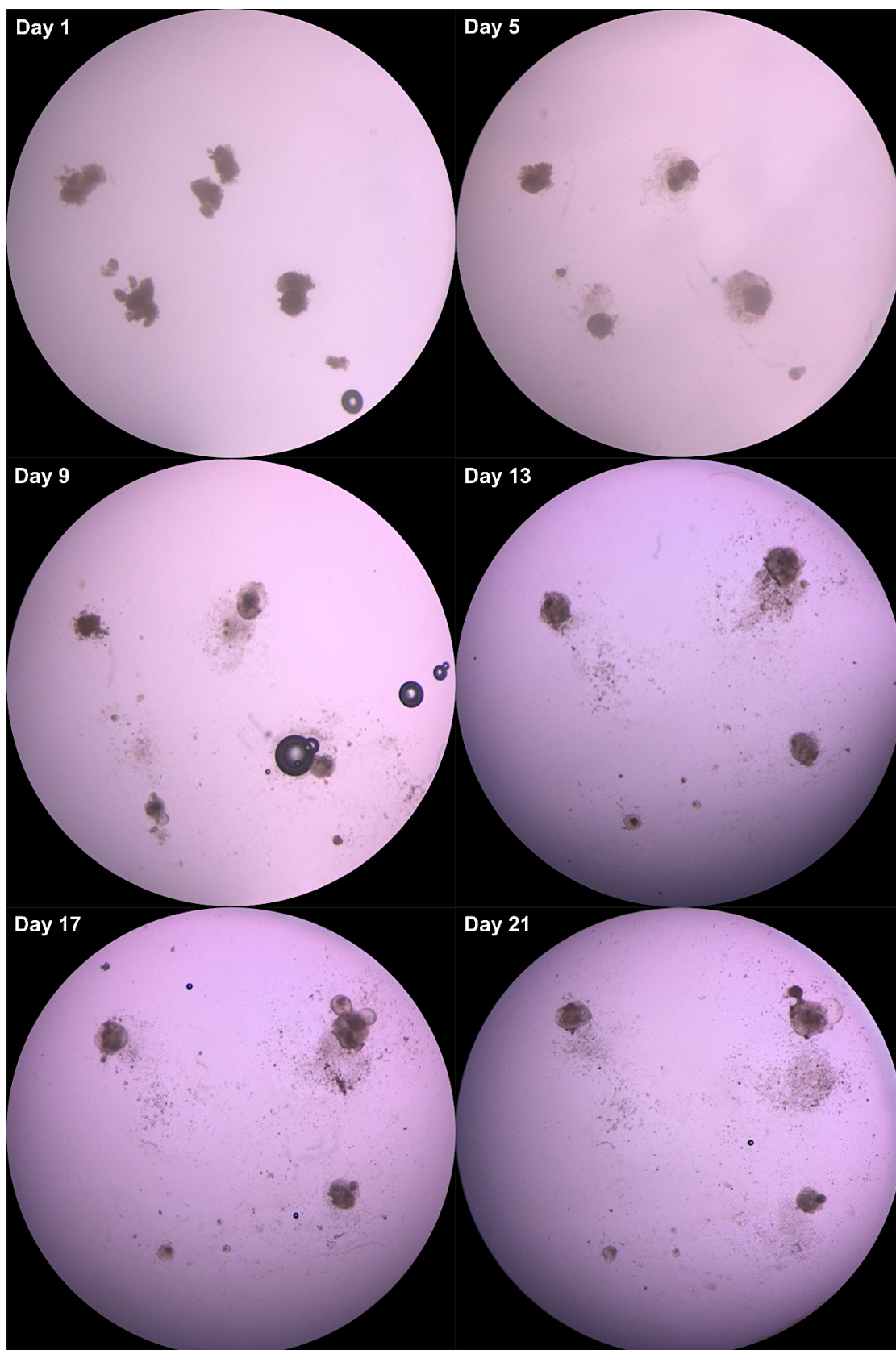


Figure 8.1 Wild-type duodenal organoids demonstrate growth over 21 days after tissue harvest. Duodenal tissue from wild-type embryos at E12.5, grown in GFR Matrigel. Growth is demonstrated over 21 days, with the initial formation of spheroids, followed by organoids with crypts, indicating differentiation. Well diameter is 12mm in each image.

8.2.2. Growth pattern of duodenal organoids

Having established the organoid model, the next aim was to assess the growth pattern of duodenal organoids, comparing wild-type, *Fgf10*^{+/-}, and *Fgf10*^{-/-} tissue. This same protocol was used, with the duodenum of E12.5 embryos (n=3 per group) harvested simultaneously between genotypes. Predictably given the E12.5 gestational age, no cases of DA were seen in *Fgf10*^{-/-} embryos. As per the above protocol, these organoids were cultured until day 21, and differences between the number, size and shape of organoids were compared.

Wild-type organoids demonstrated the same growth patterns highlighted above in all three tissue samples harvested (**Figure 8.2A** and **Figure 8.2D**).

Fgf10^{+/-} duodenum failed to progress beyond the development stage of spheroids (**Figure 8.2B** and **8.2E**), forming these in two of three tissue samples harvested. One sample failed to demonstrate any growth. The absence of crypt formation suggests the tissue cultured is of low complexity and only loosely mirroring gastro-intestinal tissue (Kardia et al., 2021).

Fgf10^{-/-} duodenal tissue importantly failed to demonstrate any evidence of growth in all three duodenal tissue samples (**Figure 8.2C** and **8.2F**).

These results suggest a basic growth pattern exists in comparison between *Fgf10* genotypes.

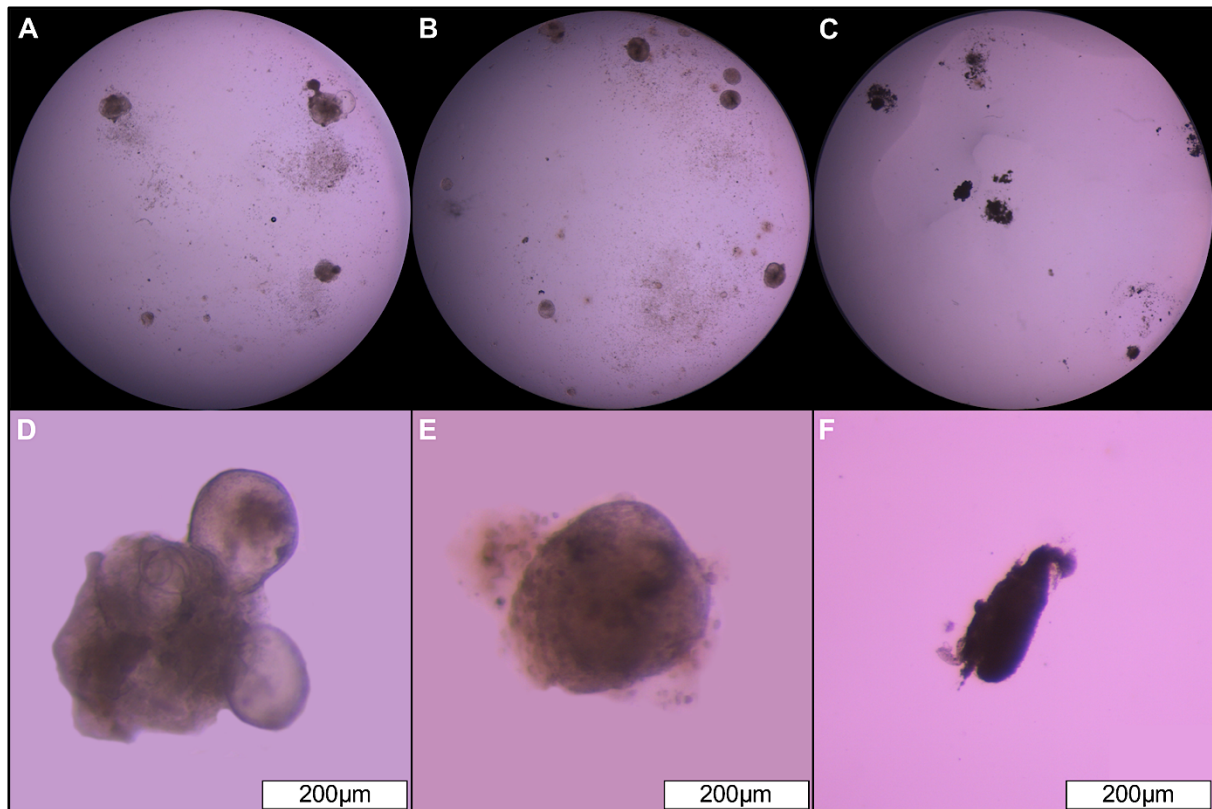


Figure 8.2 Growth comparison between wild-type, *Fgf10*^{+/-} and *Fgf10*^{-/-} duodenal organoids. *GFR Matrigel wells, cultured for 21 days, containing duodenal tissue from wild-type (A), *Fgf10*^{+/-} (B) and *Fgf10*^{-/-} (C), were compared for growth patterns. Wild-type organoids demonstrated crypt formation (D), in 2 of 3 wells. *Fgf10*^{+/-} tissue were able to form spheroids (E), but failed to progress or differentiate further. *Fgf10*^{-/-} tissue (F) did not show any growth over 21 days of culture.*

8.2.3. Organoid staining

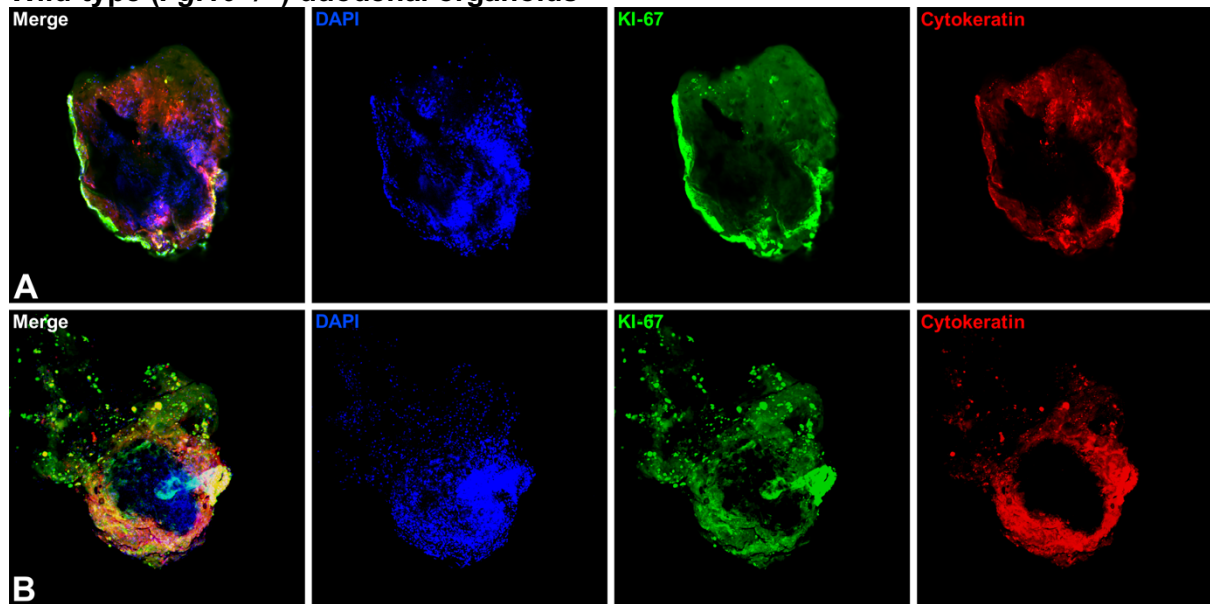
In order to verify the growth pattern findings, functional staining was used to depict organoid growth activity. Organoids were isolated and fixed (see methods in section 3.5.5) on day 14 of organoid culture to identify activity prior to the growth retardation seen after day 21 of culture. Wild-type, *Fgf10*^{+/-} and *Fgf10*^{-/-} organoids were fixed and functionally stained using antibodies selected in order to demonstrate organoid growth activity.

KI-67 antigen was targeted with KI-67 (RABBIT) antibody to demonstrate cell proliferation (Zhang et al., 2021). The epithelial intracytoplasmic cytoskeleton was targeted with cytokeratin (MOUSE) antibody to demonstrate intestinal epithelial differentiation (Bouwens, 1998, Moll, 1993). Moreover, DAPI was used to provide a background on nuclei to compare and contrast against this cell activity.

Upon imaging (**Figure 8.3**), wild-type tissue demonstrated more significant evidence of cell proliferation (red) and differentiation (green) when compared to *Fgf10*^{+/-}.

No remaining *Fgf10*^{-/-} tissue could be seen on imaging. Given the growth patterns identified, it is likely that any remaining *Fgf10*^{-/-} tissue fixed in GFR Matrigel on day 14 dissipated in the wash and staining steps outlined in section 3.5.5.

Wild-type (*Fgf10*^{+/+}) duodenal organoids



Heterozygous (*Fgf10*^{+/-}) duodenal organoids

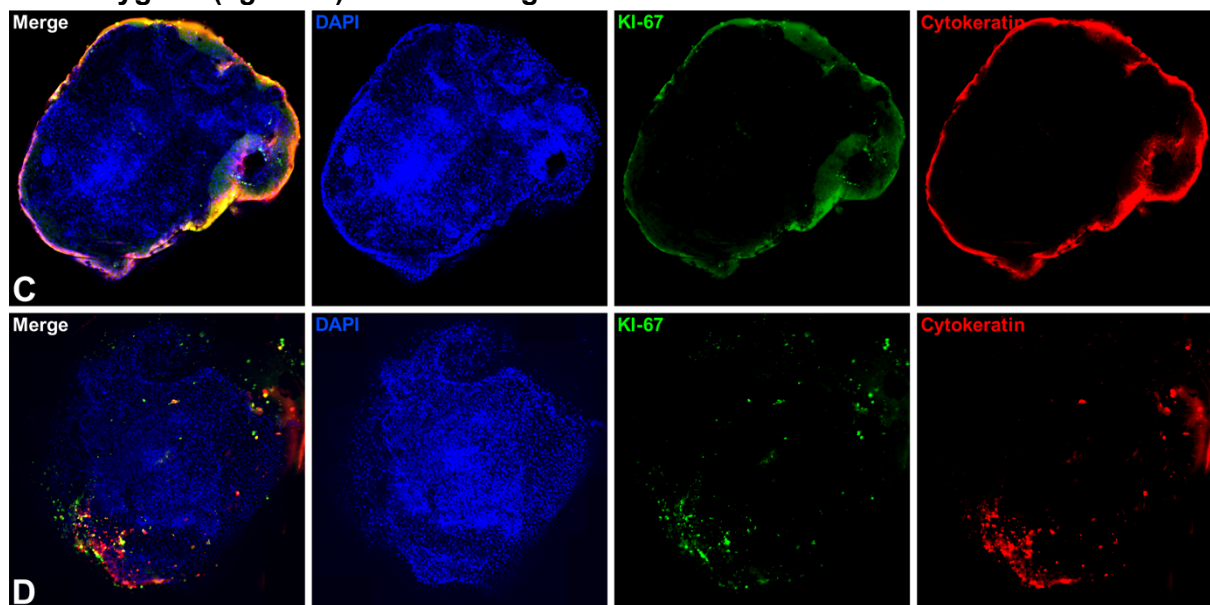


Figure 8.3 Wholemount staining of duodenal organoids demonstrate increased proliferation and differentiation in wild-type tissue. *Wholemount staining performed on duodenal organoids at day 14 of culture, demonstrate that wild-type (A and B) have increased activity of KI-67 (proliferation) and cytokeratin (differentiation) when compared to *Fgf10*^{+/-} (C and D). No *Fgf10*^{-/-} organoids were able to be grown, therefore comparison in staining was not possible.*

8.3. Discussion

This chapter has demonstrated a novel application of organoid culture to analyse growth patterns of duodenal tissue harvested from wild-type, *Fgf10*^{+/-} and *Fgf10*^{-/-} embryos. This *ex vivo* model demonstrates the capability sought after to test proteins from key genes identified in an attempt to generate growth in *Fgf10*^{-/-} tissue. The complete failure of *Fgf10*^{-/-} tissue to grow in a growth-factor reduced *ex vivo* environment suggests that the incomplete penetrance seen *in vivo* may be due to the interaction of other genes, compensating for the loss of FGF10.

Organoid growth pattern differences do not correspond with the *in vivo* animal model, in which no gross morphological gastrointestinal malformations are observed in the stomach and duodenum of *Fgf10*^{+/-} embryos. This raises the further question of the epigenetic effects of *Fgf10* haploinsufficiency.

As only small sample sizes (n=3 per group) were used to validate and test this organoid model, and growth could not be sustained beyond day 21, further work is required to validate these growth patterns. Other gastrointestinal regions (i.e. stomach, intestine) where *Fgf10* is less active should also be analysed to assess for differences in growth between genotypes. This would suggest that this phenomenon is isolated to the duodenum. Using chip organoid growth and generation of organoids from stem cell lines generated from *Fgf10*^{-/-} may also reduce variability in growth and tissue quantity, leading to longer-term organoid growth.

8.4. Conclusion

Within this chapter **aim 3** has been achieved. An *ex vivo* model has demonstrated embryonic duodenal growth; growth pattern of duodenal organoids assessed; and staining to compare cell activity performed. **Hypothesis 3** has been confirmed, with organoids grown from the duodenal tissue of *Fgf10*^{-/-} mouse embryos demonstrating a difference in growth pattern compared to wild-type duodenal tissue.

9. General discussion and future directions

9.1. Summary of results chapters

The first results chapter (chapter 4) outlined and validated the CRISPR-derived *Fgf10*^{-/-} tm1 and tm2 mouse-model as a valuable and relevant tool to support the investigation of DA in humans.

The following three chapters (chapters 5, 6 and 7) utilised this mouse model to investigate the secondary genetic changes present in DA-affected mice and the role that up- and downregulated genes and genetic pathways may play in DA aetiology. Up to chapter 6, these results present the findings for atresia in both the duodenum and caecum, with the goal of identifying common genes/pathways involved in these atresias. However, the genetic changes within the atretic caecum (including decreased FOX and HOX gene expression) were dissimilar to those observed within the duodenum. Given this would represent an entirely separate line of enquiry, it was decided to defer any additional investigation of the caecum/colon for the purpose of this thesis.

The results of chapter 7 build upon the findings of chapters 5 and 6, to show that DA-affected *Fgf10*^{-/-} duodenum undergoes a range of specific genetic changes compared to wild-type duodenum. These include changes to genes responsible for regionalisation of the gastrointestinal tract during development. For example, downregulation of genes involved in duodenal development (e.g. *Pdx1*, *Sfrp5* and *Foxa2*) and also upregulation of genes involved in gastric or pyloric specification (e.g. *Barx1*, *Isl1*, *Gata3*, *Six2*, and *Oxct1*). Also upregulated were those linked to Wnt signalling (*Sfrp5* and *Barx1*). Interestingly, genes involved in other gastrointestinal regions failing to develop, such as the pylorus (*Isl1*, *Gata3* and *Nepn*) and biliary system (*Spp1*), also underwent expressional change in *Fgf10*^{-/-} mice.

In the final chapter (chapter 8), an *in vitro* model was validated to support future studies into genetic and protein-associated growth changes within *Fgf10*^{-/-} duodenal tissue.

9.2. Hypothetical mechanisms and signalling pathways responsible for DA formation in *Fgf10*^{-/-} embryos.

As previously highlighted, *Gata3* and *Isl1* are associated with failed pylorus muscle development (Li et al., 2014), and further linked to *Barx1* and *Six2* in the *Sox9* mediated pyloric development pathway (**Figure 7.43**). The relative upregulation of these genes, along with the downregulation of normal genes involved in duodenal development (*Pdx1*, *Foxa2*) supports mis-patterning of the duodenum in *Fgf10*^{-/-} embryos, to become anteriorised and more gastric/pyloric.

Fgf10 acts on *Sox9* in the stomach (Li et al., 2014), as well as in the pancreas where it promoting proliferation and pancreatic fate maintenance in progenitor cells (Ndlovu et al., 2018). It is hypothesised that *Fgf10* is also signalling through *Sox9* in the duodenum. This is supported by the finding that *Sox9* is downregulated in most *Fgf10*^{-/-} duodenum, even despite a likely compensatory upregulation of other mesenchymal factors upstream of *Sox9* (i.e. *Gata3*, *Isl1*, *Barx1*, and *Six2*).

The overarching hypothesis for this research is that “the aetiology of DA in humans is genetically driven, and the causative genetic changes are downstream of the FGF10/FGFR2b signalling pathway”.

The results of this thesis provide a new lens through which to consider these pathways. The changes within a critical cluster of genes associated with pyloric muscle formation raise the possibility of the interplay between pyloric sphincter specification and DA formation in *Fgf10*^{-/-} embryos. Expanding upon this, the significant changes seen in gastro-duodenal regionalisation suggests that the disruption of lumen in DA could result from this anterior-posterior mis-patterning with ectopic pyloric specification.

This thesis provides a basis for continued evidence- and hypothesis-driven research. It concludes with the formation of the following new hypotheses to be investigated:

1. In *Fgf10*^{-/-} embryos, anterior-posterior mis-patterning results in the duodenum becoming anteriorised and more gastric/pyloric.
2. In *Fgf10*^{-/-} embryos with DA, the DA spectrum represents an ectopic and abnormal pyloric sphincter.
3. In *Fgf10*^{-/-} embryos with DA, spatial abnormalities of the AP patterning may localise to, or be instructive of, the locus of atresia.
4. In *Fgf10*^{-/-} embryos with DA, abnormalities in the mesenchyme/muscle development cause a failure in the support to gut/lumen maintenance.

9.3. Limitations

9.3.1. Animal model

Animal models are used to study physiological and biological processes, as they provide a platform of knowledge for later translation into human research. The generation of tm1 and tm2 *Fgf10*^{-/-} mice provided a robust and validated basis for animal research into DA. However, the lethality of complete *Fgf10* interruption beyond birth (due to the absence of lungs) and the incomplete penetrance of DA in *Fgf10*^{-/-} embryos (albeit the highest penetrance seen in the literature to date) meant a significant amount of heterozygous cross mating was required to reach the experimental numbers for genetic studies. This was most significant in the earlier gestational ages and when seeking more severe, rarer phenotypes of DA. Cross-breeding followed Mendelian genetics, meaning that for every four embryos bred, one was wild-type, two were *Fgf10*^{+/-}, and one was *Fgf10*^{-/-} (and the proportion and distribution of DA in these was as per that reported in chapter 4). Furthermore, the small quantity of duodenal tissue existing in these embryos meant a requirement to pool harvested tissue, reducing the ability to include multiple biological replicates for severe DA phenotypes in younger gestational ages.

As with any animal model in research or human disease, the ultimate goal is to translate these findings from the bench to the patient's bedside. The premise of this thesis assumes relevance between the development of the mouse and human duodenum. While general murine gastrointestinal development has been shown as markedly similar to humans (Stanford et al., 2020), substantial gene expression differences exist between the stomachs of mice and humans (Busslinger et al., 2021). Furthermore, the genetic link between DA in humans and mice initially seems quite different. Trisomy 21 is most commonly associated with DA in humans, but no association exists in mice; *Fgf10*^{-/-} is most strongly associated with DA in mice, but FGF10-associated conditions are not linked to DA in humans. The overarching hypothesis suggesting DA is downstream of the FGF10/FGFR2B axis does account for this. However, significant genetic complexity exists in interpreting results from a mouse model with a gene knockout known to not directly correlate with DA in humans. Therefore, as outlined below, significant emphasis will be placed on future research to correlate these findings to human DA-affected patients.

9.3.2. DA progression

As shown in chapter 4, DA is not present across all gestational ages, and is seen to demonstrate an increased proportion of severe phenotype as embryos mature. Therefore, it is hypothesised that DA morphology represents a spectrum of disease which evolves in the duodenum throughout embryogenesis. This creates a limitation in the grouping of *Fgf10*^{-/-} duodenum, where tissue which (at the time of dissection/harvest) does not show DA, may be destined to become atretic at a later gestational age. As well type 1 DA may be from a duodenum fated to evolve to a type 2 or 3. Furthermore, the argument could be made that duodenal tissue persisting in *Fgf10*^{-/-} embryos afflicted with type 2 or 3 DA, is subject to survivorship bias, as the duodenal segment afflicted with atresia is no longer present (and therefore neither are genetic expression changes specifically associated with it). It is for this reason that the results of all *Fgf10*^{-/-} tissue have been included, and emphasis placed upon developing an *ex vivo* model for future research.

9.3.3. Organoid model

While the organoid research outlined in chapter 8 presents a novel concept for the growth of embryonic gastrointestinal tissue and analysis of tissue growth to inform normal biology, it is still in its infancy in application. The small sample numbers and limited culture duration mean that some functional analysis can be performed, but longer-term growth analysis still needs to be enhanced. While this model serves as a potential *ex vivo* setting from which to conduct future research, that research should consider organoid models with greater standardisation. Organ-on-a-chip based *in vitro* model may better standardise the quantity of tissue from which growth is measured and form the basis for further protein/genetic analysis of organoids grown.

9.4. Challenges

9.4.1. COVID-19 and laboratory access

No area of research has escaped the interruption caused by the COVID-19 pandemic, however non-essential medical research within Melbourne, Australia, was hit particularly hard. Between the period of obtaining results from RNA-seq (chapter 6) and completing the gene validation required before moving to gestational age analysis (chapter 7), the laboratory was subject to 18 months of intermittent lockdowns denying access. This meant aspects of future research outline below (i.e. *in situ* hybridisation and human exome sequencing) which were hoped to be included within this thesis were unable to be complete during the candidature of this Doctor of Philosophy. It is the full intent of this laboratory team under the supervision of Associate Professor Teague to continue in these future directions, building upon the results of this thesis.

9.4.2. Animal welfare

Sudden and unexpected formation of eye ulcers affecting the majority of the *Fgf10*^{+/-} mice, meant an almost complete loss of this colony in the first year of this research. This was eventually linked to F10 cleaner, and its use to clean cages, surfaces and gloves in the animal house. Working with animal technicians and veterinarians it was discovered that the craniofacial anomalies present in *Fgf10* heterozygous mice predispose them to eye issues, which the F10 cleaner triggered. This setback caused a significant interruption in the initial experimental progress of tissue collection, and ethics/welfare implications threatened all experimental work with this colony. Even after F10 was identified as the causative agent, and changes implemented in animal handling and cleaning, it took several months to regenerate the required breeding stock for experimental plug-mating to recommence. This particular issue has been fed back to the international community who work with *Fgf10*^{+/-} mice in order to prevent any further unnecessary research delay and best protect animal welfare.

9.5. Future Directions

The results presented within this thesis represent a stepping stone on the path towards identifying the aetiology of DA in humans. Therefore, they must be viewed in the context of what research is to follow.

In order to expand upon the temporal expression of key genes outlined in chapter 7, spatial analysis of gene expression will be performed. This will include anterior and posterior gastrointestinal regions, as well as further definition of expression within segments of the duodenum (including pre- and post-DA regions). Through performing *in situ* hybridisation of *Barx1*, *Isl1*, *Gata3*, *Six2* and *Sox9*, it is hoped this will provide evidence of a posterior shift to the cut-off of gene expression in *Fgf10*^{-/-} embryos. Furthermore, if this shift correlates to the physical location where DA forms, this will provide substantial support that anteriorisation of the duodenum plays a role in DA formation.

In order to expand upon the temporo-spatial findings for *Fgf10* expression outlined through the use of RT-qPCR in chapter 5, greater fidelity and imaging possibilities will be afforded by the use of *Fgf10*^{CreERT2}_{tomato}^{flox/flox} [B6-*Fgf10*<tm1.1(cre/ERT2)Sbel>Gt(ROSA)26Sor<tm9(CAG-tdTomato)Hze>] and *Fgf10*^{flox/flox}_{tomato}^{flox/flox} [B6.*Fgf10*<tm1.2 Sms>] mouse strains (El Agha et al., 2012). Through performing immunohistochemistry investigation based upon these powerful developmental biology tools, stage- and tissue-specific expression of *Fgf10* will be assessed and correlated to other expression patterns sought above.

In addition to the aforementioned *Sox9* pathway, multiple other pathways and genes (including those involved in pancreatic progenitor differentiation or proliferation) were identified as affected in *Fgf10*^{-/-} in chapters 6 and 7. Profiling human DA patients for similar genetic changes seen in DA-affected *Fgf10*^{-/-} mice may provide the greatest context to view these results and ascribe meaning to interrelated changes found.

In order to determine the translational relevance of the genetic analysis within the murine DA model, this research team plans to study the genetic profile of a thought-provoking subset of human DA patients with known associated anomalies akin to *Fgf10-Fgfr2b* signalling-related defects, (e.g. craniofacial, limb and lung anomalies). It is considered that this human DA subset is at particular "risk" of demonstrating an underlying genetic basis for their DA related to the FGF10-FGFR2b signalling pathway. For this analysis, the already-established institutional DA patient database (#DB0077) will be screened for cases with potential *Fgf10-Fgfr2b* signalling-related defects and perform detailed clinical genetic phenotyping and exome sequencing of phenotypically homogenous patient groups. During the subsequent bioinformatic and genetic analyses, particular attention will be paid to key genes and pathways identified in chapters 6 and 7.

9.6. Closing remarks

Despite the best efforts of this thesis, the cause of DA in humans remains unknown. The findings presented here, while exciting and thought-provoking, humbly lay a bedrock for further research in DA aetiology. The future directions outlined above will expand upon the updated hypotheses and work to reach outside of the animal model, linking these results to human DA cases.

Finding the cause of DA remains the ultimate translation of this research, and will have immediate therapeutic relevance. It is hoped that, through achieving this, the clinician's ability to perform antenatal counselling will be improved. Furthermore, an understanding of DA aetiology will develop hypothesis-driven research, with the goal of ameliorating or even preventing the development of DA in humans.

10. Appendices

10.1. Appendix 1



The Role of Fibroblast Growth Factor 10 Signaling in Duodenal Atresia

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Introduction: Duodenal atresia (DA) is a congenital bowel obstruction requiring major surgery in the first week of life. Three morphological phenotypes are described, reflecting increasing degrees of obstruction and discontinuity of the duodenum. The cause of DA is not known. Tandler's original "solid cord" hypothesis conflicts with recent biological evidence, and is unable to account for differing DA types. In humans, a genetic etiology is supported by the association between Trisomy 21 and DA, and reports of familial inheritance patterns. Interruption of FGF10/FGFR2b signaling is the best demonstrated genetic link to DA in mice, with 35–75% of homozygous knockout embryos developing DA.

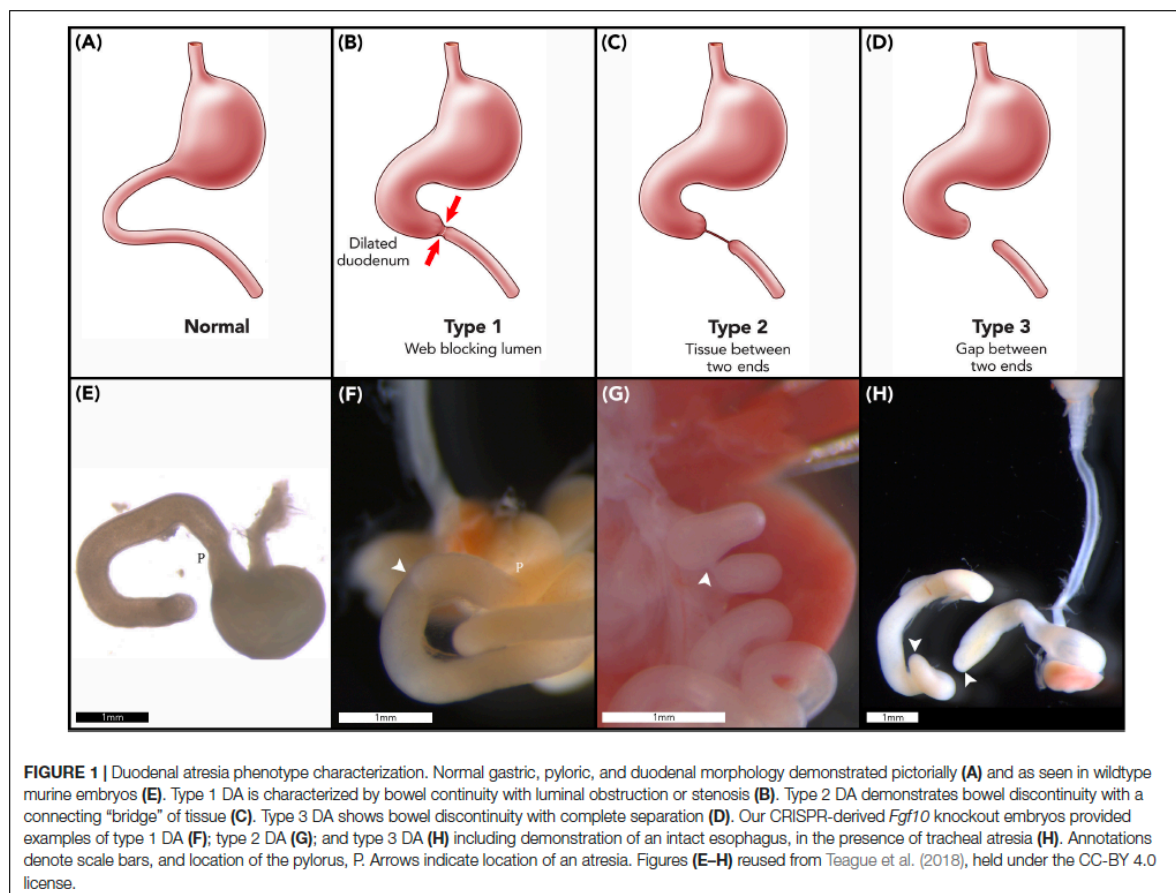
Purpose: This review examines the current evidence surrounding the etiology of DA. We focus on research regarding FGF10/FGFR2b signaling and its role in duodenal and other intestinal atresia. Further, we outline planned future research in this area, that we consider necessary to validate and better understand this murine model in order to successfully translate this research into clinical practice.

Conclusion: Determining the etiology of DA in humans is a clinical and scientific imperative. *Fgf10/Fgfr2b* murine models represent current science's best key to unlocking this mystery. However, further research is required to understand the complex role of FGF10/FGFR2b signaling in DA development. Such complexity is expected, given the lethality of their associated defects makes ubiquitous interruption of either *Fgf10* or *Fgfr2b* genes an unlikely cause of DA in humans. Rather, local or tissue-specific mutation in *Fgf10*, *Fgfr2b*, or their downstream targets, is the hypothesized basis of DA etiology.

Keywords: duodenal obstruction, congenital intestinal atresia, fibroblast growth factor 10, fibroblast growth factor receptor 2b, morphogenesis

INTRODUCTION

Duodenal atresia (DA) is a congenital foregut malformation affecting 1 in 5,000–10,000 live births (Escobar et al., 2004). Three morphological types of DA are described, reflecting increasing degrees of obstruction and discontinuity; type 1: bowel continuity but luminal obstruction or stenosis, type 2: bowel discontinuity with a connecting "bridge" of tissue, and, type 3: bowel discontinuity



with complete separation (Skandalakis and Gray, 1994; Figures 1A–D). Duodenal atresia was first described in humans by Calder (1733), but it was not until the early twentieth century that Vidal and Ernst performed the first reported successful surgical repair (Vidal, 1905; Ernst, 1916). Nowadays, DA is routinely surgically repaired in the first week of life, with re-establishment of duodenal continuity and minimal postoperative morbidity or mortality (Zani et al., 2017).

The cause of DA is not known. In 1900, Julius Tandler published a hypothesis on the origins of DA, based on his studies of normal duodenal development (Tandler, 1900). Tandler meticulously dissected 11 human embryos and theorized, on the basis of his macroscopic observations, that normal duodenal development includes a “solid cord” phase due to exuberant endodermal growth. As such, he proposed that failure of this “solid cord” to re-canalize may result in DA in humans. Even then, Tandler cautioned readers, stating: “It is clear to me that the opinion represented here does not exceed the status of a new hypothesis, and it is not meant to exceed this.” (Nichol et al., 2011). Notwithstanding, Tandler’s “solid cord” hypothesis is generally accepted worldwide by the majority of pediatric surgeons. This is despite conflicting developmental biology

findings (Cheng and Tam, 1998; Botham et al., 2012), and failure of Tandler’s theory to account for the morphological variations of DA (Merrot et al., 2006).

As will be discussed in detail in this review, murine studies have shown that interruption of the *Fgf10-Fgfr2b* signaling axis may result in DA in 35–75% of knockout mice (Fairbanks et al., 2004b; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012b; Teague et al., 2018). This also supports a genetic etiology for DA in humans, however, no specific genetic cause has been demonstrated to date. In humans, the association between DA and Trisomy 21 is well recognized, with DA occurring in 20% of cases (Khan et al., 2017). Yet, animal models of Trisomy 21 fail to demonstrate DA or other associated gastrointestinal malformations (Delabar et al., 2006). Again in humans, an autosomal recessive inheritance pattern for familial cases has been proposed, albeit based on a limited number of isolated and historical case reports (Fonkalsrud et al., 1969; Berant and Kahana, 1970; Best et al., 1989; Gross et al., 1996; Lambrecht and Kluth, 1998). Finally, whilst associations between DA and specific chromosomal anomalies have been reported (Mora et al., 2014; Zamfir et al., 2016), none relate to Chromosome 21, or to Chromosome 5 which houses the *FGF10* gene in humans.

FGF SIGNALING IN GASTROINTESTINAL DEVELOPMENT

The mammalian fibroblast growth factors (FGF) family contains 22 genes, 18 of which have been shown to bind and activate tyrosine kinase FGF receptors (FGFR), using either heparin-like molecules or klotho cofactors receptor binding (Ornitz and Itoh, 2015). These 18 FGFs have been grouped into six sub-families based on biochemical function, sequence homology and evolutionary traits (Ornitz and Itoh, 2001). The FGF7 sub-family is comprised of FGF3, FGF7, FGF10, and FGF22, and preferentially binds and activates FGFR2b (the IIIb splice variant of the FGFR2 receptor). This receptor is expressed in the epithelial layer and can be activated by FGF7 and FGF10 ligands (Ibrahimi et al., 2001). These ligands show no binding activity with the mesenchyme-expressed FGFR2c variant (Igarashi et al., 1998; Zhang et al., 2006). Conversely, FGF8 ligand preferentially binds and activates FGFR2c, with no binding activity toward FGFR2b (Macarthur et al., 1995; Ornitz et al., 1996).

The activation of FGFR2b by FGF10 is associated with mesenchymal/epithelial interactions which instruct branching and budding morphogenesis. This is a key signaling pathway for normal development of the gastrointestinal tract [e.g., stomach (Spencer-Dene et al., 2006), duodenum (Kanard et al., 2005), and large intestine (Burns et al., 2004)] and its derivatives [e.g., lung (Park et al., 1998)]. Indeed, gastrointestinal development involves multiple signaling pathways and their associated transcription factors, including FGF10-FGFR2b, which are summarized in Figure 2A and reviewed elsewhere (Kim and Shivdasani, 2016; Danopoulos et al., 2017).

FGF10-FGFR2b binding is implicated in the activation of multiple intracellular signaling pathways, including the ERK1/2, MAP kinase (MAPK) pathway, to induce downstream activation of proliferation and cell survival genes (Luo et al., 2008; Yang et al., 2011; Figure 2B). TBX4/TBX5 may induce *Fgf10* expression to promote region-specific stimulation of the mesenchymal proliferation at the initial sites of foregut growth. This growth depends on the regulation of the HOX and TBX genes, which are mediated by retinoic acid (RA) (Wang et al., 2006). Potentially, RA acts on TBX genes, such as TBX4 and TBX5, as a co-factor to activate FGF10 transcription and, as a result, mediates the FGF10-FGF8 positive feedback loop which is required for outgrowth (Nishimoto et al., 2015). Embryos that are RA-deficient fail to outgrow due to defective FGF10 signaling, resulting in a lack of FGF-target genes in the epithelium. Interestingly, the development of the foregut may be partly restored in the defective embryos by introducing RA supplementation early on in development (between E7.5 and E8.5), suggesting that RA regulation is upstream of FGF10 (Figure 2B; Wang et al., 2006).

Within the cecum and proximal colon FGF9 binds to the mesenchyme-expressed FGFR2c receptor to direct cecal mesenchyme expansion via the expression of PITX2. In conjunction with this, FGF10 acts to trigger the budding of the epithelium into the cecal mesenchyme, again through mediation of PITX2 expression. Thus, the interaction between

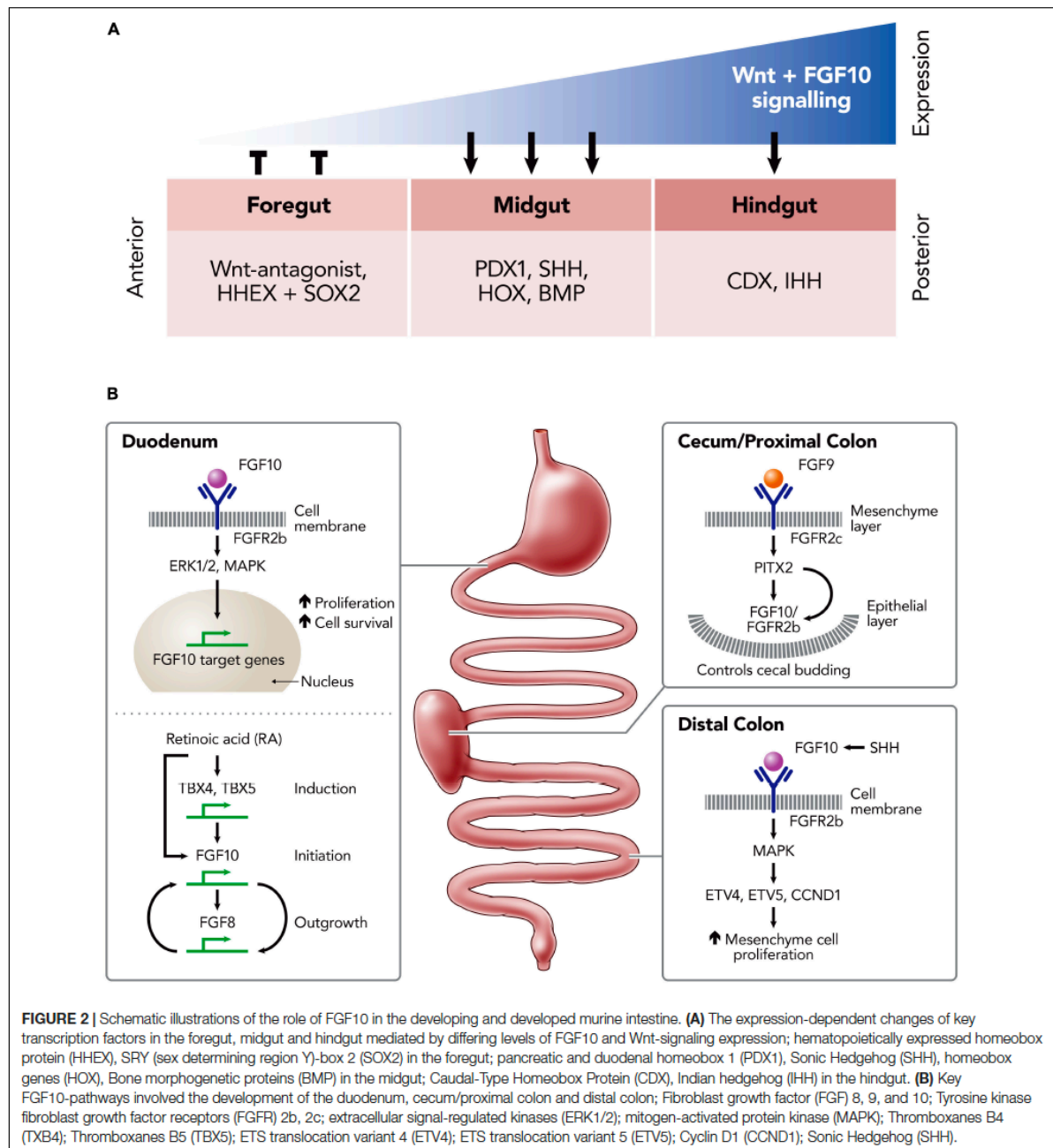
FGF10 and PITX2 is critical for the maintenance and the proliferation of the epithelial progenitor cells of the colon (Figure 2B). During embryonic development, FGF10 is also expressed in the mesenchyme of the distal colon, where it binds and activates FGFR1b and FGFR2b to promote proliferation, differentiation and survival of the epithelium. This activation maintains progenitor cells within the embryonic small intestine by targeting E26 transformation-specific (ETS)-Family genes such as ETV4, ETV5, and CCND1 through the MAPK pathway (Nyeng et al., 2011; Figure 2B).

The homozygous deletion of the *Fgf10* or *Fgfr2b* gene results in mice with multiple organ defects, including lung, limb and mammary glands (with failure to form buds 1, 2, 3, and 5). The resultant mice are non-viable after birth due to lung agenesis (De Moerloose et al., 2000; Mailleux et al., 2002).

Fgf10/Fgfr2b SIGNALING IN DUODENAL ATRESIA

Fairbanks et al. (2004b) were the first to identify a link between the *Fgf10/Fgfr2b* signaling pathway and intestinal atresia in mice. Using *Fgfr2b* knockout mice, they observed DA in 35% of null embryos when examined at gestational stage E18.5. These authors reported examples of both type 1 DA (57%) and type 3 DA (43%), but no type 2 DA (Fairbanks et al., 2004b). The same group then turned their attention to interruption of the ligand instead of receptor, knocking out *Fgf10* (Kanard et al., 2005). Interestingly, *Fgf10* knockout embryos were found to have a similar penetrance of DA (38%) compared to *Fgfr2b* knockouts, but with notably different relative frequency of DA types; type 1 DA (8%), type 3 DA (92%), and still no type 2 DA (Kanard et al., 2005). Further emphasizing the importance of the *Fgf10/Fgfr2b* pathway in intestinal development, deletion of either *Fgf10* or *Fgfr2b* also resulted in a more distally located intestinal atresia, i.e., colonic atresia (Fairbanks et al., 2004a). In contrast with the incomplete penetrance of DA, colonic atresia was present in 100% of *Fgf10* and *Fgfr2b* knockout mice embryos (Fairbanks et al., 2004a).

Importantly, the morphology of the duodenum is normal in *Fgf10* hypomorphic mice, with no examples of DA in this setting of *Fgf10* under-expression (Teague et al., 2018). Thus, even reduced Fgf10 signaling is sufficient for morphologically normal duodenal development. Similarly, Sala et al. (2006) found normal colonic development in *Fgf10* hypomorphic mice, whereas complete loss of *Fgf10* gene function resulted in colonic atresia. In the colon, *Fgf10* expression was shown to be expressed first distally before extending proximally, and was found to be critical for the maintenance and proliferation of the epithelial progenitor cells in the colon. Furthermore, crypt formation seen within the proximal colon in the absence of *Fgf10* suggested other signaling pathways were active in the development of this region, reinforcing the early anatomical and functional regionalization of the GI tract early during development. Another important finding of Sala et al. (2006) was that atresia occurred without and associated loss of mesenchyme. This finding makes vascular compromise an unlikely mechanism for colonic atresia in *Fgf10*



knockouts, as a vascular event would be expected to cause loss of both mesenchyme and epithelium.

The impact of *Fgf10* over-expression has also been investigated in both *ex vivo* and *in vivo* models. Torashima et al. (2016) showed that the addition of *Fgf10* to tissue-engineered small intestine (TESI) resulted in increased size and weight of the sample, and increased villi height and crypt

depth. In this setting, epithelial differentiation was not inhibited by over-expression of *Fgf10*, a finding attributed to the lack of normal feedback inhibitory mechanisms in the *ex vivo* TESI samples. Nyeng et al. (2011) studied *in vivo* over-expression of *Fgf10* within the developing duodenum. In these embryos, *Fgf10* over-expression caused attenuation of cell differentiation and expansion of the progenitor niche along with upregulation of

FGFR signaling targets known to exert feedback inhibition on the FGF pathway (e.g., *Sprouty2*, *Etv4*, and *Etv5*) (Minowada et al., 1999; Nyeng et al., 2011). Conversely, complete loss of *Fgf10* led to the premature differentiation of all enteric cell lineages in the duodenum (Nyeng et al., 2011). These findings support the inference that *Fgf10* is necessary to prevent premature differentiation of enteric cell lineages, and so maintain the epithelial progenitor cell population during development. In extension of this conclusion, these authors hypothesized that premature differentiation and resultant depletion of the progenitor cell population is responsible for the epithelial deficiencies of DA in *Fgf10* knockout mice (Nyeng et al., 2011).

An alternative to the premature differentiation hypothesis, is that proposed by Botham et al. (2012). These investigators concluded that exaggerated endodermal apoptosis, rather than terminal differentiation of progenitor cells was the root cause for DA in *Fgfr2b* knockout mice. Apoptosis was seen in the endoderm at E10.5, disappearance of the endoderm in the atretic region by E11.5, followed by involution of the mesoderm in the absence of further apoptosis at E13.5. This timeline corresponded with first recognition of DA in these *Fgfr2b* knockout mice, also at E13.5. Comparisons were also made between the duodenum of *Fgfr2b* knockout mice and otherwise normal, human embryo samples. In mice, loss of endoderm is complete at an earlier stage of development (E11.5, Carnegie Stage 16). In humans, however, “epithelial crowding” with corresponding apparent loss of duodenal lumen is not seen until Carnegie Stage 17 with “recanalization” only at Carnegie Stage 18. Thus, there is a temporal dislocation between luminal occlusion in atretic murine and normal human duodenal development (Matsumoto et al., 2002; Botham et al., 2012). The timeline for murine DA is supported by the findings of Nichol et al. (2012), who showed murine duodenal narrowing evident from E11.5 and DA from E13.5.

Yet another hypothesis is that of Merei (2004), who proposed that intestinal atresia may be the result of aberrant notochord signaling. Reeder et al. (2012a) used *Fgfr2b* knockout mice, reporting 42% penetrance of DA and 100% penetrance of colonic atresia. These investigators refuted the role of aberrant notochord signaling as the basis for murine DA, showing neither disruption of sonic hedgehog (*Shh*) expression nor notochordal discontinuity in *Fgfr2b* knockouts. Furthermore, organ culture of explanted wildtype murine gut in the presence of supra-physiological concentrations of *Shh* failed to induce atresia in either the duodenum or colon.

One striking feature of the DA reported in the aforementioned, seminal studies is the morphological types of DA reported in *Fgf10* and *Fgfr2b* knockout mice (Fairbanks et al., 2004b; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012b). In humans, the distribution of DA types shows similar proportions of type 1 and type 3 DA, and few cases of type 2 DA; e.g., 54% type 1, 4% type 2, and 42% type 3 DA (Khan et al., 2017). In contrast to this, when collated together, 93% of all murine DA cases reported in these studies were examples of type 3 DA, i.e., the most severe type of DA with complete discontinuity of the duodenum (Figure 1D;

Fairbanks et al., 2004b; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012b). Also, none of these studies reported any examples of type 2 DA in mice.

Of relevance to DA type and the severity of malformation is the work by Reeder et al. (2012b). Reeder et al. (2012b) investigated a role for retinaldehyde dehydrogenase 2 (*Raldh2*), a gene which encodes the enzyme responsible for the final step of vitamin A conversion to RA. *Raldh2* is critical for normal posterior foregut development (Wang et al., 2006), and is under-expressed in the duodenum of *Fgfr2b* knockout mice (Botham et al., 2012). Therefore, Reeder et al. combined heterozygous *Raldh2* deletion with homozygous deletion of *Fgfr2b* with the hypothesis of generating a more severe DA phenotype than with homozygous deletion of *Fgfr2b* alone. Contrary to this hypothesis, addition of *Raldh2* haploinsufficiency resulted in a reduced rather than increased penetrance of atresia (21 vs 45%), and a shift toward less severe atresia types: *Fgfr2b*^{−/−}; *Raldh2*[±] (80% type 1 DA, 20% type 2 DA, and no type 3) versus *Fgfr2b*^{−/−}; *Raldh2*^{+/+} (7% type 1 DA, 0% type 2 DA, and 93% type 3 DA). These authors suggested that signaling downstream of *Fgf10*/*Fgfr2b*, i.e., *Raldh2*, was responsible for the phenotypic variations in DA type morphology.

We have recently reported a more human-like distribution of DA types, including type 2 DA, in two strains of CRISPR-derived *Fgf10* knockout mouse (Teague et al., 2018). Of note, these newly reported strains; tm1 (B6-Fgf10<c.[464_470dup;506_645del]APNMu>); and tm2 (B6-Fgf10<c.495_507delAPNMu>) (Eppig et al., 2006) each demonstrate all three DA types (Figures 1F–H), and a significantly higher penetrance of DA than previous animal models [74% (Teague et al., 2018) versus 35–45% (Fairbanks et al., 2004b; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012b)]. The basis for such differences in type-distribution and penetrance when compared with previously reported *Fgf10* knockout mouse strains remains unclear. The various strains are of consistent background, namely C57/Bl6 (Fairbanks et al., 2004b; Kanard et al., 2005), and whilst the genetics differ, each represents a nonsense mutation (Eppig et al., 2006). We hypothesize that signaling molecules and pathways downstream of *Fgf10*/*Fgfr2b* are moderating the morphology and penetrance of the DA phenotype in these various strains.

Our hypothesis is supported by the findings of Al Alam et al. (2012). While an FGF9/*Pitx2*/FGF10 signaling axis has been proposed within the lung (De Langhe et al., 2008), Al Alam et al. demonstrated that the same signaling axis is active in developing gut (Al Alam et al., 2012). Using tissue specific *Fgf9* and *Pitx2* conditional knockout they demonstrated that, within the cecum, FGF9 controls mesenchymal *Pitx2* expression and, in turn, *Fgf10* expression. They also found that *Pitx2* induced the expression of *Fgf10*, even in the absence of *Fgf9*, suggesting that *Pitx2* is downstream of *Fgf9*. In further attempts to define pathways upstream of *Fgf10* involved in atresia, Reeder et al. investigated the role that *Shh* plays with *Fgfr2b* (Reeder et al., 2014). Using a Matrigel culture system and focusing on the colon of *Fgfr2b* knockout mice, they found a downregulation of *Shh* at E11.5 throughout the entire colon, and then loss of *Foxf1* expression 12 h later in the mid-to-distal colon prior to its

involution. They also found that the exogenous addition of *Shh* to the *Fgfr2b* deficient colon failed to prevent the formation of atresia, suggesting that the loss of *Shh* is not a critical event in atresia formation.

FORTH-COMING RESEARCH INTO DUODENAL ATRESIA ETIOLOGY

Based on the currently available literature regarding *Fgf10-Fgfr2b* signaling in duodenal atresia, we hypothesize that the etiology of DA in humans is genetically driven, and that the causative genetic changes are downstream of *Fgf10-Fgfr2b*. This downstream locus may account for both the incomplete penetrance of DA in the murine model, and the different morphological types (i.e., severity) of DA. It may also explain why human DA patients lack the non-survivable associations of *Fgf10* deletion such as pulmonary agenesis. Therefore, we plan to test this hypothesis by further investigating the up- and down-regulation of genes and pathways in atretic and morphologically normal duodenum. Coordinated RNA sequencing, validation, and protein interaction analyses are planned, focusing on the duodenum of CRISPR-derived *Fgf10* knockout mice with and without duodenal atresia, as well as additional wildtype controls. We consider that all three of these mice populations are necessary to discern candidate genes and pathways responsible for DA within the murine model. Also, given the complete penetrance of colonic atresia in the setting of *Fgf10-Fgfr2b* signaling interruption, we plan additional experiments to delineate the up- and down-regulation of genes and pathways in atretic and morphologically normal colon. We consider the results of colonic analyses may aid discernment of the genetic changes which hold true relevance for atresia formation, amongst the high volume of genetic changes we expect to observe in this model. Furthermore, we plan to test the aforementioned dose-dependence hypothesis, by scrutinizing gene expression differences between the three morphological types of DA in our CRISPR-derived *Fgf10* knockout mice.

In order to determine the translational relevance of the genetic analysis within our murine DA model, we plan to study the genetic profile of a thought-provoking subset of human DA patients with known associated anomalies akin to *Fgf10-Fgfr2b* signaling-related defects, e.g., craniofacial, limb, and lung anomalies. We consider this human DA subset to be at particular “risk” of demonstrating an underlying genetic basis for their DA related to the FGF10-FGFR2b signaling pathway. For this analysis, we will screen our institutional DA patient database for case with potential *Fgf10-Fgfr2b* signaling-related defects, and perform detailed clinical genetic phenotyping and exon sequencing of phenotypically homogenous patient groups. During the subsequent bioinformatic and genetic analyses, we will pay particular attention those genes and pathways evidenced to be associated with DA in our murine model.

Ultimate translation of these further studies will be to determine the cause of DA in humans, which will have

immediate and therapeutic application in the clinical context of antenatal counseling. Also, these may provide the bases for future hypothesis-driven clinical targets and trials to ameliorate or prevent the development of DA in humans.

SUMMARY

Interruption of FGF10-FGFR2b signaling is the best demonstrated genetic link to DA in mice. This corresponds in importance, and hypothesis-formulating relevance, to the association between DA and Trisomy 21 in humans. *FGF10* haploinsufficiency and *FGF10* mutations have been linked to human disease, principally to craniofacial syndromes (Entesarian et al., 2005; Milunsky et al., 2006; Rohmann et al., 2006), chronic respiratory disease (Klar et al., 2011), but no human gastrointestinal tract atresia cases to date (Tatekawa et al., 2007). In contrast to this narrative of haploinsufficiency, homozygous *Fgf10* invalidation appears necessary for DA in our animal models (Teague et al., 2018), albeit accompanied by non-survivable anomalies such as lung agenesis. Therefore, we hypothesize that DA is caused by conditional tissue-specific deletion in *Fgf10*, *Fgfr2b* or their downstream targets (e.g., spatially limited to the duodenum or foregut). Constitutional deletion of this nature is less likely to explain DA in humans for reasons of non-survivable anomalies as detailed previously.

We consider determining the etiology of DA in humans to be a clinical and scientific imperative. Therefore, further study is required to understand the exact role of FGF10-FGFR2b signaling in this. Murine models of DA based on interruption of this signaling will be key to unlocking the mystery of DA in humans. Parallel genetic studies of human DA patients will enable translation of model-derived lessons from bench to bedside.

AUTHOR CONTRIBUTIONS

MJ conceived the project, conducted the review of literature, analyzed the evidence, and wrote and finalized the manuscript. GS provided specific scientific input into the analysis of evidence, and assisted in writing and finalizing the manuscript. PC mentored MJ and assisted in finalization of the manuscript. JH mentored MJ, and assisted in the analysis of evidence and finalization of the manuscript. SK mentored MJ, and assisted in the review of literature, analysis of evidence, and finalization of the manuscript. WT mentored MJ, conceived the project, and assisted in the review of literature, analysis of evidence, and writing and finalization of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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10.2. Appendix 2



FGF10 and the Mystery of Duodenal Atresia in Humans

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Background: Duodenal atresia (DA) is a congenital obstruction of the duodenum, which affects 1 in 7000 pregnancies and requires major surgery in the 1st days of life. Three morphological DA types are described. In humans, the association between DA and Down syndrome suggests an underlying, albeit elusive, genetic etiology. In mice, interruption of fibroblast growth factor 10 (*Fgf10*) gene signaling results in DA in 30–50% of embryos, supporting a genetic etiology. This study aims to validate the spectrum of DA in two novel strains of *Fgf10* knock-out mice, in preparation for future and translational research.

Methods: Two novel CRISPR *Fgf10* knock-out mouse strains were derived and embryos generated by heterozygous plug-mating. E15.5–E19.5 embryos were genotyped with respect to *Fgf10* and micro-dissected to determine the presence and type of DA.

Results: One twenty seven embryos (32 wild-type, 34 heterozygous, 61 null) were analyzed. No wild-type or heterozygous embryos had DA. However, 74% of *Fgf10* null embryos had DA (49% type 1, 18% type 2, and 33% type 3).

Conclusion: Our CRISPR-derived strains showed higher penetrance of DA due to single-gene deletion of *Fgf10* in mice than previously reported. Further, the DA type distribution in these mice more closely reiterated that observed in humans. Future experiments will document RNA and protein expression of FGF10 and its key downstream signaling targets in normal and atretic duodenum. This includes exploitation of modern, high-fidelity developmental tools, e.g., *Fgf10*^{flox/+}–tomato^{flox/flox} mice.

Keywords: duodenal obstruction, congenital intestinal atresia, fibroblast growth factor 10, morphogenesis, models, animal, CRISPR-Cas systems

INTRODUCTION

Duodenal atresia (DA) is an important congenital cause of bowel obstruction in newborns. Often diagnosed prenatally on ultrasound scan or postnatally on abdominal radiograph, it requires intensive care after birth and major surgery in the 1st days of life. Three morphological types of DA are described, reflecting increasing degrees of obstruction and discontinuity; type 1: bowel

continuity but luminal obstruction or stenosis, type 2: bowel discontinuity with a connecting “bridge” of tissue, and type 3: bowel discontinuity with complete separation (Skandalakis and Gray, 1994). While modern surgical and neonatal management of DA achieves survival in more than 95% of cases (Khan et al., 2017), the embryological etiology of this condition remains an unanswered question amongst researchers, clinicians and patients’ families.

In 1900, the Viennese anatomist Julius Tandler meticulously studied 11 human embryos with apparently normal gut, and theorized that the duodenum underwent a normal “solid cord” phase during development, secondary to exuberant endodermal (epithelial) growth. Further, he suggested failure of this proposed cord to re-canalize accounted for DA (Tandler, 1900). At the time, Tandler himself cautiously stated, “*It is clear to me that the opinion represented here does not exceed the status of a new hypothesis, and is not meant to exceed this*” (Nichol et al., 2011). Despite this caution, Tandler’s theory was rapidly and widely adopted as dogma, and went largely unchallenged for many years. Merrot et al. (2006) were amongst the first to counter the “recanalization theory,” asserting that it failed to explain the different morphological types and other variability seen in humans with DA. Furthermore, clinical reports have suggested a genetic basis for DA, with an autosomal recessive inheritance pattern reported for some cases (Berant and Kahana, 1970; Gross et al., 1996; Lambrecht and Kluth, 1998), and the well-recognized association with Down syndrome (Trisomy 21), which occurs in approximately one fifth of DA cases (Khan et al., 2017).

Fairbanks et al. (2004) were the first to report a link between DA and fibroblast growth factor (FGF) pathways in mice, specifically fibroblast growth factor 10 (FGF10) and its receptor fibroblast growth factor receptor 2b (FGFR2b). The FGF family of signaling molecules composed of at least 22 members involved in different aspects of organogenesis (Ornitz and Itoh, 2001), of which FGF10 is associated with instructive mesenchymal/epithelial interactions, occurring during budding and branching morphogenesis. The homozygous deletion of the *Fgf10* gene results in mice which are non-viable after birth due to lung agenesis, also exhibiting defects of the limbs, anterior pituitary gland, salivary glands, inner ear, teeth, skin, and skull (De Moerloose et al., 2000; Mailleux et al., 2002). Since 2004, several reports have highlighted the importance of the FGF10-FGFR2b signaling pathway in DA as well as other congenital gut malformations such as caecal atresia (Kanard et al., 2005; Fairbanks et al., 2006; Nichol et al., 2011; Botham et al., 2012; Reeder et al., 2012).

We report here our experiences with an *Fgf10* knock-out mouse model, the strains for which were developed using novel CRISPR/Cas9 techniques. Our investigations to date have focused upon characterization of these strains, with a view toward hypothesis-driven determination of the genetic etiology of DA. Herein, we also discuss our future directions and investigations, which aim to make optimal use of modern and powerful developmental biology tools.

MATERIALS AND METHODS

Mouse Strain Derivation

Mutant animals were produced via CRISPR/Cas9 injection of C57/Bl6 oocytes by Monash University as a node of the Australian Phenomics Network (APN). In contrast to Yasue et al. (2014), who used CRISPR technique to target exon 1 of the *Fgf10* gene, our strains were derived using RNA guides targeting exon 3, designed with the aim of minimizing “off-target” events.

Thus, two novel murine strains were developed, the first with a 7 bp duplication and 140 bp deletion (B6-*Fgf10*<c.[464_470dup; 506_645del]APNMu>), and the second with a 13 bp deletion (B6-*Fgf10*<c.495_507delAPNMu>) (Eppig et al., 2006). These strains are coined here as “tm1” (464_470dup; 506_645del) and “tm2” (495_507del), respectively.

Animal Husbandry and Mating Strategies

Mice were maintained in a temperature- (24°C) and lighting- (14:10 h light-dark cycle) controlled room with free access to food and water within the accredited, institutional animal facility, and cared for in strict adherence with animal welfare and ethical requirements.

To establish breeding colonies for each strain, wild-type and heterozygous mice were mated, and liveborn pups genotyped to inform successive matings; see genotyping protocols below. *Fgf10* mutation is fatal in its homozygous (null) form due to lung agenesis (Sekine et al., 1999). Therefore, heterozygous plug-mating was reserved for experimental use only, with the aim of generating null, heterozygous, and wild-type embryos. For these experimental matings, pregnant dams were humanely culled at timed gestations to provide timed embryos ranging from E15.5 to E19.5.

Mouse Strain Genotype Characterization

Animals were genotyped by PCR analysis of DNA, extracted from tail or ear clippings. For the tm1 strain, a common forward primer was used (5'-GGAGTGTAGATCATTACATGGC-3') with a tm1-specific reverse primer (5'-GTGAGGATACCATCTCTTTCTGTCC-3') to produce a wild-type allele of 348 bp and mutant allele of 215 bp. For tm2, the same forward primer was used (5'-GGAGTGTAGATCATTACATGGC-3') with the tm2-specific reverse primer (5'-GAATTCAGGGCTATGCTTTGC-3') producing a wild-type allele of 242 and 148 bp (390 bp before *NsiI* digestion) and mutant allele of 377 bp. The full details of DNA extraction and standard PCR protocols used are provided as **Supplementary Material**.

RNA and RT-qPCR Analysis

Total RNA was extracted using the RNeasy mini column (Qiagen, Cat: 74104), treated with DNase I (Qiagen, Cat: 79254), and the concentration was determined using NanoDrop spectrophotometer (Thermo Scientific, 2000). Complementary DNA (cDNA) was synthesized using Bio-Rad iScript Advanced cDNA kit (Bio-Rad, Cat: 170-8842). Gene expression (2 µl of cDNA) was measured by GoTaq qPCR

(Promega, Cat: A6001) for real time quantitative polymerase chain reaction (RT-qPCR). Nucleotide sequences for *Fgf10* mRNA (Fw 5'-CACCTATGCATCTTTAACTGGC-3') (Rv 5'-TCTATGTTTGGATCGTCATGGGG-3') was used and expression was normalized to *Rpl32* (Fw 5'-GAGGACCAAGAAGTTCATCAGG-3') (Rv 5'-CATTGTGGACCAGGAACCTGC-3'). The results were analyzed using the 7500 SDS software and relative expression was calculated to determine the fold change. Statistical analysis was performed in Prism 7.0 (GraphPad Software). Error bars on the results are presented as a standard error of the mean (SEM), and a *p*-value of <0.05 was considered significant. Statistical significance was evaluated using Student's unpaired *t*-test. The full details of RT-qPCR protocols used are provided as **Supplementary Material**.

Mouse Strain Phenotype Characterization

Embryos of gestation E15.5–E19.5 were humanely collected, culled and micro-dissected, during which key phenotypic features were recorded using bright-field microscopy. Greatest attention was shown to the morphology of the foregut, and determination made as to the presence and type of DA where applicable. Where ambiguous, duodenal morphology, and patency was further investigated using either wholemount immunostaining or paint-filling as adapted from techniques to delineate embryonic murine inner ear morphology (Morsli et al., 1998). The entirety of the duodenum from the pylorus to the duodenojejunal flexure was preserved intact, with back-light microscopy to confirm patency. Duodenal type was determined by consensus of two investigators at the time of dissection, and then representative images secondarily re-assessed independently by two or more authors blinded to the original type determination to provide internal validation.

Ethical Considerations

Ethics approval for the experiment was granted from the Murdoch Children's Research Institute (MCRI) Animal Ethics Committee (#A792), and the MCRI Institutional Biosafety Committee (#215-2014 PC1 NLRD).

RESULTS

Population

A total of 280 embryos were collected for this study (144 tm1 and 136 tm2 embryos).

Of the 144 tm1 strain embryos, genotyping determined 42 (29.2%) embryos to be wild-type, 74 (51.4%) heterozygous, and 28 (19.4%) *Fgf10* null embryos. With respect to gestation, 70 embryos were collected at E15.5, 32 at E16.5, 23 at E17.5, and 19 at E18.5. In total, 67 tm1 embryos were further micro-dissected, that being 20 wild-type, 19 heterozygous, and all 28 *Fgf10* null embryos.

Of the tm2 strain embryos, genotyping determined 35 (25.7%) embryos to be wild-type, 68 (50%) heterozygous, and 33 (24.3%) *Fgf10* null embryos. Eighteen embryos were collected at E15.5, 29

at E16.5, 25 at E17.5, 48 at E18.5, and 16 at E19.5. In total, 60 tm2 strain embryos were micro-dissected, that being 12 wild-type, 15 heterozygous, and all 33 *Fgf10* null embryos.

Genotype Characterization

The product from classic PCR genotyping confirmed expected genetics according to the tm1 and tm2 mutations (Figure 1A). The PCR product for each strain was formally sequenced to confirm correct action of the primers in each protocol. Semiquantitative PCR showed RNA expression for heterozygote and *Fgf10* null embryos in both tm1 and tm2 strains (Figure 1A). With wild-type litter mates providing controls for *Fgf10* gene expression in both strains, the heterozygous embryos expressed approximately half the *Fgf10* (tm1 = 42.29% and tm2 = 51.16%), and no expression was seen in *Fgf10* null embryos.

General Phenotype Characterization

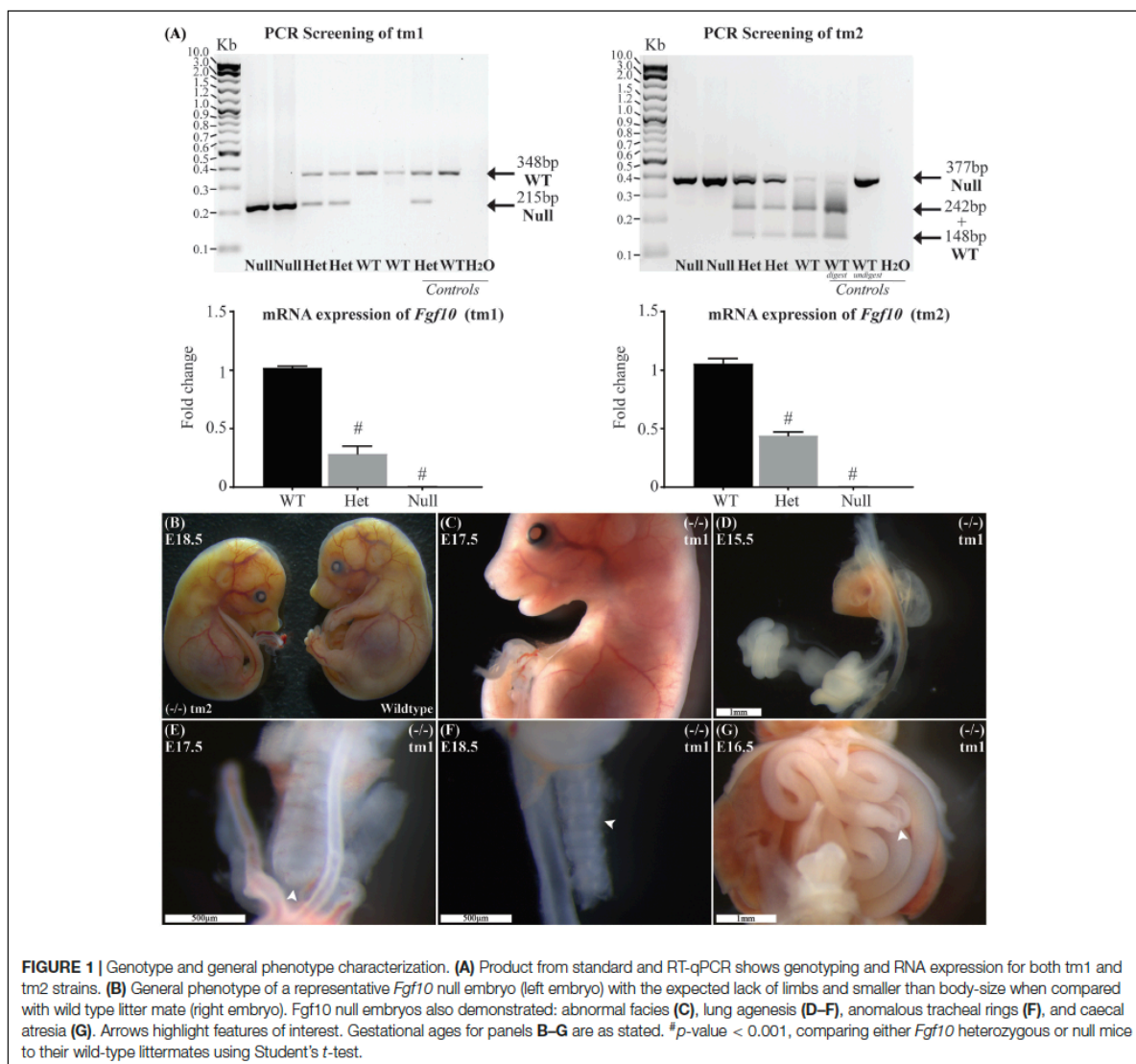
The general phenotype of both tm1 and tm2 strain *Fgf10* null embryos was consistent with previously reported *Fgf10* knock-out mouse strains (Sekine et al., 1999; Sala et al., 2011; Teshima et al., 2016). These *Fgf10* null embryos demonstrated characteristic lack of limbs and smaller than normal body size (Figures 1B,C), abnormal facies (Figure 1C), lung agenesis (Figures 1D–F) anomalous tracheal rings (Figure 1F), and caecal atresia (Figure 1G). Expected (i.e., normal) changes in organ morphology during development notwithstanding, these abnormalities in general phenotype were represented at all gestational ages of *Fgf10* null embryos assessed. Further, as our experimental question and aim placed primary focus on abdominal foregut morphology, we did not undertake detailed comparisons of these general phenotypic features during *Fgf10* null embryo development, e.g., between tm1 and tm2.

The general phenotype of tm1 and tm2 *Fgf10* heterozygotes mirrored that of wild-type (i.e., normal) litter mates, including morphologically normal limb buds, bilateral and branched lungs, normal appearance of tracheal rings and no caecal atresia. Finally, whilst the morphology and ultrastructure of some organs in *Fgf10* heterozygous mice is known to be abnormal (Jaskoll et al., 2005; El Agha et al., 2012), these were not formally assessed for the tm1 and tm2 strains, with the exception of foregut morphology as detailed below.

Duodenal Phenotype Characterization

Wild-type litter mates provided controls for normal gastric, pyloric, and duodenal morphology (Figure 2A). Without exception, *Fgf10* heterozygous embryos also demonstrated normal gastroduodenal morphology (Figures 2B,C), as distinct from *Fgf10* null embryos.

Of 61 *Fgf10* null embryos, 45 (74%) demonstrated DA. With DA observed in 21 (75%) of tm1, and 24 (73%) of tm2 *Fgf10* null embryos. *Fgf10* null embryos without DA did, however, demonstrate microgastria, which was a universal phenotypic feature for the *Fgf10* null cohort (Figures 2D,E). Both tm1 and tm2 strains provided examples for all three DA types: tm1 with 9 (43%) type 1, 4 (19%) type 2, and 8 (38%) type 3 DA; tm2 with 13 (54%) type 1, 4 (17%) type 2, and 7 (29%) type 3 DA (Figures 2F–K). The small sample sizes for each strain precluded



meaningful comparison of relative type frequency between the strains and gestational ages.

Duodenal atresia in *Fgf10* null embryos reiterated the type morphology of DA in humans. In each case, the morphology of DA was recognizably distinct from the physiological luminal narrowing at the junction between the stomach and duodenum, i.e., pylorus (Figures 2A–E). Type 1 DA is characterized in these examples (Figures 2F,G) by continuity of the outer (serosal) aspect of the fetal gut wall, whilst the lumen is obstructed by a web at the site of atresia. Other examples of type 1 DA (not shown in Figure 2) demonstrate stenosis of the gut lumen, such that the lumen is notably and abnormally narrowed, whilst epithelial continuity persists. Type 2 DA (Figures 2H,I,M) represents a more severe duodenal anomaly, in which the gut wall and lumen are discontinuous, albeit a bridge or span of tissue maintains

a physical connection between the two ends. Finally, type 3 DA (Figures 2J–L) is the most severe duodenal phenotype, recognized here by complete disconnection of the atretic gut ends.

Other Intestinal Phenotype Characterization

Given the recognized association between DA and esophageal atresia in humans, DA-affected *Fgf10* null embryos were analyzed for esophageal continuity. Figure 2L (*tm1*) and Figure 2M (*tm2*) provide representative images of the universally intact esophagus for these embryos, i.e., no esophageal atresia. The 100% penetrance of caecal atresia in *tm1* and *tm2* *Fgf10* null embryos has been noted above.

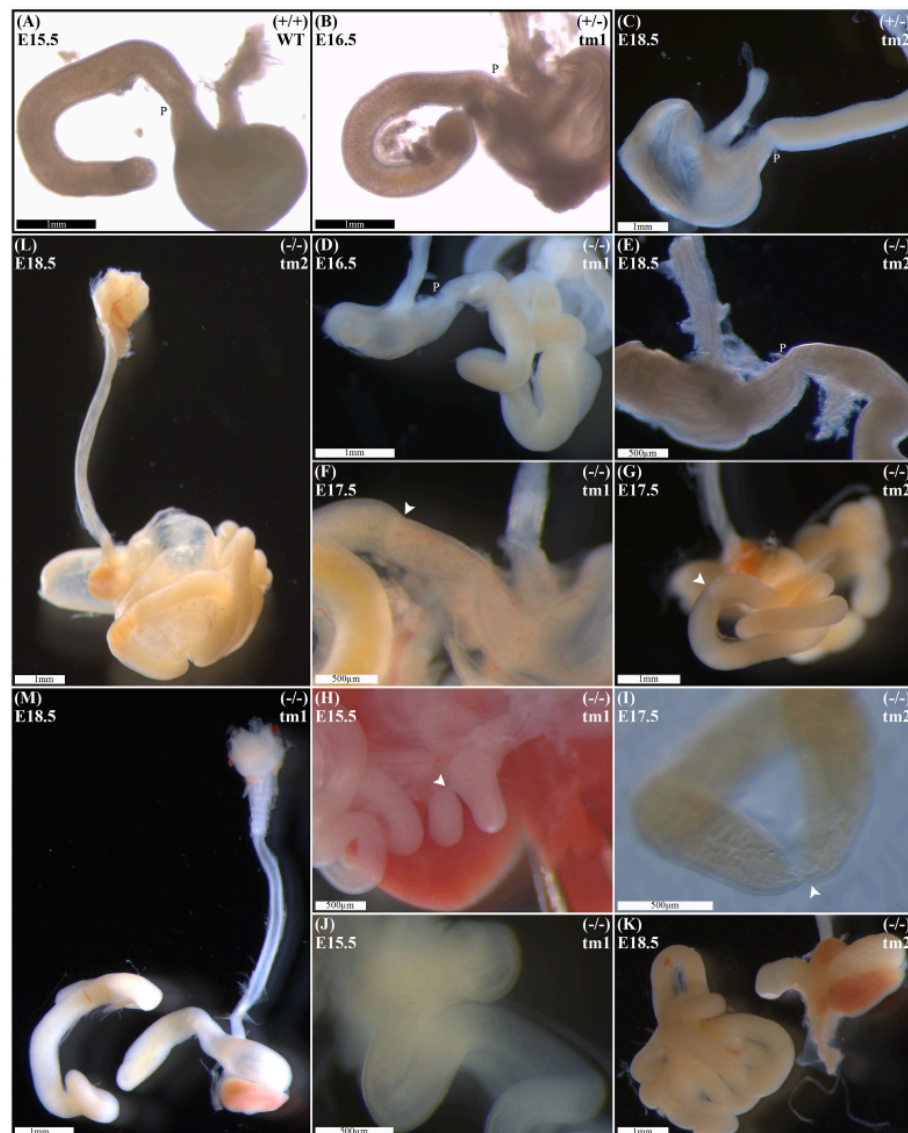


FIGURE 2 | Duodenal phenotype characterization. Normal gastric, pyloric, and duodenal morphology was demonstrated by wild type embryos (A), as well as *Fgf10* heterozygous embryos for tm1 (B), and tm2 (C) strains. Null *Fgf10* embryos universally demonstrated microgastria, but duodenal morphology varied according to presence and type of DA. Null embryos provided examples of: normal continuity and morphology of the duodenum for tm1 (D) and tm2 (E); type 1 DA, tm1 (F) and tm2 (G); type 2 DA, tm1 (H) and tm2 (I); and type 3 DA, tm1 (J) and tm2 (K). (L) Type 2 DA demonstrating a “double bubble” with significantly dilated proximal duodenum; tm2. (M) Type 3 DA demonstrating an intact esophagus, in the presence of tracheal atresia; tm1. Annotations denote genotype, scale bars, and location of pylorus, P. Arrows indicate location atresia in type 1 DA and type 2 DA examples. Gestational ages for each panel are as stated.

DISCUSSION

A Necessary Model

A limited understanding of the etiology of DA presently restricts the design and assessment of strategies to prevent or ameliorate the phenotype of DA in humans. As it is not possible to directly

investigate the pathogenesis of DA in human embryos, a suitable animal model is required to advance knowledge toward such translation. Although the association between DA and Down syndrome is the best understood genetic link with DA in humans, the animal models for Down syndrome are not applicable here as they universally fail to demonstrate DA or indeed any other

Trisomy 21-associated gastrointestinal abnormalities (Delabar et al., 2006).

Like Trisomy 21 in humans, interruption of FGF10/FGFR2b signaling is the best demonstrated genetic link to DA in mice (Fairbanks et al., 2004; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012). Despite this, mice lacking expression of either *Fgf10* or its receptor gene did not provide a promising model for future study due to relatively poor penetration of the DA phenotype with DA present in only 35–45% (Fairbanks et al., 2004; Kanard et al., 2005; Botham et al., 2012; Reeder et al., 2012). Further, these strains failed to reiterate in mice the full spectrum and distribution of DA types seen in humans within a single strain with, at times, unexplained contradiction in the distribution of DA types demonstrated (Kanard et al., 2005; Reeder et al., 2012).

An Improved and Promising Model

The DA penetrance and types evident in our novel CRISPR-derived, *Fgf10* knockout mouse strains, reported here as tm1 and tm2, represent an important and positive development in the field. In distinction to previous reports, our novel *Fgf10* null embryo strains demonstrate both a significantly higher penetrance (74%), as well as examples for all three morphological types of DA.

The basis for the differences in DA penetrance and DA type distribution between our and previously reported *Fgf10* knockout mouse strains remains unclear. The strains are of consistent background, namely C57/BL6 (Fairbanks et al., 2004; Kanard et al., 2005), and whilst the genetics differ, each represents a nonsense mutation (Eppig et al., 2006). The exact genetics, and its interplay on downstream targets, may well be playing a role in penetration of the DA phenotype. Interestingly, Reeder et al. (2012) observed that the addition of haploinsufficiency of retinaldehyde dehydrogenase 2 (*Raldh2* ±) to *Fgfr2IIIB* homozygous null embryos resulted in a reduced penetrance of DA, accompanied by a less severe DA phenotype. Of note, type 2 DA was evident in *Fgfr2IIIB*(−/−); *Raldh2*(±) but not in *Fgfr2IIIB*(−/−) mouse embryos (Reeder et al., 2012). Reeder et al. (2012) results could be interpreted as demonstrating a relationship between DA penetrance and type distribution in the murine model. If this were so, a model demonstrating all DA types might be expectedly undermined by weak penetrance. However, our results demonstrate both improved penetrance as well as expression of all three DA types, affirming applicability of the tm1 and tm2 strains presented here as a promising model of DA.

Whilst the performance of the tm1 and tm2 strains is enhanced, neither strain demonstrated complete penetrance of DA. Incomplete penetrance of the duodenal phenotype is distinct from other phenotypic features of *Fgf10* null embryos, which demonstrate complete penetrance, e.g., absent limbs, absent lungs, and caecal atresia. Presently undefined developmental differences notwithstanding, we consider the incomplete penetrance of DA indicates redundancy in the requirements for “normal” duodenal morphogenesis. As such, tm1 and tm2 *Fgf10* null embryos without DA provide a rich population for future study, to discern signature signaling differences when compared with wild-type, heterozygote and

DA-affected *Fgf10* null embryos. Understanding such differences may reveal key etiological components responsible for DA, as well as attributes for exploitation in future translations to achieve normal duodenal development in humans with a genetic predisposition to DA.

A Hypothesis-Driven and Disciplined Model

Fundamentally, we hypothesize that the etiology of DA in humans is genetic, with the causative genetic changes located downstream of the FGF10/FGFR2b signaling pathway. This downstream locus may account for the incomplete penetrance of DA in the murine model as discussed previously. Further, interruption of downstream signaling may account for both the lack of either *FGF10* or *FGFR2b* gene deletion in human DA cases previously screened for this (Tatekawa et al., 2007), as well as the absence of non-survivable associations of *FGF10* deletion in humans with DA, such as pulmonary agenesis.

However, before we may begin to scrutinize and develop this hypothesis within our *Fgf10* knockout model, our rigor must be first directed to gaining a better understanding of the temporo-spatial expression patterns relevant to the *Fgf10*/Fgfr2b signaling pathway in the murine fore and midgut. Existing similar expression patterns have focused on the more anterior foregut (Nyeng et al., 2007, 2008), the question of gut boundary regionalization (as reviewed in San Roman and Shivdasani, 2011), or lacked sufficient temporo-spatial resolution to adequately inform further investigation within our DA model (Fon Tacer et al., 2010). To understand the expression pattern of *Fgf10* in the developing duodenum, we plan to exploit the fidelity and imaging possibilities afforded by the *Fgf10*^{CreERT2}tomato^{flox/flox} [B6-*Fgf10*<tm1.1(cre/ERT2)Sbel>Gt(ROSA)26Sor<tm9(CAG-tdTomato)Hze>] and *Fgf10*^{flox/+}tomato^{flox/flox} [B6-*Fgf10*<tm1.2 Sms>] mouse strains (El Agha et al., 2012). Using these powerful developmental biology tools, we will assess stage- and tissue-specific expression of *Fgf10*, and correlate this with coincident expression patterns for *Fgfr2b* and their collective downstream targets. Expression patterns will be assessed using immunohistochemistry, as well standard and quantitative PCR methods.

Having established normal expression patterns, we will then be able to compare and contrast expression of the same signaling pathway components in tm1 and tm2 *Fgf10* heterozygote and *Fgf10* null embryos, both those with and without DA. The use of staged-gestation embryos, including embryos from earlier gestation than the previous threshold of E15.5, will allow us to determine when and how in development molecular divergence between these genetically distinct mice arises. These temporo-spatial molecular characteristics will then be correlated with concurrent normal or abnormal morphogenic changes in the developing duodenum.

A Testable and Translational Model

The ultimate aim of our DA research is to identify targets for translational therapies to prevent or ameliorate DA in humans. In humans, DA is associated with a wide range of

associated anomalies, the presence of which negatively impact prognosis (Hemming and Rankin, 2007; Choudhry et al., 2009). We have established an ethics-approved (#DB077) clinical database for all children managed with DA at The Royal Children's Hospital, Melbourne since 2000. Within this cohort of more than 100 DA patients (unpublished data), we have identified a thought-provoking subset with associated anomalies akin to Fgf10-Fgfr2b signaling-related defects, namely craniofacial, limb and lung anomalies (Sekine et al., 1999; De Moerloose et al., 2000; Teshima et al., 2016). We consider this human subset to be at particular "risk" of an underlying genetic changes impacting the FGF10/FGFR2b signaling pathway, and plan to undertake detailed clinical genetic phenotyping and exon sequencing of phenotypically homogenous patient groups. Candidate mutations thus identified will be tested by introducing these variants into mice using CRISPR, and confirming whether this (or these) mutations do indeed generate DA in these increasingly bespoke murine models. Moreover, a putative genetic mutation (or mutations) responsible for DA would represent a landmark development in the understanding of normal gut morphogenesis and DA, opening the door to superior genetic and antenatal counseling, as well as potential future therapies for DA-affected fetuses and families.

AUTHOR CONTRIBUTIONS

WT conceived the project and prepared the study design, methodology, molecular biology techniques, performed the microsurgical tissue dissection, image capture, analyzed and interpreted the results, and wrote and finalized the manuscript. MJ assisted in study design, molecular biology techniques, microsurgical tissue dissection, image capture, results analysis and interpretation, preparation, and finalization of the manuscript. LH assisted in design and performance of mouse strain derivation using CRISPR technique. IS assisted in design

and performance of mouse strain derivation using CRISPR technique and finalization of the manuscript. AC assisted in design and performance of molecular biology techniques. GS performed the molecular biology techniques. SK assisted in study design, results interpretation, and finalization of the manuscript. RL assisted in study design, establishment of methodology, molecular biology techniques, and finalization of the manuscript. JH mentored WT, assisted in study design, results interpretation, and finalization of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00530/full#supplementary-material>

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10.3. Appendix 3

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Quality of life outcomes in children born with duodenal atresia

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ABSTRACT

Purpose: The aim of this study was to determine long term quality of life (QoL) outcome for children who underwent surgery for duodenal atresia (DA).**Methods:** Patients were identified from a prospective database of neonatal DA cases managed at a tertiary pediatric surgical centre. The QoL was measured using the validated PedsQL™ 4.0 core score and PedsQL™ gastrointestinal module; higher score equates to better QoL. Participants' scores were compared to published control cohorts, age-matching the core score. Trisomy 21 was identified a priori as a possible confounder, informing subgroup analyses for children with and without trisomy 21.**Results:** Fifty-five families were invited to participate, with 38 surveys returned (39% male; median age 6.7y, range 2.7–17.3y). Seven participants had trisomy 21. There were no differences in QoL measures between all DA participants and controls. The PedsQL™ core score was significantly lower for DA participants with trisomy 21, but there was no accompanying difference in PedsQL™ gastrointestinal score.**Conclusions:** Children undergoing DA surgery in the neonatal period typically grow up to have a QoL comparable to a healthy population. Children with DA and trisomy 21 were more likely to have reduced overall QoL, albeit without an associated difference in gastrointestinal QoL score.**Level of Evidence:** Prognosis study – level II (prospective cohort study).

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Duodenal atresia (DA) is a congenital abnormality of the duodenum with an incidence of 0.9 to 1.2 per 10,000 live births [1, 2]. Surgical repair of DA has been recognized as the mainstay of treatment since the introduction of the duodenojejunostomy [3] and duodenoduodenostomy [4] for successful surgical repair in the early and mid-1900s. DA has a well-recognized association with trisomy 21 (T21), with approximately 4% of T21 patients also having DA [5], whilst approximately 30% of DA patients are found to have T21 [2, 6].

Post-operative morbidity and prolonged hospitalization in neonates with DA has been attributed to associated anomalies, prematurity and low birth weight [7]. Late complications recognized in children undergoing surgical repair of DA include duodenal motility disorders, adhesive bowel obstruction and gastro-esophageal reflux disease [6, 7]. Although the morbidity and mortality associated with DA has been

studied [7–9], the quality of life (QoL) of children born with DA after surgical management in early life has not been quantitated.

The aim of our study is to assess the QoL in children who underwent surgery duodenal atresia. Our primary outcome is to assess their QoL compared to a healthy cohort of children. Our secondary outcomes were to evaluate the QoL of children as rated by children compared to their parents, and to determine if there were other patient characteristics, such as type of duodenal atresia, had an impact on QoL.

1. Methods

1.1. Database

We have established an ethics-approved (#DB077) clinical database for all children managed with operatively confirmed DA at The Royal Children's Hospital, Melbourne since July 2000. This database includes information on demographics, antenatal history, delivery, postnatal case, associated anomalies, morphological type of DA, and operative details, including procedure performed and post-operative care.

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1.2. Quality of life outcome questionnaires

The Pediatric Quality of Life Inventory (PedsQL™) questionnaires are a collection of validated surveys that can be used to assess the quality of life of children with and without a breadth of medical conditions. The surveys have different versions that target different age groups but cover the same key themes but adjust their language and relevant activities to match the developmental age of the child. The PedsQL™ 4.0 core score is used to assess global quality of life and covers four domains: physical, emotional, social and school functioning. The first domain forms a physical subscale score, and the remaining domains form a psychosocial subscale score. For toddlers aged 2 to 4 years there is only a parent questionnaire containing 21 items. For the older age groups: young children (ages 5 to 7 years), children (ages 8 to 12 years) and teens (ages 13 to 17 years), there is an additional and separate questionnaire for the child to complete, with 23 items covering the same four domains. Irrespective of who completes the questionnaire, all questionnaires report on the child's quality of life.

The PedsQL™ gastrointestinal (GI) module is similar, in that there is only one parent questionnaire for toddlers aged 2 to 4 years, and a parent and child questionnaire for the other age brackets. However, each questionnaire contains 74 items over 14 domains covering a breadth of gastrointestinal symptoms the participant or parent may have encountered over the last 1 month.

Responses to the items in both questionnaires are given a score of 0 to 4. Items are then reverse scored and linearly transformed to give a score of 0 to 100. The PedsQL™ 4.0 physical and psychosocial subscale scores were similarly scored to give a result of 0 to 100. In both tools, a higher score equates to a better quality of life.

1.3. Survey Collection

DA patients 2 years or older as at the commencement of the study (May 30, 2018) were identified from the database, and their parents invited to participate. In accordance with local ethics committee recommendation, patients who had not visited our health service in the 5 years prior were first sent a tracing letter. Patients' families who responded to the tracing letter with current contact details, or who had been seen by our health services in the last 5 years, were then invited to participate and sent a PedsQL™ 4.0 generic core scale and PedsQL™ GI module questionnaire appropriate for the patient's age. Surveys were returned by provided, postage-paid envelopes. If no questionnaire was returned within 4 weeks of receipt, two attempts were then made to contact the parents via phone to return the survey or complete the survey over the phone.

1.4. Data analysis

Data presented is the results of the parental surveys, and are presented as means, median and standard deviations, unless otherwise stated. The results of the PedsQL™ scores were compared to published control groups [10, 11] using t-tests, with the core scores age matched. We could not identify any studies using the PedsQL™ score to examine quality of life in children with T21, thus we were unable to use a specific T21 only control cohort for this subgroup of patients. Matched parent and child results on the PedsQL™ scores were compared using Wilcoxin signed rank test. Categorical variables were compared using Fisher's exact test and numerical values were compared with Mann–Whitney U Test. Correlation between continuous variables were analyzed using regression analysis and quantitative variables between three or more categorical groups were compared using one-way analysis of variance (ANOVA).

In recognition of T21 as a possible confounder of QoL outcomes, a priori determined subgroup analyses were performed comparing healthy controls and DA participants with or without T21. Further, drop-out analysis was performed to address potential bias or differences between DA

cases participating in the study and those not participating. Data were analyzed using SPSS Version 25.0 (Armonk, NY: IBM Corp); p value <0.05 was considered statistically significant for all tests.

1.5. Ethical considerations

Ethical approval for this study was obtained from The Royal Children's Hospital Human Research Ethics Committee (HREC 38054A).

2. Results

2.1. Participant characteristics

We identified 110 potential participants in our database who met our inclusion criteria. However of these, 12 children were deceased and 43 families were either not contactable by the tracing letter or had letters returned as not-deliverable. Thus, 55 families were contacted and invited to participate. Of these, 38 returned completed survey questionnaires, which correlates to a response rate of 69% (38/55). Twenty three children with DA were aged 5 years or older, and their families received both a parent and child survey questionnaires. Seventeen families returned the additional child survey questionnaire. Of the remaining six families who did not return additional child survey, five had children with DA and T21.

Median age at the time of the study was 6.7 years (2.7 to 17.3) and 39% were male. Further population type including type of DA, operation performed (including anastomosis type for cases managed by duodenoduodenostomy), incidence of T21 and other associated anomalies are displayed in Table 1.

Drop-out analysis to assess for possible bias between our participants (n = 38) and non-participants (n = 72) is also summarized in Table 1. The median age in the participant group (median = 6.7y) was a significantly younger than the non-participant group (median = 10.0y, U = 896, p = 0.02). Otherwise, there were no significant differences between our participant and non-participant groups with respect to morphological type of DA, operation performed, VACTERL association, CHARGE syndrome, cardiac abnormalities or genetic anomalies, including T21.

Table 1
Patient characteristics of duodenal atresia.

Characteristic	Participants (n = 38)	Non-participants (n = 72)
Age in years		
Median (range)	6.7 (2.7–17.3)	10.0 (2.5–17.9)
Sex, n (%)		
Male	15 (39)	39 (54)
Female	23 (61)	33 (46)
Type of DA, n (%)		
Type I	16 (42)	40 (56)
Type II	2 (5)	2 (3)
Type III	17 (45)	27 (38)
Not recorded	3 (8)	3 (4)
Operation, n (%)		
Duodenoplasty	5 (13)	8 (11)
Duodenoduodenostomy	33 (87)	64 (89)
Diamond	26 (68)	50 (69)
Side-by-side	2 (5)	7 (10)
End-to-end	5 (13)	4 (6)
Not recorded	0 (0)	3 (4)
Additional anomalies, n (%)		
VACTERL (3+)	1 (3)	7 (10)
CHARGE	1 (3)	0 (0)
Cardiac		
Minor	10 (26)	17 (24)
Major	6 (16)	8 (11)
Genetic		
None	20 (53)	42 (58)
T21	7 (18)	14 (19)
Other	4 (11)	3 (4)
Not tested	7 (18)	14 (19)

2.2. Primary outcomes

Results of the PedsQL™ scores are summarized in Table 2. Overall, our results demonstrate that children undergoing surgical repair of DA as neonates have a similar quality of life compared to age-matched published controls [10] as measured by the PedsQL™ core score. On subgroup analysis however, participants with DA and T21 scored significantly lower than the control group ($p = 0.03$). This is likely due to participants with T21 scoring significantly lower than the control group in the social subscore ($p < 0.01$); Table 2. Gastrointestinal symptoms, as scored by the PedsQL™ GI score, were similar between our participants with DA and healthy controls [11] ($p = 0.35$). Further, in contrast to the core scores, subgroup analyses comparing PedsQL™ GI score in DA participants with or without T21 and the control group did not reveal any significant differences; Table 2.

2.3. Secondary outcomes

There was no difference between the parent reported and the child reported quality of life measured by the PedsQL™ core scores ($Z = 0.804$, $p = 0.421$). However, measuring gastrointestinal symptoms using the PedsQL™ GI score, parents reported fewer concerns and symptoms than their children (median = 93.2 vs 88.9, $Z = 2.05$, $p = 0.036$).

Age was not a significant factor in determining predicting a participants PedsQL™ core score ($r_s = -0.15$, $p = 0.36$) or PedsQL™ GI score ($r_s = -0.18$, $p = 0.28$). Similarly, morphological type of DA was not predictive of a participant's PedsQL™ core score ($F(2, 33) = 0.55$, $p = 0.59$) or of their PedsQL™ GI score ($F(2, 32) = 0.39$, $p = 0.68$).

3. Discussion

Despite requiring hospitalization and operative management early in life, this study shows children managed for DA have an overall QoL comparable with age-matched healthy controls. Furthermore, this

positive outcome for DA patients is also seen when considering GI specific QoL parameters. The long term clinical outcomes of DA have been retrospectively examined [6], however, there is no published QoL data for patients with DA. This prospective QoL study offers new and positive insights into the long-term prognosis of this neonatal condition.

DA is among a number of surgical neonatal conditions for which survivors show QoL outcomes comparable with healthy controls. Neonatal conditions such as congenital diaphragmatic hernia, Hirschsprung Disease (HD), gastroschisis, omphalocele and esophageal atresia all have similar QoL outcomes to healthy children, as measured by the PedsQL™ generic core scores [12–15]. This is in contrast to neonatal conditions such as necrotizing enterocolitis, for which survivors do show worse QoL outcomes than healthy children [14]. When compared to children with chronic childhood gastrointestinal illnesses (both functional and organic), who score lower on PedsQL™ GI module as compared to healthy controls, children who have undergone surgery for DA have similar PedsQL™ GI module scores to healthy children [11, 16]. These findings will inform parent counseling by clinicians as to the expected long-term recovery of GI function in children with surgically-repaired DA.

In our study, age was not a predictive factor in determining QoL scores. However, in some congenital surgical conditions, such as HD, a downtrend in PedsQL™ core scores with age has been reported [12]. This distinction is likely related to the timing, and upper GI (DA) vs lower GI (HD) symptom implications of these two congenital conditions. Complications of DA surgical repair are likely to be maximal in the early postoperative course, i.e. neonatal period, and most likely within the index admission. Thereafter, long-term complications such as gastro-esophageal reflux are unlikely to worsen or gain renewed QoL importance with advancing age. This is in contrast to HD, where toilet training in the toddler years, constipation (or indeed fecal incontinence) in the early school years and psychosocial development while managing abnormal bowel habits in the later school years, each have explicable and negative effects on the QoL scores [12].

Our participants with T21 and DA had a significantly lower PedsQL™ core score compared to healthy controls, in distinction to the normal QoL for DA participants without T21. This T21-specific reduction in QoL was not evenly distributed in all domains, being limited to core score and social score, but not demonstrated in the PedsQL™ GI module. The normal GI symptom scores in children with both DA and T21, suggest that the QoL impairment otherwise evident in this subgroup may not be directly attributable to DA. The impact of T21 or QoL is however important in our patient group, and further assists clinicians and other healthcare professionals which patients and families may be in greatest need of support. For this reason, we consider future studies that examine the general QoL in congenital gastrointestinal conditions with a higher incidence of T21, e.g. HD, anorectal malformations and esophageal atresia, should undertake planned subanalysis of participants with T21 to address this as potential confounding factor biasing their results.

Prospectively collecting the clinical data in our study ensured we had almost complete data sets for all DA patients approached for inclusion in this study. However, in a small number of cases, the operation records did not specify DA morphological type and/or the specific type of anastomosis performed (i.e. diamond, side-by-side or end-to-end). Despite this potential limitation, our drop-out analysis of study participants and non-participants confirmed no difference in type of DA, comorbidities or type of surgery between the participants and non-participants. Given this consistency, disease-related factors do not appear to have influenced participation in this study. This notwithstanding, the participation or non-response bias may yet limit the applicability of our results, where families whose children have poor outcomes may consequently disengage from the opportunity to participate in such research. It could also be argued, that families of children with poor outcomes may seek out participation to make clear their child's plight, and so participation bias is not a given.

In conclusion, this study shows that children undergoing surgical repair of DA in early life typically have comparable QoL outcomes with

Table 2
Total PedsQL™ 4.0 Generic Core Scale and subscores, and PedsQL™ GI module scores, for all participants ($n = 38$) with subanalysis of those with ($n = 7$) and without ($n = 31$) trisomy 21.

	Participants	Control	p-Value
PedsQL™, mean (SD)			
Overall	80.8 (19.5)	83.1 (14.7)	0.45
T21	62.6 (21.5)	82.8 (14.7)	0.03
Non-T21	84.9 (16.8)	86.5 (14.8)	0.59
Physical, mean (SD)			
Overall	85.6 (21.9)	85.0 (18.8)	0.86
T21	67.4 (30.2)	84.3 (18.8)	0.16
Non-T21	89.7 (17.7)	85.1 (18.9)	0.16
Psychosocial*, mean (SD)			
Overall	78.0 (19.7)	82.0 (14.6)	0.22
T21	60.6 (20.8)	81.8 (14.6)	0.03
Non-T21	82.0 (17.5)	82.1 (14.6)	0.98
Emotional, mean (SD)			
Overall	76.2 (20.4)	81.5 (16.2)	0.11
T21	72.9 (20.6)	81.2 (16.1)	0.29
Non-T21	76.9 (20.6)	81.6 (16.5)	0.22
Social, mean (SD)			
Overall	78.0 (26.9)	84.0 (18.8)	0.18
T21	46.3 (28.2)	84.3 (18.8)	<0.01
Non-T21	85.2 (21.2)	83.9 (18.4)	0.75
School, mean (SD)			
Overall	78.6 (24.4)	80.4 (18.5)	0.66
T21	56.4 (32.5)	80.1 (18.4)	0.11
Non-T21	83.9 (19.1)	80.5 (18.6)	0.39
Gastrointestinal Module, mean (SD)			
Overall	87.6 (15.6)	90.0 (12.7)	0.35
T21	84.2 (46.6)	90.0 (12.7)	0.37
Non-T21	88.3 (15.7)	90.0 (12.7)	0.57

* The psychosocial subscore is the average of all the items in the emotional, social and school subscores.

their healthy peers. Those children with T21 as well as DA represent an important subgroup, whose long-term needs may differ from other DA patients, even if not as a result of DA-related outcomes per se. Our study is the first to address such questions of QoL and specific gastrointestinal symptom QoL outcomes in DA patients utilizing validated PedsQL™ tools. The findings from this study may assist pediatric surgeons and other healthcare professionals, and improve the nature and relevance of counseling for families of children with DA when discussing long term outcomes, especially for those facing the challenges of both DA and T21.

Ethics

This study was approved by the Royal Children's Hospital (Melbourne) Human Research Ethics Committee (HREC 38054A).

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Disclosure

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