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The Developmental Origins of Tracheo- Oesophageal Malformations:

Investigating the molecular and morphological basis of
foregut abnormalities in Adriamycin treated mice and in a
newly established 3D foregut culture system.

Danielle Mc Laughlin, MB BCh B.A.O., MRCSI

A thesis submitted to the University of Dublin in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

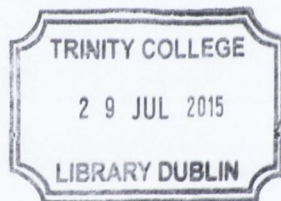
January 2015

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Summary

Oesophageal Atresia/Tracheo-oesophageal fistula (OA/TOF) is a congenital malformation that requires urgent and complex surgery soon after birth and is associated with life-long respiratory and gastro-intestinal morbidity. Although genetic aetiology has been identified for some syndromic forms, in the majority of cases the cause of this important clinical condition remains unknown. These malformations arise in the oesophagus and trachea during early development, when they share a common embryological precursor, the foregut. Proper foregut separation into these derivatives provides a critical framework for eventual respiratory and swallow function. A clear understanding of the aetiological basis for oesophageal defects within the developmental window in which they arise will aid future diagnostic and therapeutic strategies.

As early development cannot be analysed in humans, animal models have enabled the investigation of complex congenital malformations, allowing the origin of birth defects to be comprehended. Use of these models has led to the identification of crucial regulatory factors in early development for proper foregut separation and the realisation that correct molecular patterning of foregut regions, designating tissue as either oesophageal or respiratory in nature, is synonymous with successful oesophagus and trachea formation.

Adriamycin treatment of embryonic mice produces tracheo-oesophageal malformations that closely mimic the human condition. In this model, structural anomalies of the foregut and the notochord, an important source of regulatory signals in the embryo, occur together. Foregut and notochord defects also arise simultaneously in mice which lack the *Noggin* gene. *Noggin* is an antagonist of Bone Morphogenic Protein (BMP) signalling, a key regulatory pathway acting on various tissues during development. BMP signal is transduced within cells, following ligand to receptor binding, by the phosphorylation of SMAD proteins which control downstream target gene transcription. The presence of notochord defects and the detection of disturbed BMP related gene expression together with foregut malformations have led to the hypothesis that abnormal BMP signalling as well as notochord defects contribute essentially to the pathogenesis of OA/TOF

In this thesis I have examined the role of both BMP signalling and the notochord in foregut development employing both *in vivo* and *in vitro* approaches. I examined key indicators of BMP signalling in Adriamycin treated mice and found that BMP over activity occurs in a specific area of the developing foregut. This was apparent in terms of overall BMP response, highlighted by the detection of phosphorylated SMAD, and in the spatial expression analysis of a newly compiled group of BMP target genes which are expressed

in the foregut. I observed that the site of BMP signalling abnormalities within the foregut were intimately related with the position of adjacent notochord defects.

I further explored the effect of both BMP activity and the notochord on foregut separation by first establishing an *in vitro* model which supports foregut development in 3D explant culture. This culture system was assessed using known markers of normal foregut separation as well as a morphological staging system produced during the course of this work. This confirmed that isolated foregut explant culture successfully recapitulated foregut separation into oesophagus and trachea in a manner that is highly faithful to what is seen normally *in vivo*.

Physical manipulation of the notochord and molecular manipulation of BMP signalling levels in foregut culture revealed that excess BMP signal, in the form of Bmp4, but none of the other conditions, resulted in foregut malformations. The comparison of Bmp4 treated foreguts to well-established models of abnormal foregut development, including Adriamycin treated mice, confirmed that Bmp4 treatment was sufficient to induce an OA/TOF phenotype. In addition I demonstrated that excess Bmp4 signal induced a change in the nature of the foregut tissue away from oesophageal and towards a respiratory type, which may be a crucial mechanism in the pathogenesis of tracheo-oesophageal malformations.

The work presented in this thesis provides new insight regarding the pathogenesis of OA/TOF and in particular identifies BMP signalling to be crucial for proper foregut separation through regulation of tissue identity within foregut regions. This has been revealed by the effective use of complimentary *in vivo* and *in vitro* approaches which together provide a system to identify further key regulatory factors in the molecular control of foregut morphogenesis.

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I dedicate this thesis to my family and especially to my brother Joseph, thinking of you always.

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Mc Laughlin D., Murphy P., Puri P., “The molecular and morphological impact of notochord manipulation on the foregut in 3D explant culture.”, *In Proceeding of 16th European Congress of Paediatric Surgery*, Dublin, Ireland, 2014 [Presented June 21st 2014, Dublin, Ireland].

Mc Laughlin D, Murphy P, Puri P., “Altered Tbx1 gene expression is associated with abnormal oesophageal development in the Adriamycin mouse model of Oesophageal Atresia/Tracheo-Oesophageal fistula”, *In Proceeding of the Aesculap Prize Session, 26th Symposium on International Paediatric Surgical Research*, Cape Town, South Africa, November 2013. [Presented Nov 29th 2013, Cape Town, South Africa]. Awarded 3rd prize.

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Mc Laughlin D., Murphy P., Puri P., “Disruption of BMP signaling in the foregut of Adriamycin mouse model of Oesophageal Atresia/Tracheo-Oesophageal fistula”, *In Proceeding of the Young Investigator Award Session, 15th European Congress of Paediatric Surgery*, Leipzig, Germany, 2013 [Presented June 6th 2013, Leipzig, Germany].

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List of Abbreviations

3D 3 Dimensional

AMM Adriamycin Mouse Model

AP antero-posterior

ARM Adriamycin Rat Model

BMP, Bmp (r) Bone morphogenic Protein (receptor)

CO₂ Carbon Dioxide

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxyribonucleic acid

DV dorso-ventral

E Embryonic Day

E-Cadherin Endothelial Cadherin

EMAP Edinburgh Mouse Atlas Project

EMT Epithelial to mesenchymal transition

EMI Epithelial to mesenchymal interaction

FBS Fetal bovine serum

FGF, Fgf (r) Fibroblast growth factor (receptor)

GFP Green fluorescent protein

Hnf3b Hepatocyte nuclear factor 3 beta

IHC Immunohistochemistry

MGI Mouse Genome Informatics

Nkx2.1 Neurokinan 2 homeobox 1

OA Oesophageal atresia

OPT Optical Projection Tomography

PBS Phosphate buffered saline

PFA Paraformaldehyde

pSMAD phosphorylated SMAD

mRNA messenger Ribonucleic acid

RA(R) Retinoic acid (receptor)

SHH, Shh Sonic Hedgehog

SOX2, Sox2 Sex determining region Y (Sry)– box 2

Tbx T-box

TGF Transforming growth factor

TOF Tracheo-oesophageal fistula

TS Theiler Stage

UV Ultraviolet

VACTERL Vertebral Anorectal Cardiac Tracheo-oEsophageal Renal Limb

Symbols and measures

M	molar
mM	minimolar
min	minute
hr	hour
ml	millilitre
nm	nanometre
g	gram
µl	microlitre
µg	microgram
ng	nanogram
µm	micron
α	alpha
β	beta
bp	base pair
°C	degrees centigrade

Chapter 1

Introduction

1.1 Overview

The basic life sustaining functions of nutrient ingestion and oxygen intake depend on anatomical apparatus which share a common foundation in development. The foregut arises from folding of the endoderm germ layer following gastrulation, forming a single precursor structure for the generation of both the ventral trachea and the dorsal oesophagus (Wells and Melton, 1999). Proper foregut separation into these derivatives establishes a critical developmental framework for eventual respiratory and swallow function. In humans a spectrum of tracheo-oesophageal anomalies, encompassed by the term oesophageal atresia/tracheo-oesophageal fistula (OA/TOF), arise in approximately 1 in 3500 births annually (Pedersen et al., 2012) in isolation or in association with other congenital birth defects. These malformations require urgent and complex surgery soon after birth and are associated with life-long respiratory and gastro-intestinal morbidity (Holland and Fitzgerald, 2010). The typical blind ending proximal oesophagus and abnormal connecting fistula between the stomach and trachea which occur in afflicted neonates are thought to result from faulty foregut separation in the early embryo (reviewed in Ioannides and Copp, 2009). Specific genetic aetiology has been identified for some syndromic forms of this condition, however for OA arising in isolation or occurring within the VACTERL (vertebral, ano-rectal, cardiac, tracheo-oesophageal, renal and limb) association of birth defects, the cause remains obscure (Solomon, 2011, Shaw-Smith, 2006).

Animal models enable analysis of the aetiology and pathogenesis of complex congenital malformations, allowing the developmental basis of birth defects to be comprehended, and have led to major advances in the surgical and therapeutic management of these conditions (Mortell et al., 2006). Analysis of molecular and morphological behaviour in animal models of abnormal foregut development crucially aids in determining the pathogenic mechanisms responsible for OA/TOF (Mc Laughlin et al., 2013). OA associated single gene mutations in humans have been confirmed in mouse models to induce structural foregut defects (Fausett and Klingensmith, 2012). Adriamycin treatment of rodents produces a remarkably human-like VACTERL phenotype in embryos, including a spectrum of tracheo-oesophageal malformations, providing a powerful tool for the detection of altered molecular signals associated with morphological foregut disturbance (Dawrant et al., 2007b, Ioannides et al., 2002, Diez-Pardo et al., 1996, Beasley et al., 2000, Mc Laughlin et al., 2013). A distinct feature in both the Adriamycin Rat

Model (ARM) and Adriamycin Mouse Model (AMM) are structural abnormalities of the notochord, informally referred to as branches, which represent failure of the notochord to properly delaminate away from the foregut in the dorsal midline towards the neural floor plate (Hajduk et al., 2012, Qi and Beasley, 1999c). Notochord abnormalities are also found in the Noggin knockout mouse model in association with tracheo-oesophageal malformations (Que et al., 2006). Noggin is an extracellular antagonist of Bone Morphogenic Protein (BMP) signalling (Zimmerman et al., 1996). Diverse and vital roles for BMPs, members of the TGF-beta superfamily of highly evolutionary conserved signalling molecules, are well established across embryogenesis. BMP signalling manipulation results in faulty foregut separation (Domyan et al., 2011, Li et al., 2008b) however the means by which these signals act to induce structural change are not well understood.

This thesis aims to examine the pathogenesis of OA/TOF in a developmental context by studying the molecular control of foregut separation in the early embryo. In particular I investigated the role of BMP signalling and notochord defects in foregut malformations, using the AMM as well as an innovative *in vitro* approach. In this chapter I synthesise current knowledge of foregut development as it pertains to the pathogenesis of OA/TOF. I summarise the key embryological features of foregut and notochord development (section 1.1), review the aetiology of human OA/TOF (section 1.2), examine the importance of the AMM model of OA/TOF (section 1.3), explore mechanisms by which foregut development may be disturbed and in particular at a molecular level by analysing evidence from various animal models of OA/TOF (section 1.4), and introduce the use of *in vitro* techniques to investigate foregut development (section 1.5).

1.2 Key embryological features of foregut and notochord development

1.2.1 Early embryogenesis

Gastrulation organises cells from the single epiblast layer into 3 layers; endoderm, mesoderm, and ectoderm, by differentiation of precursor cells during cell migration through the primitive streak (reviewed in Solnica-Krezel and Sepich, 2012). Subsequently, in the cup shaped embryo (from E7.5 in mice), the endoderm sheath undergoes extensive folding anteriorly, posteriorly and ventrally to form a primitive gut tube, the cranial or anterior end of which becomes the foregut, and the midgut and hindgut arising caudally (posteriorly), following turning of the embryo on its anterior to posterior axis (Figure 1.1) (Wells and Melton, 1999). The oesophagus and trachea share a common embryological predecessor in the foregut, a simple tubular structure of endoderm cells surrounding a lumen, encompassed within the anterior trunk mesoderm in the post-gastrulation embryo. A series of complex morphological manoeuvres orchestrated by various molecular signalling pathways are responsible for the development of two anatomically and functionally distinct structures by division of this single precursor.

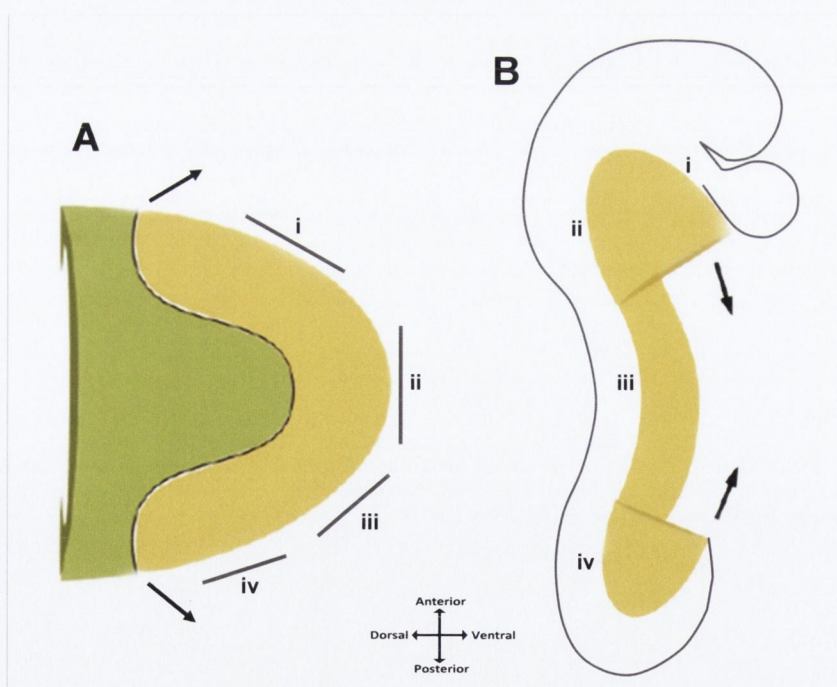


Figure 1.1: Formation of the foregut. Schematic of E7.5 cup shaped embryo (A) showing endoderm (yellow) sheath underlying the mesoderm and ectoderm. Roman numerals mark corresponding areas in the primitive foregut (B) once folding occurs (arrows) with directionality as shown. Regions i and ii are fated to become ventral and dorsal endoderm anterior foregut respectively. Adapted from Wells and Melton, 1999.

Patterning signals in the early embryo designate cell fate identity in specific regions of the foregut endoderm, directing organogenesis of the thyroid, thymus, and lungs from ventral endoderm and oesophagus from dorsal endoderm of the anterior foregut (as reviewed by Zorn and Wells, 2009a). Specification of cell fate may occur as early as migration through the primitive streak and is established by the end of gastrulation according to cell fate mapping experiments (Lawson et al., 1986). Initial induction of anterior endoderm identity by Nodal, a fundamental signalling pathway in vertebrate development, presages formation of the early gut tube (Thomas et al., 1998). Correct antero-posterior (AP) and dorso-ventral (DV) patterning of the foregut endoderm then refine domains for subsequent organogenesis and depend on crosstalk of signals between the foregut endoderm and the surrounding mesoderm (epithelial-mesenchymal interaction) to establish these precise regions (Masters, 1976, Shannon et al., 1998). For example the specification of the respiratory primordia from the ventral anterior foregut requires signalling from the adjacent cardiac mesoderm (Serls et al., 2005). Molecular factors crucial for proper anterior foregut separation into ventral trachea and dorsal oesophagus have been identified in animal models with structural foregut defects, and it is apparent from these studies that the foregut requires finely balanced input from many developmentally important signalling pathways (as discussed in more detail in section 1.5.3).

1.2.2 Notochord development

One of the most striking and pivotal discoveries in Adriamycin treated rodent embryos was abnormality of the notochord, a transient but crucial structure in developing vertebrate embryos. The notochord is a specialised structure of mesodermal origin, defining members of the chordate phylum by its presence. The notochord is derived from the cells of the node, the primary organiser, as it regresses during gastrulation. These cells initially lie dorsally adjacent to the endoderm in the midline. They organise into a rod shaped structure as the endoderm layer is folding to form the foregut, defining the anterior-posterior axis of the embryo and allowing axis elongation by providing structural rigidity (Adams et al., 1990). The notochord rod then moves dorsally away from the dividing foregut, progressing from anterior to posterior, towards the neural tube in a process termed delamination, and comes to lie adjacent to the neural floor plate, eventually being resorbed to form part of the nucleus pulposus of the intervertebral discs (Jurand, 1974). This process is summarised in Figure 1.2.

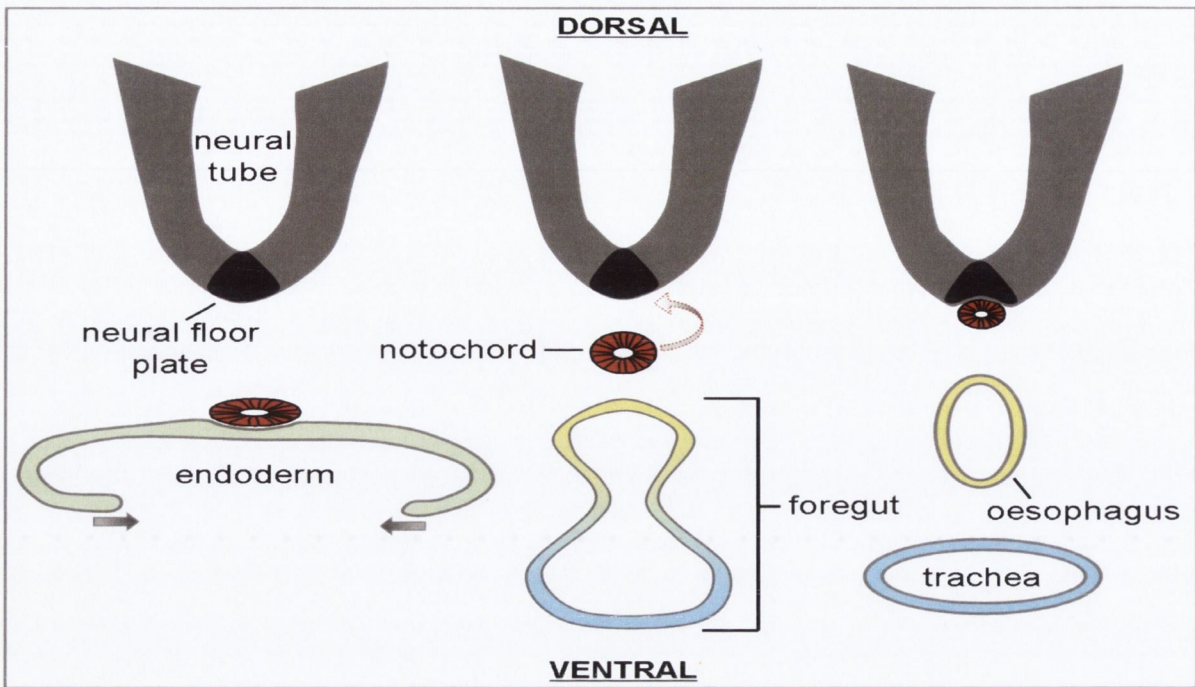


Figure 1.2 Schematic representation of notochord delamination during foregut separation. The notochord (in red) has formed an organised rod shaped structure dorsally adjacent to the endoderm layer (in green) as the latter undergoes folding to form the foregut (converging grey arrows). As the foregut divides to form the oesophagus dorsally and the trachea ventrally, the notochord moves dorsally to lie adjacent to the neural floor plate towards which it is known to express powerful signalling (red dashed arrow) while also regressing in size.

The notochord is a potent source of signalling during organogenesis, and strongly influences molecular patterning and morphology of adjacent tissues such as the dorsally located neural tube (van Straaten et al., 1988). The notochord acts a primary organiser, patterning cells for dorso-ventral, anterior-posterior and left-right orientation (reviewed in Cleaver and Krieg, 2001). Notochord signals direct somite development, illustrated by failure of somite formation when the notochord is ablated, and transplanted notochord can induce the formation of a secondary neural floor plate in adjacent lateral neural tube (Bitgood and McMahon, 1995, Straaten et al., 1988). The notochord expresses developmentally important signalling genes including Sonic hedgehog (Shh) and Activin, known to permit the development of foregut derived organs such as the pancreas (Hebrok et al., 1998). Despite their close proximity, the influence of the notochord on the ventrally located foregut as it separates to form oesophagus and trachea is undetermined.

1.2.3 Foregut separation

In humans, foregut separation into oesophageal and respiratory derivatives occurs during the 3rd through 7th weeks of gestation (Gray and Skandalakis, 1972), corresponding to embryonic day (E) 9 to E11.5 in the mouse (Theiler, 1989a). The early developmental errors from which tracheo-oesophageal malformations arise are not well understood. Even the normal embryological steps leading to foregut division defy consensus (Kluth and Fiegel, 2003). Numerous theories compete to clarify these complex morphogenetic processes and, when explaining the developmental origins of OA/TOF, individual groups reflect varied interpretations of normal events. Classically the onset of foregut separation is described as the emergence of the respiratory primordia (at E9.5 in mice, day 28 in humans), commonly referred to as lung buds, which bulge from the ventro-lateral walls of the foregut into the surrounding mesoderm and later elongate, displaying branching morphogenesis to achieve lung formation (Figure 1.3). The molecular designation of the site of lung bud formation in the foregut is reflected in the expression of the respiratory specific marker *Nkx2.1* in the ventral foregut endoderm from E8 (Maeda et al., 2007). The induction of lung budding depends on high levels of Fibroblast growth factor (FGF) from the adjacent cardiac mesoderm and is additionally regulated by BMP, Retinoic Acid (RA), Sonic Hedgehog (SHH) and Wnt signalling (Malpel et al., 2000, Chen et al., 2007, Pepicelli et al., 1998, Weaver et al., 2000)

Subsequent to early lung bud formation, the foregut divides from the pharynx anteriorly to the site of the lung buds posteriorly, allowing mesoderm to intervene. This generates a ventral trachea leading to the respiratory structures and a dorsal oesophagus in continuity with the stomach (Kaufman and Bard, 1999). Very little is definitively understood regarding either the molecular or cellular mechanisms by which this process of division is achieved. DV patterning of the foregut at this level is illustrated in mutually exclusive domains for *Sox2* dorsally and *Nkx2.1* ventrally as further explored in section 1.5.3.1. The specific molecular influences that designate dorsal foregut identity are unknown however it has been suggested that pathways which regulate *Sox2* expression, such as SHH and BMP, may contribute to oesophagus formation (Que et al., 2007, Domyan et al., 2011).

In the mouse distinct oesophagus and trachea emerge by E10.5 (Cardoso and Lu, 2006). The mechanism by which this division occurs is suggested to be a process of septation of the lumen by inwardly advancing lateral foregut endoderm wall ridges which

cross the lumen and meet to create a tracheo-oesophageal septum (Gray and Skandalakis, 1972). Subsequent posterior or bidirectional elongation of the oesophageal and tracheal tubes is proposed to complete the process of foregut division (Williams et al., 2003). In this chain of events, failure of septation could account for the development of tracheo-oesophageal malformations.

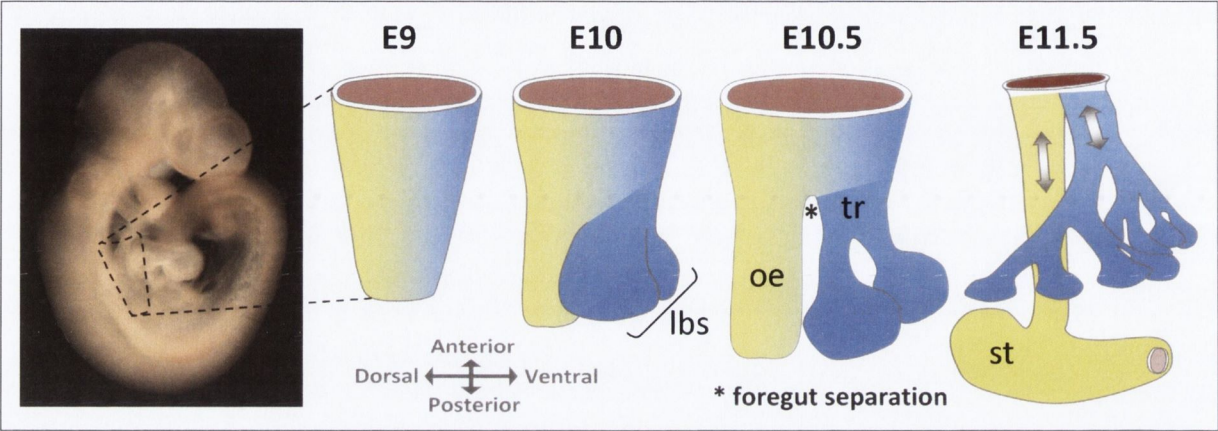


Figure 1.3 Foregut separation into oesophagus and trachea in mice. Pre-patterning of the foregut into dorsal (yellow) and ventral (blue) domains is reflected by the shading. The lungbuds (lb) arise from the ventro-lateral foregut walls between E9 and E10 and elongate together with the division of the foregut by septation of the lumen (asterix) to produce distinct oesophagus (oe) and tracheal (tr) structures. Further elongation of the oesophagus and trachea may occur posteriorly or bidirectionally (see arrows) to complete the process. stomach *st*.

The compartmentalisation of the foregut lumen by a septum is not universally agreed upon (Zaw-Tun, 1982). O’Rahilly and Muller argue that the trachea forms by lengthening of a respiratory outgrowth from a single site in the ventral foregut (referred to as the Water and Tap theory) (O’Rahilly and Muller, 1984). The designation of cells to respiratory lineage in ventral foregut, proximal to the origin of the respiratory primordia, as indicated by the expression of the respiratory specific *Nkx2.1* gene at that site (Desai et al., 2004), would however suggest that cells anterior to the level of lung budding will go on to form tracheal tissue. The anterior undivided foregut, measured from the pharynx to the upper limit of respiratory structures, has been shown to significantly shorten between E11.5 and E12.5, suggesting that progressive septation is a key mechanism (Ioannides et al., 2010).

Section 1.3 The aetiology of human OA/TOF

1.3.1 OA and the VACTERL association

OA/TOF occurs in approximately 1: 3,500 newborns every year (Pedersen et al., 2012). The term OA/TOF encompasses a variety of tracheo-oesophageal malformations of obscure aetiology, which, despite the comparative frequency with which they arise, remain highly complex to manage both medically and surgically. In 85% of cases the upper oesophagus is blind ending and an anomalous connection exists between the trachea and the stomach, the trachea-oesophageal fistula (Spitz, 2007, Pedersen et al., 2012) (Fig 1.4 A). This and other anatomical variants including; oesophageal atresia without a fistula, both upper and lower oesophageal remnant fistula to the trachea, upper oesophagus to trachea fistula, and fistula without oesophageal atresia, were classified by Gross in 1953 (Gross, 1953). These anatomical disturbances prevent swallow and allow gastrointestinal contents to spill into the respiratory tree. This life-threatening condition requires immediate surgery at birth to allow feeding and prevent damage to the vulnerable neonatal lungs.

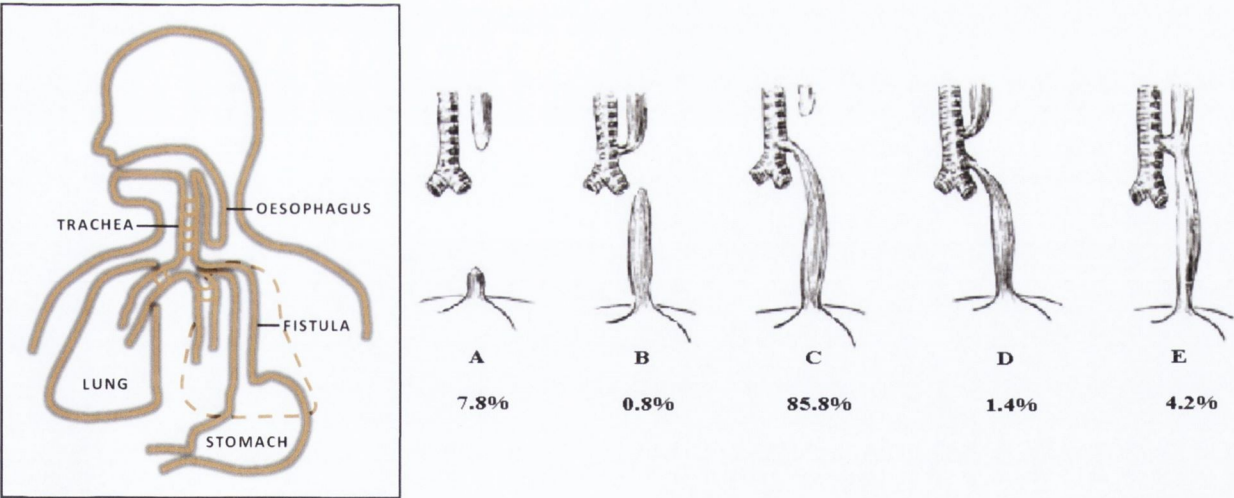


Figure 1.4: Anatomical varieties of OA/TOF showing frequency of occurrence by Gross classification. On the left in orange the human anatomical context for a Gross Type C OA/TOF is shown. The fistula refers to any abnormally communicating structure between the trachea (shown with transverse cartilage rings) and the upper gastrointestinal system Other human OA/TOF phenotypes are shown in the right panel (A to E) reflecting various types of fistula and are described in the text. Adapted from Gross 1953.

Initial surgical repairs were pioneered in the 1940's (Myers, 1986). Although survival rates have since benefitted immensely from advances in surgical technique and neonatal care, long term morbidity is frequent and problematic (Kovesi and Rubin, 2004). Chronic respiratory problems, oesophageal dysmotility, oral aversion, and gastro-oesophageal reflux disease (GERD) often confer poor quality of life on affected children post-operatively (Spitz, 2007, Tomaselli et al., 2003). Some of these problems may be sequelae of surgical intervention, however there may be an inherent inability of the malformed upper gastrointestinal structures to fulfil the long-term aim of surgical correction, which is to re-establish a functional oesophagus. Furthermore in some cases when the native oesophagus cannot function, despite the evolution of complex oesophageal replacement techniques (Kunisaki and Foker, 2012), no absolute surgical cure exists.

OA/TOF arises in isolation only 50% of the time; the remainder of cases present with other defects, most frequently with the VACTERL association of congenital anomalies in up to 30% of cases (de Jong et al., 2010, Pedersen et al., 2012). VACTERL occurs in up to 1:10,000 neonates per year (Botto et al., 1997), and is defined by the occurrence of at least 3 of Vertebral, Ano-rectal, Cardiovascular, Tracheal, Esophageal, Renal and Limb defects simultaneously (Solomon, 2011). VACTERL is classified as an association, defined by Lubinsky in 1986 as “derivatives of nonspecific teratologic events acting on developmental fields” (Lubinsky, 1986). These defects coalesce in a non-random fashion, and single or multiple diffuse insults may affect multiple organ anlagen during a critical stage in early development. The nature of the insult in VACTERL and the mechanism by which it induces a variety of tissue anomalies remains unknown.

1.3.2 Genetic aetiology of OA

A minority of OA/TOF cases arise in syndromes with known genetic background, for example, CHARGE syndrome, Feingold syndrome and Fanconi anaemia (Shaw-Smith, 2006) , however no unifying aetiological factor has been identified for OA/TOF occurring in isolation or along with VACTERL. The low rate of familial recurrence suggests that an overall monogenetic causation for OA/TOF is unlikely (Brunner and van Bokhoven, 2005). The human syndromes or genetic mutations that have been reported to manifest OA/TOF are summarised in Table 1.1.

Table 1.1 Genetic aetiology of syndromic forms of OA/TOF

Gene	Chromosomal Locus	Syndrome name	Reference
SOX2	3q26.3	AEG	Williamson et al., 2006
FOXF1	16q24.1	-	Stankiewicz et al., 2009
NOG	17q22	-	Marsh et al., 2000
GLI3	7p13	Pallister Hall	Johnston et al., 2005
MYCN	2p24.1	Feingold	Marcelis et al., 2008
CHD7	8q12	CHARGE	Jongmans et al., 2006
TBX1	22q11	Di George	Lee et al., 2008
FANC	Xp22.2	Fanconi anemia	Auerbach, 2009 Holden et al., 2006
RARA	17q21.1	-	Mendelsohn et al., 1994
MID1	Xp22.3	Opitz B/GGG	De Falco et al., 2003
MKKS	20p12	McKusick-Kaufman / Bardet Biedl	Slavotinek et al, 2000 Stone et al, 2000
-	-	Fryns	Ayme et al., 1989
-	-	Congenital Microgastia–limb reduction complex	Lurie et al., 1995
PCSK5	9q21.3	-	Szumska et al., 2008
Chromosome 18		Trisomy 18	Schnizel et al, 2001
Chromosome 21		Trisomy 21	Bianca et al, 2002
Chromosome 13		Trisomy 13	Depaepe et al., 1993
X Chromosome		Trisomy X	Robert et al, 1993

The further investigation of these OA associated syndromes by genetic manipulation in mice has resulted in the generation of valuable models of abnormal foregut development which recapitulate the human OA phenotype, allowing embryological investigation into the aetiological basis of OA. SOX2 loss of function mutation is responsible for Anophthalmia Esophageal Genital (AEG) syndrome in humans (Williamson et al., 2006) and when mutated in mice Sox2 is shown to also result in OA/TOF (Que et al., 2009). SHH pathway mutations are implicated in human OA by the presence of tracheo-oesophageal malformations together with GLI3, MYCN and FOXF1 gene mutations, all of which are downstream elements of SHH signalling and when these or Shh gene are manipulated in the mouse, foregut malformations ensue (as reviewed by Shaw-Smith, 2010). Evidently the pathogenic basis of OA can be extrapolated across human and mouse species, for the described genes at least, in a reliable manner.

1.3.3 Environmental factors

Environmental factors may play a role in the pathogenesis of OA/TOF in humans, one example of which is Methimazole, a thioamide treatment agent for hyperthyroidism which has been purported to increase the rate of OA embryopathy when maternally ingested during pregnancy (Di Gianantonio et al., 2001). Evidence for a causative association between methimazole and OA is limited and not borne out in further studies (Pedersen et al., 2012). Recent work examining the rate of OA in newborns of mothers with hyperthyroid status suggested that association exists between the maternal hyperthyroid state during pregnancy and OA, confounding those earlier observations (Martin et al., 2014). With maternal diabetes during pregnancy many congenital malformations arise more frequently, and an increased rate of OA in children was reported by one study (Oddsberg, 2011). Exposure to exogenous sex hormones during the first trimester of pregnancy were also reported to be associated with increased risk of OA/TOF in newborns, as was maternal alcohol consumption and smoking (Wong-Gibbons et al., 2008). It was suggested from a study carried out in Finland that microbial infection may be an aetiological factor for oesophageal atresia (Kyyronen and Hemminki, 1988). In-utero exposure to diethylstilbestrol (DES) may also be linked to OA risk in the next generation by affecting the oocytes of the exposed embryo, later to be mother to an OA affected child (Felix et al., 2007). Within this heterogeneous group of proposed environmental factors none have consistently been identified across multiple studies or have been definitively shown to have a causative association with OA.

Section 1.4 The importance of the AMM model of OA/TOF

1.4.1 The history of the AMM

The desire to comprehend the basic developmental errors from which congenital malformations arise drives clinicians and developmental biologists to seek ways to examine these conditions in an embryological context. The establishment of animal models of congenital anomalies is a valid means to circumvent the lack of human research materials (Rosenthal and Brown, 2007). By identifying or inducing pathology of interest in a small mammal with multiple progeny per breeding cycle, short gestation period and low maintenance costs, a feasible vehicle for research purposes can be established.

A constellation of anomalies, including gastrointestinal, bone, renal and cardiovascular defects were found in the offspring of treated pregnant rats during teratogenicity studies for the anti-cancer drug Adriamycin (Thompson et al., 1978). This phenotype was remarkably similar to the VACTERL association of congenital malformations in humans and included OA/TOF-like anomalies. These findings have been exploited to establish important animal models of OA/TOF, the Adriamycin Mouse Model and the Adriamycin Rat Model (Ioannides et al., 2002, Diez-Pardo et al., 1996, Dawrant et al., 2007a). Although an animal model can do no more than mimic the true human condition, the striking phenotypic similarity of the deformities in ARM and AMM to the human VACTERL anomalies make it a particularly appropriate research implement.

Adriamycin, generically named Doxorubicin, is a glycosidic antibiotic of the anthracycline group, isolated from *Streptomyces peucitius*, and is commonly used for treatment of cancers such as sarcoma, lymphoma, leukemia, neuroblastoma and breast cancer (Tan et al., 1973, O'Bryan et al., 1973, Young et al., 1981). It has been shown to enter the nucleus, where it intercalates with DNA and causes protein associated single and double strand DNA breaks (Cullinane et al., 1994). It is suggested that the major mechanism of Adriamycin involves formation of interstrand crosslinks with DNA, interfering with DNA replication and transcription, through inhibiting nucleic acid synthesis (Cullinane et al., 2000, Meriwether and Bachur, 1972, Momparler et al., 1976). No teratogenic effects have been recorded in the children of mothers who received Adriamycin whilst pregnant to date excepting a single case report of a child with anorectal malformation (Murray et al., 1984). This may be due to dosage, timing of administration outside the critical window of organogenesis or interspecies differences. Adriamycin has also been used to induce fibrosis mediated cardiotoxicity and nephropathy models in adult

rodents (Gianni et al., 2008, Fogo, 2003). The molecular influence by which Adriamycin induces faulty organogenesis is unknown. Altered apoptosis or the production of reactive oxygen species are possible cellular mechanisms of Adriamycin action however no definitive evidence is available to date (Dunkern and Mueller-Klieser, 1999, Gutteridge and Halliwell, 2000).

Following the inadvertent discovery of a range of defects in the offspring of Adriamycin treated pregnant rats by Thompson et al (Thompson et al., 1978), the potential of employing this teratogen to establish a model of OA/TOF and VACTERL was not realised until Diez-Pardo et al published their results 20 years later (Diez-Pardo et al., 1996). The Adriamycin Rat Model (ARM) was subsequently reproduced by several groups in a reliable fashion. Early publications described the phenotype of treated embryos in detail; histopathological assessments of the anomalies were preferentially made utilising near term embryos. Comparisons between control and treated embryos at earlier gestation times, more relevant to the window of organogenesis, became feasible as imaging modalities advanced, and the focus of research moved towards mechanical disturbances of development. However, extensive molecular analysis is required to discern which signalling pathways are involved in the regulation of foregut division in both normal and Adriamycin treated embryos. Towards this goal the model was successfully adapted to the mouse (Ioannides et al., 2002, Dawrant et al., 2007a). This was beneficial given the superior knowledge and range of molecular tools available. Extensive transferable knowledge from transgenic mouse lines and gene databases are readily available in the mouse, it being the developmental biologists' vertebrate model of choice (Bogue, 2003).

1.4.2 Phenotype in Adriamycin treated embryos

The AMM has been reliably replicated to investigate OA/TOF and validated to display a full range of VACTERL anomalies (Dawrant et al., 2007b). Although the direct action of Adriamycin in causing these defects is unknown, the close anatomical resemblance of treated embryos to the human phenotype of VACTERL associated OA/TOF encourages investigation of the model for embryological errors in tandem with continuing endeavours to clarify normal oesophageal development. Adriamycin has also been shown to induce VACTERL anomalies in association with caudal regression syndrome in treated chick embryos by Naito et al, who reported distribution of drug to caudal epithelium and to the foregut (Naito et al., 2009). This suggests that the mechanism by which Adriamycin acts

could be consistent across vertebrate species.

While a 100% rate of at least one VACTERL anomaly can be achieved in the ARM (Gillick et al., 2008), between 28% and 93% of embryos exhibited tracheo-oesophageal malformations in individual studies (Gillick et al., 2003, Diez-Pardo et al., 1996, Merei et al., 1997b, Qi et al., 2001, Xiaomei et al., 2012). A spectrum of these anomalies has been described in the ARM; the near term embryo most frequently (80%) displaying an upper OA pouch with distal TOF (Merei et al., 1997b). This defect is identical to Gross classification C type OA/TOF, the most common anatomical variant seen in humans (86%) (Harmon and Coran, 1998). The blind-ending upper oesophageal pouch was seen to arise from the dorsal wall of the distal pharynx, lined with stratified squamous epithelium and end at the level of cricoid cartilage (Merei and Hutson, 2002). Late outgrowth of the upper pouch from the dorsal wall of the distal pharynx was described at E15 in the ARM (Beasley et al., 2004, Possogel et al., 1998). Pure OA without fistula, complete laryngotracheal cleft (undivided oesophago-trachea), tracheal atresia, tracheal agenesis, and stomach agenesis have also been recorded in the model (Merei et al., 1998, Qi and Beasley, 1999a, Qi and Beasley, 1999b). The lungs of treated embryos were hypoplastic and had decreased airway branching, but histological features of airway organisation were similar to controls (Xiaomei et al., 2012). Other tracheo-oesophageal anomalies including foregut duplications and bronchopulmonary foregut malformations, such as an ectopic lung or bronchus arising from the esophagus, are reported in the ARM, indicating these may have a similar developmental aetiology to OA/TOF in the ARM (Qi and Beasley, 1999a, Qi et al., 2001).

In CBA/Ca mice Ioannides et al induced tracheo-oesophageal malformations in 47% of embryos at a dose of 4mg/kg/day of Adriamycin on E6 and 7 (Ioannides et al., 2002). At an increased dose of 6mg/kg administered on E7 and 8, 50% of surviving embryo's had tracheo-oesophageal anomalies when examined at E18 (Dawrant et al., 2007a). Comprehensive morphological examination of the tracheo-oesophageal malformations in the ARM at a range of gestational stages using 3D imaging of wholemount specimens was conducted by Hadjuk et al using fluorescently labelled antibodies against specific markers expressed in the foregut (Hadjuk et al., 2011b). This approach permitted detailed morphological assessment and detected a higher incidence of foregut malformations than previous groups in up to 64% of embryos across E10 to E12 gestation. The spectrum of malformations perceived included: complete atresia of the entire foregut, foregut stenosis (Figure 1.5 B), OA with upper pouch, total laryngotracheal cleft

(Figure 1.5 A), tracheal atresia, fistula with proximal origin at the carina or bronchus and stomach agenesis

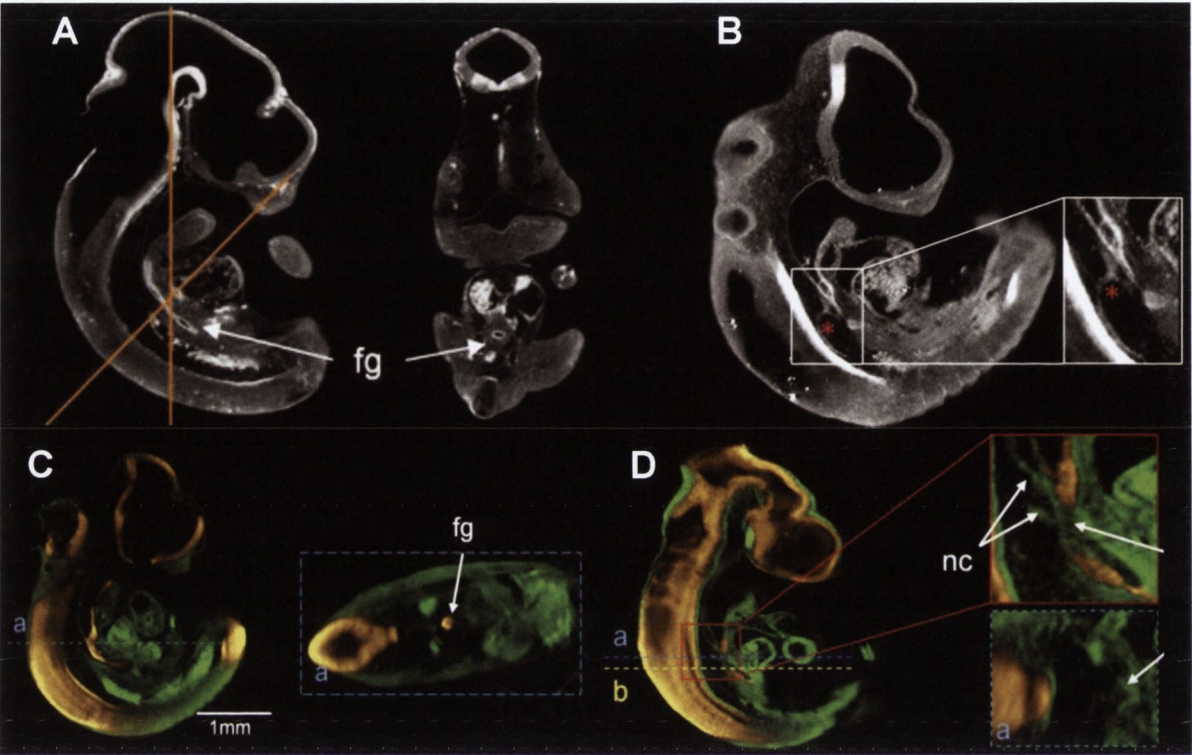


Figure 1.5 Morphological and molecular features of Adriamycin treatment in mice. 3D reconstructions of OPT scans in E10 AMM (A, B, D) and Control (C) embryos for immunolocalisation of Hnf3β (in greyscale A and B) or Sox2 in situ hybridisation (in yellow C and D). Grey and green reflect background. A and B show abnormal foregut (fg) morphology in association with notochord branching (asterisk in B boxed region). In D loss of Sox2 from dorsal foregut domain is highlighted with arrows in boxed magnified regions. Scale bar as shown. Adapted from Hajduk et al, 2011.

1.4.3 The tracheo-oesophageal fistula

The fistula is used in the successful surgical reconstruction of OA. It is freed at the point of tracheal connection in the case of a distal TOF and anastomosed to the upper oesophageal pouch where feasible (Holland and Fitzgerald, 2010). Post operatively it is planned that this repair would facilitate co-ordinated swallowing as a primary outcome. In reality, this lower oesophageal remnant may have an innate incapacity to fulfil these expectations. Disordered lower oesophageal sphincter motility and GORD frequently complicate OA/TOF in children post-operatively and cause long-term morbidity (Tomaselli et al., 2003). These may be surgical complications or could be congenital in nature. It is vitally important to comprehend what the functional capabilities of the fistula might be in a

developmental context and to this end debate exists as to whether it arises from oesophageal or respiratory lineage.

In humans the fistula is an abnormal connection, most commonly seen to arise from the carina (the point of tracheal bifurcation into the bronchi) and extend to the stomach. It has been shown to contain cartilage as well as oesophageal musculature in its walls and to be lined by respiratory and oesophageal epithelium (Yoo et al., 2011, Hokama et al., 1986). Similarly in the Adriamycin treated rodents the fistula most often arises from the left main bronchus and can also originate from the posterior wall of the trachea. Crisera et al have reported that the fistula arises as the posterior branch of a tracheal trifurcation with the main bronchi, elongates posteriorly and then connects with the stomach (Crisera et al., 1999a). Spilde et al also reported a discontinuity between the fistula and the stomach (Spilde et al., 2002a), while other authors reported that the fistula developed from posterior foregut, maintaining a connection with the stomach throughout (Sasaki et al., 2001, Merei et al., 1997b).

Nkx2.1 is a gene expressed specifically in respiratory designated or definitive tissue which has been detected in resected fistula specimens (Spilde et al., 2002b, Minoo et al., 1999). In the ARM the fistula has been shown to be Nkx2.1 protein positive (Crisera et al., 1999b). In the AMM, Ioannides et al reported that the fistula was Nkx2.1 protein negative and expressed Sox2, which is usually restricted to oesophageal tissue following foregut division (Ioannides et al., 2002). Hadjuk et al detected Nkx2.1 gene expression in the fistula at E11 in the AMM but not at later stages (Hajduk et al., 2011b). Respiratory tissues exhibit specialised properties including branching morphogenesis (Hogan et al., 1997). In the ARM the fistula is not normally seen to branch in vivo but it can be induced to branch when cultured in respiratory mesenchyme and in response to fibroblast growth factors (FGF) (Xiaomei et al., 2012, Crisera et al., 2000b, Spilde et al., 2004). The evidence to date suggests that the fistula may arise from a bi-potential origin which can be induced under certain conditions to establish a respiratory identity when exposed to the appropriate mesenchymal signals.

1.4.4 Other VACTERL anomalies in Adriamycin treated embryos.

Utilising the ARM, many groups have successfully generated embryos with a wide range of VACTERL malformations and Dawrant et al additionally validated the AMM as a model for the association (Dawrant et al., 2007b). Renal abnormalities are prominent in

human VACTERL and urogenital defects can be detected in up to 100 % of ARM embryos and are also present in the AMM (Dawrant et al., 2007b, van Heurn et al., 2002, Gillick et al., 2008). The most commonly reported defect is unilateral or bilateral hydronephroureter in association with either bladder agenesis or bladder hypoplasia (Liu et al., 1999, Liu and Hutson, 2001, Mortell et al., 2005, Franca et al., 2004). Other anomalies include renal agenesis, uretro-urethral/urogenital fistula, and uretral agenesis. Telemelcos and Hutson performed microscopic examination of affected kidneys showing abnormal nephron architecture with thin medulla and dilation of collecting ducts and tubules (Temelcos and Hutson, 2004). Ano-rectal malformations are present in a significant proportion of treated embryos. Imperforate anus in association with short tails and recto-urethral or recto-vaginal fistulas has been described in males and females respectively (Merei, 2002), mimicking the human malformation closely (Peña and Levitt, 2006). Cloacal malformations have also been described in the model (Liu and Hutson, 2000).

All neonates diagnosed with OA/TOF undergo screening for cardiovascular defects as this is the most frequent anomaly occurring in association with OA/TOF and is a significant prognostic factor (Spitz et al., 1994). Cardiovascular anomalies in the ARM include ventricular septal and atrial septal defects and right sided aortic arch; aberrant left innominate artery, retro-oesophageal subclavian artery and single umbilical artery have also been described (Merei, 2003, Qi et al., 1997a). Skeletal anomalies seen include asymmetry, wedging and butterflying of the vertebra; rib and sternal abnormalities; and limb deformities due to delayed ossification resulting in bowing of long bones (Kotsios et al., 1998, Abu-Hijleh et al., 2000, Xia et al., 1999). Interestingly single or multiple gastrointestinal atresias, including duodenal atresia, are frequently seen in this model (Merei, 2004, Fourcade et al., 2001). Duodenal atresias are commonly reported occurring in association with VACTERL anomalies in humans and many experts advocate that they should be named as part of the association (Rittler et al., 1996).

Although the ARM and AMM demonstrate the full gamut of VACTERL anomalies, the focus of research in the model has been tracheo-oesophageal malformations. Beyond the phenotypic observations summarised here, the remainder of the anomalies arising in treated embryos have not been subject to in-depth research. However, given that a common insult has been applied, findings in relation to perturbed foregut division will have relevance to the developmental origins of all these defects and in future it may be useful to examine for similar disturbances in the anlagen of these structures in this model.

1.5 Mechanisms of disturbed foregut development.

Signalling and cell behaviour are inextricably linked. Throughout development molecular cues permit ordered cell differentiation to achieve proper organogenesis. Signals received by the cell dictate function and morphology (Tam et al., 2003). Conversely the shape of a cell and the organisation within it, which reflect its developmental history and signalling context, influence how it responds to further stimuli. Historically much of the focus in models of faulty foregut separation has been to identify pathogenic changes in cell activity without looking to the molecular control of these behaviours. The emergence of sophisticated molecular techniques and advancements in imaging modalities is now bridging this gap.

1.5.1 Cellular mechanisms

While it appears that foregut separation is facilitated by septation, controversy remains regarding the cellular mechanisms by which this might be achieved. Programmed cell death or apoptosis has been proposed to be disturbed in OA/TOF (Qi and Beasley, 1998). Qi and Beasley described a temporo-spatial pattern of apoptosis in the normally dividing foregut encompassing 3 phases, initiated by proliferation of the respiratory primordia to bud from the ventro-lateral foregut wall. Apoptosis in cells at lateral foregut epithelial ridges resulted in collapse of the lateral foregut walls and separated the oesophagus and trachea. Rapid proliferation of cells in both the oesophagus and trachea along with interposition of mesenchyme between the structures completed foregut division (Qi and Beasley, 2000, Williams et al., 2003). In the ARM, lack of apoptosis at the lateral epithelial ridges was perceived to result in failure of the foregut to divide (Williams et al., 2000). A similar change in the pattern of apoptosis at the lateral foregut wall was reported in the ARM in addition to reduced cellular proliferation in paraxial and perinotochordal mesoderm (Orford et al., 2001). Conversely, Zhou et al found evidence of increased programmed cell death in the ARM (Zhou et al., 1999), and Gillick et al did not find any change in the pattern or rate of apoptosis between treated and control embryos (Gillick et al., 2002). Ioannides et al did report a change in the pattern of apoptosis in the AMM as well as in *Shh* and *Nkx2.1* knockout transgenic mice, which also exhibit tracheo-oesophageal malformations. The group additionally examined the *Apaf1* mutant mouse

which lacks apoptosis and perceived successful foregut division in the absence of apoptosis (Ioannides et al., 2010). In the Noggin knockout mouse apoptosis was not disturbed in the abnormal foregut or notochord (Fausett et al., 2014). Therefore it seems that although apoptosis is occurring in these rapidly changing tissues and may exhibit altered patterns in Adriamycin models, it is unlikely that programmed cell death is the primary mechanism by which normal foregut separation is achieved.

Cell to cell interactions are fundamental in the control of cell behaviour. Notochord cells may reprogram the identity of the foregut endoderm by prolonged direct cellular contact. This extended interaction is conceivably generated by defective intracellular adhesion. Fibronectin, which functions in intracellular adhesion, is over-expressed in anomalous notochord branches in the ARM (Mortell et al., 2004b). Li et al proposed that prolonged cellular adhesion permits the notochord to appropriate dorsal foregut cells, inducing atresia in the prospective oesophagus by decreasing cell numbers and these findings were reprised by a subsequent group (Li et al., 2007, Fausett et al., 2014). Expression of intracellular adhesion proteins, pan-cadherin and N-CAM was up regulated in the proximal atretic oesophagus, fistula and the trachea of the ARM, indicating that irregular cell adhesion behaviour is not confined to the notochord in Adriamycin treated embryos (Tugay et al., 2003). The establishment of cell polarity, (the sorting of molecules into sub-cellular domains), in epithelial cells is a crucial step in enabling cell function (Tepass, 2012). Cadherins, as well as inducing intracellular adhesion, are vital in determining cell migratory behaviour and tissue differentiation (Kikuchi et al., 2009), and are involved in stabilising cell polarity (Grindstaff et al., 1998, Tepass et al., 2000). In the AMM E-cadherin, usually specific to epithelial cells, was abnormally present in the extracellular matrix of cells in aberrant notochord branches (Hajduk et al., 2012).

Laminins are ubiquitously present in cell basement membrane (Hamill et al., 2009). In the AMM the notochord was hindered from establishing an independent basal lamina from the foregut and abnormal streaks of laminin connected these structures (Hajduk et al., 2012). Arrangement of notochord cells in a rigid structure is thought to facilitate rod elongation (Adams et al., 1990). Failure to synthesise an intact basal membrane could affect turgor within the notochord rod, impair delamination, and induce structural defects. It has been suggested that failure of the notochord to delaminate in Adriamycin treated embryos may affect the foregut structure mechanically by impingement or by traction (Qi and Beasley, 1999c, Merei, 2004). Traction, generated by excessive adhesion, could be propagated by differential growth rates in adjacent tissues inducing structural deformity in both foregut and notochord (Sutliff and Hutchins, 1994).

Altered mesodermal organisation surrounding the foregut endoderm could result in trachea-oesophageal malformations. The mesoderm maintains reciprocal signalling with adjacent endoderm and establishes fields into which epithelial organs can proliferate and subsequently forms specialised tissue mesenchyme. Epithelial mesenchymal interactions such as these are shown to be crucial to organogenesis in the lung and many other tissues (Hogan and Yingling, 1998, Thesleff et al., 1995). In the ventral foregut mesoderm, condensed cellular patterns are associated with the outgrowth of the lung buds (Morrissey and Hogan, 2010). In the ARM disturbances occur in the mesodermal cell pattern and architecture around the foregut, indicating failure of these condensed mesoderm fields to form, which may physically and molecularly deter organogenesis (Possogel et al., 1998, Sasaki et al., 2001).

1.5.2 The notochord

Notochord defects are not unique to Adriamycin models. In humans, notochordal defects have been described in embryos with VACTERL anomalies including OA/TOF, although reports in the literature are sparse (Elliott et al., 1970), and Split Notochord Syndrome describes notochord anomalies, neural tube defects and gut duplications or cysts (Bentley and Smith, 1960, Meller et al., 1989). In the Ethylenethiourea induced model of anorectal malformations in rats the notochord displays structural abnormalities (Qi et al., 2003), and Noggin knockout transgenic mice display notochordal branches in association with tracheo-oesophageal malformations (Que et al., 2006). The classic *Patch* mutant mouse (mutation of the *Pdgfr* gene) also demonstrates notochord abnormalities and foregut defects (Snider et al., 2011). The significance of these abnormalities has not yet been clarified, however, given the powerful patterning influence of the notochord, structural deformity may be echoed by associated signalling irregularities directed towards adjacent structures such as the foregut (Orford et al., 2001, Gillick et al., 2003, Hajduk et al., 2011b). It remains to be determined whether the notochord and foregut share a molecular relationship affecting oesophageal formation.

A link between the notochord and the advent of foregut separation into oesophagus and trachea is suggested by the presence of distinctive notochord defects or branches co-existing with tracheo-oesophageal malformations in the Adriamycin Mouse Model (Hajduk et al., 2011b). The structural defects of the notochord encountered have been given many terms including branching, splitting, hypertrophy, bifurcation, duplication,

tethering or bending, all referring to the appearance of the notochord failing to delaminate away from the foregut at points along its length (Williams et al., 2001). In our group, with the benefit of 3D wholemount imaging and specific immunohistochemistry to label the notochord, Hajduk et al detected branching in up to 94% of AMM embryos. The vicinity and thickness of these branches could be spatially and quantifiably related not only to the site and severity of foregut abnormalities (Figure 1.5 B), but also to the location and extent of disturbed gene expression in foregut endoderm and mesoderm (Hajduk et al., 2011b).

The importance of possible molecular interactions between notochord branches and foregut abnormalities was suggested by regionally confined disturbance of DV patterning in the foregut endoderm of the AMM. Loss of Sox2 dorsal marker and ectopic expression of Shh (normally ventrally restricted) directly adjacent to notochord branches in the dorsal foregut endoderm were observed, as well intense Shh gene expression in the foregut adjacent tips of the notochord branches (Hajduk et al., 2011b). DV patterning across the foregut, as further discussed in section 1.5.3.1, establishes a framework for proper foregut separation. The correlation of the site and extent of notochord abnormalities with these adjacent molecular disturbances suggests they may act in the pathogenesis of foregut malformations. Precedent for notochord derived signalling influencing foregut morphogenesis is found in the requirement of Activin from the notochord to induce pancreas formation, acting by suppression of foregut endodermal Shh (Hebrok et al., 1998).

The mechanism by which the notochord may signal to the foregut during oesophageal and tracheal formation is poorly understood. It has been suggested that altered Shh signal due to the proximity and extent of notochord tissue in relation to the foregut is sufficient to induce foregut malformations (Ioannides et al., 2003). Gene mutations in the Shh pathway resulting in OA phenotypes in animal models are well described (Motoyama et al., 1998, Litingtung et al., 1998) and can be found in a small proportion of syndromic human OA (Johnston et al., 2005). Findings in the Noggin knockout mouse implicate notochord defects together with BMP signalling abnormalities in the pathogenesis of foregut abnormalities (Que et al., 2006). This finding was recently further expanded to propose that attenuation of BMP signalling by Noggin specifically in the notochord is necessary to allow both proper notochord delamination and foregut development (Fausett et al., 2014).

1.5.3 Molecular signalling

During development morphogenesis is influenced by complex interactions of multiple signalling pathways. Positional information, including instructions for differentiation, proliferation, migration, and many other aspects of cell function are derived from these signals. Animal models displaying phenotypes of tracheo-oesophageal malformations have been used to elaborate interactions between the multiple pathways implicated in molecular foregut patterning, control of foregut division and lung morphogenesis. The host research groups have significant experience with the Adriamycin Mouse Model (AMM) and have employed it to investigate foregut structural derangements together with candidate gene involvement in OA/TOF (Hajduk et al., 2011b, Hajduk et al., 2011a, Hajduk et al., 2010b, Hajduk et al., 2010a, Hajduk et al., 2012, Mc Laughlin et al., 2014b, Sato et al., 2008).

Diverse signalling pathways act to regulate the anatomical and functional transformations required for foregut separation. Just some of these vital influencing pathways include SHH, Retinoic Acid (RA), BMP, FGF and Wnt signalling (Goss et al., 2009, Desai et al., 2006, Lebeche et al., 1999, Litingtung et al., 1998, Que et al., 2006). Disruption of these developmentally important pathways can result in foregut or oesophageal malformation phenotypes in humans and in animal models. Candidate molecular factors that have been identified in the aetiology of OA/TOF in animal models are summarised in Table 1.2

Table 1.2 Molecular disturbances in models of OA/TOF

Gene/ Gene product	Foregut Phenotype	Other affected structures	Reference
Ttf-1/Nkx2.1	Undivided foregut	Lung agenesis	Kimura 1996
Shh	OA/TOF	Lung, Skeletal, CNS Renal, Cardiac, Hindgut	Litingtung 1998, Chiang 1996 Hajduk 2011
Foxf1 (haplo)	OA/TOF	Lung Skeletal	Mahlapuu 2001 Hajduk 2011
Gli 2/3 (haplo) Gli 2/3	OA/TOF Foregut agenesis	Single lung Lung agenesis	Motoyama 1998
Mycn	OA/TOF		Moens 1992
RAR α/β	TOF	Lung	Mendelsohn 1994
Dietary RA deficiency (rats)	Undivided foregut	Lung	Dickman 1997 Wilson 1953
Raldh2	Absence lung buds		Desai 2006
Pcsk5	Lack tracheo- oesophageal septum	Lung	Szumaska
Pdgrfa	OA/TOF Notochord branching		Snider 2011
Sox2	OA/TOF	Lung, CNS	Que 2007, Ferri 2004 Hajduk 2011
Tbx4 (chick)	TOF		Sakiyama 2003
Hoxc4	OA		Boulet Capeocchi 1996
Hoxa5	Tracheal atresia		Aubin 1997
Wnt5a	Tracheal defect	Lung	Li 2002
Wnt 2/2b	Undivided foregut	Lung agenesis	Goss 2009
B canteinin *	Undivided foregut	Lung agenesis	Harris–Johnson 2009
Traf4	Tracheal defect		Shiels 2000
Noggin †, ‡	OA/TOF Notochord branching	Lung Limbs, CNS	Que 2006, Li 2007 P.Hajduk 2011 (Thesis)
Bmp4 *	Tracheal agenesis		Li 2008
Bmpr1a/b*	Tracheal agenesis		Domyan 2011
Bmpr1a †	OA/TOF Notochord branching		Fausett 2014

* conditional knockout anterior ventral foregut endoderm, / indicates double mutant
† conditional knockout (Noggin) or overactivation (Bmpr1a) notochord and neural floor plate
‡ conditional knockout notochord, floor plate and foregut endoderm
Full gene insufficiency unless otherwise stated, haplo indicates haploinsufficiency
Unless otherwise specified species is mouse. Related genes are listed in order and reflected by the shading.

1.5.3.1 The importance of DV patterning reflected by Sox2 and Nkx2.1

Molecular designation of foregut into dorsal and ventral domains is a key event in subsequent oesophageal and trachea formation. The importance of strict regionalised patterning in the foregut endoderm is particularly well illustrated by reciprocal gene expression of transcription factors Sox2 (dorsally) and Nkx2.1 (ventrally) in the undivided foregut, demarcating the future oesophageal and respiratory tissue domains respectively (Que et al., 2007). Encroachment of the ventral domain by Sox2 characterises models of tracheal agenesis and inappropriate dorsal presence of Nkx2.1 accompanies oesophageal atresia (Li et al., 2008b, Que et al., 2007). Transgenic mice with knockout of either of these genes have tracheo-oesophageal malformations (Minoo et al., 1999, Que et al., 2007). In humans SOX2 gene mutation causes AEG syndrome in which OA/TOF is present (Williamson et al., 2006). In mice Sox2 knockout mice have OA/TOF and dorsally expanded Nkx2.1 expression. At E8 in mice, Nkx2.1 gene expression is already localised ventrally in the foregut endoderm and is known to have a key role in designating respiratory tissues (Maeda et al., 2007). Promotion of Nkx2.1 expression by Fgfs from ventral foregut mesoderm is essential for lung formation (Serls et al., 2005) and the Nkx2.1 null mutant mice have complete laryngo-tracheal cleft with lung hypoplasia and ventral expansion of Sox2 expression (Minoo et al., 1999). In virtual sections of 3D reconstructed AMM embryos Hadjuk et al found dorsal Sox2 expression to be lost at a specific level in the foregut adjacent to notochord branches (Figure 3.5 D) (Hajduk et al., 2011b). It is apparent that DV patterning of the foregut cells is essential in regulating organogenesis and in particular that correct localisation of Sox2 and Nkx2.1 is required for proper foregut separation.

1.5.3.2 SHH Pathway

SHH signalling is fundamental to developmental processes in vertebrates and invertebrates (Hooper and Scott, 2005). It functions in antero-posterior, dorso-ventral and right left patterning in the embryo (Johnson et al., 1994, Hamada et al., 2002). It is expressed in both the notochord and foregut endoderm and disturbed Shh levels affect the development of foregut derived organs such as the pancreas (Kim et al., 1997, Litingtung et al., 1998). Transducers of the SHH pathway include receptors Smoothed and Patched (Ptc), signalling complex proteins that act as transcriptional activators or repressors Gli 2 and 3 and effector gene Foxf1, which is expressed in ventral foregut mesoderm. Mice with gene inactivations in for Shh, Gli2 and 3 and Foxf1 all exhibit tracheo-oesophageal malformations (Litingtung et al., 1998, Motoyama et al., 1998, Mahlapuu et al., 2001). In

humans *SHH* signaling mutations have been identified in patients with OA/TOF, ano-rectal malformation and holoprosencephaly (Cohen, 1989), *GLI3* mutation causes Pallister Hall Syndrome with OA/TOF phenotype (Johnston et al., 2005), and microdeletion of the gene cluster which includes *FOXF1* is associated with a VACTERL phenotype (Stankiewicz et al., 2009).

Ioannides et al detected a specific DV pattern of Shh expression in the foregut of normal mice. Shh was confined to the ventral endoderm prior to foregut division, a shift then occurred to dorsal restriction of expression at the site of foregut separation. Shh expression persisted in the oesophagus and was absent in respiratory tissues. In the foregut endoderm of AMM with tracheo-oesophageal malformations Shh was diffusely present with no identifiable DV restriction (Ioannides et al., 2003). With Optical Projection Tomography (OPT) it was possible to visualise Shh expression patterns in the foregut in detail (Sato et al., 2008). Hajduk et al confirmed the abnormal diffuse expression pattern of Shh in the AMM foregut and additionally found an ectopic site of Shh expression in the dorsal foregut which correlated with the vicinity and thickness of notochord branches. *Foxf1* expression pattern in the mesoderm was also altered, with a loss of ventral restriction and a more diffuse expression pattern around the AMM foregut associated with the position of the notochord branches (Hajduk et al., 2011b).

Shh self regulates its own expression in target tissues and is known to act in a concentration gradient dependant manner (Zeng et al., 2001, Bumcrot and McMahon, 1996). In the neural crest there was reduced auto-induction of Shh expression when the notochord was ectopically positioned closer (Goh et al., 1997). The concentration of Shh received by the foregut in the ARM could arguably be higher due to either increased volume of notochord tissue or the proximity of the notochord branch to the foregut and in response the endodermal expression of Shh may decline (Mortell et al., 2004a). *Gli2* mRNA levels were lower in the fistula than in the oesophagus in the ARM and the fistula, although Shh negative, responded to exogenous Shh by branching in culture (Spilde et al., 2003a, Spilde et al., 2003c).

1.5.3.3 Wnt pathway

Wnt signalling plays a major role in organising the embryonic body plan (Logan and Nusse, 2004). It has been shown that Wnt sustains notochord progenitor cell fate and extension of the notochord during embryogenesis (Ukita et al., 2009). Defined roles in foregut division have not been ascertained for Wnt ligands, some of which are expressed in the foregut mesoderm (*Wnt2* and *2b*) and endoderm (*Wnt7b*) (Rajagopal et al., 2008, Goss et al.,

2009). Experimental Wnt pathway inactivation inhibits foregut separation (Harris-Johnson et al., 2009). Wnt2/2b knockout mice display complete lung agenesis without foregut separation and altered DV patterning of the foregut with absence of Nkx2.1 expression in ventral endoderm (Goss et al., 2009). Wnt7b inactivation results in lung hypoplasia (Shu et al., 2002). Miller et al found that crosstalk of Wnt2 mesodermal and 7b endodermal signalling from complimentary expression domains was crucial in anterior foregut organogenesis both in vivo and in vitro (Miller et al., 2012). In the AMM however, Wnt2 expression was not found to be different in the mesoderm of the foregut versus controls, although it was detected in the mesoderm around the fistula, and Wnt7b expression was not altered (Hajduk et al., 2011a).

1.5.3.4 Retinoic acid signalling

Retinoic acid (RA) is the active metabolite of vitamin A. Offspring of rats reared on a vitamin A deficient diet exhibit severe congenital malformations including lung agenesis and an undivided foregut (Dickman et al., 1997, Wilson et al., 1953). Various retinoic acid nuclear receptors (RARs) transduce retinoid signals, and the double mutant knockout mice for RAR α and RAR β receptors have absent oesophago-tracheal septum (Mendelsohn et al., 1994). When cultured with an RAR antagonist, lung budding failed in foregut explants (Mollard et al., 2000). Deletion of retinaldehyde dehydrogenase 2 (Raldh2), an enzyme essential for RA synthesis in mouse embryos, also leads to absence of lung buds (Desai et al., 2006). Nkx2.1 expression was significantly reduced in the ventral foregut endoderm in embryos with deficient RA signalling (Desai et al., 2004).

1.5.3.5 FGF signalling

FGF signalling is synonymous with the regulation of lung organogenesis (Warburton and Bellusci, 2004), however its role in foregut separation is undefined. In the ARM, FGF signalling in the fistula was investigated in order to determine whether this structure exhibited properties of respiratory tissue. The fistula was positive for Fgf1 and Fgf10 but not Fgf7 mRNA. FGF receptor Fgf2rIIIb, to which Fgf7 and 10 obligately bind, was shown to be absent or down regulated in the fistula compared to the respiratory tree (Spilde et al., 2004, Spilde et al., 2003b, Crisera et al., 2000b). ARM fistula epithelium in recombinant culture displayed branching in response to Fgf1 (Crisera et al., 2000a). In normal mice, Fgf10 gene expression was detected in mesenchyme at the tips of the lung buds and the onset of Fgf 10 expression was shown to be delayed in AMM embryos (Hajduk et al., 2010a).

1.5.3.6 BMP signalling

The phenotypic similarities exhibited between the AMM and the Noggin knockout mouse model, in the co-occurrence of OA/TOF and notochord branching, generates particular interest in the role of the highly evolutionary conserved BMP pathway in foregut separation. Ectopic expression of Noggin, a Bone Morphogenic Protein (BMP) antagonist, in notochord branches and dorsal endoderm of Adriamycin treated embryos recently detected by the host group (Hajduk, 2011) further indicates that deregulation of the BMP pathway may contribute to the pathogenesis of OA/TOF .

BMP ligands are members of the Transforming Growth Factor beta (TGF β) superfamily of signalling proteins that specifically bind to paired type I and II serine threonine kinase transmembrane receptors and initiate signal transduction by phosphorylation of intracellular SMAD proteins to influence transcription of target genes (Miyazono et al., 2010). Although originally identified and named for activity in osteogenesis and limb formation BMP signalling is vital across various tissues during development including heart and lung and powerfully influences diverse cell processes ranging from apoptosis to proliferation (reviewed by Chen et al., 2004). Bmp4 patterning across the lung buds has been shown to be related to the control of branching morphogenesis in lung formation (Weaver et al., 2000, Bragg et al., 2001). Bmp4 expression is reported to be localised to the ventral foregut mesoderm (Que et al., 2006), Bmp7 expression can be detected in the notochord and foregut endoderm and Noggin is expressed in the notochord during this gestational window (Danesh et al., 2009). Bmp2 activity has recently been shown to be necessary for foregut formation by ventral folding morphogenesis (Gavrilov and Lacy, 2013).

Noggin, an extracellular BMP antagonist which competitively inhibits ligand to receptor binding, is normally expressed from E7.5 in the notochord and the neural tube floor plate (Danesh et al., 2009). In Noggin gene knockout mice the foregut failed to separate and notochord defects were detected. This phenotype could be rescued by reducing the gene dose of Bmp ligands 4 or 7 (Que et al., 2006, Li et al., 2007). Conditional knockout of Bmp4 gene induces severe morphological foregut disturbance in the form of tracheal agenesis and disturbed DV patterning of the foregut by Sox2 expansion into ventral domains (Li et al., 2008b). This is reprised in the Bmp receptor (Bmpr) type1a and 1b double knockout mutant (Domyan et al., 2011). Bmpr1a conditional knockout in the notochord and neural floor plate replicates the phenotype of the Notochord knockout mouse. Evidently BMP signalling is important for proper foregut separation and

is a prime candidate for further investigation in the specific pathogenesis of oesophageal atresia.

1.4.3.7 Other genes implicated in OA/TOF models

T-box transcription factor 4 (Tbx4) is co-expressed with Fgf10 in ventral foregut mesoderm (Gibson-Brown et al., 1998). In chicks, Sakiyama et al reported ectopic mesodermal induction of Tbx4 and Fgf10 resulting in ectopic expression of Nkx2.1 in endoderm and subsequent ectopic lung budding. Failure of foregut division was seen in these embryos and ectopic Tbx 4 or Fgf10 could each induce the expression of the other in the mesoderm, indicating a feedback loop exists between these genes (Sakiyama et al., 2003). However in mice, inactivation of the Tbx4 gene did not prevent lung bud formation (Naiche and Papaioannou, 2003), and in the AMM, Tbx4 expression was unchanged between treated and control embryos (Hajduk et al., 2010b).

Hox genes are a large family of major patterning genes which confer axial positional information in the vertebrate and are best described in the development of the axial skeleton (Mallo et al., 2010). Hox subgroups have been shown to be expressed in the foregut mesoderm and endoderm (Bogue et al., 1996, Pitera et al., 1999). Various Hox gene mutations induce tracheal or esophageal malformations in transgenic mice (Szumska et al., 2008, Aubin et al., 1997, Boulet and Capecchi, 1996). Calonge et al recorded reduced protein and RNA levels of Hoxa3, Hoxb3, Hoxd3, Hoxc4 in AMM lungs (Calonge et al., 2007).

These pathways do not act independently of each other but instead co-operate in a complex regulatory circuit. Shh stimulates Wnt2 and Bmp4, restricts Fgf10 as well as up-regulating noggin expression in the foregut mesoderm (Pepicelli et al., 1998, Lebeche et al., 1999, Weaver et al., 2003). Bmp4 and Fgf10 have opposing roles in the control of lung budding and Fgf10 activates Bmp4 signalling (Bellusci et al., 1997, Weaver et al., 2000) and Bmp4 downregulates Foxf1 expression (Mahlapuu et al., 2001). Wnt7b signalling promotes the expression of Bmp4 and Fgfr2 in the epithelium (Rajagopal et al., 2008, Shu et al., 2002), and Fgf10 is retinoic acid signalling responsive in the foregut mesoderm (Desai et al., 2006, Chen et al., 2007).

1.5 The use of in vitro techniques to investigate foregut development

Embryonic culture is useful technique for the investigation of early development in an observable and controllable manner. Removal of tissues of interest from the early embryo for in vitro explantation has been widely used to examine organogenesis and to investigate the embryological basis for disease, revealing the molecular control of morphogenesis. For example the developing lung is amenable to explant culture in a drop of liquid medium (Hogan et al., 1997). Explant culture provides a highly adaptable and cost effective approach for functional assays such as those performed on lung explants to determining the molecular basis for lung branching morphogenesis (Weaver et al., 2003). Successful techniques of explant culture applied to foregut include culture in liquid media, 3D culture in gel (Deutsch et al., 2001, Que et al., 2006) and Roller Bottle culture (Tremblay and Zaret, 2005). These later two techniques have been used to examine the specification of liver and pancreas development from the foregut endoderm by co-culture with soluble factors such as Fgfs or with mesoderm tissue.

Specific to foregut separation, foregut culture has been described in liquid medium to investigate the effect of signalling manipulation on oesophageal development (Que et al., 2007). This group used E10 foregut explant culture to determine that treatment with Fgf10 inhibited separation into trachea and oesophagus in order to reinforce findings from transgenic mouse lines. Culture of foregut from E8.5 embryos has previously been performed using liquid media as a model for lung bud morphogenesis (Desai et al., 2006) again describing the inhibitory effect of Fgf10 on the advent of foregut separation. This technique was also used to demonstrate that foregut malformations observed in *Raldh2* deficient mice could be rescued, in terms of branching morphogenesis of the lung buds, by supplementation with RA or Fgf10 (Wang et al., 2006). Crisera et al used explant culture of the foregut structures including the fistula from Adriamycin treated mice at E13.5 to examine the effect of FGF signalling in these structures again focussing on branching morphogenesis as a primary outcome (Crisera et al., 2000a), however these experiments did not fully maintain tissue morphology. In vitro techniques have been employed, utilising knowledge from established models of OA/TOF, to gain a better understanding of the pathomechanisms involved in foregut malformations. However successful generation of oesophagus from the undivided foregut by any in vitro technique has not been described to date.

The co-culture of embryonic tissues not normally adjacent to each other or the ablation of key sources of signals to developing tissue can reveal the molecular

relationships between embryonic structures and directly investigate the impact of these on the resulting morphological outcomes. This classical developmental biology approach is exemplified by well-known experimental manipulations of the notochord in chick embryos, revealing crucial roles for signalling from the notochord in the development of the neural floor plate and somites. The application of donor notochord to the lateral neural tube had a ventralising influence resulting in the formation of a secondary neural floor plate (van Straaten et al., 1988). Ablation of the notochord in early chick embryos resulted in failed somite formation and disturbances of right left and DV patterning of the embryo reflected by severe morphological disruption (as reviewed by Cleaver and Krieg, 2001), providing strong evidence for the importance of the notochord to early embryo development.

1.6 The Objectives of this Thesis

This thesis aims to examine the aetiology of OA/TOF in a developmental context, to build on what is already understood of the molecular control and morphology of foregut separation in the early embryo. The work presented in this thesis particularly focuses on the role of BMP signalling and the notochord in the pathogenesis of foregut malformations. Three broad objectives provide the framework for these investigations and are presented in the subsequent chapters:

- *To test the hypothesis that BMP signalling disturbances contribute to the pathogenesis of foregut malformations by examining pSMAD activity and downstream BMP target gene spatial expression in Adriamycin treated embryos (Chapter 3).* This work tests if BMP signalling disturbances occur in early stages of abnormal foregut development in the AMM. 3D scanning using Optical Projection Tomography (OPT) facilitated the novel use of pSMAD immunolocalisation in whole embryos and the identification of specific foregut expression profiles for BMP responsive genes as well as T box genes (Tbx) 1 and 2 (in Chapter 4) in the AMM.
- *To establish an in vitro system for foregut development and to examine a putative signalling relationship between the notochord and foregut during development by manipulating their physical relationship in vitro (Chapter 5).* This work tests the hypothesis that signalling from the notochord influences normal foregut division. This was addressed by first establishing a culture system that would allow foregut separation into oesophagus and trachea. A precise morphological staging system based on features of normal foregut development generated by 3D scanning and the detection of DV patterning markers together assessed the morphological and molecular characteristics of cultured foregut. These approaches were then used to determine the effect of notochord manipulations on foregut separation.
- *To test the hypothesis that BMP signalling disruption can induce oesophageal malformations (Chapter 6).* Using the culture system reported on in Chapter 5 together with the application of exogenous proteins, the morphological and molecular impact of BMP over activity (Bmp4) or antagonism (Noggin) on foregut division was analysed with OPT scanning and Confocal microscopy, examining the outcome of treatment both in whole foregut and down to a sub cellular level.

Chapter 2

Methods

General methods used throughout this thesis are detailed in this chapter. Additional experimental design and methods for chapter specific work are presented in the relevant chapter (as detailed in section 2.9).

2.1 Adriamycin treatment of Embryos: generation of Adriamycin Mouse Model (AMM) embryos.

All animal work was performed under licence B100/4447 from the Irish Medicines Board under the European Communities (and Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005, following institutional approval by The Animal Research Ethics Committee, Trinity College Dublin. Male and female CBA/Ca mice (Harlan UK, Bicester, England) housed in the Bioresources Unit, TCD, were mated overnight. Finding a vaginal plug at the end of this mating period was taken as embryonic day 0 (E0) of gestation. Pregnant dams received two intraperitoneal injections, 24 hours apart, on E7 and E8. The Adriamycin treated dams received a dose of 6mg/kg of Adriamycin (Doxorubicin, EBEWE Pharma Ges.m.b.H. Nfg.KG, A-4866 Unterach, Austria) in 0.9% Sodium Chloride. The control dams received an equivalent volume of 0.9% Sodium Chloride. The Adriamycin was prepared to a concentration of 0.3mg/ml just before the first injection and was stored in the dark at 2-8°C until the second injection was given. This established a standard weight determined volume for the intraperitoneal injection given to the mice at 0.02ml/g. Following sacrifice of pregnant dams by CO₂ inhalation and cervical dislocation, embryos were harvested by caesarean section on E9 to 11.5 in chilled PBS, fixed in 4% paraformaldehyde in PBS (4% PFA) for a gestational stage dependant time period (for E9 1 hour, for E10 4 hours, for older stages overnight) at 4°C, and dehydrated through a series of increasing methanol concentrations in PBT (PBS with 1% Triton) as follows; 25% Methanol/75% PBT, 50% Methanol/50% PBT, 75% Methanol/25% PBT; and stored at -20°C in 100% Methanol.

2.2 Cd1 Embryo collection

Cd1 embryos were required for explant experiments (as described in section 2.7), to establish normal gene expression patterns of candidate BMP target genes and for general testing of experimental protocols and probes. Following overnight mating of male and female Cd1 mice (Harlan UK) housed at the Bioresources Unit, TCD the detection of a vaginal plug was designated embryonic day 0.5 of gestation (E0.5). For general testing Cd1 embryos were harvested from E9 through E11.5, fixed in 4% PFA and dehydrated and stored as described for Adriamycin treated embryos in section 2.1.

2.3 RNA probe generation

An I.M.A.G.E (Integrated Molecular Analysis of Genome and their Expression) consortium (<http://www.imageconsortium.org/>) expressed sequence tag (EST) mouse cDNA clone bank (IRAV) stored in the Department of Zoology, Trinity College Dublin was used to generate the plasmid DNA of the genes of interest or plasmids were obtained from external sources as specified (Table 2.1). To amplify and purify the plasmids an inoculate of the EST bank *E.coli* bacterial clone was grown on Luria Bertoni (LB) agar plates (1% Peptone, 0.5% Yeast Extract, 1% NaCl, 1.5% Bacto-Agar in ddH₂O) supplemented with 100µg/ml ampicillin (Sigma). Details of the cDNA clones used to generate RNA probes are given in Table 3.1. Single colonies of each *E.coli* bacterial clone were expanded in LB broth cultures (100ml; plus ampicillin 100µg/ml) and then plasmid DNA was extracted and purified using a PureYield™ Plasmid Midiprep system (Promega) according to manufacturer's protocol and quantified using the Qubit DNA assay kit (Invitrogen). Each plasmid DNA was sequence verified (Source Biosciences) using vector primer sites flanking the insert. Sequences were analysed using tools provided by the National centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) to ensure it represented a cDNA clone of the correct gene and to identify the size and exact identity of the clone that was present (detailed in Table 3.1).

Plasmid DNA was linearised with appropriate restriction enzymes to produce template DNA for *in vitro* transcription. Antisense and sense digoxigenin (DIG)-labelled RNA probes were generated, according to vector and insert orientation, from 1µg linearised plasmid DNA using T7, T3 and SP6 RNA polymerase binding sites in the relevant plasmid vector (all reaction components from Roche) in a 20µl volume at 37°C for 3hrs. Other components included DigRNA labelling mix (Roche), RNase inhibitor (Roche), 10x Transcription Buffer (Roche) and 0.2M DTT (DL-Dithiothreitol). RNA production and integrity was checked by running 1µl on a 1% agarose gel. If visible RNA production was present (at equal or up to approximately 5 fold intensity compared to the DNA band) the DNA template was degraded by incubation with 1µl of RNase free DNase (Roche) for 15mins at 37°C. The *in vitro* transcribed RNA was purified on a G25 column (Amersham Biosciences) as per manufacturer's instructions, quantified by Qubit RNA assay kit spectrophotometry, as per manufactures instructions and stored at -20°C in an equal volume of hybridisation solution (2% blocking reagent (Boehringer), 50% deionised formamide, 5x Saline-sodium citrate (SSC) (Sigma), 0.5% 3-Cholamidopropyl-dimethylammonio-1-propanesulfonate (CHAPS), 50µg/ml Heparin, 100µg/ml yeast RNA, 0.1% Triton and 5mM ethylenediamine tetra-acetic acid (EDTA).

Table 2.1 Details of cDNA clones used to generate RNA probes for in situ hybridisation.

Gene	Extent of Probe on Genbank	Source
Id1	Nucleotide 1 to 909 on NM 010495.2	I.M.A.G.E. bank
Id2	Nucleotide 25 to 1270 on NM 010496.3	I.M.A.G.E. bank
Dpysl2	Nucleotide 10 to 2126 on NM 009955.3	I.M.A.G.E. bank
Msx1	Nucleotide 847 to 1741 on NM 010835.2	I.M.A.G.E. bank
Junb	Nucleotide 54 to 1816 on NM 008416.3	I.M.A.G.E. bank
Prx2	Nucleotide 1 to 1105 on NM 009116.2	Prof Frits
Bambi	Nucleotide 19 to 1393 on NM 026505.2	I.M.A.G.E. clone
Dlx2	* NM 010054.2 (500 bp)	I.M.A.G.E. bank
Snai1	Nucleotide 1 to 1584 on NM 011427.2	I.M.A.G.E. bank
Snai2	* NM 011415.2 (1500 bp)	I.M.A.G.E. bank
Crabp2	Nucleotide 42 to 900 on NM 007759.2	I.M.A.G.E. bank
Cdkn1a	Nucleotide 48 to 750 on NM 007669.4	I.M.A.G.E. bank
Fn1	* NM 010233.1 (500 bp)	I.M.A.G.E. bank
Kdm6b	* NM 001017426.1 (2000 bp)	I.M.A.G.E. bank
Twist1	Nucleotide 1 to 1625 on NM 011658.2	I.M.A.G.E. bank
Bmp2	Nucleotide 53 to 1215 on NM 007553.2	Prof Robert E
Bmp7	Nucleotide 904 to 1987 on NM 007557.2	I.M.A.G.E. bank
Tbx1	Nucleotide 82 to 1455 on NM 011532.1	I.M.A.G.E. clone
Tbx2	Nucleotide 796 to 2269 on NM 009324.2	I.M.A.G.E. clone

* sequencing incomplete, approximate insert size in base pairs (bp) in brackets

2.4 *In situ* Hybridisation

Stored embryos were rehydrated through a reverse series of methanol concentrations in PBT for a minimum of 10 minutes culminating in 2 PBT washes for 15 minutes. Excess membranes were dissected away from the embryo and the eye and hindbrain were pierced to avoid trapping of probe. The embryos were then staged and allocated appropriately. The embryos were digested in 10 µg/ml of Proteinase K in PBS for 10 minutes at room temperature, followed by 2 10min PBS washes and fixed in 0.25% Gluteraldehyde in 4% PFA for 20 minutes on ice followed by 2 further PBS washes at RT for 5min. Embryos were then equilibrated with hybridisation solution (detailed in section 2.3 above) until they sank and then prehybridised in fresh hybridisation solution at 65°C for 1 hour or overnight.

Antisense and sense RNA probes were retrieved from storage on ice and denatured by heating at 80°C for 3 minutes in hybridisation solution. Embryos were hybridised with probe at a concentration of 1 µg/ml overnight at 65°C in a waterbath in 3ml round bottomed hybridisation tubes. After hybridisation the probe was removed and stored at -20°C for reuse of any un-hybridised probe (up to 3 times). The following post hybridisation washes were performed at 65°C to remove unbound probe: 1 x 5mins in post-hybridisation wash (PHW) solution (50% formamide, 5x SSC and 0.5% CHAPS), then serial 30min washes in decreasing concentrations of PHW and increasing concentrations of Wash 1 (W1) solution (2x SSC and 0.1% CHAPS) as follows: 75% PHW/25% W1, 50% PHW/50% W1, 25% PHW/75% W1. This was followed by two 30min washes in W1 followed by two 30min washes in Wash 2 containing 0.2xSSC and 0.1% CHAPS. Two ten min washes were then performed in TNT (100mM Tris-HCL pH7.5, 150mM NaCl, 0.1% Triton X-100) at room temperature (RT).

Embryos were blocked with Maleic Acid buffer (100mM Maleic Acid, 150mM NaCl) containing 3% blocking powder (Boehringer) for 3hrs at 4°C followed by incubation in fresh blocking buffer with 1/3000 dilution of anti-digoxigenin (DIG) Fab fragments conjugated with alkaline phosphatase (AP) (# 11 093 274 910, Roche, Germany) for 12hrs at 4°C on a Stuart Scientific SSM4 rocker. Post antibody incubation washes were performed in TNT rocking at RT for 5mins x2 followed by 1hr x3 and then 16hr x1 at 4°C. This was followed by NMT (0.1M Tris pH9.5 / 0.1M NaCl / 0.05M MgCl₂) washes at RT for 15 min x3 to establish the optimal pH for the subsequent chromogenic reaction with Nitro-blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) stain in NMT. This reaction was carried out in the dark at RT, refreshing with new stain every 1

hour, and monitored to halt staining by washing in PBS when the appropriate staining level was achieved. Any unbound staining reagents were removed with further washes in PBS including an overnight wash at 4°C. Staining was fixed by 4% PFA overnight at 4°C and embryos were then photographed and processed for OPT scanning as in section 2.6.

2.5 Wholemound Immunolocalisation

Stored samples were rehydrated as described for *in situ* hybridisation and were washed twice in 1XTBST (10mM Tris-HCL pH8, 150mM NaCl, 0.05% Tween 20) and blocked in 10% goat, sheep or donkey serum (Sigma-Aldrich) in 1XTBST overnight at 4°C rocking. Samples were subsequently incubated with the appropriate concentration of primary antibody (see Table 2.1) for a minimum of 4 days at 4°C rocking. Post antibody washes were performed with 6, 30 minute 1XTBST washes and subsequently samples were incubated with the secondary antibody of choice at the appropriate concentration (Table 2.1) in 10% serum in 1 X TBST for 1 to 2 days at 4°C on a rocker. 4, 30min 1XTBST washes were performed to remove unbound secondary antibody and samples were then transferred into PBS and processed for OPT wholemount scanning as in section 2.6.

Table 2.2 Primary and Secondary antibodies.

Antibody	Supplier	Cat #	Dilution	Application
<i>Primary</i>				
Anti Hnf3 β rabbit polyclonal IgG	Upstate Cell Signaling	07-633	1:500	wholemound section
Anti Sox2 rabbit polyclonal	Millipore	AB5603	1:1000 1:500	section (IHC) section (IF)
Anti Nkx2.1 mouse monoclonal	Santa Cruz	sc-53136	1:500	section
Anti pSMAD 1/5/8/ rabbit polyclonal IgG	Cell Signalling	9511	1:100 1:50 1:300	wholemound section (IF) section (IHC)
Alexa Fluor 568 goat anti-mouse IgG	Invitrogen	A11031	1:200	section
Anti Noggin goat polyclonal IgG	R&D Systems	AF719	1:50	section
<i>Secondary</i>				
Cy3 goat anti-rabbit	Jackson ImmunoResearch	111-165-144	1:200	section
Alexa Fluor 488 Goat anti-Mouse IgG	Invitrogen	NCRC	1:200	section
Alexa Fluor 555 Donkey anti-Goat IgG	Invitrogen	NCRC	1:100	section
Goat anti Rabbit AP	Invitrogen	G21079	1:200	section
Goat anti-Mouse AP	Santa Cruz	sc-2008	1:200	section
Alexa Fluor 488 donkey anti-rabbit IgG	Invitrogen	A21207	1:200	section
DAPI	Invitrogen	P36931	75 μ l per slide	section

2.6 OPT Scanning

Optical Projection Tomography (OPT) is a technique for visualising internal expression patterns in whole embryos or tissues, maintaining the morphological context in which gene or protein localisation can be analysed and comprehended (Sharpe *et al.*, 2002). Following wholemount *in situ* hybridisation or immunolocalisation (with either colour staining or fluorescent labelling) samples were examined under a light microscope and prepared for scanning by the removal of debris and trapped air bubbles. Samples were embedded in 1% Low Melting Point agarose (Sigma Aldrich) and orientated carefully. Cut out agarose blocks were attached to metal mounts and dehydrated in methanol overnight and cleared in 1:2 Benzyl Alcohol: Benzyl Benzoate (Sigma) for a minimum of 6 hours. 400 images were generated by imaging samples through a 360 degree rotation with a prototype OPT scanner built at the MRC Human Genetics Unit, Western General Hospital, Edinburgh, installed at Trinity College Dublin. Usually each specimen was scanned in 3 channels, viewed in visible light (for colorimetric staining), under UV illumination with a Texas Red filter (for immunolocalisation using Cy3, 560/40nm excitation and 610LP nm emission) and with a Green Fluorescent Protein 1 filter (for background auto-fluorescence, 425/60nm excitation and 480nm emission). Raw images were integrated to reconstruct 3D digital representations and further analysis using custom software provided by the Edinburgh Mouse Atlas Project (MA3DView and MAPaint) on a Linux workstation. Virtual sections could be “cut” through the 3D reconstructions in any orientation plane to view the internal pattern. Whole 3D representations could be viewed externally following “volume rendering” which shows expression patterns in depth through the specimen (examples shown in Figure 3.3) or following “surface rendering” which produces contours across pixels above a set threshold level, allowing visualisation of the shapes of morphological structures or expression domains (examples shown in Figure 5.3). Post-analysis image processing was performed using IP Lab software.

2.7 Cryosection Immunohistochemistry

PFA fixed and dehydrated embryos samples were again rehydrated as described for *in situ* hybridisation (Section 2.4). Rehydrated samples were embedded in 1.5% routine-use agarose gel made in 7.5% sucrose in PBS, in the appropriate orientation. The agarose

blocks were trimmed to a trapezoid noting the orientation of the sample and equilibrated in 30% sucrose overnight or until they sank at 4°C while rocking, to cryoprotect the tissues. 5 to 10 micron (µm) sections were cut using a Leica cryostat at -30°C and collected onto Superfrost Plus microscope slides (VWR). Sections were dried and then stored at -20°C. Prior to use, slides were allowed to come to RT for at least 20 minutes to allow full tissue adhesion to the slides and were washed with PBS. Where required (pSmad, Nkx2.1) antigen retrieval was performed by heating the slides in 0.01M Citrate Buffer pH7.5 at 90°C in a water bath for 10 minutes to improve antigen exposure for antibody binding. These slides were allowed to come to room temperature and then washed in PBS 3 times for 5 mins each. All sections were blocked with 5% serum (from species that secondary antibody was raised in where possible) in PBTT (PBS with 0.1% Triton100X, 0.1% Tween 20) in a humid chamber at room temperature for 1 hour. Sections were incubated with 150 µl of the appropriate concentration of primary antibody in blocking solution at 4°C in a humid chamber with parafilm covers overnight. Following 3, 15 minute PBS washes in a slide box at room temperature on a rocker, the slides were incubated with the appropriate concentration of secondary antibody diluted in blocking solution for 1 hour in a humid chamber. 3 further 10 minute PBS washes were performed at room temperature on a rocker. For alkaline phosphatase conjugated secondary antibodies, staining was developed with NBT/BCIP as described for *in situ* hybridisation following a 15 minute wash in NMT. The stain was fixed with 4 % PFA for 1 hour and, following 2 PBS washes for 5 mins, coverslips were mounted on top with Aquapolymount (Polysciences) and allowed to dry in the dark for 48 hours before photographing. For fluorescent labelled secondary antibodies, the slides were mounted with Anti-Fade Gold with DAPI (Invitrogen). Slides were examined and images captured using a compound fluorescent microscope or the Zeiss Confocal microscope at the National Children's Research Centre (NCRC). Further image processing was performed using Image J software (NIH).

2.8 3D Explant Culture

Cd1 mice were mated overnight, the finding of a vaginal plug in a female the following morning was designated D0 of gestation. Pregnant dams were sacrificed on gestation day 9 and embryos were harvested by caesarean section and dissected from uteri in 1X DMEM/F12 1:1 (#21331-020 Gibco) containing 1% Penicillin Streptomycin and 6% D-Glucose at room temperature. The embryos were accurately staged using Theiler staging

and more finely using somite counting (Theiler, 1989a). Theiler stage 14 embryos were the targeted stage as they possess an undivided foregut.

2.8.1 Trunk explant

Tissue for explant was prepared by removing structures anteriorly above the level of the 1st branchial arch and posteriorly below the 8th somite by sharp dissection under an Olympus dissecting microscope in harvest medium in 6cm dishes.

2.8.2 Foregut isolation

A detailed protocol was established following extensive optimisation and is described in Appendix A. Foregut explants were prepared by microdissection of the foregut from Cd1 E9 embryos which were first staged by somite counting and allocated to somite groups by rounding as follows: less than 15 somites, 15 (15 to 17), 18 (18 and 19), 20 (20 and 21), 22 (22 and 23), 24 (24 and 25), 28 (26, 27 and 28) and 30 somites (29 and 30). Embryos were staged and dissected in the harvest medium with added 10% heat deactivated Fetal Bovine Serum (FBS) in 6cm dishes under an Olympus SZX9 dissecting microscope at maximum magnification (57x). The tissue prepared for explant consisted of foregut endoderm and the well demarcated ventral foregut mesoderm together with a variable extent of the dorsal foregut mesoderm with or without related structures as required and extended anteriorly to just below the branchial arches and posteriorly to the lower limit of the upper limb bud/protrusion. The head and unnecessary anterior structures and all tissue below the upper limb bud protrusion were initially removed. Splitting of the dorsal neural tube along the midline facilitated removal of the neural tube and the lateral somites while allowing the dorsal foregut mesoderm to remain intact. Foreguts were then shelled out of embryos by first removing the lateral ectoderm using the heart to stabilise the embryo with Dumostar Biologie tipped #7 forceps (#11297-10 Fine Science Tools). Lateral ectoderm flaps were raised and extended anteriorly with the tip of a tungsten needle from the limb bud protrusion along the embryo where the cut removing the neural tube and somites exposed the underlying mesoderm. These flaps were removed when they reached the anterior and ventral ectoderm limits by downward pressure of the length of the tungsten needle producing a clean cut. Additional blunt dissection using a tungsten needle in this manner was used to remove the dorsal foregut mesoderm tissue with or without the notochord and neural floor plate and also finally to remove the heart.

2.8.3 Collagen gel plastic ring construct and explant assembly.

Sterile plastic rings were fashioned from a Pasteur pipette (#Z350869 Sigma) using a straight edge blade. A smooth cut was used to generate rings with a height of 0.2 to 0.3 cm and an internal diameter of 0.4cm. Rat tail collagen (#354236 Corning) with added 1 in 10 DMEM 10X (#D2429 Sigma) was brought to gelling pH by adding 0.8 M NaHCO₃ (approx 3.8µl per 100 µl of collagen/DMEM solution, but titrated for each new batch) and vortexed to mix thoroughly. 20µl of this collagen mix was used to fill each plastic ring and these constructs were allowed to gel at room temperature standing on their side. Explants were transferred on forceps tips (for thorax) or by borosilicate glass capillaries, internal diameter 0.86mm (#BF150-86-10 Sutter) (for foreguts) in a drop of medium onto the collagen gel base within the plastic rings which were first positioned flat in Nunc 4 well culture dishes (# 176740 Thermo Scientific). Additional manipulations such as notochord transplant or bead placement (see sections 5.2.3 and 6.3.3 respectively) were performed at this stage. Excess medium was removed by pulled micro-capillary under mouth control. An overlay drop of 10 µl of newly made collagen mix was added to completely cover the explant and allowed to gel at room temperature. Repositioning of the explant and added materials was done prior to complete collagen gelling as necessary using tungsten needles. Wells containing explants within the collagen plastic ring construct were flooded with culture medium (1X DMEM/F12 1:1 with added 1% Penicillin Streptomycin, 6% D-Glucose, 10% FBS and 200mM L-glutamine) once collagen gelling was complete (approx 30 mins). Plastic ring constructs were confirmed to float under the medium-air interface and explants were cultured in a Sanyo CO₂ incubator (Model MCO-15AC) at 37°C and 5% CO₂ concentration in air for 24 to 72 hours. Explants were photographed pre and post culture by an Olympus Camedia digital camera (C-5050). Explants were harvested from culture with 2 x 10 min chilled PBS washes followed by fixation in 4% PFA for 2 hours within their collagen plastic ring constructs at 4°C. After fixation explants were washed in PBS and the collagen containing the explant was detached at its boundary from the plastic ring using tungsten needles under a dissecting microscope. Explants within their collagen gels were then dehydrated through a series of increasing Methanol concentrations in PBT and stored at -20°C in 100% methanol.

2.9 Methods described elsewhere

Selection of genes for BMP downstream targets (Section 3.2.2)

Analysis of location of Tbx1 gene expression in the oesophagus (Section 4.2.2)

Notochord dissection (Section 5.2.2)

Foregut Staging (Section 5.2.3)

Isolated foregut 3D explant culture with beads (Section 6.2.1)

Individual explant assessment (Section 6.2.3)

Statistical analysis for bead experiments (Section 6.2.4)

Chapter 3

Bone Morphogenic Protein signalling disruption in foregut and notochord of Adriamycin treated embryos.

3.1 Introduction

A characteristic feature of Adriamycin treated mice are notochord branching defects which are related by both position and dimension to the location and extent of molecular signalling disturbances in the abnormal foregut as well as the site and severity of the tracheo-oesophageal malformations (Hajduk et al., 2011b). Similar notochord abnormalities are found in the *Noggin* knockout mouse model in association with tracheo-oesophageal malformations (Que et al., 2006). *Noggin* is an extracellular antagonist of Bone Morphogenic Protein (BMP) signalling (Zimmerman et al., 1996). Human chromosomal defects in the region of the *NOGGIN* locus have been reported in children affected by OA/TOF but in rare cases only (Marsh et al., 2000). In *Noggin* null mice the phenotype can be rescued by reducing the gene dose of *Bmp* ligands 4 or 7 (Que et al., 2006, Li et al., 2007) suggesting a role for tightly regulated BMP signalling in normal foregut morphogenesis. In Adriamycin treated mice *Noggin* gene expression was found to be highly disturbed in the notochord branches and adjacent dorsal foregut endoderm in Adriamycin treated embryos (Hadjuk, P., Thesis, 2011) (Figure 3.1). While *Bmp4* gene was not abnormally expressed in the AMM, additional disruption may arise in the BMP pathway at other levels or in other BMP ligand encoding genes. This disturbance of *Noggin* expression, while somewhat at odds with the proposal that BMP over activity induces simultaneous foregut and notochord abnormalities in the *Noggin* knockout mouse (Que et al., 2006), underlines the potential importance of tightly controlled BMP signalling during oesophageal development.

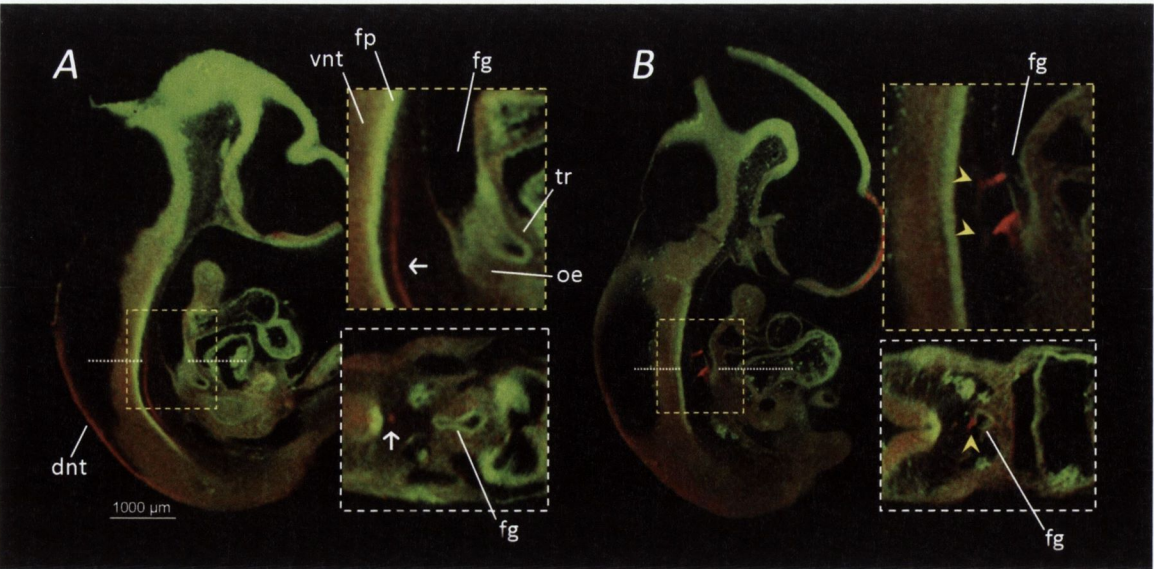


Fig 3.1 *Noggin* expression in AMM embryos. See legend facing.

Figure 3.1 contd: Simultaneous immunolocalisation of Hnf3b (in green) and in situ hybridisation compares the expression pattern of Noggin (in red) in Adriamycin treated (B) versus control (A) embryos at E10 in virtual sections of 3D reconstructed OPT scans. Boxed areas are enlarged in upper right panels; transverse sections, taken in the planes indicated by dotted lines are shown in the lower right panels. Note the abnormal shape and reduced size of the foregut lumen in the Adriamycin treated embryo (B) at the level of notochord branching (indicated by yellow arrowheads). White arrows highlight normal notochord position for comparison. Adriamycin treated embryos show abnormal intense Noggin expression in the foregut adjacent extremity of the notochord branches (B) and in the adjoining foregut which has failed to divide. Unpublished data generated by P. Hajduk (in prep).

Diverse and vital roles for BMPs, members of the TGF-beta superfamily of highly evolutionary conserved signalling molecules, are well established across embryogenesis. BMP signalling is imperative in the induction of cell fate determination in mesoderm derived structures and in the balance of epithelial to mesenchymal transition (EMT) (Kishigami and Mishina, 2005, Hogan, 1996). Dynamic BMP signalling confers DV identity from the earliest stages of embryonic development (De Robertis and Kuroda, 2004), when Bmp4 acts as a ventralising morphogen (Sasai and De Robertis, 1997). Bmp ligand genes are expressed in the foregut region as it forms and subsequently divides. Bmp4 expression is reported to be localised to the ventral foregut mesoderm (Que et al., 2006), Bmp7 expression can be detected in the notochord and foregut endoderm and Noggin is expressed in the notochord during this gestational window (Danesh et al., 2009). Bmp ligands are implicated to have roles in foregut separation however the means by which these signals can act to induce structural change is not well understood. Conditional knockout of the Bmp4 gene induces severe morphological foregut disturbances in the form of tracheal agenesis (Li et al., 2008a). In the Bmp receptor (Bmpr) type1a and 1b double knockout mutant the foregut fails to divide (Domyan et al., 2011). Bmp2 activity has recently been shown to be necessary for foregut formation by ventral folding morphogenesis (Gavrilov and Lacy, 2013). Evidently a tight balance of BMP signalling is critical for proper foregut separation.

BMP2, 4 and 7 signalling activity is transduced via phosphorylation of Smad intracellular proteins 1, 5 and 8 upon specific Bmpr1 binding (Figure 3.2), therefore detection of phosphorylated Smad proteins 1, 5 and 8 (pSMAD) is an effective indicator of overall BMP activity in tissues (Massagué and Wotton, 2000). This offers an approach to characterise BMP activity during the key developmental window for foregut separation wherein dynamic tissue rearrangements are pre-empted by dorso-ventral molecular patterning.

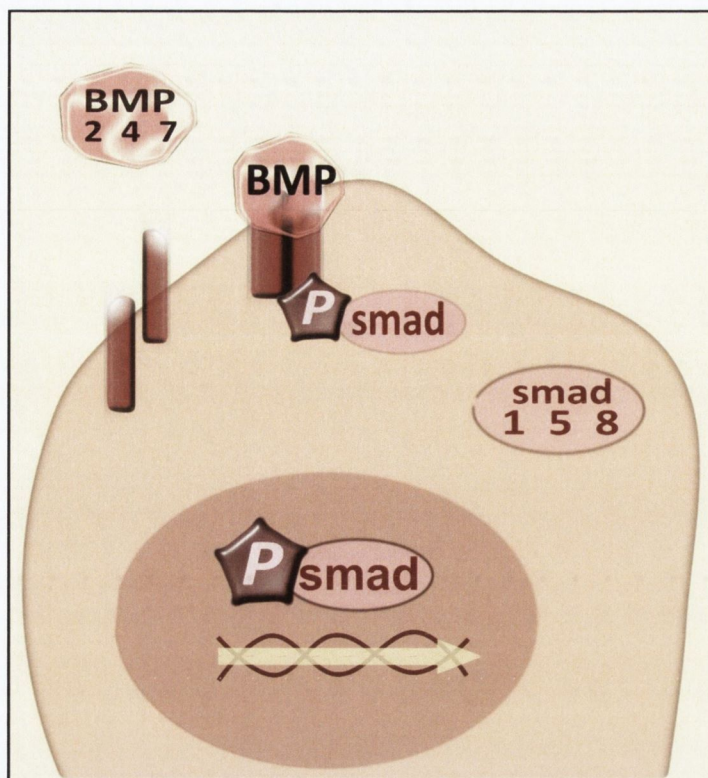


Figure 3.2: A diagrammatic representation of BMP signal transduction. Bmp ligand (2 4 or 7) binding to paired trans-membrane receptors leads to phosphorylation of intracellular smad proteins and subsequent relocalisation of phosphorylated smad (pSMAD) to the nucleus where it influences downstream target gene transcription (arrow).

The phenotypic similarities exhibited between the AMM and the Noggin knockout mouse raise compelling arguments for the investigation of the BMP pathway in the AMM. Previously reported Noggin gene expression disturbance in the AMM foregut and notochord further implies that balance in BMP signalling may be a crucial molecular mechanism in normal oesophageal development.

The main objective of the work presented in this chapter is;

To test the hypothesis that BMP signalling is disturbed in the AMM and contributes to the pathogenesis of foregut malformations.

This was addressed by:

- 1) The analysis of BMP signalling during foregut development. Normal, control and Adriamycin treated embryos were examined by a novel technique of pSMAD immunolocalisation in whole embryos with OPT, together with pSMAD immunohistochemistry on cryosections, to detect overall BMP activity.
- 2) The identification of BMP target genes expressed in the developing foregut. A survey of array data and spatial gene expression archives identified candidate BMP responsive genes with potential foregut region expression which were subsequently detected in normal embryos by wholemount in situ hybridisation and 3D imaging.
- 3) The comparison of foregut region spatial expression profiles of these BMP downstream target genes in Adriamycin treated embryos and controls by wholemount in situ hybridisation and OPT scanning to interrogate the downstream read-out of BMP signalling.
- 4) The examination of Bmp2 and Bmp7 gene expression profiles in AMM compared to controls by wholemount in situ hybridisation and OPT scan.

3.2 Methods

3.2.1 Overview of pSMAD immunolocalisation techniques.

Control and Adriamycin treated embryos were obtained, processed and stored as described in section 2.1 and additional Cd1 embryos as described in section 2.2. Stored embryos were prepared either for wholemount pSMAD immunolocalisation (as described in Section 2.4) together with OPT scanning (as described in Section 2.6) or immunohistochemistry on cryosections (as described in section 2.7) followed by examination on a compound fluorescent microscope. Cryosections of AMM, control and normal embryos were additionally assessed by immunofluorescent labelling for pSMAD together with DAPI nuclear stain and examined using Confocal laser microscopy (as described in section 2.7). The antibody preparations used in this chapter are given in Table 2.2. OPT scans of wholemount pSMAD immunolocalisation were 3D reconstructed and further processed as described in section 2.6.

3.2.2 Selection of BMP downstream target genes.

Extensive literature review and interrogation of array dataset repositories such as ArrayExpress (<https://www.ebi.ac.uk/arrayexpress/>) and Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) were undertaken to identify quantitative datasets of differentially expressed genes under conditions of altered BMP 2, 4 or 7 signalling in mammalian tissues or cells irrespective of investigative platform. Resulting lists of potential BMP responsive genes were screened using virtual gene expression data depositories such as GXD (<http://www.informatics.jax.org/expression.shtml>) and EMAGE (<http://www.emouseatlas.org/emage/>), in addition to primary literature review, to select out and prioritise investigation of genes with any prior indication of expression in the foregut or notochord during the E9 to E11.5 developmental window of foregut division. Preliminary testing of selected genes in normal embryos by *in situ* hybridisation identified candidates with confirmed foregut expression which were advanced to testing in AMM versus control specimens.

3.2.1 Overview of in situ hybridisation technique.

Control and Adriamycin treated embryos were obtained, processed and stored as described in section 2.1 and additional Cd1 embryos as described in section 2.2. RNA probes were generated for each candidate gene as described in section 2.3. Probes used for in situ hybridisation in this chapter are given in Table 2.1. Stored embryos were prepared for in situ hybridisation, as detailed in section 2.4, and subsequently processed for OPT scanning as described in section 2.6. Except where otherwise expressly stated a minimum of 5 AMM and 3 control specimens were examined at each gestational stage for each gene analysed.

3.3 Results

3.3.1. Localisation of pSMAD 1/5/8 illustrates BMP signalling distribution in the foregut region during normal development.

Normal Cd1 embryos underwent pSMAD detection on cryosections and in whole specimens by immunolocalisation, utilising OPT scanning to reconstruct and analyse the pattern in whole embryos (n=5 per technique, per stage). Localisation of pSMAD in Theiler staged whole embryos across E9 (TS14) to E11.5 (TS19) (Theiler, 1989b) was established by 3D digital reconstruction of OPT scans (Figure 3.3 A). Known sites of high BMP activity including the fronto-nasal process, branchial arches, limb buds, otic vesicles and the dorsal neural tube displayed strong pSMAD signal (Danesh et al., 2009). Comparison of digital sections from 3D reconstructions with physical sections at E9 (Figure 3.3 matched pairs are B,G and C,H), E10 (Figure 3.3 matched pairs are D,I and E,J) and E11 (Figure 3.3 matched pair is F,K) revealed conformity across both approaches in pSMAD distribution at known sites of prominent BMP activity as well as in the foregut region, validating the novel use of whole embryo pSMAD detection.

Additional digital sections from 3D reconstructions demonstrate the benefit of maintaining embryonic architecture while examining molecular patterning (Figure 3.3 L to O). Visualisation of any plane within the 3D reconstructed object permitted detailed examination of the notochord and the foregut, in which normal pSMAD distribution was characterised at each gestational stage. While distributed uniformly throughout the length of the notochord at each stage, the level of pSMAD signal in the notochord was more intense at E10 than at E9 or E11 (Figure 3.3 D, I and M versus B, G and L or F and K respectively). In the foregut, pSMAD was detected in the endoderm and in the ventral foregut mesoderm; a distinct dorso-ventral distribution pattern of pSMAD was noted in the foregut endoderm, with strong ventral predominance (Figure 3.3 C,H,L and D,I,M). Following foregut division at E9.5 pSMAD was detected in the trachea and lung bud endoderm and in the ventro-lateral tracheal and lung bud mesoderm but was not detected in the oesophagus, elaborating the ventral predominance of BMP/pSMAD 1/5/8 signalling (Figure 3.3 E,J,N and F,K).

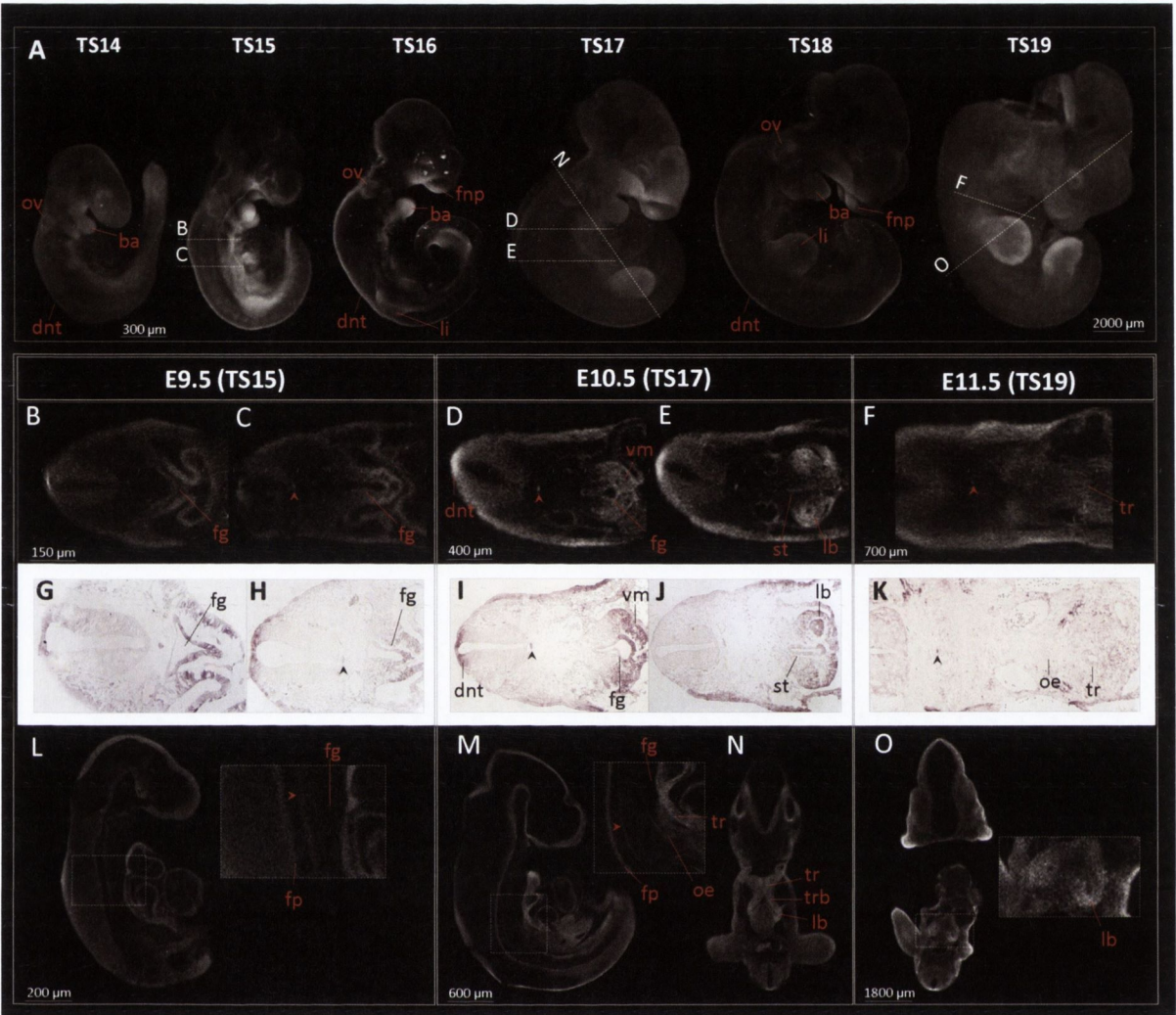


Figure 3.3: BMP activity revealed by pSMAD immunolocalisation in mouse embryos at E9 to E11.5. Techniques used were wholemount pSMAD detection using OPT in A (entire panel) to F and L to O, and immunohistochemistry for pSMAD on cryosections (G to K). Panel A are volume rendered digital reconstructions from OPT scans of Theiler Staged whole embryos demonstrating strong pSMAD signal in the branchial arches (ba), limb buds (li), dorsal neural tube (dnt), otic vesicle (ov) and the frontonasal process (fnp).

Virtual sections of 3D reconstructed OPT scans in transverse (B to F), sagittal (L and M) and oblique (N and O) planes are indicated by dashed lines in panel A (labelled accordingly). Sections G to K provide stage and anterior-posterior position matched comparison to virtual sections B to F. Arrowheads indicate the notochord, which demonstrates stronger pSMAD signal at E10.5 (D,I,M) than at E9.5 (C, H, L) or E11.5 (F and K). Clear ventral predominance of pSMAD signal in the foregut endoderm (fg) is illustrated across all gestation stages and strong pSMAD localisation is also noted in the ventral foregut mesoderm (vm), trachea (tr) endoderm, lung bud (lb) endoderm and mesoderm but not in the oesophagus (oe). *fp*, neural floor plate. Dorsal is to left in A through M. Scale bars as shown

3.3.2 *Wholemout visualisation of abnormal pSMAD immunolocalisation in Adriamycin treated foregut and notochord.*

Wholemout immunolocalisation, combined with OPT imaging, as a tool for pSMAD detection conferred the advantage of preserved embryo morphology, enabling integrated analysis of spatial BMP activity and structural foregut abnormalities in the AMM. The pSMAD distribution was identical between normal CD1 embryos and experimental control embryos across all regions and stages. Adriamycin treated embryos did not differ from controls in the observed pattern of pSMAD at the fronto-nasal process, the branchial arches, the limb buds and the dorsal neural tube (Figure 3.4 compare A to B, C to D, and E to F).

At E9, 5 control and 11 AMM whole embryos were assessed. Control embryos displayed an undivided foregut with a ventral laryngo-tracheal groove indicating the site of future tracheal formation (Figure 3.4 A). In the AMM, the notochord cross sectional area was frequently expanded laterally and dorso-ventrally and demonstrated ventral displacement and extensive abnormal connection with the dorsal foregut (4/11 embryos) (Figure 3.4 B). pSMAD detection was prominent in both the abnormally shaped notochord and in the adjoining dorsal foregut endoderm (Figure 3.4 B). In 3/11 embryos discrete notochord branching defects were present where notochord tissue bridged ventrally across the intervening mesoderm to connect to the dorsal foregut endoderm while still in continuity with the dorsally positioned notochord proper. These notochord branches displayed higher pSMAD signal than the notochord proper and areas of ectopic pSMAD were detected in the adjoining dorsal foregut endoderm. In embryos with either expansion or discrete branching of the notochord the foregut was hypoplastic in the region of notochord abnormalities and pSMAD signalling disturbances (Figure 3.4 B). In 3/11 embryos the notochord was abnormally expanded with strong pSMAD immunolocalisation but did not connect with the normally shaped foregut in which the pSMAD pattern was not perceptibly disturbed. In 1 embryo the notochord and foregut appeared normal and pSMAD patterning disturbances were not detected.

At E10 7 control and 11 AMM embryos were assessed. In control embryos a distinct septation between the ventral trachea and dorsal oesophagus indicated the onset of foregut division and early lung buds were present. E10 AMM embryos had multiple, discrete notochord branches occurring in 3 broadly classifiable antero-posterior regions: proximal foregut (anterior to the region of normal foregut separation), mid anterior foregut (where foregut separation normally occurs) and hindgut (at all levels posterior to the early duodenum). 4/11 embryos had 1 branch in each region, 3/11 had a branch each in the

proximal and mid foregut regions, 3/11 had only one branch at the mid foregut and 1/11 had no notochord branching. In notochord branches pSMAD localisation appeared more intense than in the notochord proper. In the dorsal foregut endoderm directly adjoining the notochord branches, discrete areas of ectopic pSMAD signal were discerned (Figure 3.4 D). At the level of notochord branching and pSMAD abnormalities the upper foregut was hypoplastic, foregut division had failed to occur and the formation of lung buds was delayed. The nature of abnormal pSMAD localisation in the AMM was noted to be highly similar to ectopic Noggin gene expression previously detected by our group (Hadjuk, P., Thesis, 2011) (compare Figure 3.4 D to Figure 3.1 B).

At E11 7 control CBA and 10 AMM embryos were assessed. In control embryos notochord regression was inferred by lower pSMAD signal than at E10 (Compare Figure 3.4E to C). Oesophagus and trachea formation, with early bronchial morphogenesis, were noted as expected for this stage of development. In the AMM the upper foregut was narrowed and the oesophagus was absent (Figure 3.4F boxed regions). An abnormal connection between the tracheal bifurcation and the stomach via the lower foregut remnant, referred to as the fistula and typical of the model, was visualised in most embryos (7/10). Notochord branching at this stage consisted of single fine branches in the pharyngeal foregut region which were adjacent to, but did not connect with the dorsal foregut wall (7/10 embryos) (Figure 3.4 F). pSMAD signal in these branches was comparable to the notochord proper however remarkably intense ectopic foci of pSMAD signal were detected in the dorsal foregut endoderm at a corresponding level to the notochord branch (Figure 3.4 F boxed regions). In 2/10 embryos substantial notochord branches persisted in contact with the dorsal foregut endoderm. High levels of pSMAD in these branches as well as ectopic pSMAD signal in the dorsal foregut were noted, similar to treated embryos at E10. Ectopic pSMAD was detected in the fistula endoderm in 1 embryo only. 1 AMM embryo did not display any phenotypic morphological abnormalities or pSMAD disturbances at E11. At all gestational stages ectopic pSMAD in the dorsal foregut endoderm did not occur in the absence of notochord branching in the AMM.

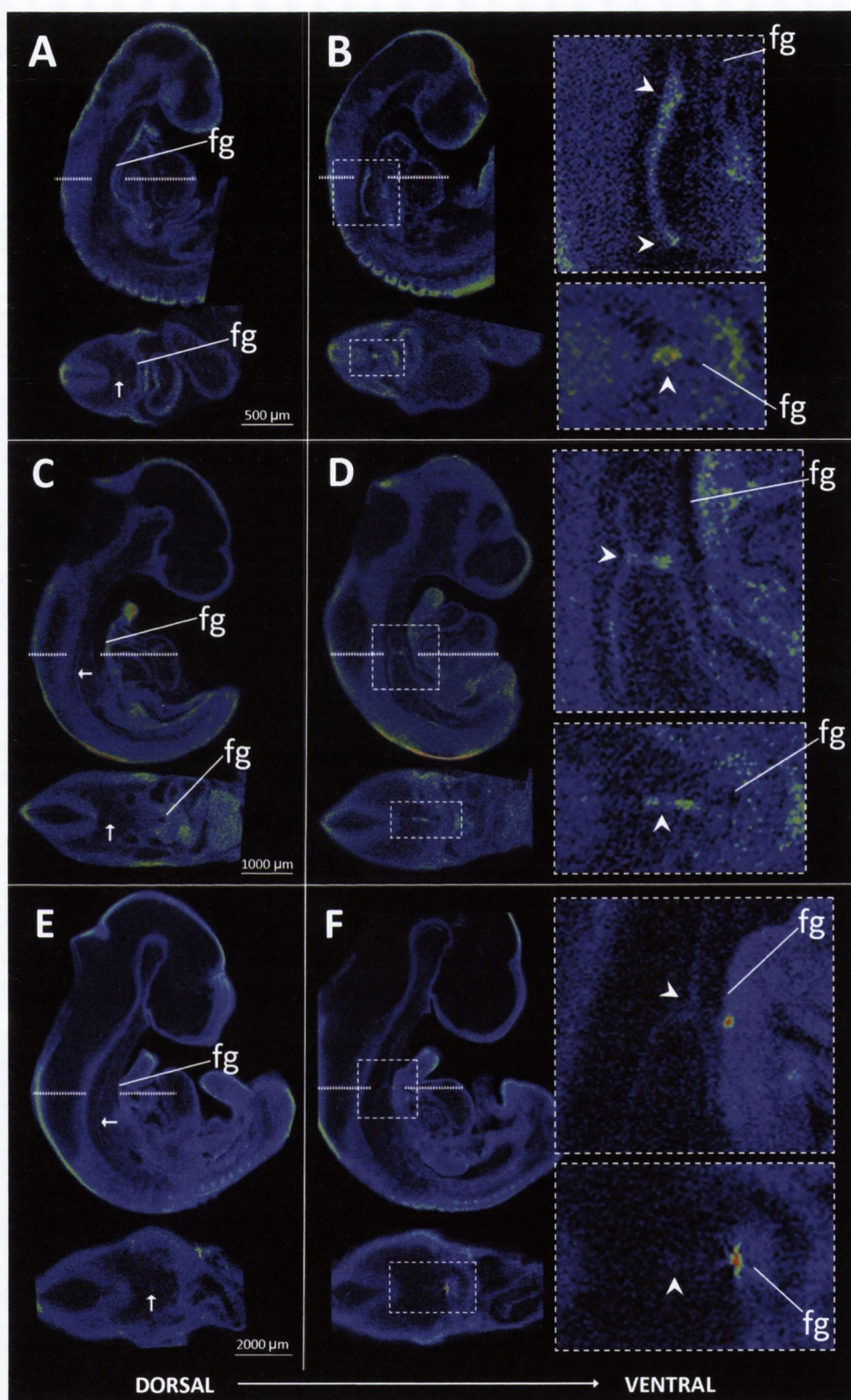


Figure 3.4: Immunolocalisation of pSMAD in AMM embryos demonstrates abnormal BMP signalling in the foregut. Heat map representing pSMAD immunolocalisation in whole embryos in 3D reconstructed OPT scans at E9 (A and B), E10 (C and D) and E11 (E and F). Strong to weak signal is indicated by Red to Green to Blue. Arrow heads indicate notochord branching in AMM embryos (B,D and F) and arrows indicate normally positioned notochord in controls (A, C and E). Transverse sections are at the marked planes in the sagittal image above. Boxed regions are magnified to the right. Note abnormal notochord morphology as described in the text: expanded with extensive connection to the dorsal foregut (in B); discrete branches bridging across the mesoderm from notochord rod to foregut wall (in D); branch not connecting to the foregut (in F). Intense focus of BMP activity/pSMAD in the dorsal foregut endoderm is shown in F (magnified boxed region). Scale bars as shown.

3.3.3 High resolution analysis of abnormal pSMAD localisation in the notochord and foregut of the AMM.

Following observation of abnormalities in wholemount pSMAD immunolocalisation in the AMM, colourmetric and immunofluorescent detection techniques were employed on cryosections to confirm the tissue distribution of BMP activity in tracheo-oesophageal malformations and to observe the pattern at a cellular level. A minimum of 3 control and 5 AMM embryos were compared for each gestational stage. Mirroring findings in whole embryos, abnormally high levels of pSMAD were detected in the dorsal foregut endoderm and in adjacent notochord branches in Adriamycin treated embryos across all gestational time points (Figure 3.5 B). Strong pSMAD labelling coincided with abnormal morphology of the foregut including altered shape of the endoderm, foregut narrowing or complete occlusion of the dorsal foregut lumen (Figure 3.5 E, B and D respectively). Clusters of abnormally shaped and intensely pSMAD stained cells within the dorsal foregut wall were identified in the AMM at E9, E10 and E11, adjacent to notochord branches. At E9 and E10 these clusters were included in the contact area between the dorsal foregut wall and the notochord branch (Figure 3.5 E). At E11, where the notochord branch was not in continuity with the dorsal foregut, these cells persisted within the abnormally shaped dorsal foregut endoderm (Figure 3.5 D). These aberrant cell organisations corresponded with the remarkably intense foci of pSMAD immunolocalisation in the dorsal foregut wall described in the semi-quantitative (heat-map) analysis of wholemount specimens (compare Figure 3.5 D and Figure 3.3 F). Using immunofluorescent detection of pSMAD together with DAPI (DNA/nuclear) counterstaining showed that while pSMAD localises to the nuclei in adjacent areas of the foregut, these abnormal cells show highly intense extra-nuclear pSMAD localisation (Figure 3.5 F).

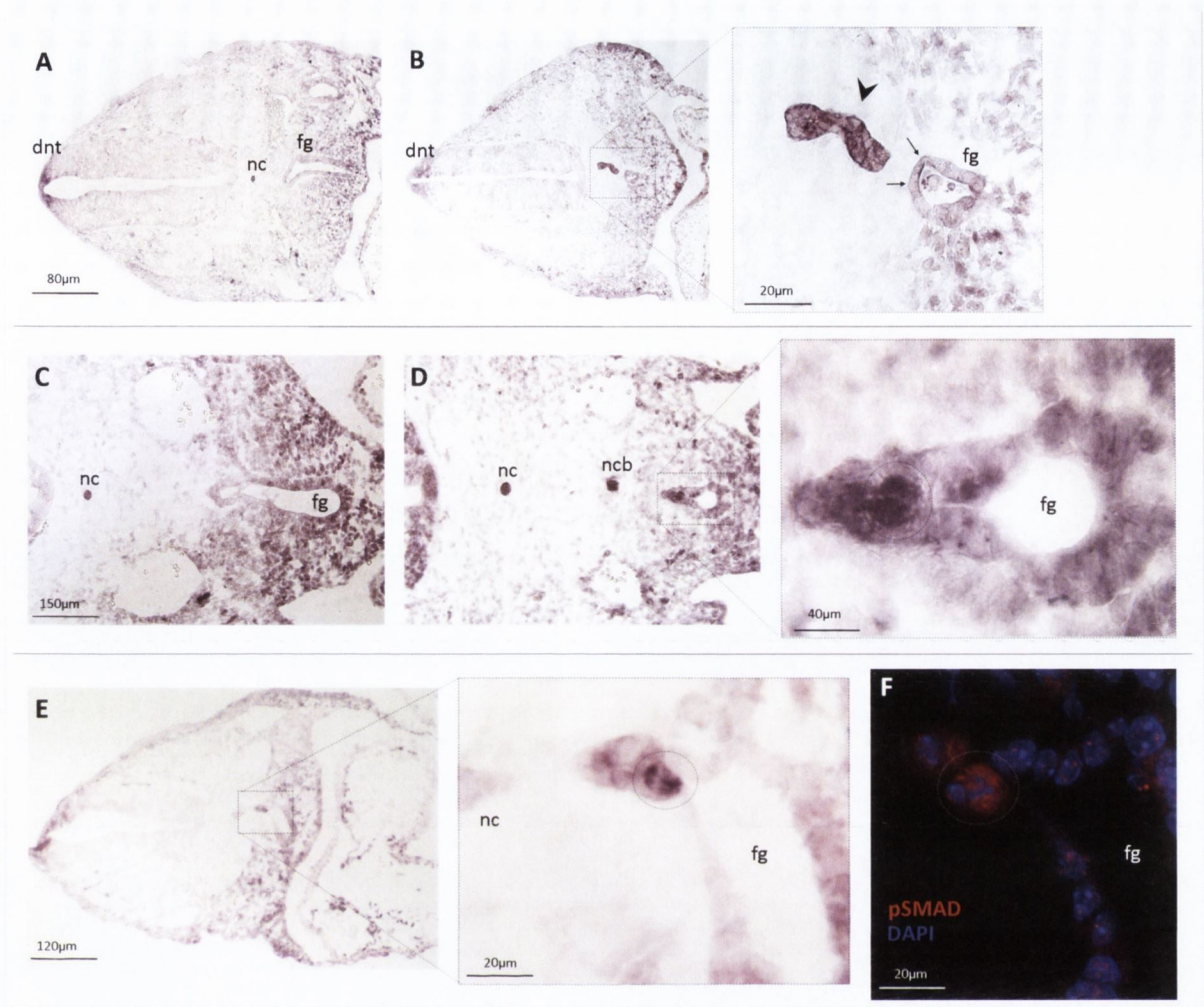


Figure 3.5: High resolution analysis of abnormal pSMAD localisation in the AMM. pSMAD immunolocalisation on cryosections in AMM (B,D,E,F) compared to control (A,C) embryos across E9 (A, B), E10 (E, F) and E11 (C,D). F shows immunofluorescent labelled markers imaged by Confocal microscopy, all others are of immunohistochemistry. Boxed regions are magnified as indicated. Abnormal foregut (fg) morphology is described in text. Notochord (nc) and notochord branch (ncb and arrowhead in B) show strong pSMAD signal in B and D. Intensely pSMAD positive cell clusters are circled (D,E,F). Observe extra nuclear pSMAD (in red) in F compared to other foregut cells in view (DAPI in blue marks nucleus). E and F are serial sections of the same specimen. *dnt* dorsal neural tube.

3.3.4 Disturbed BMP downstream target gene expression in the AMM foregut region.

Given the evidence for both altered Noggin expression and pSMAD localisation in the abnormal notochord and foregut of Adriamycin treated embryos, the role of BMP signalling in foregut malformations was further examined in the functional read-out of this pathway at the level of downstream gene targets. A list of BMP responsive genes was compiled (as described in Methods) from analysis of BMP2, 4 or 7 signalling response assays in a variety of cell and developmental contexts. Gene expression databases were used to sub-select 15 genes potentially expressed in the foregut region at E9 to E11.5 (Table 3.1). *In situ* hybridisation was used to definitively assess expression of each gene in the foregut region of on normal embryos. Strong ectodermal expression of 2 genes, Fibronectin 1 (Fn1) and Twist homolog 1 (Twist1), interfered with clear visualisation of patterns of expression in the foregut region using the wholemount technique. Only 2 of the genes screened, Cyclin-dependent kinase inhibitor 1A (Cdkn1a) and KDM1 lysine specific demethylase 6B (Kdm6b), did not show foregut specific expression and so were precluded from analysis in AMM. Ultimately 11 candidate genes were confirmed to exhibit foregut region expression and were selected for expression analysis in AMM.

Table 3.1: Selection of potentially foregut expressed BMP target genes.

Gene symbol	Encoded protein	BMP downstream target gene: evidence	Potential foregut expression: evidence
Id1	Helix-loop-helix transcription factor	ten Dijke et al., 2003 Rodriguez-Martinez, 2011	Jen et al., 1996 Gray et al., 2004
Id2	Helix-loop-helix transcription factor	Fessing et al., 2011 Ahmed et al 2011	Jen et al., 2006 Gray et al., 2004
Dpysl2	Dihydropyrimidinase-related protein	Fei et al., 2010	Ito et al, 2008
Msx1	Homeodomain transcription factor	Mukhopadhyay et al., 2005 Zhang et al., 2003	Houzelstein et al., 1997 Coudert et al., 2005
JunB	Activator protein-1 transcription factor	Clancy et al., 2003 Kowanetz et al., 2004	Licht et al 2006
Prx2	Homeobox transcription factor	Peng et al., 2003 Higuchi et al., 2013	Tribioli et al., 2002 Leussink et al., 1995
Bambi	Transmembrane glycoprotein	Grotewold et al 2001	Grotewold L, et al, 2001
Dlx2	Homeodomain transcription factor	Rodriguez-Martinez et al., 2011	Clouthier DE, et al., 2000
Snai1	Zinc finger transcriptional repressor	De Jong et al., 2002	Gray PA, et al., 2004
Snai2	Zinc finger transcriptional repressor	Clancy et al., 2003	Sefton M, et al., 1998
Crabp2	Retinoic acid binding protein	Vallier et al., 2009	Ruberte E, et al., 1992
Fn1	Cell ahesion glycoprotein	Lavery et al., 2009	George EL, et al., 1993
Cdkn1a	Cyclin-dependent kinase inhibitor	Vallier et al., 2009	Ludke TH, et al., 2013
Twist1	Basic helix-loop-helix transcription factor	Shelton et al., 2008	Loebel DA, et al., 2002
Kdm6b	Lysine-specific demethylase	Fei et al., 2010	Hoffman BG, et al.,2008

3.3.4.1 Id1 and Id2

Inhibitor of DNA binding (Id) 1 and 2 genes encode Helix Loop Helix family transcription factors and were identified as early responders to BMP pathway activation (ten Dijke et al., 2003, Katagiri et al., 2002, Miyazono and Miyazawa, 2002), directly activated by pSMAD protein complex formation (Lopez-Rovira et al., 2002, de Jong et al., 2002). Id1 and Id2 were previously described as expressed in the foregut region (Jen et al 1996). We detected prominent Id1 expression across E9-E11 in the dorsal neural tube, in an antero-posteriorly limited region of the ventral neural tube, in the branchial arches and the limb buds, all in fact sites of reported high BMP activity, in both control and treated specimens (Figure 3.6 A and C). In the foregut of control embryos, Id1 expression was localised to the ventral foregut endoderm at E9 (not shown) and was additionally detected in the ventro-lateral foregut mesoderm, in the endoderm of the ventrally forming trachea and in the lung bud meso-endoderm at E10 and E11 (Figure 3.6 A and B and not shown). In Adriamycin treated embryos, Id1 was focally ectopically expressed in the dorsal foregut endoderm immediately adjacent to notochord branching at E9, E10 (Figure 3.6 C and D; dashed circle and arrow) and E11, highly resembling the pattern of abnormal pSMAD localisation in the dorsal foregut endoderm (compare Figure 3.6 C and Figure 3.4 D). Id2 gene analysis revealed highly similar expression patterns to Id1 in control and similar disruption in the AMM at all stages examined (Appendix B Figure A1).

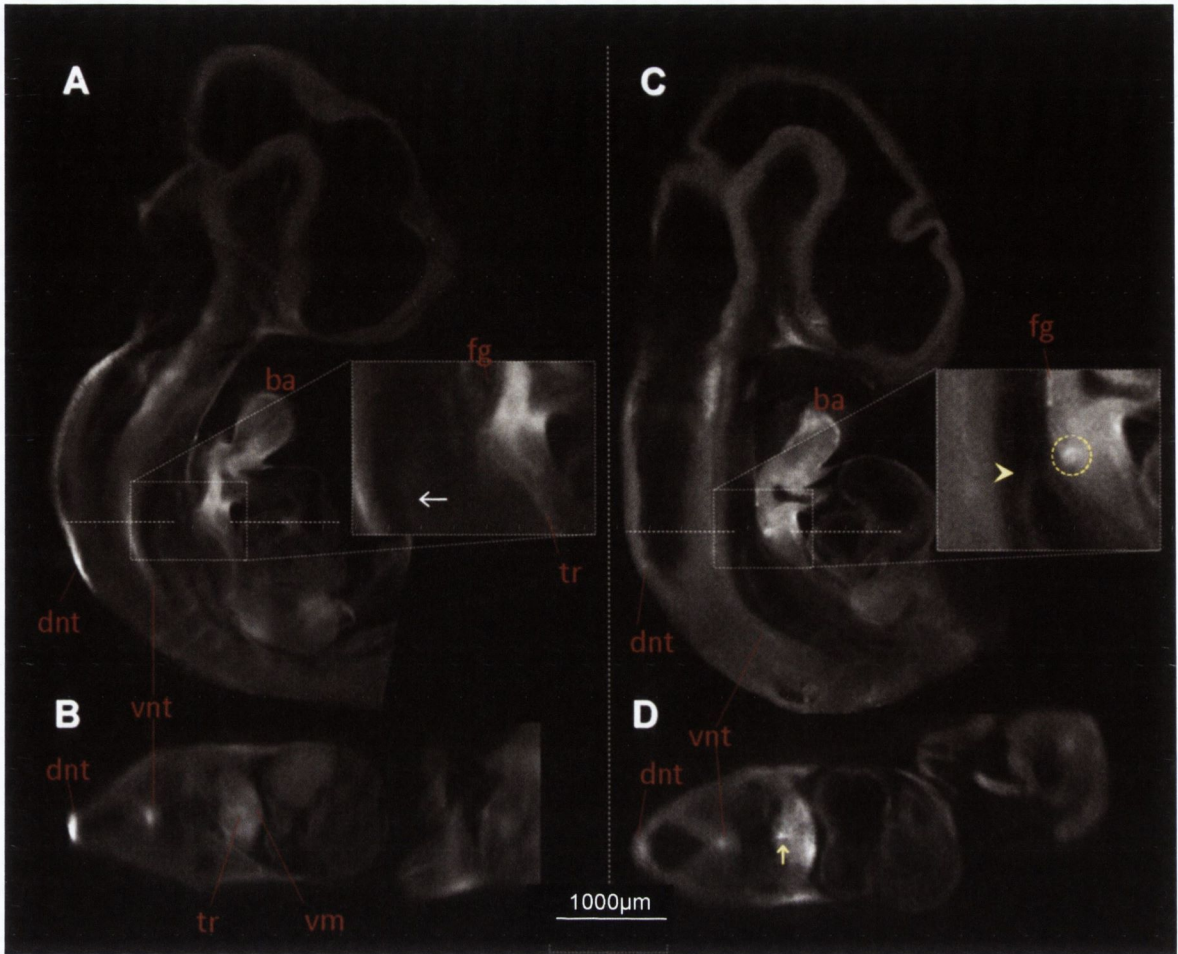


Figure 3.6: Expression of Id1 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Id1 in situ hybridisation (ISH) in embryos at E10 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowheads indicates notochord branching, yellow arrow and circles highlight abnormal gene expression. In control white arrows indicate normal notochord position. *dnt* dorsal neural tube, *vnt* ventral neural tube, *tr* trachea, *ba* branchial arch, *vm* ventral foregut mesoderm. Scale bar as shown

3.3.4.2 Dpysl2

Dihydropyridimase-like gene 2 (*Dpysl2*) has been identified as a BMP/SMAD induced target gene in embryonic cell fate determination (Fei et al., 2010) with a role in axonal outgrowth and central nervous system development (Sun et al., 2010). *Dpysl2* was previously described to be expressed in developing lung and in the embryonic enteric nervous system (Ito et al., 2000). *Dpysl2* expression was observed in the ventro-lateral neural tube and generally throughout the ectoderm across all stages in both control and Adriamycin treated embryos. In controls, discrete endodermal expression was detected in the anterior oesophagus at the point of foregut separation as well as in the lung buds at E10 (Figure 3.7 A, B and C) but was not observed in the foregut or oesophagus at E11, only persisting in the lung bud endoderm (Appendix Figure). In Adriamycin treated embryos at E10 and E11, *Dpysl2* expression was absent from the lung buds and there was an obvious intense upregulation of expression at the point of abnormal foregut separation at E10 (Figure 3.6 D, E and F) as well as abnormally persistent expression in a corresponding location at the fistula-trachea connection at E11 (Appendix B Figure A2).

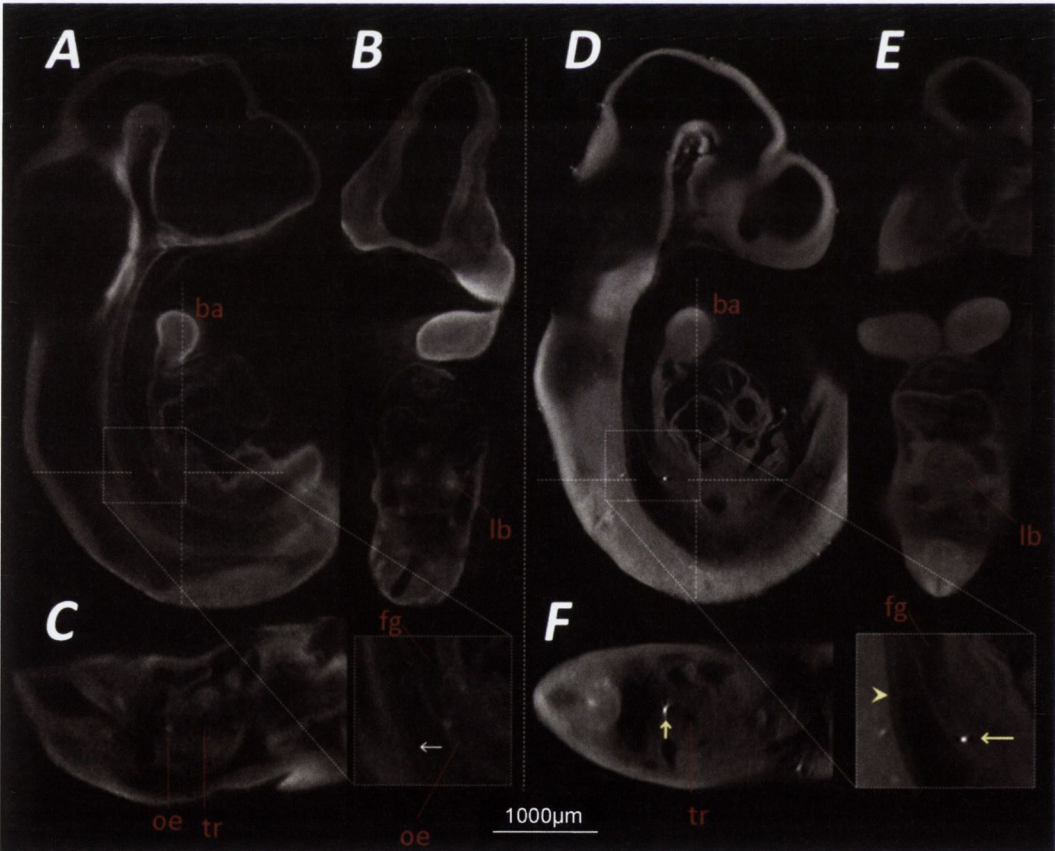


Figure 3.7: Expression of *Dpysl2* in AMM embryos. Virtual sections of 3D reconstructed OPT scans of *Dpysl2* ISH in embryos at E10 in control (A,B,C) and Adriamycin treated (D,E,F). Dashed lines indicate planes of transverse and coronal section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows highlight abnormal gene expression. In control white arrows indicate normal notochord position. *dnt* dorsal neural tube, *vnt* ventral neural tube, *tr* trachea, *ba* branchial arch, *vm* ventral foregut mesoderm, *oe* oesophagus, *lb* lung bud. Scale bar as shown.

3.3.4.3 Msx1

The Msh homeobox 1 (*Msx1*) gene encodes a homeodomain transcription factor and acts downstream of *Bmp4* via pSMAD 1/5/8 (Park et al., 2013, Tucker et al., 1998, Vainio et al., 1993) in limb morphogenesis, craniofacial and cardiac development (Lallemand et al., 2009, Vieux-Rochas et al., 2013, Chen et al., 2008). Prominent *Msx1* gene expression occurred in the branchial arches and in the dorsal neural tube in all embryos. In the foregut of E10 control embryos *Msx1* expression was confined to the ventral endoderm. At this stage *Msx1* expression was also present in the tracheal endoderm clearly delineating its distal extent at the tracheal bifurcation (Figure 3.8 A and B). In Adriamycin treated embryos at E10 foregut hypoplasia and failure of oesophagus formation was observed in keeping with typical features of the model. Complete loss of *Msx1* expression at the distal trachea at the site of tracheal bifurcation occurred in association with these malformations (Figure 3.8 C). Abnormal extension of *Msx1* gene expression into the ventral mesoderm at the level of notochord branching was observed at the narrowest point of the hypoplastic foregut structure (Figure 3.8 C and D).

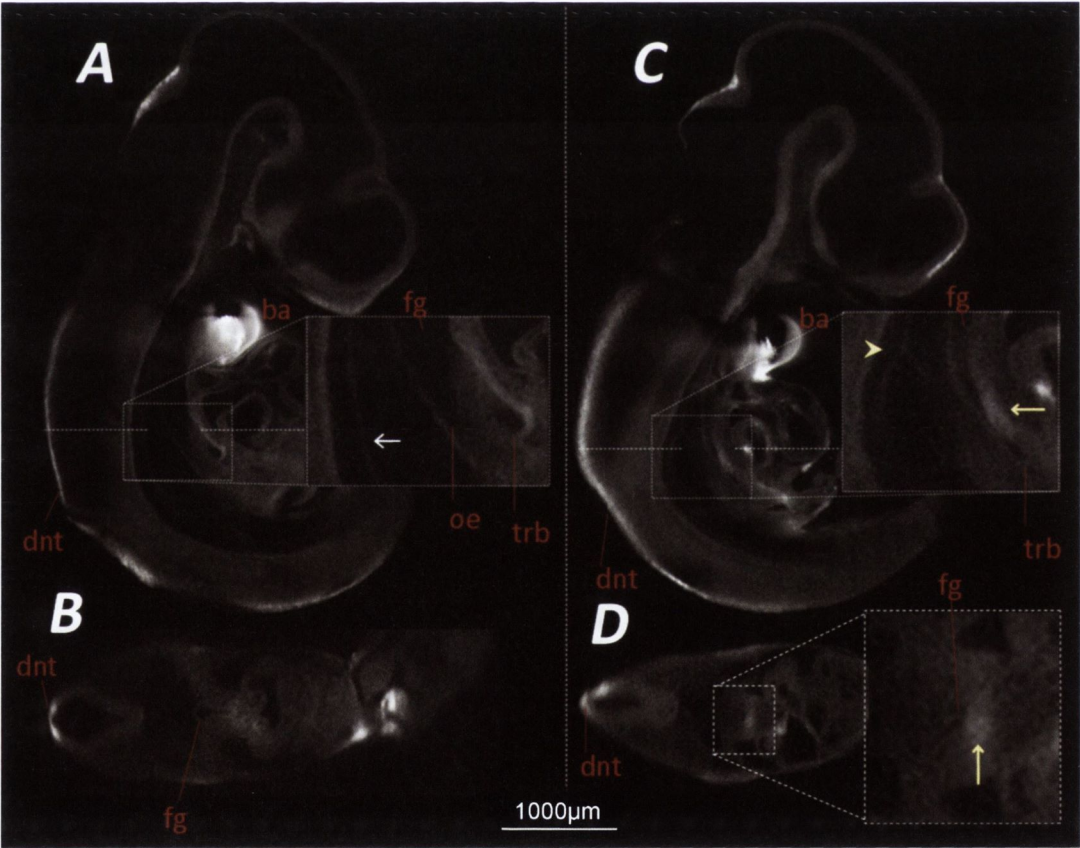


Figure 3.8: Expression of *Msx1* in AMM embryos. Virtual sections of 3D reconstructed OPT scans of *Msx1* in situ hybridisation (ISH) in embryos at E10 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowhead indicates notochord branching, yellow arrows highlight abnormal gene expression. Observe extreme narrowing of the foregut in C and D. In control white arrows indicate normal notochord position. *dnt* dorsal neural tube, *trb* tracheal bifurcation, *ba* branchial arch, *oe* oesophagus, *fg* foregut. Scale bar as shown

3.3.4.4 JunB

The Jun B proto-oncogene (JunB) encodes an activator protein-1 family transcription factor recognised to be an important BMP target gene in bone differentiation and chondrogenesis (Feng et al., 2010) and in mediating EMT response (Gervasi et al., 2012). In control and treated embryos JunB was strongly expressed in the ventral neural tube, branchial arches and in the apical ectodermal ridge of the limb buds. Expression of this gene in the foregut mesoderm of control embryos was spatially restricted to the lateral and ventral regions but was uniformly present throughout the foregut endoderm at E9 and E10, and was also noted in the lung bud endoderm at the latter stage (Figure 3.9 A and B). Also in E10 controls a markedly intense and confined territory of JunB expression was observed in the endoderm/mesoderm interface at the distal tip of the bifurcating trachea (Figure 3.9 A, B and C). In Adriamycin treated embryos at E10, loss of the discrete intense JunB expression at the distal margin of the trachea, coinciding with the absence of foregut separation, was conspicuous (Figure 3.9 D and E). Foregut and tracheal endoderm and the foregut mesoderm continued to express JunB in treated embryos; however, focally elevated expression of JunB detected in the dorsal foregut endoderm, directly adjacent to the notochord branch, was clearly reminiscent of ectopic pSMAD and Id1 expression in the AMM dorsal foregut (compare Figure 3.9 D to Figure 3.6 C and Figure 3.4 D). Interestingly, in 3 of 5 treated embryos examined at E10, expression of JunB in the neural tube was abnormally extended dorsally, co-locating with abnormal ventral neural tube morphology. Additional intense expression of JunB occurred at the ventricular surface of the ventral neural tube, the antero-posterior extent of which appeared to be grossly defined by the length of the notochord branch (Figure 3.9 boxed region).

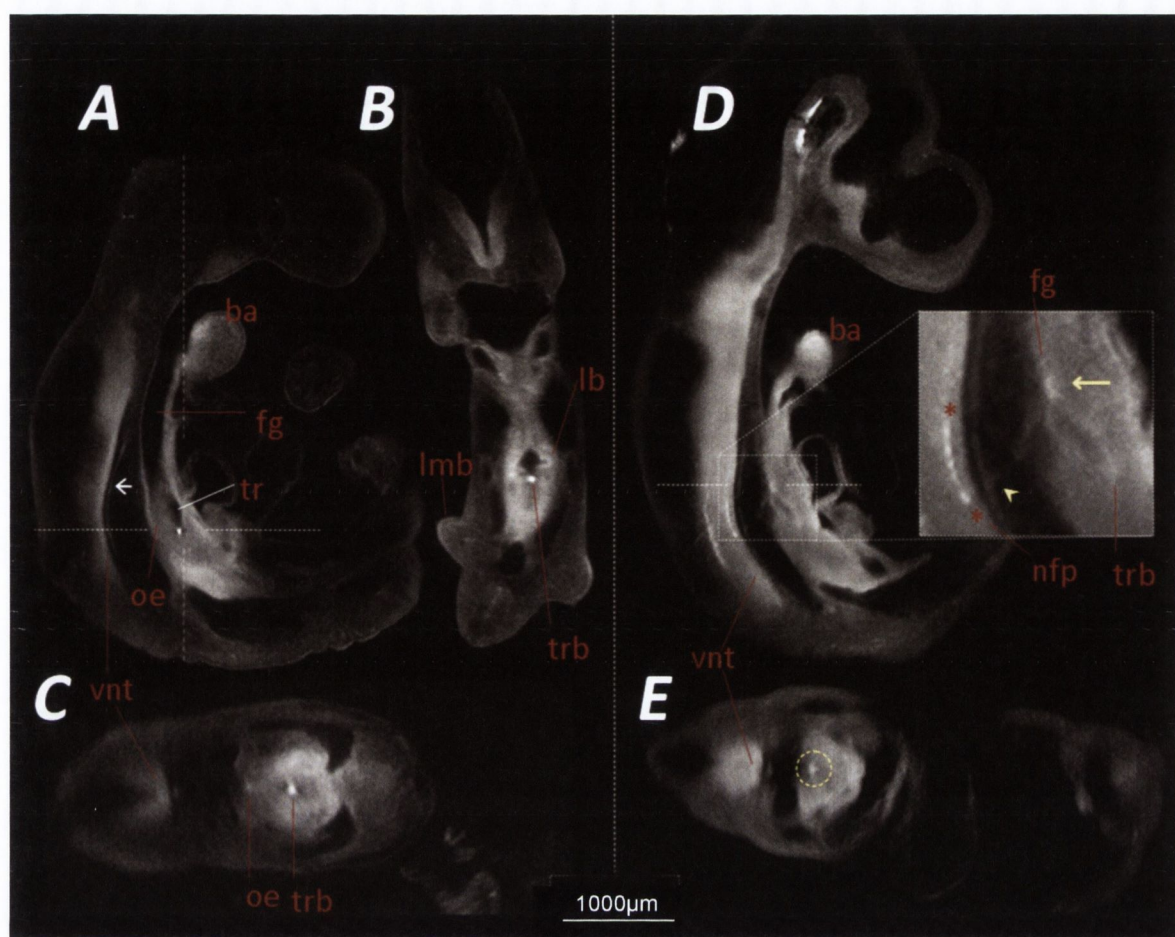


Figure 3.9: Expression of JunB in AMM embryos. Virtual sections of 3D reconstructed OPT scans of JunB ISH in embryos at E10 in control (A,B,C) and Adriamycin treated (D,E). Dashed lines indicate planes of transverse and coronal section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows and circles highlight abnormal gene expression. In control white arrows indicate normal notochord position. *vnt* ventral neural tube, *npf* neural floor plate, *tr* trachea, *ba* branchial arch, *oe* oesophagus, *lb* lung bud, *trb* tracheal bifurcation, *oe* oesophagus, *fg* foregut, *lmb* limb bud. Scale bar as shown. Asterisks indicate region of abnormal JunB signal in the ventral neural tube.

3.3.4.5 Prx2

The Paired related homeobox 2 (Prx2) gene product is a transcription factor, identified as a downstream BMP target in the cranial placodes during EMT (Higuchi et al., 2013) and is suggested to define sites where epithelio-mesenchymal interactions (EMI) occur (Meijlink et al., 1999). In all examined embryos, Prx2 was expressed in the ventral neural tube including the neural floor plate as well as in the limb buds and in the lateral ectoderm of the thoracic region. At E9 and E10 Prx2 expression was prominent in lateral foregut mesoderm in control and treated embryos extending into the ventral mesoderm at the level of the trachea at the latter stage (Figure 3.10 A to C). At E11 in controls, Prx2 expression in the ventral mesoderm persisted at the level of the trachea however was no longer detectable in the lateral foregut mesoderm (Figure 3.10 G to I). I did not observe any changes in the Prx2 expression domains in the Adriamycin treated embryos at E9 and E10, despite the presence of phenotypic foregut malformations, however more intense Prx2 signal in treated embryos versus controls was consistently observed in the foregut mesoderm (Figure 3.10 D to F), indicating an upregulation of the gene. Although ISH detection is not quantitative, control and AMM groups were subjected to precisely the same detection and staining conditions in parallel. At E11, in association with proximal foregut hypoplasia and directly adjacent to notochord branching, strong lateral foregut mesoderm expression of Prx2 persisted abnormally and ectopic expression of Prx2 was detected in the dorsal foregut endoderm and mesoderm (Figure 3.10 J to K). Disturbed Prx2 gene expression in the dorsal foregut occurred in direct association with notochord branching (Figure 3.10 J and L boxed regions).

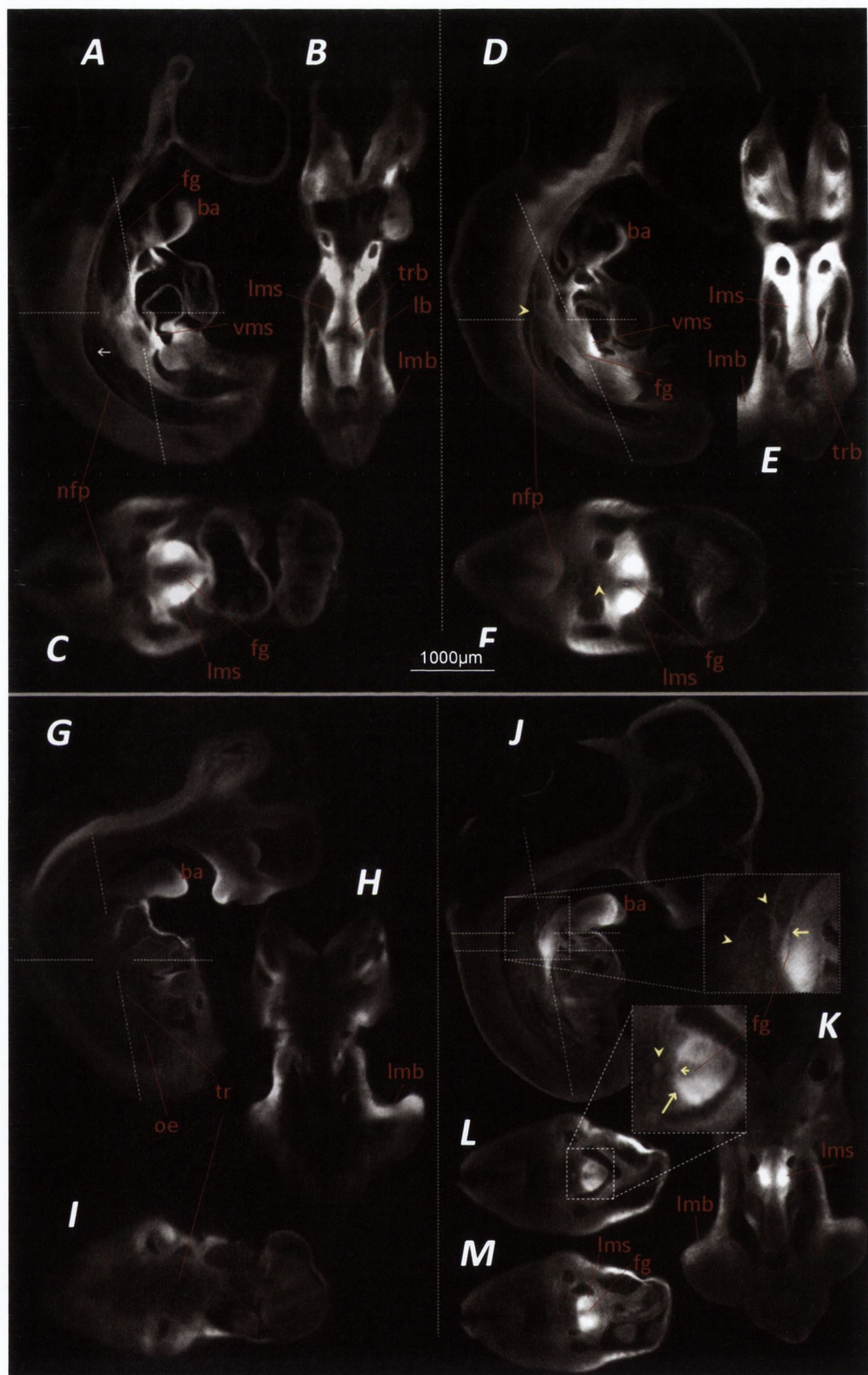


Figure 3.10: Expression of Prx2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Prx2 ISH in embryos at E10 in control (A,B,C) and Adriamycin treated (D,E, F) and at E11 in control G,H,I) and AMM (J to M). Dashed lines indicate planes of transverse and coronal section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows highlight abnormal gene expression. In control white arrows indicate normal notochord position. *vnt* ventral neural tube, *nfp* neural floor plate, *tr* trachea, *ba* branchial arch, *oe* oesophagus, *lb* lung bud, *lms* lateral foregut mesoderm, *vms* ventral foregut mesoderm, *trb* tracheal bifurcation, *oe* oesophagus, *fg* foregut, *lmb* limb bud. Scale bar as shown.

3.3.4.6 Dlx2

The transcription factor encoded by the Distal-less homeobox 2 (Dlx2) gene acts under the regulation of BMP signalling in facial and branchial arch patterning as well as in osteogenesis (He et al., 2014, Depew et al., 2005, Harris et al., 2003). In control and AMM embryos (n = 3 per stage for each control and AMM) Dlx2 was expressed in the dorsal neural tube, in the notochord and in the foregut and tracheal endoderm (Figure 3.11 A). In AMM embryos at the level of notochord branching focal areas of strong Dlx2 expression were detected in the dorsal foregut and pharyngeal endoderm at E9 and E10 (Figure 3.11 C, D, E and F). At E11 no appreciable abnormalities of Dlx2 expression were noted in AMM embryos.

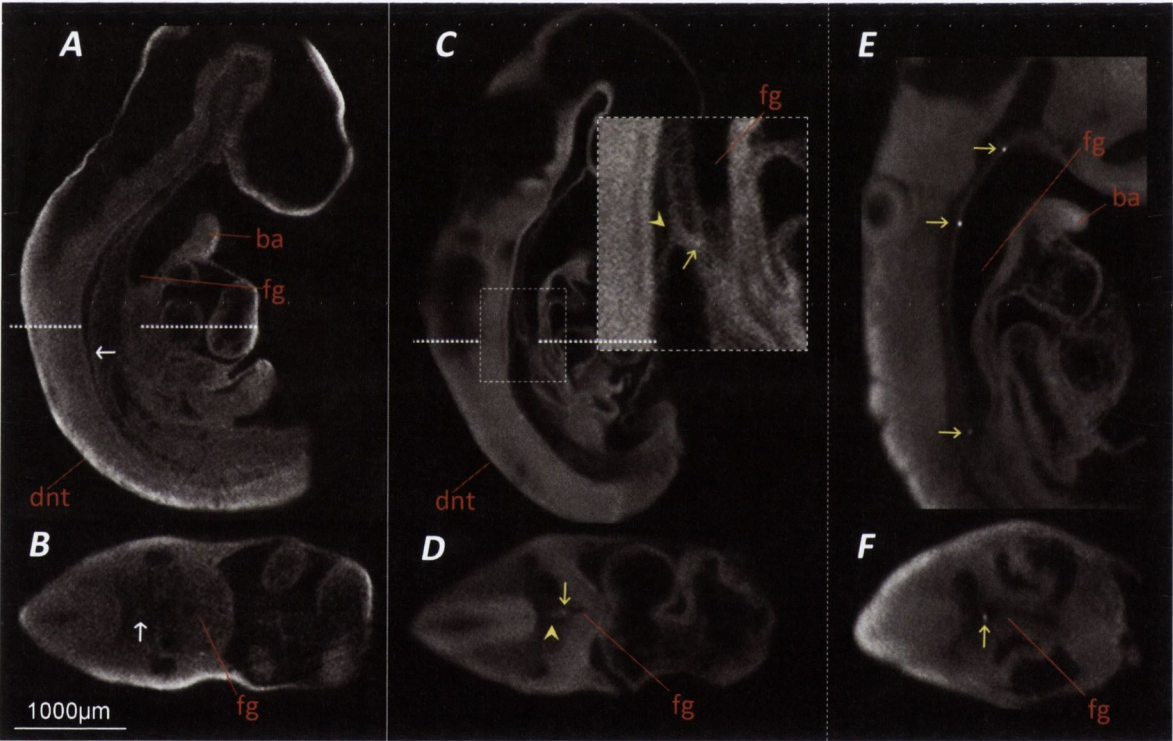


Figure 3.11: Expression of Dlx2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Dlx2 ISH in embryos at E10 in control (A,B) and Adriamycin treated (C,D) and at E9 in AMM (E,F). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows highlight abnormal gene expression. In control white arrows indicate normal notochord position. *ba* branchial arch, *fg* foregut. Scale bar as shown.

3.3.4.7 Bambi

Bone morphogenic protein and activin membrane bound inhibitor (Bambi) is a BMP antagonist that mimics the *Bmpr1* intracellular domain, binding and sequestering BMP (Onichtchouk et al., 1999). Bambi is co-expressed with *Bmp4* during craniofacial, eye, kidney, and limb development (Grotewold et al., 2001). In control and AMM embryos ($n=3$ per stage for each) Bambi was found to be expressed in the otic vesicles, apical ectodermal ridge in the limb buds, neural tube and the neural floor plate (Figure 3.12). Interestingly co-expression was not seen with *Bmp4* in all sites of the foregut region. *Bmp4* is expressed strongly in the ventral foregut mesoderm (P. Hajduk, unpub results, in prep) whilst Bambi was expressed in the ventral foregut mesoderm at a low level as well as in the lung bud endoderm and mesoderm and was additionally expressed in the foregut and trachea endoderm. Unlike *Noggin*, an extracellular BMP antagonist, no disturbances of Bambi expression were detected in the AMM foregut despite the presence of typical abnormalities of the notochord and foregut in examined embryos (Figure 3.12 C and D).

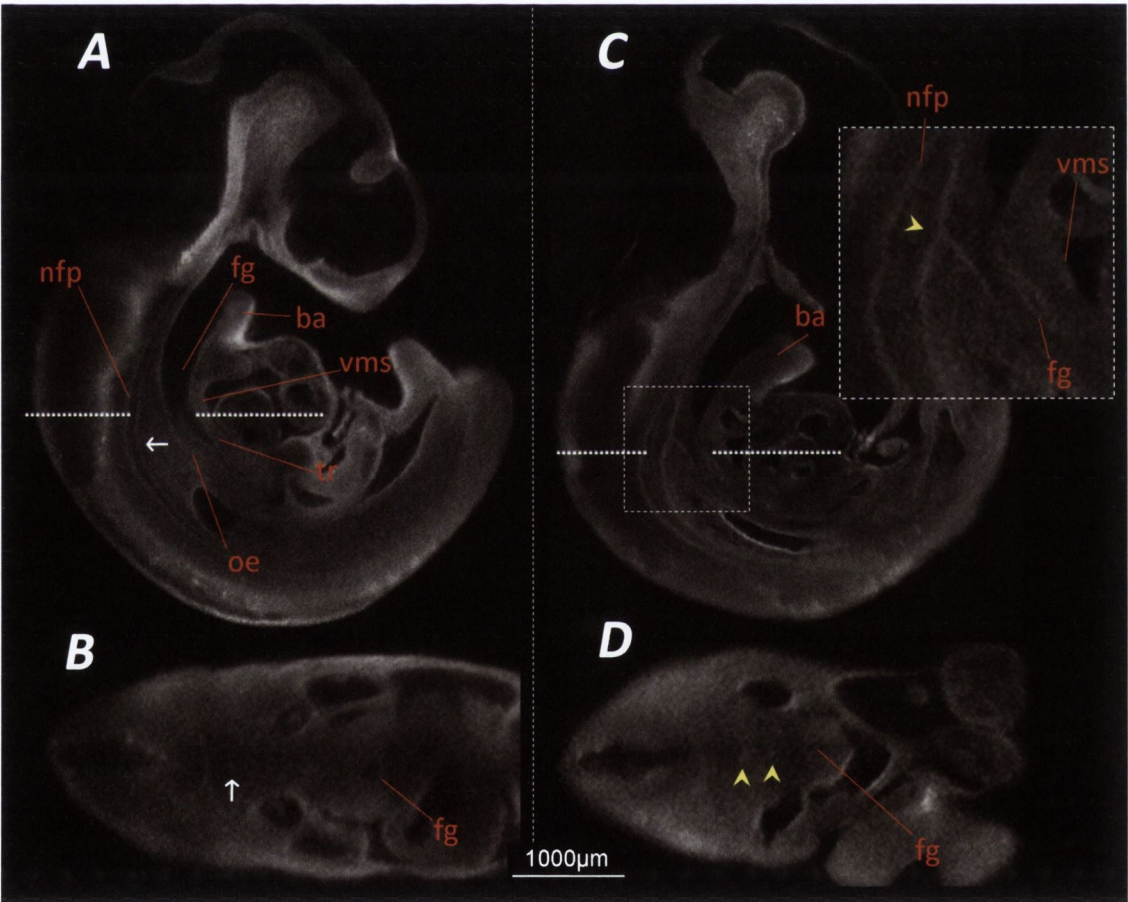


Figure 3.12: Expression of Bambi in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Bambi ISH in embryos at E10.5 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching. In control white arrows indicate normal notochord position. *nfp* neural floor plate, *fg* foregut, *oe* oesophagus, *tr* trachea, *ba* branchial arch, *vms* ventral foregut mesoderm, *ba* branchial arch, *fg* foregut. Observe the abnormal narrow foregut in C boxed region. Scale bar as shown.

3.3.4.8 Snail and 2

Snail family zinc finger 1 (*Snail*) and 2 (*Snai2*) are important effectors in BMP mediated EMT and in particular drive E-Cadherin down-regulation to promote mesenchymal cell fate (Richter et al., 2014, Carver et al., 2001). In E10 control and AMM embryos *Snail* is strongly expressed in the tail, limb buds and fronto-nasal process but is not seen to be prominently expressed in the foregut of control embryos (Figure 3.13 A and B). In the AMM at E10 strong *Snail* expression was detected along a variable extent of the dorsal endoderm of the abnormal undivided foregut in 3 of 5 affected embryos (Figure 3.13 C and B) but was not present in 2 embryos with typical AMM phenotype. At E10 in control and AMM embryos *Snai2* did not exhibit distinctive foregut expression however in the AMM notochord, at the level of branching there was prominent *Snai2* expression, which was not present in control specimens (n=3 for each control and AMM) (not shown).

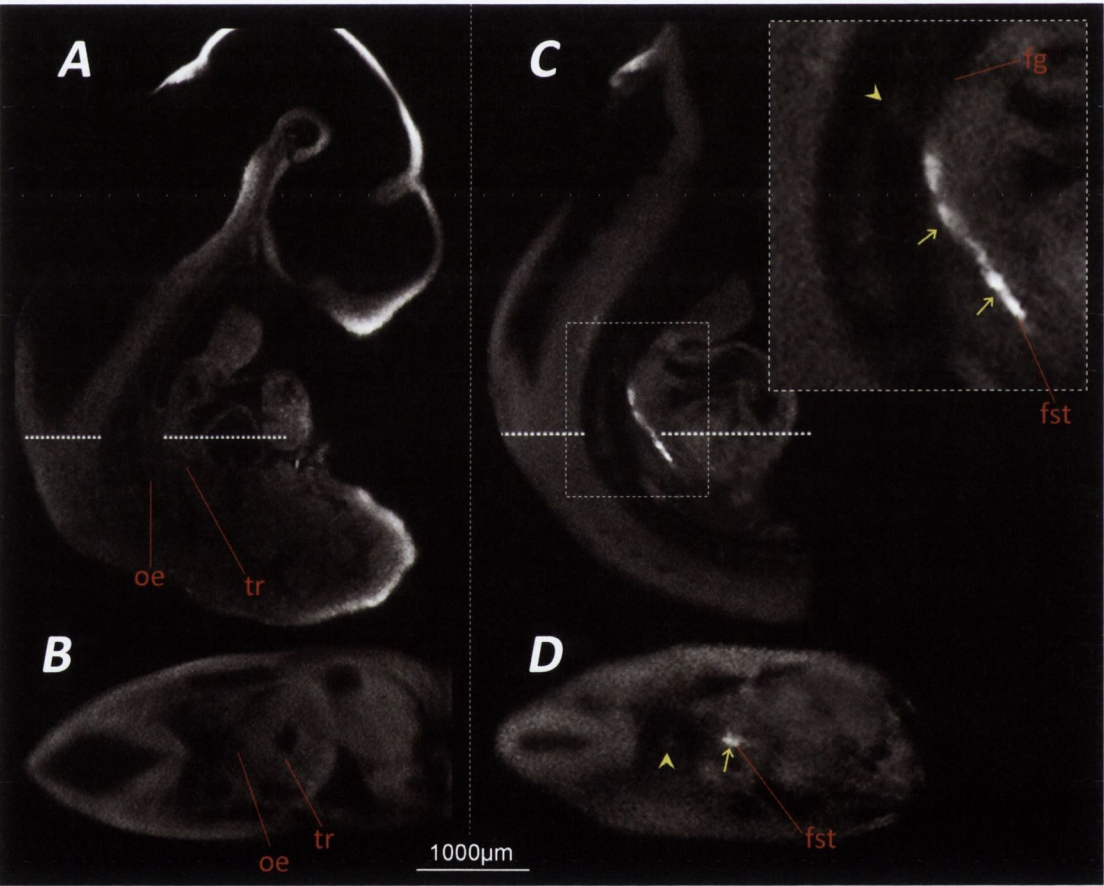


Figure 3.13: Expression of *Snail* in AMM embryos. Virtual sections of 3D reconstructed OPT scans of *Snail* ISH in embryos at E10.5 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching and yellow arrows highlight abnormal gene expression. In control white arrows indicate normal notochord position. *fg* foregut, *oe* oesophagus, *tr* trachea, *fst* fistula/undivided foregut.. Scale bar as shown.

3.3.4.9 Crabp2

Cellular retinoic acid binding protein II (Crabp2) regulates Retinoic Acid signalling but has been shown to be influenced by BMP ligands as a result of crosstalk between these important signalling pathways (Means and Gudas, 1996). Crabp2 is expressed in the dorsal neural tube, the somites, limb buds, branchial arches and otic vesicles and in the foregut, trachea and lung bud endoderm at E9 through E11 in control embryos (Figure 3.14 A and B). In the AMM at E9 through E11 there was a generalised attenuation of Crabp2 signal throughout the embryo and focally prominent expression localised to the tips of the notochord branches (n=3 per stage for each control and AMM). (Figure 3.14 C and D).

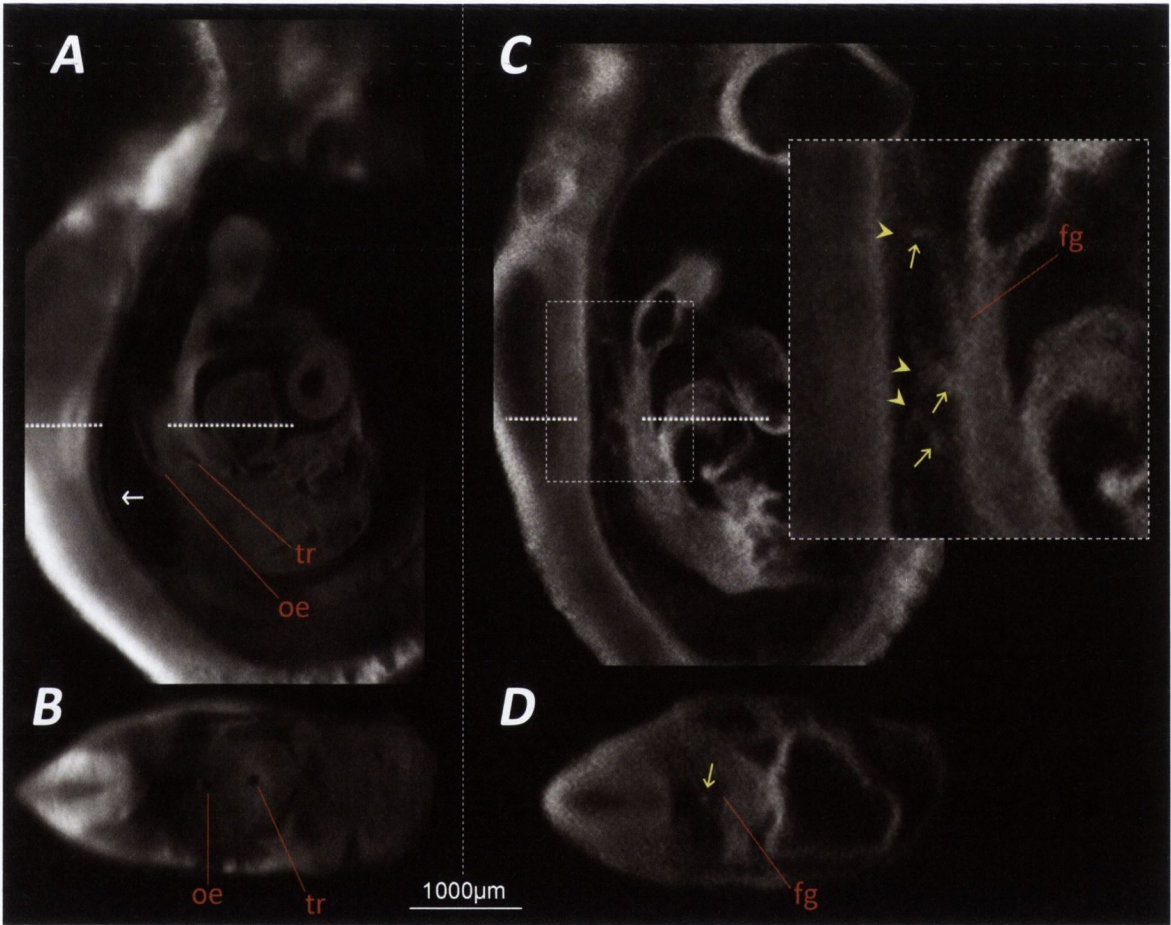


Figure 3.14: Expression of Crabp2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Crabp2 ISH in embryos at E10 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching and yellow arrows highlight abnormal gene expression In control white arrows indicate normal notochord position. *fg* foregut, *oe* oesophagus, *tr* trachea. Observe 3 distinct notochord branches in C boxed region. Scale bar as shown.

3.3.5 *Gene expression of BMP ligands 2 and 7 is not spatially disrupted in Adriamycin treated embryos.*

Noggin null mutants display foregut malformations and notochord abnormalities with phenotypic similarity to the AMM which can be rescued by reduced gene dose of Bmp 4 and 7, consequently the spatial gene patterning of Noggin and these ligands in the Adriamycin mouse model was of particular interest. Expression of Bmp2, 4 and 7 have all previously been reported in the developing foregut region (Madabhushi and Lacy, 2011, Danesh et al., 2009). Noggin and Bmp4 spatial gene expression profiles were previously examined by P. Hajduk as described (section 3.1). Therefore to complete the assessment of the BMP signalling environment I assayed the spatial expression domains of Bmp2 and 7 in control and Adriamycin treated embryos by in situ hybridisation. OPT scanning and 3D digital reconstruction. As in all other experiments, this allowed complete morphological analysis and revealed typical AMM foregut abnormalities in treated embryos.

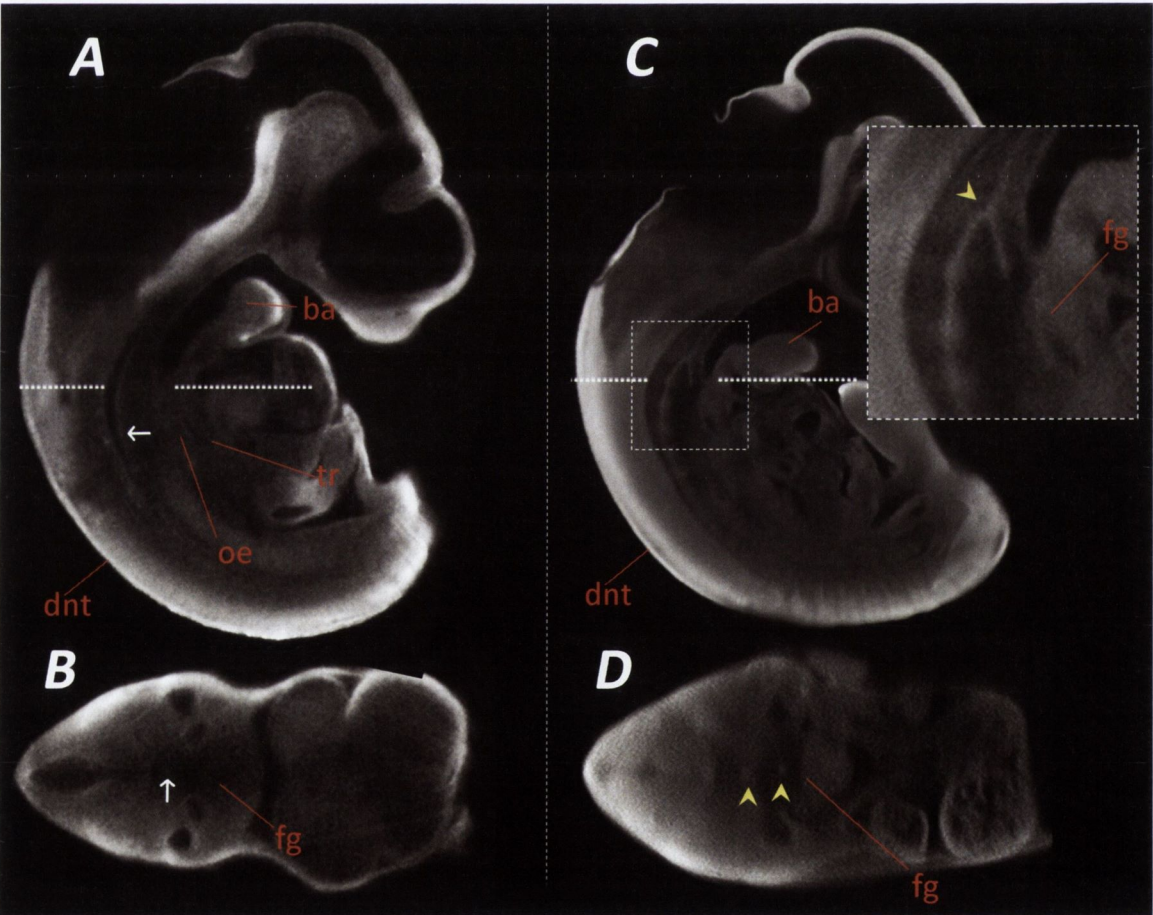


Figure 3.15: Expression of Bmp7 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Bmp7 ISH in embryos at E11 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate notochord branching. Observe the abnormally narrow foregut in C boxed region. In control white arrows indicate normal notochord position. *fg* foregut, *oe* oesophagus, *tr* trachea, *ba* branchial arch, *dnt* dorsal neural tube. Scale bar as shown.

In the foregut of control embryos Bmp7 was observed to be predominantly expressed in the dorsal endoderm at E9 and E10 as well as in the definitive oesophagus at E11 and was present in the notochord at each of these gestational stages (Figure 3.15). Bmp2 was expressed uniformly throughout the foregut, tracheal and oesophageal endoderm across the gestational stages examined in control embryos (Figure 3.18). Comparison to Adriamycin treated embryos across all stages showed no change in the spatial expression of any of the ligand encoding genes although typical morphological abnormalities of the notochord and foregut were confirmed in examined embryos.

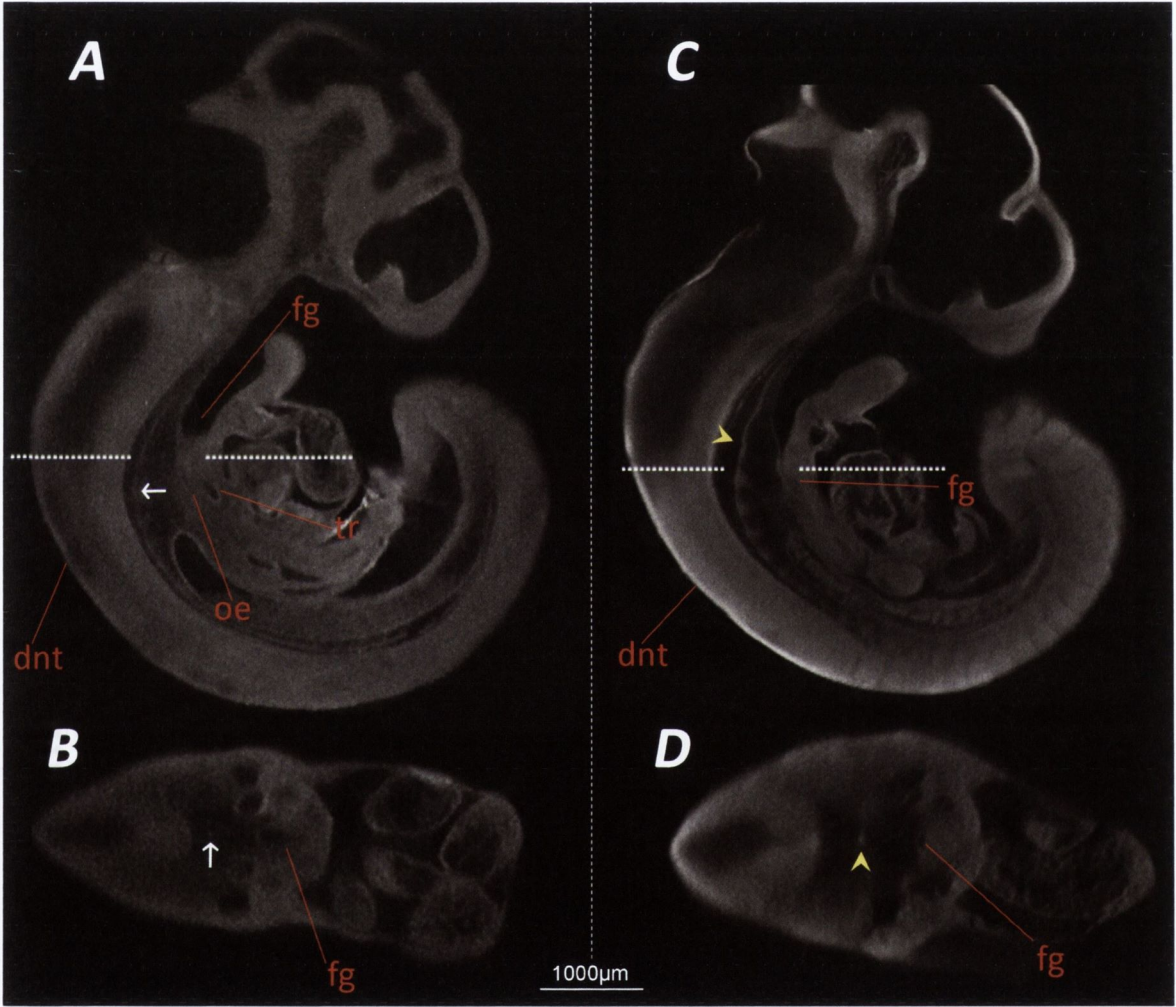


Figure 3.16: Expression of Bmp2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Bmp2 ISH in embryos at E11 in control (A,B) and AMM (C,D). Dashed lines indicate planes of transverse section from the main sagittal image, boxed regions are magnified as shown. In AMM yellow arrowheads indicate abnormal notochord. In control white arrows indicate normal notochord position. *fg* foregut, *oe* oesophagus, *tr* trachea, *dnt* dorsal neural tube. Observe the notochord although not branched is widened, abnormally ventral and exhibits irregular shape in association with abnormal foregut in C. Scale bar as shown.

3.4 Discussion

Foregut separation is a fascinating example of the delicate balance in molecular signalling and complex morphological rearrangements required to achieve correct organogenesis. The data reported in this chapter reveal that multilevel disruption in the BMP signalling pathway occurs in association with tracheo-oesophageal malformations and notochord branching in Adriamycin treated mouse embryos. I established immunolocalisation of pSMAD in whole embryos together with Optical Projection Tomography as an approach to comprehensively characterise the distribution of overall BMP activity in the developing foregut and other embryonic tissues, permitting description of the remarkably focal nature of BMP pathway disturbances in the AMM in a morphological context. Further examination of BMP pathway activity at a downstream level by assessing spatial expression patterns of BMP target genes also revealed disruption in these foregut areas.

Striking correspondence between disruptions at multiple BMP pathway levels is reflected in comparing pSMAD localisation, Noggin gene and downstream target gene (in particular *Id* genes and *JunB*) spatial expression profiles as summarised in Table 3.2. These analogous patterns indicate highly localised disturbance of BMP signalling in the foregut endoderm and immediately adjacent notochord branches. The focal nature of the BMP disturbance in the AMM was illustrated by the presence intensely pSMAD positive cells in the dorsal foregut endoderm at a corresponding level to notochord branching. These cells appeared initially involved in the area of prolonged notochord-endoderm contact and then persisted within the dorsal foregut endoderm. These cells manifested abnormal patterning in terms of the localisation of intense pSMAD outside the nuclear domain and also exhibited disrupted cellular shape, organisation and behaviour, appearing to cluster in the endoderm at sites of foregut lumen narrowing and occlusion. In the adjacent normally shaped and organised foregut cells pSMAD was seen to localise normally to the nuclei. The relevance of these intensely pSMAD positive cells in the pathogenesis of foregut malformations may be inferred by their positional association with the notochord branches as well as further molecular disturbances, in genes downstream of pSMAD, in this area. These cells may originate molecular signals inducing downstream BMP gene activation, as demonstrated by the co-existence of ectopic *Id* gene expression at these sites. These signals may also exert an effect on the foregut structure which was observed to be morphologically abnormal in the immediate vicinity (Figure 3.5 D).

Table 3.2 Summary of foregut region expression for BMP ligands, target genes and pSMAD in normal and AMM embryos.

Gene	CONTROL		AMM	
	Foregut	Notochord	Foregut	Notochord
Bmp4	Ventral mesoderm	no	no change	no change
Bmp2	Endoderm	Low level	no change	no change
Bmp7	Dorsal endoderm and oesophagus	yes	no change	no change
Noggin	Endoderm (low level)	yes	High in dorsal endoderm beside nc branches	High in branches
pSMAD*	Ventral endoderm and mesoderm	yes	High in dorsal endoderm beside nc branches	High in nc branches and where widened
Id1 / Id2	Ventral endoderm and mesoderm	no	High in dorsal endoderm beside nc branches	no change
Dpysl2	Focal anterior oesophagus and lung buds endoderm	no	High in anterior oesophagus, Lost in lung buds.	no change
Msx1	Endoderm, especially tracheal bifurcation	low level	Lost at tracheal bifurcation	no change
			Extended into ventral mesoderm at level nc branch	
JunB	Endoderm especially lung buds and very intense at tracheal bifurcation	low level	High in dorsal endoderm beside nc branches Lost at tracheal bifurcation	no change
Prx2	Lateral mesoderm.	low level	E10: high intensity mesoderm. E11: abnormal persistence in mesoderm, high in dorsal endoderm beside nc branches	Abnormally persisting at E11
Dlx2	Endoderm	yes	High in dorsal endoderm beside nc branches.	High at interface nc branch & fg
Snai1	no	low level	High in dorsal endoderm beside nc branches.	no change
Snai2	no	yes	no change	More prominent
Crabp2	Endoderm	no or low level	Lower in endoderm	High in nc branches
Bambi	Endoderm	yes	no change	no change
DV restriction specified if present as dorsal or ventral. no = not detected, yes = present, not restricted Similar patterns in AMM are colour coded. * refers to protein immunolocalisation, all others are <i>in situ</i> hybridisation, <i>nc</i> notochord, <i>fg</i> foregut.				

Interrogation of widely accessible data repositories for the identification of BMP downstream genes relevant to foregut development, utilised the increasing availability of microarray and RNA sequencing data as well as high throughput studies of gene expression (eg. Gray et al., 2004) and gene expression databases curated by expert groups such as Mouse Genome Informatics (MGI) and the Edinburgh Mouse Atlas Project (EMAP) to provide resources for research. This approach ultimately led to novel descriptions of highly specific patterns of expression in the developing foregut for selected genes (Table 3.2). *Id* genes are described as synonymous with BMP signal transduction in many developing tissues. These data confirm that in the foregut of both normal and Adriamycin treated embryos pSMAD distribution and *Id1* and *Id2* gene expression were spatio-temporally synchronous. Endodermally restricted foregut BMP downstream gene activity was notably disturbed at the tracheal bifurcation (*JunB*, *Msx1*) and the level of foregut separation (*JunB*, *Dpysl2*) as well as in the lung buds (*Dpysl2*) in the AMM. Signal attenuation of these 3 genes in more posterior foregut locations co-occurred with signal intensification in more anterior regions, suggesting that graded response to BMP signal or auto-regulation may be a feature of these expression profiles. In contrast mesodermally expressed BMP downstream gene *Prx2* displayed an extensive region of signal intensification around the foregut. I also noted shifts in the tissue restriction profiles between endoderm and mesoderm for both *Msx1* (abnormally extended into the tracheal mesoderm) and for *Prx2* (abnormally expressed in the dorsal foregut endoderm) arising in both instances at the level of notochord branching. Failure to maintain proper tissue specification at these locations is suggested by these findings.

Key regions of the foregut may be highly sensitive to well-ordered tissue fate decisions in order to achieve properly formed oesophageal and respiratory structures (Jacobs et al., 2012). Subtle spatial redistribution of BMP sensitive cell behaviours such as adhesion or migration at these vulnerable sites might be reasonably anticipated to have considerable morphological implications. For example loss of *JunB* and *Msx1* gene expression at the distal extent of the trachea occurs in the AMM. These genes mediate BMP driven cell differentiation towards mesenchymal identity. Delayed mesoderm formation has been shown to occur in *Xenopus* embryos due to attenuated *Bmp4* activity via competitive Activin inhibition (Dyson and Gurdon, 1997). It is at the tracheal bifurcation in the AMM and in humans that the tracheo-oesophageal fistula typically has its anterior communication. Failure of mesoderm to establish between the endoderm of the trachea and more posterior foregut could account for the existence of this anomalous connection.

Interestingly Adriamycin has previously been shown to promote cell to cell adhesion via the upregulation of E-Cadherin (CDH1) in breast cancer cells, driving these cells away from proliferative and migratory states (Yang et al., 1999) and it has previously been shown that E-Cadherin abnormally persists between the cells of the notochord in Adriamycin treated embryos, as they fail to properly delaminate from the dorsal foregut endoderm (Hajduk et al., 2012). Adriamycin induced E-cadherin over expression and a subsequent cell behaviour shift towards increased adhesion could account for observed altered notochord behaviour in the AMM; i.e. it remains abnormally ventral and in prolonged contact with the dorsal foregut. Classically however E-Cadherin expression is driven down as part of BMP induced and pSMAD mediated transformation towards mesenchymal cell fate (Richter et al., 2014). The co-location therefore of E-Cadherin with intense pSMAD activity in the notochord and dorsal foregut endoderm suggests a possible unravelling of appropriate EMT response with uncoupling of the dynamic between BMP and E-Cadherin.

Overall these data suggest that localised abnormal activation of BMP signalling in the dorsal foregut region is associated with oesophageal atresia. This conclusion is supported by evidence from the Noggin knock out model where BMP pathway over activity resulted in tracheo-oesophageal malformations and notochord defects, a phenotype that could be rescued by reducing the gene dose of Bmp 4 and 7 (Que et al., 2006, Li et al., 2007). In a further investigation of the mechanism of Noggin gene knockout effects, exploring the hypothesis that Noggin is required to attenuate BMP signal in the developing foregut, Fausett et al (2014) found that conditional activation of Bmpr1a in the notochord and neural floorplate exhibited a similar OA/TOF phenotype to conditional knockout of the Noggin gene in these structures. Also reported however was data to suggest that the Noggin knockout model differs from the AMM and other genetic mouse models of foregut defects in that it does not exhibit altered dorso-ventral patterning, implying that pathogenesis of foregut abnormalities occurring in association with notochord defects may not reduce to a single aetiological process. In the AMM our group have found that Noggin gene expression is abnormally strong in the notochord branches and the foregut endoderm (Hajduk, P., Thesis, 2011), which would further indicate an alternative molecular signature to that generated by Noggin gene knockout in the mouse. In models of OA such as the AMM, altered dorsal foregut endoderm patterning including loss of Sox2 expression and ectopic Shh expression suggest abnormal ventralisation (Hajduk et al 2011). Conversely Noggin usually acts as a powerful dorsalising influence (Smith and Harland, 1992). These findings may be reconciled when we consider that Noggin gene expression can be

enhanced under conditions of increased BMP activity (Lavery et al., 2009), perhaps as a mechanism to re-establish BMP activity balance, which may account for the co-location of Noggin with ectopic pSMAD in the abnormally ventralised dorsal foregut endoderm. While it is clear that notochord and foregut defects may occur in the presence of strong Noggin gene expression, it remains to be determined whether Noggin over activity can be causally associated with abnormal foregut morphology.

Spatial expression profiles for Bmp 2, 4 or 7 were not altered in the AMM indicating that disruption of Bmp signalling may not occur at the ligand level. Several factors may be considered when examining these findings. The expression profiles of these developmentally important genes are reported to be highly dynamic and can flux rapidly in response to each other (Scholl et al., 2012, Lavery et al., 2009). Adriamycin treatment of mice at E7 and E8 gestation is sufficient to generate the model but does not induce a 100% rate of abnormalities. It is not possible to assess for morphological foregut or notochord disturbance until they manifest at E9. The current approach therefore, by including confirmation of phenotypic anomalies, may consequently fail to identify altered Bmp ligand-encoding gene expression behaviour in the correct window following Adriamycin treatment. At earlier gestational stages in the AMM, if disrupted levels of Bmp gene expression are present it will not be possible to relate these findings directly to altered foregut morphology. Additionally in situ hybridisation can only detect the RNA gene product and does not provide information regarding the ligand product activity. Multiple levels of post-transcriptional control in the outcome of gene signalling are not captured using these techniques. Genetic or in vitro approaches to manipulate BMP activity may more robustly examine whether focal BMP disruption, as suggested by ectopic pSMAD localisation in the AMM, can induce morphological foregut defects. The importance of BMP signalling to development however, requires complex temperospatial limits to be applied to genetic manipulations of BMP ligands in the early embryo. In Chapter 5 the establishment of an in vitro system that can be used to examine the effect of signalling manipulations on foregut development is detailed, which is then used to examine the role of BMP signalling in foregut development in Chapter 6.

Adriamycin has been previously shown to directly induce the expression of Id genes in mouse cell lines (Glackin et al., 1992, Kurabayashi et al., 1994). It is apparent however that upstream pSMAD activity is strongly focally disrupted in treated embryos which display foregut and notochord abnormalities. The known direct mechanism of action of Adriamycin is primarily in the nucleus, and is suggested to involve formation of interstrand crosslinks with DNA, interfering with DNA replication and transcription,

through inhibiting nucleic acid synthesis (Meriwether and Bachur, 1972, Cullinane et al., 2000). This suggests that an unidentified but pivotal factor upstream of pSMAD is targeted by Adriamycin treatment.

Diffusible signal from the defective notochord could mediate the Adriamycin effect by induction of a localised molecular and morphological response in the adjoining foregut. In embryos without notochord defects pSMAD localisation or downstream gene expression was not altered. In addition the site of molecular disturbance in the foregut was directly adjacent to notochord abnormalities at each level of the BMP pathway examined. SHH signalling has been shown to act via BMP/pSMAD signal transduction in limb bud osteogenesis (Yang et al., 1997, Drossopoulou et al., 2000) and when provided from the notochord in the correct developmental window, permits foregut derived pancreas organogenesis (Kim et al., 1997). SHH signalling pathway gene mutations cause oesophageal abnormalities in humans and in mouse models (Litingtung et al., 1998, Motoyama et al., 1998, Mahlapuu et al., 2001, Johnston et al., 2005, Stankiewicz et al., 2009). Ectopic expression of Shh at the level of notochord branching (Hajduk et al., 2011b), occurring adjacent to the area of altered BMP activity in the foregut, suggests that notochord derived Shh could act through BMPs in the pathogenesis of foregut malformations. This explanation however over-simplifies the relationships that exist between pathways such as BMP and SHH which are more accurately characterised as complex regulatory circuits. BMP widely interacts with other highly embryologically relevant signalling families previously implicated in foregut development such as Fibroblast Growth Factors (Warburton and Bellusci, 2004), WNT (Harris-Johnson et al., 2009), T-box (Mc Laughlin et al., 2014a), Homeobox (Szumska et al., 2008) and Retinoic Acid (Mendelsohn et al., 1994) (or reviewed in Mc Laughlin 2014). These highly evolutionary conserved developmental regulatory pathways carefully co-ordinate to direct proper tissue designation. Disruption of any manner where this balance could be crucially important, such as at foregut separation, may culminate in phenotypically similar outcomes.

Altered Bmp pathway activation provides an attractive aetiological hypothesis in the examination of the molecular and cellular mechanisms that may be responsible for the pathogenesis of tracheo-oesophageal malformations. It has been suggested that elevated BMP signal could act in the dorsal foregut endoderm via Sox2 inhibition to promote inappropriate ventral identity, by which mechanisms it acts in the ventral foregut endoderm to bestow and limit respiratory tissue fate (Domyan et al., 2011). This mechanism of altered cell fate induction or other Bmp responsive cell behaviours may provide a common pathogenesis to reconcile various models of oesophageal atresia.

3.5 Conclusion

The novel use of wholemount pSMAD immunolocalisation together with 3D OPT scanning complemented by established immunohistochemistry techniques effectively illustrated DV restriction of BMP activity across the developing foregut. In the AMM this pattern of BMP activity was focally disturbed in the foregut, as reflected by intense ectopic pSMAD localisation within the dorsal foregut in clusters of abnormally organised cells. Interrogation of the functional read out of BMP signalling in the AMM by identifying and analysing foregut expressed BMP target genes identified disturbances in similar foregut regions. This combination of approaches revealed multi-level disruption of the BMP signalling pathway occurring in association with tracheo-oesophageal malformations.

Chapter 4

Altered Tbx1 gene expression in the Adriamycin mouse model of Oesophageal Atresia/Tracheo- Oesophageal Fistula.¹

¹ The data presented in this chapter are largely as published in:

Mc Laughlin, D., P. Murphy, and P. Puri, Altered Tbx1 gene expression is associated with abnormal oesophageal development in the Adriamycin mouse model of oesophageal atresia/tracheo-oesophageal fistula. *Pediatr Surg Int*, 2014. 30(2): p. 143-9.

4.1 Introduction

T-box genes are a family of transcription factors with a highly conserved DNA binding domain which are richly expressed in tissues undergoing active embryonic induction of organogenesis. Although T-box genes including Tbx1 and Tbx2 are well described modulators of pharyngeal and cardiac development (Papaioannou and Silver, 1998), the role of Tbx gene activity in the adjoining foregut, has not been determined. In the screen to identify BMP downstream gene targets for examination in the AMM (described in Chapter 3) Tbx1 and 2 were identified to respond to BMP activity (Wang et al., 2010, Fessing et al., 2010) and appeared to exhibit expression in the developing foregut (Zohn et al., 2007, Christoffels et al., 2004). As major regulatory genes, T-box genes do not act solely at the discretion of BMP signalling and so cannot be considered dedicated BMP downstream target genes, however the identification of Tbx1 and Tbx2 by the screening approach highlighted a potential role for these genes under the influence of BMP signalling in foregut development.

Tbx1 is a major determinant gene in 22q11 deletion syndromes (22q11DS), a common cause of congenital cardiovascular malformations (van der Bom et al., 2011), encompassing Di George syndrome, velocardiofacial syndrome and conotruncal anomaly face syndrome (Merscher et al., 2001, Liao et al., 2004). The characteristic cardiac outflow tract and pharyngeal apparatus abnormalities are phenocopied in Tbx1 mutant mice (Lindsay et al., 2001) in which hypoplastic foregut and irregularity of tracheal origin at the laryngo-tracheal groove have also been detected (Mesbah et al., 2012, Jerome and Papaioannou, 2001). Tbx2 and Tbx3 gene expression levels have been shown to modulate the penetrance of the phenotype in 22q11DS. These genes are co-expressed in the ventral foregut and exhibit functional redundancy (Mesbah et al., 2012). Tbx4 and Tbx5 are synexpressed in the developing respiratory system also with evidence of redundancy (Chapman et al., 1996). Tbx4 over-expression in chicks has been reported to result in tracheo-oesophageal malformations (Sakiyama et al., 2003), however when examined in the AMM Tbx4 gene expression, localised in the lung mesenchyme, was not disturbed (Hajduk et al.).

OA/TOF frequently occurs together with other birth anomalies and most commonly with VACTERL, however OA/TOF has also been reported in association with Di George Syndrome (Lee et al., 2008). Additionally CHARGE syndrome (Coloboma, Hear defects, Atresia of choanae, Retardation of growth and development, Genital hypoplasia, and Ear

abnormalities) due to CHD7 gene mutation has up to 30% associated incidence of OA/TOF (Bergman et al., 2011). Distinct phenotypic and genetic overlaps between CHARGE and 22q11DS have recently been reported (Corsten-Janssen et al., 2013). These reports illustrate an association between OA/TOF and TBX1 gene mutation in humans. Moreover, 22q11DS locus mutations have been identified in patients with clinical VACTERL association (Iafolla et al., 1991, Meins et al., 2003, Schramm et al., 2011).

The previously described importance of Tbx transcription factors in the regulation of morphogenesis of foregut adjacent structures such as the heart and pharynx, the presence of structural foregut defects in the Tbx1 knockout mouse, as well as newly emerging evidence of a clinical link between TBX1 and OA/TOF in humans together suggests that Tbx1 and 2 may be integral in the molecular control of foregut morphogenesis. This warranted an in depth examination of the role of Tbx1 and 2 in foregut development.

The objective of this chapter was *to examine the hypothesis that Tbx1 and Tbx2 genes function in normal foregut development and are involved in the pathogenesis of tracheo-oesophageal malformations*. Using *in situ* hybridisation and OPT scanning I aimed to describe the normal gene expression profiles for Tbx1 and Tbx2 in the foregut region and subsequently identify any disturbances in those profiles in the morphologically abnormal AMM foregut.

4.2 Methods

4.2.1 Overview of *In situ* hybridisation for Tbx1 and Tbx2

Control and Adriamycin treated embryos were obtained, processed and stored as described in section 2.1 and additional Cd1 embryos as described in section 2.2. RNA probes were generated for each candidate gene as described in section 2.3. Probes used for *in situ* hybridisation in this chapter are given in Table 2.1. Stored embryos were prepared for *in situ* hybridisation, as detailed in section 2.4, and subsequently processed for OPT scanning as described in section 2.6. For each gene a minimum of 5 AMM and 3 control specimens were examined at each gestational stage analysed.

4.2.2 Positional analysis of Tbx1 gene expression in the oesophagus

Height intervals were measured between the level of gene expression in the anterior oesophagus and the distal extent of the trachea, a constant reference point present in all embryos at E10. Relative height intervals were calculated as a fraction of individual total embryo height on its maximal anterior-posterior axis. Statistical analysis to compare heights between groups of $n=5$ was performed by student t-test using SPSS software with significance level (p) set at 0.05.

4.3 Results

4.3.1 Tbx1 expression at highly localised sites in the normal oesophageal endoderm.

In all embryos prominent Tbx1 expression was detected in the otic vesicles and the pharyngeal arch arteries at E10 and could be seen to be expressed in the foregut region on external visualisation of embryos following in situ hybridisation.(Fig. 4.1 A and B). Strong expression in the somites was additionally detected at E11 (Fig 4.2 E and G). Detailed analysis of virtual sections through 3D digitally reconstructed embryos generated by OPT revealed remarkably focal domains of Tbx1 gene transcripts in the oesophagus in both normal Cd1 and control CBA embryos at E10. These areas of expression were confined to the dorsal and ventral poles of the proximal oesophageal endoderm (Figure 4.1 C and D) slightly posterior to the site of tracheo-oesophageal separation. These foci of expression did not persist at E11 in control specimens. In Adriamycin treated embryos these localised expression points persisted but were displaced posteriorly consistent with a tracheo-oesophageal malformation phenotype (Fig 4.1 E). This is clearly demonstrated by comparing the position of expression with the distal extent of the trachea. At E10 relative height intervals from the focal site of Tbx1 oesophageal expression to the distal extent of the trachea were significantly reduced in treated embryos (mean 0.007, range -0.008 to 0.029,) compared to both normal (mean 0.039 range 0.02–0.053) and control embryos (mean 0.056 range 0.019–0.099) (Figure 4.1).

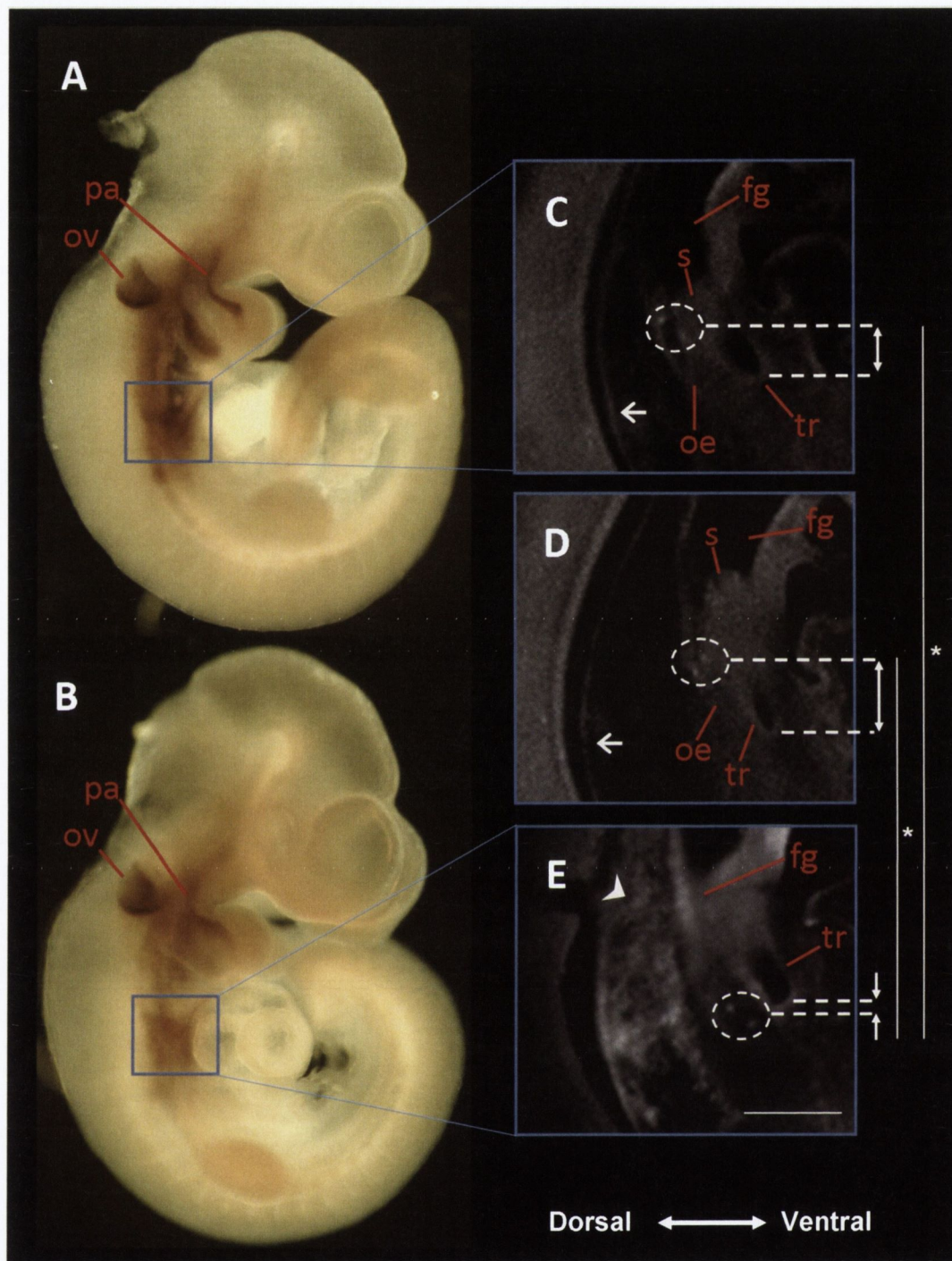


Figure 4.1: Expression of *Tbx1* in the foregut of normal and AMM treated embryos. Photomicrographs (A,B) and virtual sections of 3D reconstructed OPT scans (C,D,E). all demonstrating expression of *Tbx1* by in situ hybridisation in E10 embryos. *Tbx1* is similarly expressed in the region of the foregut (fg), the developing pharyngeal artery (pa) and otic vesicle (ov) in normal embryos (A) compared with Adriamycin treated embryos (B). The region for C to E sagittal sections are indicated by the boxed regions in A and B. *Tbx1* is focally expressed at the dorsal and ventral poles of the proximal oesophageal (oe) endoderm in normal (C) and control (D) embryos and posteriorly displaced in Adriamycin treated embryos (E) (circled in white). Relative height intervals between the level of focal expression and the distal extent of trachea (tr) (broken white lines) as shown by arrows are compared, the distance being significantly less in treated embryos compared to the other groups (* $p < 0.05$) ($n = 5$ for each normal, control and treated). Notochord branching is indicated by arrowheads, *s* tracheo-oesophageal septum. Scale bar in E = 200 μm and applies also for C and D.

4.3.2 Ectopic Tbx1 gene expression in the foregut region of Adriamycin treated mice.

In addition to positional disturbances of the anterior oesophageal foci of Tbx1 at E10, Tbx1 expression was disturbed along the dorsal foregut endoderm of Adriamycin treated embryos versus controls (Figure 4.2 A and B). In severely affected embryos bulky notochord branching was associated with highly abnormal Tbx1 expression in the hypoplastic foregut (Figure. 4.2 C and D). Severe foregut malformations included complete foregut atresia or extreme foregut hypoplasia, as present in the sample shown in Figure 4.2 C where no foregut lumen can be appreciated. At E11, ectopic expression of Tbx1 in the AMM was detected immediately dorsal to the trachea-oesophageal fistula in the region where the oesophagus is located in control embryos (Figure 4.2 G and H arrows compared to E). I observed that the anterior oesophageal foci of Tbx1 expression reported at E10 (cf. section 4.3.1) did not persist at E11 in control specimens. At E11 in the AMM the focal nature of abnormal Tbx1 expression was reminiscent of these oesophageal foci, and positionally reflected the site where the oesophagus would be in normal embryos (see comparison as detailed in Figure 4.2) however the ectopic expression at E11 was mesodermally and not endodermally located.

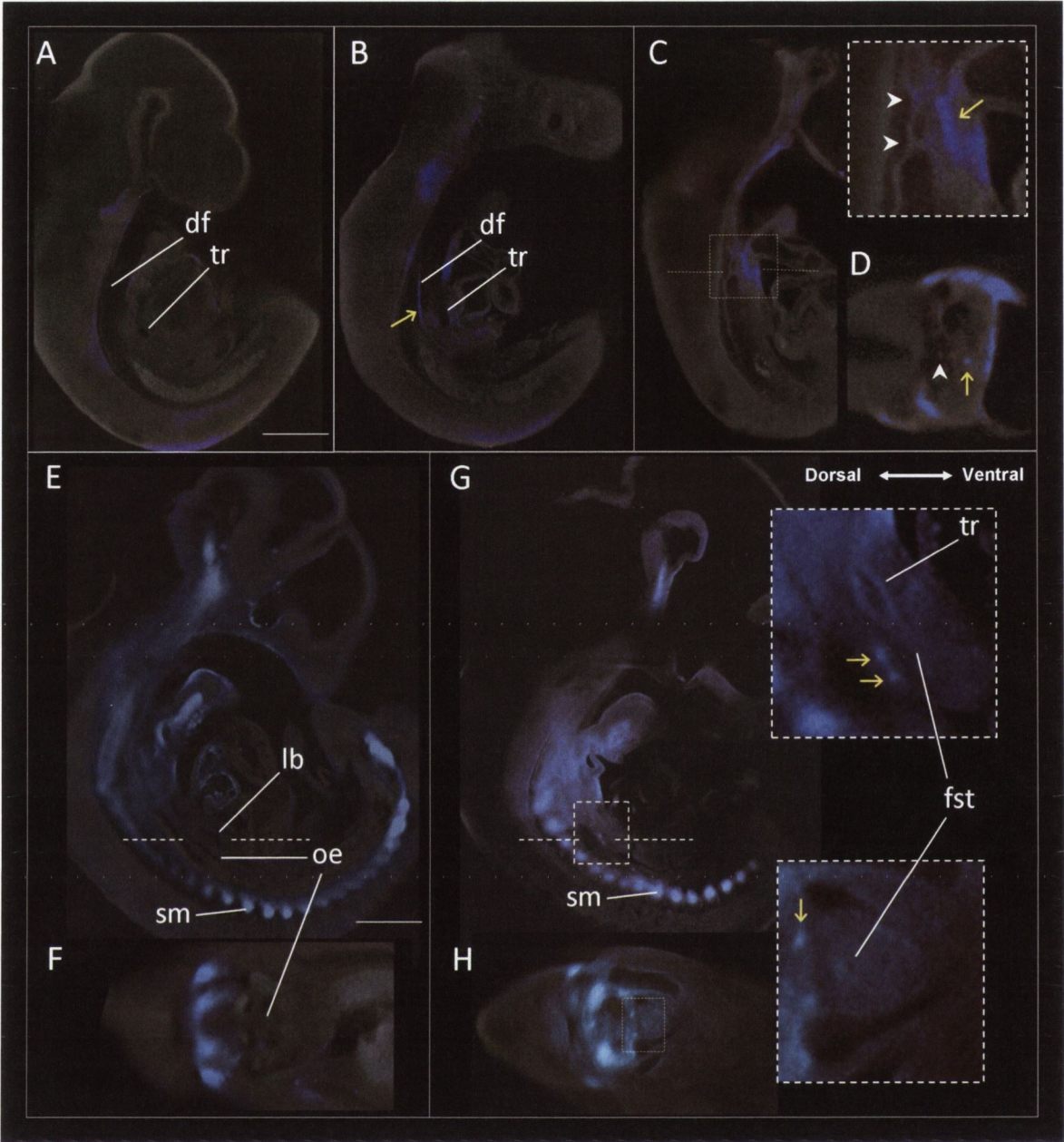


Figure 4.2: Abnormal Tbx1 expression in Adriamycin treated embryos shown by OPT scanning and 3D reconstruction. At E10 Tbx1 (pseudocoloured in blue) is ectopically expressed (as indicated by yellow arrow) in the dorsal foregut (df) of AMM (B) versus control embryos (A) and is strongly aberrantly present in the hypoplastic foregut endoderm severely affected embryos adjacent to notochord branching (C and D). In C the foregut lumen is completely occluded and cannot be appreciated (compare to A) and the extent of the abnormal expression (yellow arrow) spans 2 bulky notochord branches (indicated by the arrowheads). At E11 anomalous foci of Tbx1 expression (marked with yellow arrows) are detected dorsal to the tracheo-oesophageal fistula (fst) (G and H) occupying an area where the oesophagus is normally present (see control specimen in E and F). *lb* lung bud, *sm* somite, *oe* oesophagus, *tr* trachea. Scale bar in A = 600 μ m also applies to B and C, and E = 1500 μ m also applies to G.

4.3.3 Altered *Tbx2* gene expression in severely affected Adriamycin treated embryos.

Tbx2 was strongly expressed in the branchial arches, otic vesicles and ectoderm of the lateral body wall, as well as in the ventral foregut mesoderm at E10 in normal and control embryos but was not expressed in the foregut endoderm (Figure 4.3 A and B). In the AMM, at E10, altered *Tbx2* expression was only detected in the foregut severely affected embryos with bulky notochord branching (2 of 5). Very fine domains of ectopic foregut endodermal expression of *Tbx2* were present in discrete areas of severely hypoplastic foregut in these two embryos at the level of notochord branching and were associated with loss of ventral foregut mesoderm expression (Figure 4.3 C and D).

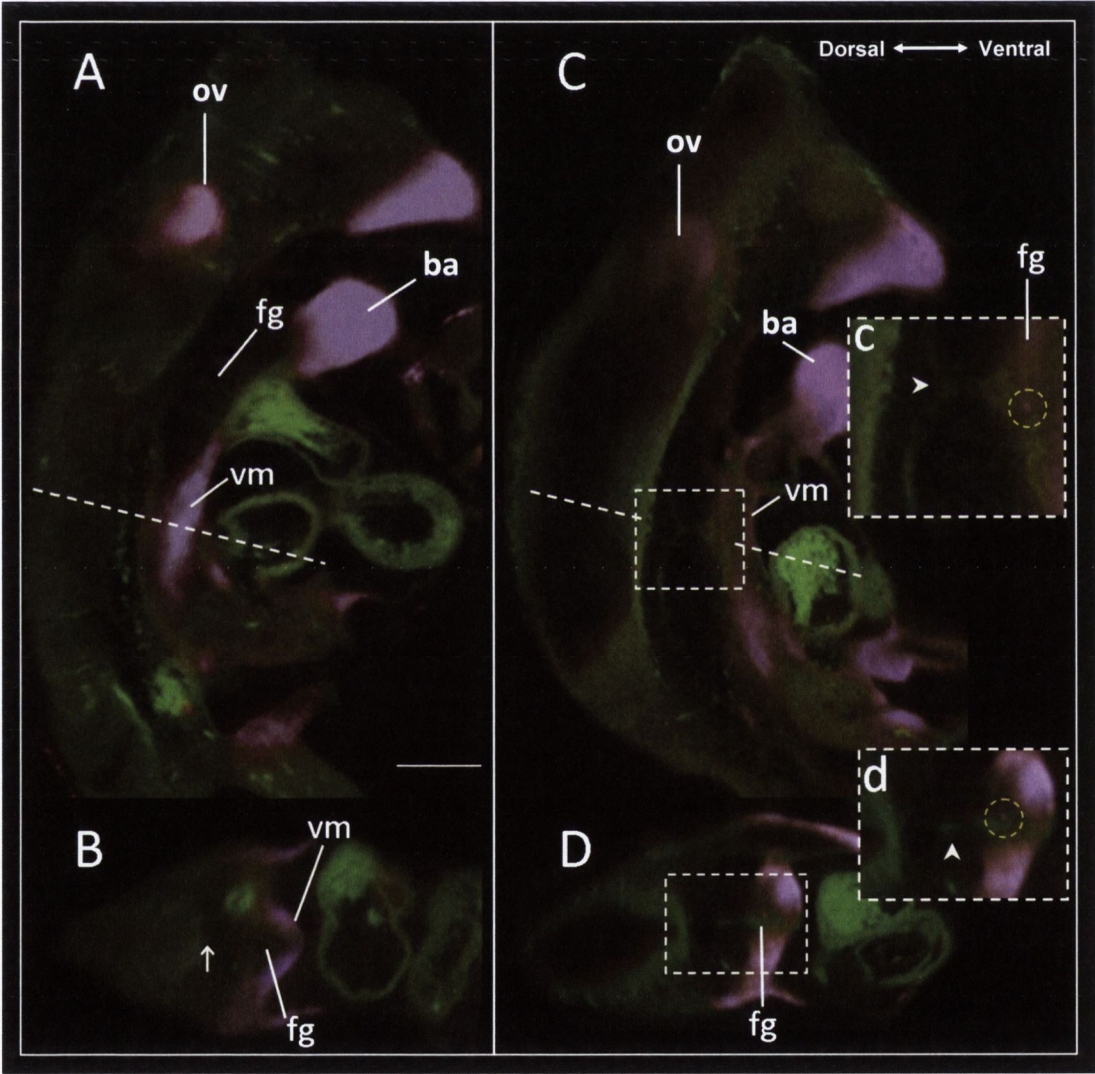


Figure 4.3: Abnormal *Tbx2* expression in severely affected Adriamycin treated embryos. Discrete ectopic expression in hypoplastic foregut (circled in yellow) and downregulation of expression in ventral foregut mesoderm (vm) of *Tbx2* (psuedocoloured in purple) in shown in severely affected AMM embryos (C and D) versus controls (A and B) at E10. The foregut lumen (visible in B) is occluded in D in association with bulky notochord branching (marked with arrowhead). Scale bar in A = 400 μ m. *ov* otic vesicle, *ba* branchial arches

4.4 Discussion

These data offer a novel description of a *Tbx1* expression domain localised in the anterior oesophagus, which, in addition to extensive abnormalities of *Tbx1* expression in the AMM foregut, suggest a contributory role for *Tbx1* in foregut development and in the pathogenesis of tracheo-oesophageal malformations. T-box genes, in particular *Tbx1*, are known to play an integral role in pharyngeal apparatus and cardiac outflow tract development and have been shown to interact with major developmental signalling pathways (Liu et al., 2009, Zhang et al., 2006, Bachiller et al., 2003, Fong et al., 2005, Manning et al., 2006). The strikingly focal pattern of normal *Tbx1* gene expression confined to the dorsal and ventral poles of the anterior oesophagus, in addition to extensively altered *Tbx1* expression in the AMM foregut, suggest the *Tbx1* transcription factor may additionally modulate normal oesophageal development.

The mechanism by which the oesophagus lengthens remains controversial. *Tbx1* has been shown to promote cell proliferation (Chen et al., 2009) and the extremely focal *Tbx1* expression in the oesophagus domain could indicate active proliferation causing elongation by outgrowth from a confined region of the anterior oesophagus. *Tbx1* is also an essential regulator of neural crest cell migration in the developing pharynx and in other tissues (Vitelli et al., 2002, Moraes et al., 2005) and may be necessary for enteric nervous system development in the anterior oesophagus, although the neural crest contribution to this region is not fully defined. Interestingly abnormal Vagal nerve development has previously been reported in *Tbx1* knockout mice (Calmont et al., 2011) as well as in the AMM and human OA/TOF (Cheng et al., 1999, Qi et al., 1997b) although the highly restricted nature of *Tbx1* expression at the anterior oesophagus is difficult to interpret in this context.

Intense foci of abnormal *Tbx1* expression occurred in the AMM at E11 in the mesoderm immediately dorsal to the fistula. In this area the oesophagus is present in normal embryos. Our group have not previously observed a disruption at this site in foregut expressed genes either in the current or previous work. Abnormal persistence of foregut expressed genes at E11 as well as shifts in expression of genes normally restricted to the endoderm into the mesoderm have both however been observed to occur in the AMM (cf. section 3.3.4.5). This disturbed expression at E11 could possibly reflect the persistence of the foci of oesophageal expression observed at E10 in normal embryos, abnormally located in the mesoderm, in the absence of the oesophageal endoderm at that site.

Both gain and loss of function mutations in *Tbx1* result in cardiac outflow tract and pharyngeal apparatus deformities consistent with a 22q11DS phenotype (Zweier et al., 2007, Cheng et al., 1999). Tight regulation of *Tbx1* gene dosage appears to be required for correct morphogenesis of these structures (Freyer et al., 2013). *Tbx1* expression is abnormally switched on in the ventral foregut of *Tbx2/3* double knockout mice who also display 22q11DS type deformities. This *Tbx1* up-regulation is associated with loss of *Shh* gene expression in the ventral foregut (Mesbah et al., 2012) demonstrating a role for T-box signalling in DV patterning of the foregut. In the AMM, the normal pattern of *Shh* expression is lost in the dorsal endoderm of the abnormally undivided foregut at E11 (Hajduk et al., 2011b), which correlates with abnormal expression of *Tbx1* detected at that site in this work.

Tbx2 gene dose has been shown experimentally to influence the phenotypic severity of 22q11DS malformations and may be a co-factor of penetrance in humans although *Tbx3* gene expression can protect from *Tbx2* gene loss (Mesbah et al., 2012). In the AMM ectopic *Tbx2* gene expression in the foregut endoderm with loss of expression in the ventral foregut mesoderm was only detected in severely affected embryos with bulky notochord branching and hypoplastic foregut. Abnormal *Tbx1* expression was present in all AMM embryos and was more conspicuously altered in severely affected embryos than *Tbx2* expression (compare Fig 4.2 C to Fig 4.3 C). *Tbx2* may therefore not be centrally involved in the development of OA/TOF but still be susceptible to derangement in response to upstream events when extensive disruption occurs. The ectopic foci of abnormal *Tbx2* signal in the dorsal AMM foregut is reminiscent of the nature of other molecular disturbances in the AMM foregut as reported in Chapter 3 and in particular the analysis of pSMAD localisation in the AMM (cf. Figure 3.3 E). These correlating expression profiles may reflect the ability of *Tbx2* to respond to BMP signalling in the foregut.

In Adriamycin treated embryos, abnormal *Tbx1* expression localises to the dorsal foregut endoderm at E10. While this may be due to the limits of examining a specific developmental window, *Tbx1* may be a direct target of Adriamycin disruption in the foregut or form a crucial part of the downstream response via interactions with other key modulators such as BMPs in the development of tracheo-oesophageal malformations. Interplay of *Bmp4* signalling and *Tbx1* activity is necessary for appropriate induction of cell differentiation in pharyngeal arch artery formation and multiple cross-regulatory mechanisms between these genes have been identified (Bachiller et al., 2003, Fulcoli et al., 2009, Nie et al., 2011). In the dorsal foregut endoderm of the AMM the pattern of

abnormal expression of Tbx1 is highly similar to ectopic Noggin gene expression (Hajduk P., Thesis, 2011) (cf. Figure 3.1) and localises to the same region as ectopic pSMAD immunolocalisation (cf. Figure 3.4 D) and abnormal expression of BMP downstream target genes Id1 and JunB (cf. Figure 3.6 C and R). In this region of the AMM foregut altered expression of Shh and Sox2 has also been reported (Ioannides et al., 2002). These simultaneous patterning disturbances imply that BMP signalling disruption in tracheo-oesophageal defect pathogenesis which may impact cross regulation between important regulatory genes in development.

4.5 Conclusion

Restriction of Tbx1 gene expression in remarkably restricted domains at the dorsal and ventral poles of the anterior oesophagus may reflect important mechanisms of oesophageal development occurring at that site which are regulated by Tbx1. Abnormal Tbx1 and Tbx2 gene expression in the foregut correlated with the phenotypic severity in AMM embryos, further indicated that T-box activity, and in particular Tbx1, may be involved in the pathogenesis of tracheo-oesophageal malformations, possibly in response to BMP signalling.

Chapter 5

A 3D explant system for foregut development: the molecular and morphological impact of notochord manipulation.

5.1 Introduction

During early embryogenesis, as the foregut is being formed by ventral folding of the endoderm germ layer, the notochord lies in close proximity to the dorsal foregut endoderm. Notochord cells are specialised mesoderm derivatives which directly arise from the potent signalling cells of the node and form a rod-shaped structure at the midline defining the anterior-posterior embryonic axis (Jurand, 1974). The close anatomical relationship between the notochord and the dorsally located neural tube is echoed by an important signalling relationship between these structures, first demonstrated by the ability of notochord transplants to induce a secondary neural floor plate (a ventral neural tube structure) in adjacent lateral neural tube cells (van Straaten et al., 1988). This was later characterised at the molecular level and shown to involve Shh signalling as a key component (Echelard et al., 1993, Marti et al., 1995). The ability of the notochord to provide DV patterning instructions to the adjacent neural tube could be extrapolated to propose that the notochord also has a crucial molecular relationship with the foregut, when the proximity of these structures is considered. It has yet to be proven directly if DV patterning arises from the notochord to contribute to foregut separation.

Foregut separation into dorsal oesophagus and ventral trachea is presaged by DV patterning of the foregut endoderm which designates tissue domains for the development of these derivate structures (as reviewed by Zorn and Wells, 2009b). A link between the notochord and foregut separation was suggested by the presence of distinctive notochord defects or branches co-existing with foregut malformations and molecular patterning disturbances in the Adriamycin Mouse Model (Hajduk et al., 2011b). These notochord defects are positionally related to the site of the morphological abnormalities in the foregut and the width of the notochord branch was found to correlate with the severity of the foregut malformation. Additionally the foregut showed disturbed DV patterning, reflected in altered expression of Sox2 and Shh, at the level of notochord branching, as well as the presence of altered gene expression in the foregut adjacent notochord branches. Again the extent of altered molecular signalling in the foregut was associated with the width of the notochord branch. A precedent for notochord to foregut signalling influencing morphogenesis is seen in the requirement of notochord derived Activin to induce pancreas formation from the foregut by suppression of endodermal Shh (Hebrok et al., 1998).

The presence of AMM-like notochord defects together with foregut malformations in the Noggin knockout mouse model further suggests the importance of the notochord in

foregut development and in fact conditional knockout of *Noggin* or over-expression of *BMPR1a* in the notochord and the neural floor plate was sufficient to induce both notochord and foregut defects (Fausett et al., 2014). In the classical *Patch* mutant mouse, caused by a deletion of the Platelet Derived Growth Factor Receptor alpha gene, notochord and foregut abnormalities co-exist with neural crest defects (Snider et al., 2011). In humans notochord abnormalities consistent with the abnormal branching seen in the Adriamycin mouse model, have also been reported to occur in association with congenital malformations of the hind and foregut, and with *VACTERL* anomalies including *OA/TOF*, although reports in the literature are understandably sparse (Elliott et al., 1970). *Split Notochord Syndrome* in humans describes notochord anomalies, neural tube defects together with gut duplications or cysts (Bentley and Smith, 1960, Meller et al., 1989).

The mechanism by which the notochord may signal to the foregut during oesophageal and tracheal formation is poorly understood. It has been suggested that altered *Shh* signal due to the proximity and extent of notochord tissue in relation to the foregut is sufficient to induce foregut malformations (Ioannides et al., 2003). Gene mutations in the *Shh* pathway, resulting in *OA* phenotypes in animal models are well described (Motoyama et al., 1998, Litington et al., 1998) and can be found in a small proportion of syndromic human *OA* (Johnston et al., 2005). Findings from the *Noggin* knockout mouse implicate notochord defects together with BMP signalling abnormalities in the pathogenesis of foregut abnormalities. My results in the AMM further indicate overactivity of BMP signalling in the abnormal foregut and notochord based on disturbances of pSmad, *Noggin* and BMP downstream target gene expression (cf. Chapter 3). The remarkably focal nature of the BMP signalling abnormalities in the dorsal foregut endoderm is particularly striking and has a close spatial relationship to the distinctive notochord defects present in the model (cf. Figure 3.5). Fausett et al (2014) proposed that *Noggin* attenuation of BMP signalling in the notochord is necessary to allow both proper notochord delamination and foregut development.

The apparent interdependence of BMP signalling and the notochord branch effect in foregut morphogenesis is difficult to further decipher through analysis of phenotype in whole embryos from the 2 key models displaying this phenomenon. In the Adriamycin mouse we see many important signalling pathways have abnormal expression profiles in the foregut under the effects of this teratogen (as reviewed by Mc Laughlin et al., 2013). The *Noggin* knockout mouse does not exhibit foregut patterning abnormalities and it has been proposed that sequestration of cells away from the dorsal foregut endoderm by the abnormally adherent notochord, may account for failure of the oesophagus to develop

(Fausett et al., 2014). I proposed to address the complexity of this issue by teasing out whether physical manipulation of the notochord can have a direct effect on foregut morphology and molecular patterning which will better inform our understanding of the signalling relationship between these structures.

In order to perform these investigations I first undertook to establish a model in which notochord manipulation in relation to the foregut could be performed under conditions which successfully recapitulate oesophageal and tracheal formation from foregut. *In vitro* tissue explantation is a recognised method of manipulating embryonic organ system development in an observable and controllable manner. Successful *in vitro* techniques applied to foregut tissue include culture in liquid media and Roller Bottle culture (Tremblay and Zaret, 2005). However in order to support physical manipulations a model with a stable 3D culture environment is required and has not previously been used for the foregut. E10 foregut culture has been described in liquid medium to investigate the effect of signalling manipulation on oesophageal development (Que et al., 2007), however the explants used in these experiments exhibited foregut division at the time of culture onset. Culture of foregut from E8.5 embryos has previously been performed using liquid media as a model for lung bud morphogenesis (Desai et al., 2006). Oesophageal formation was not reported using this method. I proposed to use a robust physical support for the tissue to allow stable co-culture with notochord. Gels have been widely used for cell or tissue support in culture for a variety of applications and several commercial options are available. Experiments manipulating the embryonic notochord have previously been reported in chick embryos. Ablation of notochord or grafting of notochord transplant could induce structural and molecular abnormalities of the neural tube and somites (van Straaten and Hekking, 1991).

Designation of foregut into dorsal and ventral domains is a key event in subsequent oesophageal and trachea formation. The importance of strict regionalised patterning in the foregut endoderm is particularly well illustrated by the relationship between dorsal marker Sox2 and ventral marker Nkx2.1 (Wells and Melton, 1999). Encroachment of the ventral domain by Sox2 characterises models of tracheal agenesis and inappropriate dorsal presence of Nkx2.1 accompanies oesophageal atresia (Li et al., 2008b, Que et al., 2007). DV domains have been established in the foregut at E8.5 gestation as indicated by Sox2 and Nkx2.1 localisation (Serls et al., 2005, Sherwood et al., 2009). At this gestational stage however the foregut is yet to be completely formed by folding morphogenesis of the endoderm germ cell layer. Thus at E9 gestation (TS14) the foregut is formed and exhibits DV patterning but is undivided, providing a natural point at which the ability of

manipulation in culture to alter the morphological or molecular course of foregut separation can be tested. In addition E9 tissue is amenable to established processing techniques for wholemount immunolocalisation with OPT scanning for analysis.

The specific aims of the work presented in this chapter were:

- 1) To establish an explant culture system capable of supporting foregut development as reflected by successful division into oesophagus and trachea. I assessed various gel supports and constructs and the use of whole trunk or isolated foregut explants by gross morphological assessment for external features of development and explant viability. I also used OPT together with wholemount immunolocalisation of the Hnf3 β endoderm marker to assess for foregut separation.
- 2) To assess the characteristics of foregut separation in explant culture. I used Hnf3 β immunolocalisation for morphological assessment and immunohistochemistry for Sox2 and Nkx2.1 as markers of DV foregut patterning to examine foregut development in explant culture. I also developed a morphological staging system, based on the anatomical features of foregut development in vivo, to assess explant foregut separation.
- 3) To analyse the impact of notochord manipulation on foregut division in culture. I compared the morphological and molecular features of explants with notochord retained, notochord removed or with notochord added using techniques as described in Aims 1 and 2.

5.2 Methods

5.2.1 *Overview of explant culture and analysis*

Embryos were obtained and tissues prepared for explant culture as described in detail in Section 2.8. Briefly optimisation of the explant culture technique was performed over the course of two years and 12 experiments and I progressed from using whole embryo trunk for explant (as detailed in 2.8.2) to the use of isolated foregut (as detailed in Section 2.8.3 and Appendix A) as fine dissection skills were acquired. 7 initial rounds of whole thorax explant culture, maintained in vitro for up to 72 hours using various gels and constructs for 3D support, were assessed daily for the presence of contracting cardiac cells to reflect explant viability. These various 3D supports are outlined in Table 5.1. At the end of the culture period (24 to 72 hours) external morphological features were examined and recorded to examine for advances in development. In particular the somites were examined for regression and the otic vesicles were examined for otic pit closure and endolymphatic duct development (Theiler, 1989a). Following optimisation of whole thorax explant culture, which culminated in the use of Rat Tail Collagen within plastic ring buoys to support explants (as detailed in section 2.8.4), wholemount Hnf3b immunolocalisation (as described in Section 2.4) together with OPT scanning (as described in Section 2.5) was used on a further set of whole thorax explants (Experiment no. 8, Table 5.1) to assess for foregut division at 24, 48 and 72 hour time points.

In isolated foregut explants, using the optimised 3D explant culture system, Hnf3b immunolocalisation in wholemount specimens (as above) and immunohistochemistry on serial cryosections (as described in section 2.6) allowed morphological and molecular analysis of foregut development following 48 or 72 hours of culture. A serial section approach was employed for IHC using well established markers of foregut DV patterning. Adjacent sections on different slides were analysed for localisation of Hnf3b (endoderm marker), Sox2 (dorsal marker) and Nkx2.1 (ventral marker). Morphological matching of sections was then used to allow direct comparison of patterning domains.

5.2.2 *Notochord manipulation*

During foregut isolation (as described in Section 2.8.2) the notochord with or without the neural floor plate were either maintained in the tissue for explant or removed along with

the dorsal foregut mesoderm by dissection using tungsten needles. For notochord transplantation, notochords were harvested from somite-number matched littermate control embryos by first detaching the notochord and the neural floor plate along with the neural tube from the dorsal foregut mesoderm by tungsten needle dissection as described in the foregut isolation technique. Notochords and a minimal amount of neural floor plate were then dissected away from the neural tube. Notochords were transferred by borosilicate glass capillaries under mouth control onto the base of a collagen plastic ring construct containing a foregut explant. The notochord transplant was positioned at the dorsum of the anterior part of the foregut explant using tungsten needles. The explants and notochord were secured in place using an overlay drop of collagen, with position correction prior to complete collagen gelling using tungsten needles as necessary. Explants with transplanted notochords, maintained notochord or removed notochord were cultured for 48 hours and harvested for further processing as described for foregut explants.

Various problems were encountered when dissecting and handling notochord tissue. At E9 the notochord was fragile compared to later stages (e.g. E10) and the close anatomical relationship between the notochord and the neural floor plate hindered complete notochord isolation, in fact this was rarely achieved without damaging the notochord beyond usefulness. In addition a high attrition rate due was encountered during transfer of the notochord or even transplant positioning with the tungsten needles or coated boro-silicate glass capillary under mouth pipette control due to sticking of the notochord to implements and dishes. Furthermore, when grafting a notochord transplant to an explanted foregut, the notochord appeared repelled by the foregut. While ideal conditions for notochord dissection were not established, successful notochord transplants were achieved in a number of specimens as detailed in the results.

5.2.3 *Foregut Staging*

Normal Cd1 embryos were harvested from E9 to E11 gestation and processed for Hnf3 β wholemount immunolocalisation as described in Section 2.4 and OPT scanning (as described in Section 2.5). OPT scans were examined by surface render, which uses thresholded signal from the marker of interest to map the surface of the labelled tissue in 3D (cf Section 2.5). Representative anatomical features of foregut separation which were highly stage specific as assessed by accurate Theiler staging of embryos based on externally visible morphological features including somite number (Theiler, 1989a) were recorded and used to establish a staging system based on foregut morphology. The

resulting system was applied to isolated foregut explants analysed by Hnf3 β wholemount immunolocalisation and immunohistochemistry on cryosections to determine the stage of foregut development achieved in comparison to normal development.

Samples used in the staging assessment were also used as controls in later results and included foreguts both with and without their native notochord as well as notochords treated with PBS soaked alginate beads. These culture conditions did not affect staging as detailed below and in the subsequent chapter (Section Table 6.1). Not all samples underwent initial somite counting before culture therefore only a proportion of all samples underwent staging.

5.3 Results

5.3.1 3D explant culture supports foregut separation into oesophagus and trachea.

A summary of each experiment including the outcome of gross morphological assessment is given in Table 5.1. In brief for whole thorax explant, 8 sets of culture experiments with a total of 86 explants were performed. I assessed the use of various commercial gels as a 3D support for tissue in culture by comparing the resulting tissue viability and morphology. Neither the use of Hydrogel (Expt 1 and 2, Table 5.1) nor Matrigel (Expt 3 and 4, Table 5.1) resulted in good morphological outcomes and were supplanted by the use of Rat tail collagen which provided improved explant viability. Further experiments assessed the use of various methods of explant assembly within collagen. Direct embedding within a collagen droplet on the base of the culture well gave variable results (Expt 5, Table 5.1). Spreading of tissue inside the collagen along the well base in the course of the culture period disrupted external morphological features. Pre-placement of a collagen droplet on the base of the well to provide a cushion on top of which the explant was embedded within collagen gave some improvement (Expt 6, Table 5.1) however it was concurrently noted that explants which detached from their gels and floated within the medium had enhanced morphology and tissue viability.

I devised the use of plastic rings within which a collagen gel base was prepared for the subsequent embedding of explants (Figure 5.1 A) This construct floats just below the medium-air interface while providing stable 3D support and resulted in greatly superior morphological and tissue viability outcomes at up to 72 hours of culture (Expt 6 and 7, Table 5.1), as demonstrated by progressive development of the otic vesicles (Figure 5.1 B and C) and the somites (Figure 5.1 D and E) and as well as persistent contraction of cardiac cells. Following the completion of optimisation a further thorax explant experiment was performed (Expt 8, Table 5.1). In these specimens, assessed by Hnf3 β wholemount immunolocalisation and OPT scanning, I observed foregut division into ventral and dorsal tubular structures in most of the whole thorax explants after 48 hours of culture (5/7), however proper lung bud formation was only discernable in a minority of specimens (2/7).

Table 5.1 Viability and development of thorax explants in 3D culture.

Experiment No.	3D Support		In Culture		Heartbeat		Development		
	Gel	Assembly	Time (hrs)	No. samples	Present	Absent	Gain	No gain	Not assessed
1	Hydrogel	Base of well	24	3	*	**			***
			48	3		***		***	
			72	3		***		***	
2	Hydrogel	Base of well	24	16	*****	*****	**	*****	****
			48	10	*****	*****	***	*****	**
			72	6		*****		*****	
3†	Matrigel	Base of well	24	8	*****				*****
			48	5		*****			*****
			72	3		***			***
4	Matrigel	Base of well	24	12	*****		*****	*****	
			48	8	*****		*****	***	
			72	4	*	***	**	**	
5	Rat Tail Collagen	Base of well	24	8	*****		*****		
			48	5	*****	**	***	*	
			72	3	**	**	***		
		Coverslips	24	4	****		****		
			48	3	*	**	***		
6	Rat Tail Collagen	Collagen cushion	72	1		*		*	
			24	8	*****	**	***	**	**
			48	6		*****		*****	**
		Plastic rings	72	4		****		****	
			24	8	*****		*****		**
			48	5	***	**	*****		
7	Rat Tail Collagen	Plastic rings	72	3		***		***	
			24	19	*****	*	*****	*	*
			48	14	*****	****	*****	*	*
8	Rat Tail Collagen	Plastic rings	72	8	*****	**	***	*****	
			24	13	*****	*	*****		*
			48	7	*****	*	*****	*	

Specimens were analysed at 24 hour intervals while in culture and individual assesments are represented by an asterisk.
Total number of samples for each experiment are highlighted in blue. Harvest of specimens from this total number occurred at 24 hour intervals as indicated by no.samples in culture at the subsequent time points (for example in Exp2, of a total of 16 explants, 6 were harvested at 24 hours and 10 remained available in culture at 48 hours for assessment etc).
The position of some samples precluded developmental analysis if not being harvested (Not assessed).
† medium incorrectly made

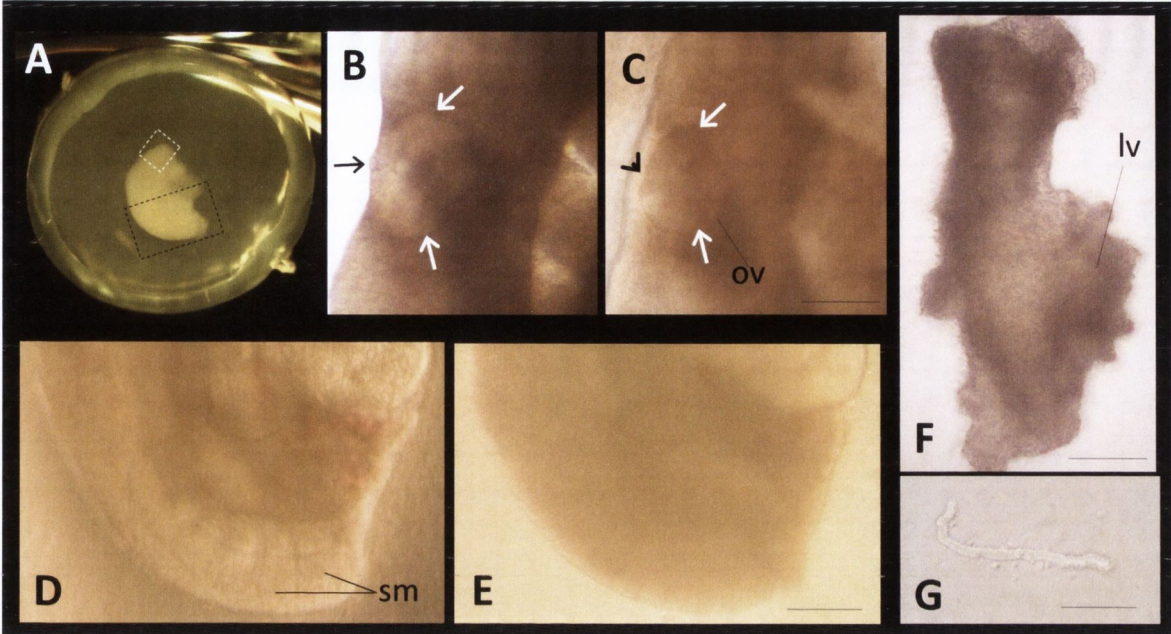


Figure 5.1; External morphology in 3D explant culture. Photographs of explants (A to G). In A a whole thorax explant is embedded within collagen gel plastic ring construct and floating in medium at D0 of culture. The white boxed area reflects the region of B (at 0 hours in culture) and C (at 24 hours culture) examining ear development. The otic placode in B (white arrows) goes on to form the otic vesicle (ov) in C following closure of the otic pit (black arrow in B) and early endolymphatic duct formation (black arrow head in C). The black boxed region in A reflects the region of D (at 0 hours in culture) and E (at 48 hours culture) showing regression of the anterior thorax somites during culture. F shows an isolated foregut sample in medium, note the minimal amount of mesoderm maintained with the explant except at the hepatic diverticulum (lv) for future liver development. In G a length of isolated notochord has been maintained in 3D gel culture for 48 hours. Scale bars in E = 100 μ m, C and F= 50 μ m, G = 20 μ m.

Successful isolation of foreguts for explant culture subsequently supplanted the use of whole thorax explants (Figure 5.1 F). The final optimised technique resulted in highly consistent results as exemplified in Figure 5.2. The ideal stage for foregut isolation was found to be 18 to 24 somites. None of the foreguts explanted prior to the 15 somite stage survived in 3D culture (n = 6). In comparison to thorax explants, the use of isolated foregut tissue from 18 to 24 somite embryos (n =12) achieved foregut division into oesophagus and trachea as well as lung bud formation (Figure 5.2 J and K) and as further detailed in Section 5.3.2 below. This was shown by analysis of Hnf3 β localisation on explant sections (Figure 5.2 compare D and E) and in whole embryos by 3D imaging (Figure 5.2, compare I with J,K). After 48 hours of culture foregut explants also demonstrated a stomach, dorsal and ventral pancreatic buds as well as liver formation (Fig 5.2 K)

In two specimens which did not possess bilateral lung buds, a ventral trachea (with a single lung bud) was present and completely distinct from the dorsal oesophagus (Appendix Figure 5 A). This morphological variant may represent an effect of the explant technique due to exposure of the foregut to altered anatomical and signalling environment or could be the result of damage to the delicate ventro-lateral foregut endoderm during explantation. Interestingly both of these samples were observed to have a reduced liver domain, reinforcing the possibility of ventral foregut damage.

Attempts to culture and expand notochord cells for use in co-culture experiments were not successful. Classical tissue dissociation techniques with both mechanical disruption and trypsinisation failed to produce viable plated cells. Lengths of completely isolated notochord could be maintained in 3D collagen gel culture, (but not without this 3D support) for up to 72 hours without gross change (Figure 5.1 G). In 3D culture, notochord morphology was maintained as was Hnf3 β and Sox2 marker positivity when compared to normal embryos (Figure 5.6). Unfortunately immunolocalisation of Brachyury, as an established marker of notochord, underwent several trials in cryosections of normal Cd1 embryos without success.

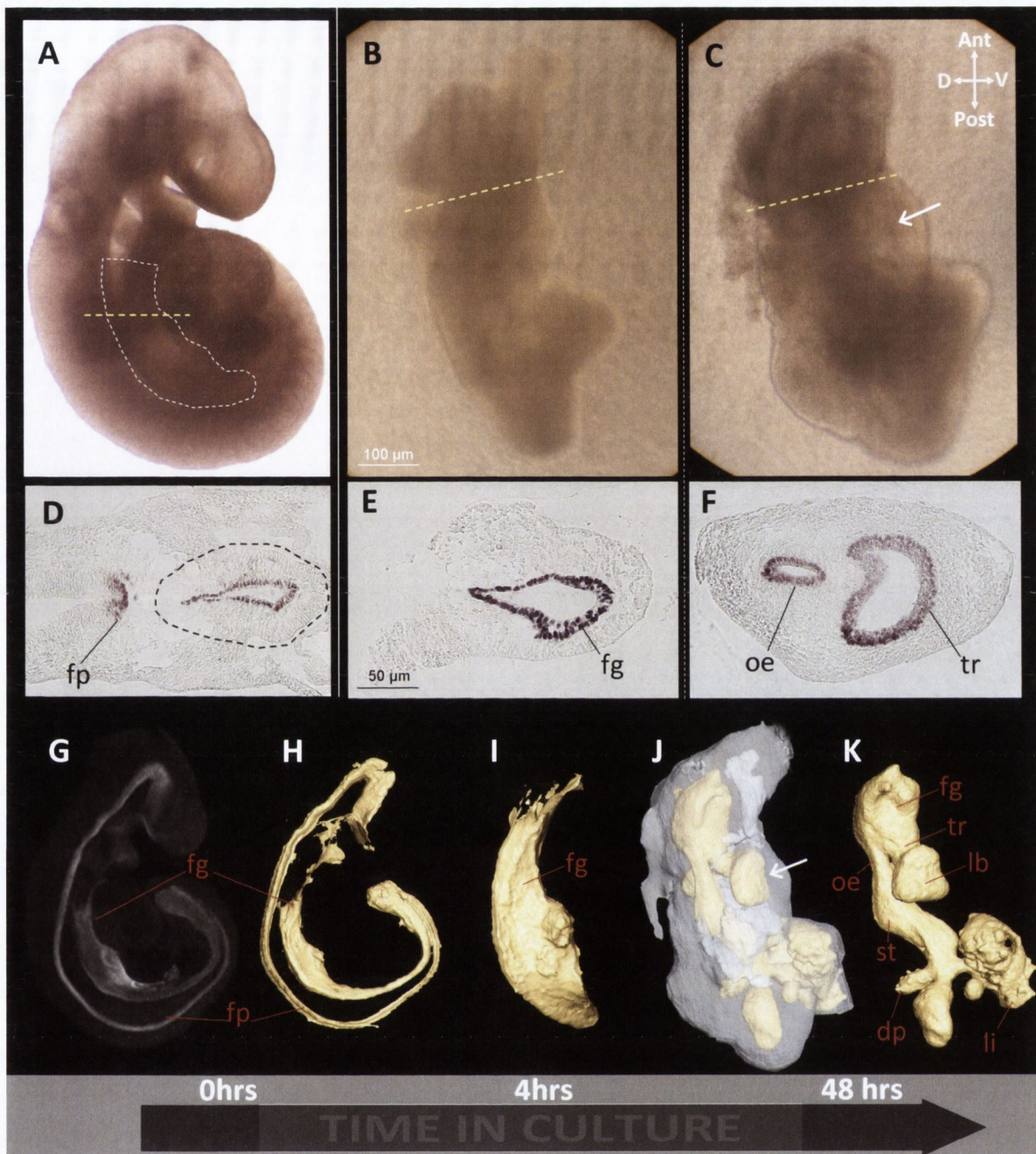


Figure 5.2 Isolated foreguts explanted into culture at E9 achieve foregut separation.

Photographs show an E9 embryo (A), a foregut explant in culture at 4 hrs (B) and at 48 hrs (C) as indicated by the timeline. Observe the shape and morphological features of the same explant specimen in B and C which grows over 48 hours in culture. *Hnf3β* immunolocalisation in cryosections (D to F) and in wholmount specimens with OPT scanning (G to K) reveal morphology and allow comparison between normal E9 embryos (D, G, H), foregut explants at 4 hours of culture (E,I) and at 48 hours of culture (F,J,K). In A and D the dashed outlines show the dissection area to obtain isolated foregut (compare to B and E respectively). Plane for sections D to F are given by dashed yellow lines in A to C. G is an example of a volume render visualisation of 3D reconstructed OPT scan (in greyscale) and H is the same scan analysed using surface render (in yellow), note the matching features. In E9 embryos the foregut (fg) is undivided (D,G, H). At 4 hours in culture the foregut is similarly undivided (E,I). J is surface rendered OPT scan of the specimen shown in B and C, note the corresponding shape (non foregut tissues are depicted in white and rendered transparent), and in particular the area highlighted by the arrow where shading in the photograph corresponds to the structures marked by *Hnf3β* (in yellow). K is sample J viewed with the non foregut tissue extracted. Distinct oesophagus (oe), trachea (tr), lung bud (lb), stomach (st), dorsal pancreatic bud (dp) and liver (lv) are all seen to have formed over the the culture period (compare to I and H). Foregut separation is also detected in cryosection F (compare to E). Scale bars as given apply across each panel.

5.3.2 *Normal foregut development can be staged based on morphological features.*

I established a morphological staging system based on the characteristics of the developing foregut *in vivo*, revealed by Hnf3b immunolocalisation in whole Cd1 embryos at TS15 (E9.5) through TS18 (E11). This was facilitated by OPT scanning and 3D reconstruction using surface rendering (cf. Section 5.2.3). The relative position of the lung buds to the foregut and the presence and morphology of distinct oesophagus, trachea and stomach as they arise from the single foregut structure, were key features that characterised each of the 4 Thieler stages in normal embryos (n= 4 per stage) (Figure 5.3 panel A).

At TS15 in normal embryos the lung buds have arisen from the ventro-lateral foregut wall as endodermal outpouchings which maintain a wide communication with the foregut lumen while the foregut remains undivided and the stomach has not yet formed (Figure 5.3 A TS15). At TS16 (E10) the lung bud position is entirely ventral in relation to the foregut and the lung buds are distinct from the foregut tube while maintaining communication with it by the early bronchi, however complete foregut separation has not yet occurred and the stomach is not yet clearly discernible. At TS17 (E10.5) distinct oesophageal and tracheal structures are present with complete detachment of endoderm walls by intervening mesoderm. The early stomach has also taken shape at this gestational stage. At TS18, elongation and narrowing of both the oesophagus and trachea were observed as was well defined stomach morphology (Figure 5.3 A TS18).

5.3.3 *Isolated foregut explants achieve comparable morphological outcome to in vivo.*

This staging system was then applied to isolated foregut explants in a standardised fashion to provide a measure of the degree and normality of development achieved *in vitro* using the 3D culture system versus normal foregut development. All of the foregut explants staged using this approach were placed in culture at TS14 and underwent somite counting pre-dissection, so that progress in development could be accurately assessed.

Complete foregut separation into oesophagus and trachea as well as early stomach formation consistent with TS17 staging was achieved in 11 of 14 samples assessed (Figure 5.3 C). The remaining 3 samples displayed completely ventral lung buds but not distinct oesophagus and trachea formation and were therefore staged as TS16 although the early stomach was discernable in both of these samples (Figure 5.3 B).

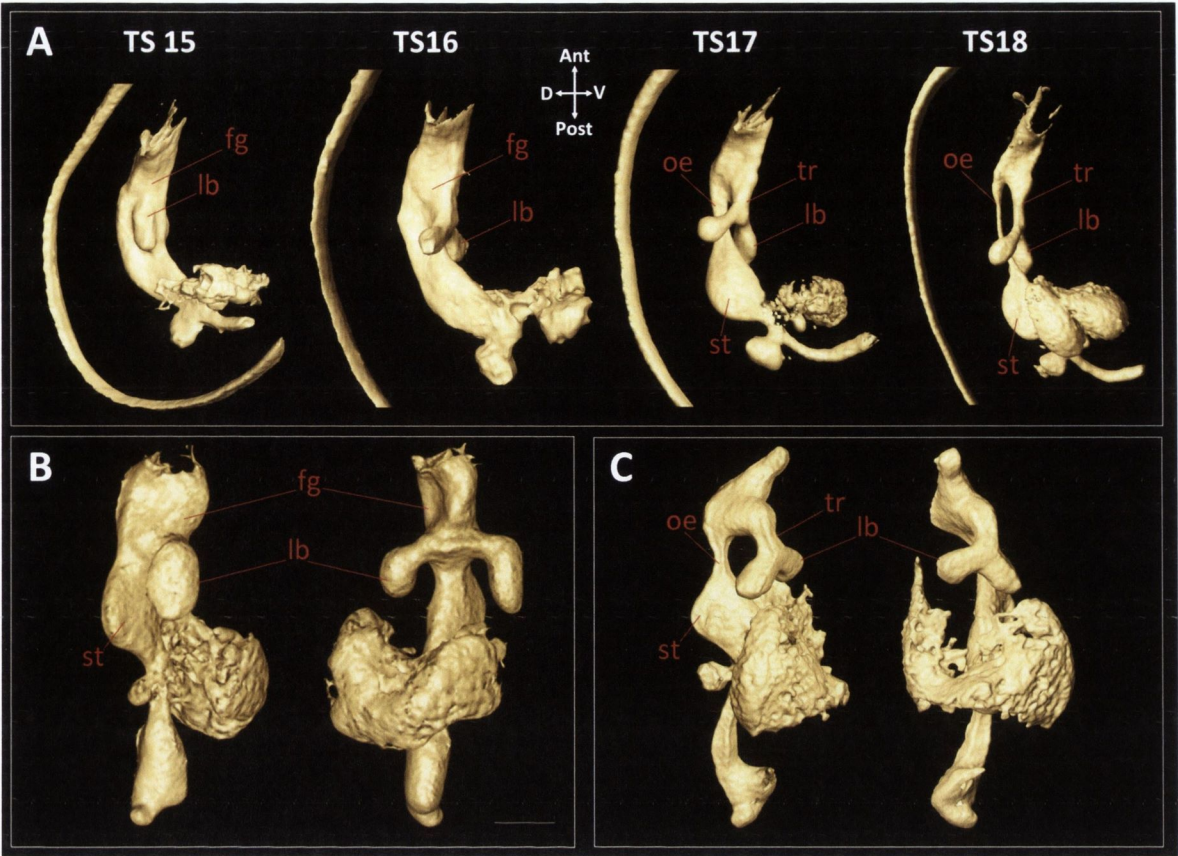


Figure 5.3 Developing foregut morphology in vivo and in 3D explant culture; a staging system for foregut development. Wholemout Hnf3 β immunolocalisation was detected by OPT scanning and depicted by surface render (in yellow) to reveal the anatomy in dividing foreguts. Accurately Theiler Staged (TS) normal embryos in panel A show lung bud (lb) formation from the foregut (fg) at TS 15 (E9.5) in a ventro-lateral position and at TS16 (E10) in a ventral position relative the foregut. Distinct oesophagus (oe), trachea (tr) and stomach (st) formation become apparent at TS17 (E10.5) and are more defined and elongated at TS18 (E11). B and C are two foregut explant specimens obtained from TS14 (E9) embryos viewed at the lateral (left in each panel) and ventral aspects (on the right in each panel) exhibiting foregut separation following 48 hours in culture. Foregut explants were assigned TS staging by comparing the lung bud position and the presence of distinct oesophagus, trachea and stomach. B was staged as TS16, (compare to Panel A TS16) and C as TS17 (compare to A TS17).

A small proportion of samples (n=2) demonstrated foregut division into a ventral trachea and dorsal oesophagus but did not possess bilateral lung buds (Appendix B Figure 3 A). Only samples which had lung buds present were assessed for staging given that lung bud position was a key feature of stage. The somite count at the time of explant was between 18 and 24 somites for all samples. As expected, staging at 48 hours of culture appeared to stratify according to the initial somite count. 1 out of 3 18 somite foreguts achieved TS17 stage, 3 of 4 20 somite foreguts and all 22 and 24 somite foreguts (n = 3 and n = 4 respectively) achieved TS17 at the end of the culture period. The staging results for all the specimens assessed are summarised in Figure 5.4.

Additional foreguts cultured from 28 and 30 somite embryos (at early TS15) (n=3 each) demonstrated narrow elongated oesophagus and trachea consistent with development to TS18 stage after 48 hours in culture (Figure 5.4, Appendix B Figure 3 C). The pre-culture existence of lung buds in these older embryos precluded their use in further experiments except where stated. In foreguts which were cultured for 72 hours starting with 22 somite stage embryos (n=2) TS 18 staging was also achieved (Appendix B Figure 3 B). All of the TS18 staged explants also displayed evidence of early branching within the lung buds, which is normally detected after TS18.

This analysis shows that 48 hours of culture in the 3D system supported an advance in development from TS14 to TS17 which approximately corresponds to 36 hours of foregut development *in vivo* (TS14 generally corresponds to E9 and TS17 to E10.5). This was considered adequate, and the ideal developmental window, to examine for foregut molecular and morphological disturbances resulting from manipulation of the culture environment. The additional development gained by a further 24 hours in culture was at the expense of normal morphology with kinking in the foregut derived structures due to limited expansion space and was not used in further experiments.

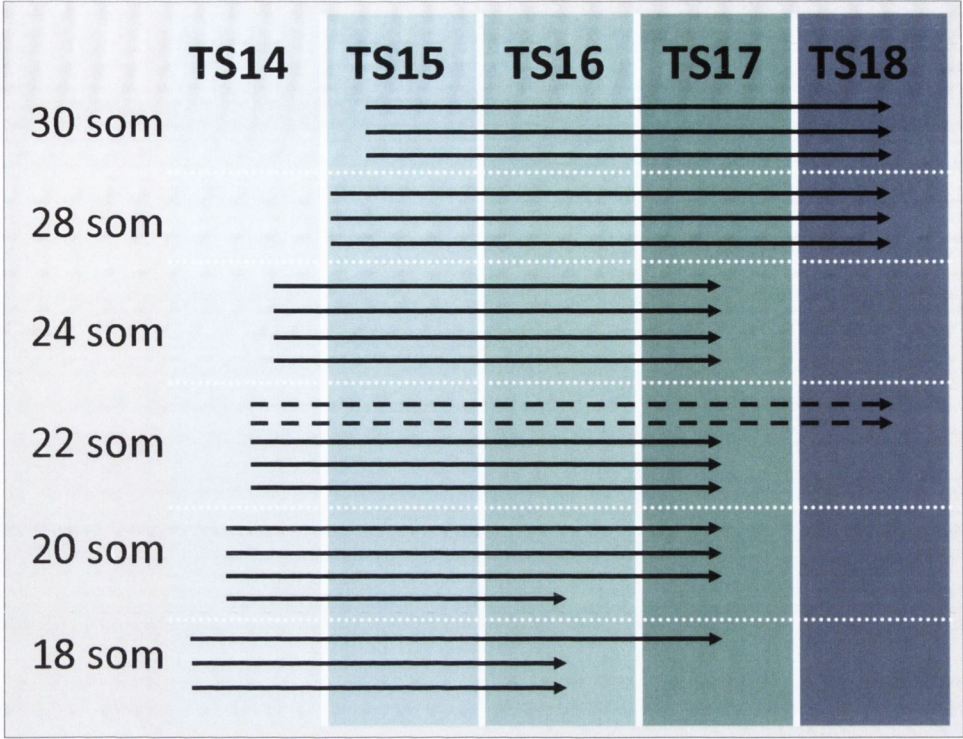


Figure 5.4 Foregut explants show morphological progression in culture which reflects pre-culture somite count. Each arrow represents an individual foregut explant specimen with initial somite count prior to explant on the y axis and the extent of development gained, as assessed by morphological staging, to end of culture period (48 hours for all except dashed arrows which were cultured for 72 hours).

5.3.4 Isolated foregut explants exhibit normal molecular patterning.

A serial section approach allowed detailed characterisation of dorso-ventral patterning in isolated foregut explants as demonstrated by the distribution of Sox2 and Nkx2.1 proteins in the foregut endoderm ($n = 7$). In the undivided foregut of explants at 4 hours of culture, and stage matched normal embryos *in vivo*, strict domains for Sox2 dorsally and Nkx2.1 ventrally were observed throughout the foregut (Figure 5.4B), except in the most anterior part of the foregut which was entirely Sox2 positive and did not express Nkx2.1. The patterns of Sox2 and Nkx2.1 protein expression in foregut explants were comparable with stage matched *in vivo* normal embryos at TS17 (Figure 5.4 compare C ii to D). Sox2 was present in the circumference of the foregut endoderm at the anterior extreme of the explant (cf. Figure 6.7 C) but, at the level of the dividing foregut, was restricted to the dorsal endoderm (Figure 5.4 C i), and was present in the oesophagus (Figure 5.4 C ii) and in the stomach (Figure 5.4 C iii and iv). Sox2 expression in the foregut extended to the level of the early duodenum at the dorsal and ventral pancreatic buds (Figure 5.4 C v) but not beyond. Nkx2.1 was absent in the most anterior regions of the explanted foregut (cf. Figure 6.7 C), was restricted to a ventral domain in the dividing foregut (Figure 5.4 C i) and present in the trachea and in the lung buds (Figure 5.4 C ii and iii). A very small domain of Nkx2.1 positive cells was present in the ventral wall of the oesophagus or upper stomach in most specimens and coincided with lower Sox2 signal in that limited area in the adjacent section (Figure 5.4 C iii), illustrating the disparate domains maintained by these transcription factors.

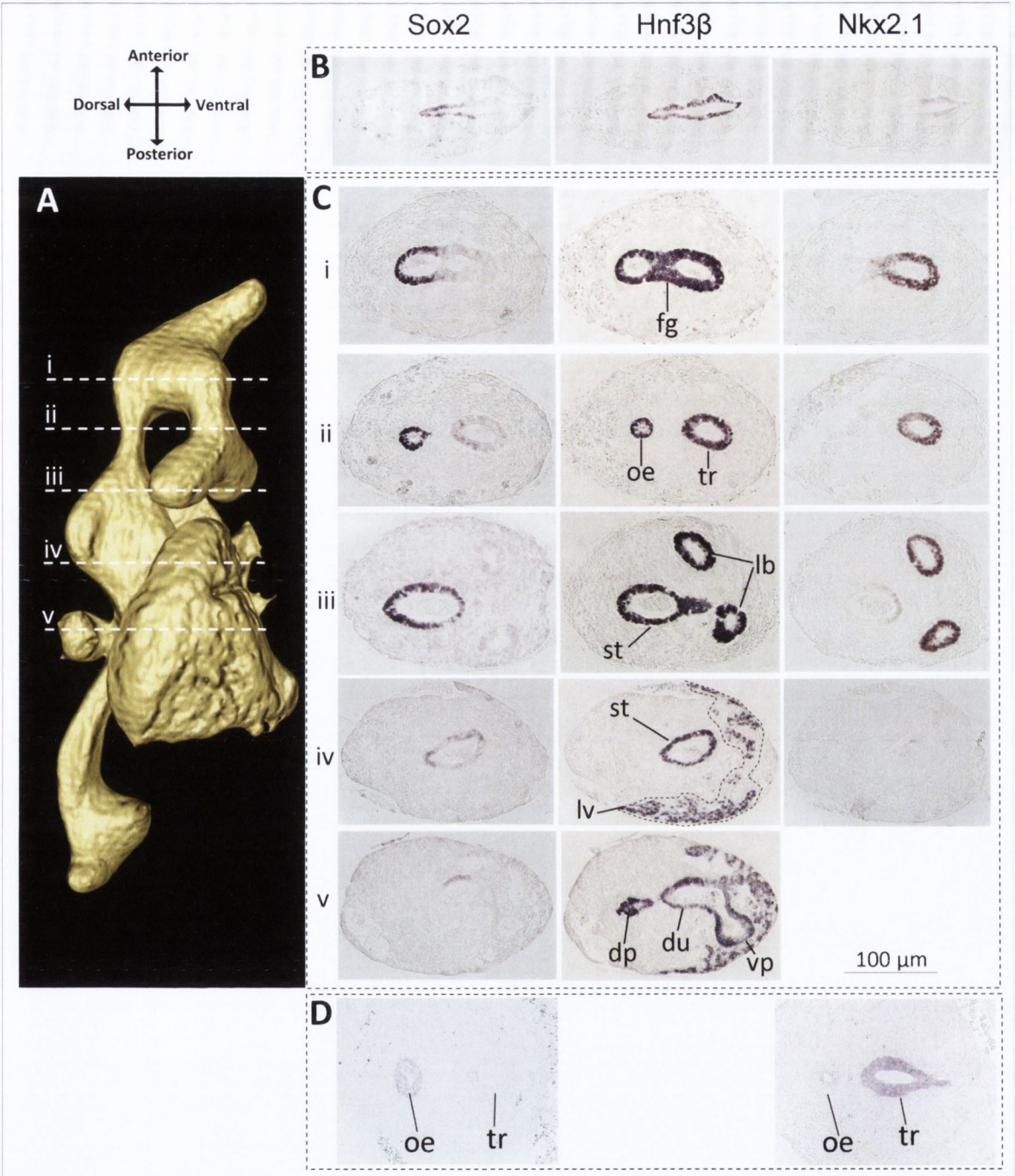


Figure 5.5 Dorso-ventral molecular patterning in isolated foregut explants.

Panel A is a surface rendered 3D reconstruction of Hnf3β wholemount immunolocalisation as detected by OPT, reprised here to show the plane of sections shown in C as labelled by roman numerals in both. Immunohistochemistry (as indicated) for Sox2, Hnf3β and Nkx2.1 is shown in Panel B, an isolated foregut explant harvested at four hours of culture; Panel C, a foregut specimen after 48 hours in culture (indicative of n=7) and Panel D, in the divided foregut of a normal embryo in vivo at E10.5 (TS17). Sox2 is restricted to dorsal domains of the undivided foregut at E9 (B) and the oesophagus at E10.5 (D) complimented by a ventrally restricted pattern of Nkx2.1 localisation in the undivided foregut endoderm (B) at E9 and the trachea at E10.5 (D). Domains of DV patterning were maintained in the foregut explants as shown in Panel C (compare C ii and D). Hnf3β IHC is additionally shown for matching of serial sections at each level. Distinct dorsal Sox2 and ventral Nkx2.1 domains were observed at the level of the dividing foregut (fg) in C i). Sox2 was also prominent in the oesophagus (oe) C ii) and stomach (st) C iii), to the level of the early duodenum (du) C v) but in not more posterior areas. Nkx2.1 was correspondingly prominent in the trachea C ii) and the lung buds (lb) C iii). Scale bar as given applies to all sections.

5.3.5 Notochord manipulation does not impact foregut division.

To test the hypothesis that an appropriate level of signalling from the notochord is required for foregut division, isolated foregut explants were cultured with (n = 6) and without (n = 10) endogenous notochord tissue at 18 to 24 somite stages (Figure 5.5 A and B respectively). Isolated foregut explants which included the native notochord also contained a small amount of neural floor plate tissue. No differences in the morphological and molecular characteristics of the foregut were observed in the absence of the notochord (Figure 5.5 A and B). In particular oesophagus, trachea and lung buds developed correctly from the foregut and the dorso-ventral patterning of Sox2 and Nkx2.1 (not shown) in the foregut and its derivatives was comparable between the groups. Staging of the explants was not affected by the presence of the notochord. 4/6 foreguts with endogenous notochord retained and 7/10 without notochord were staged as TS17.

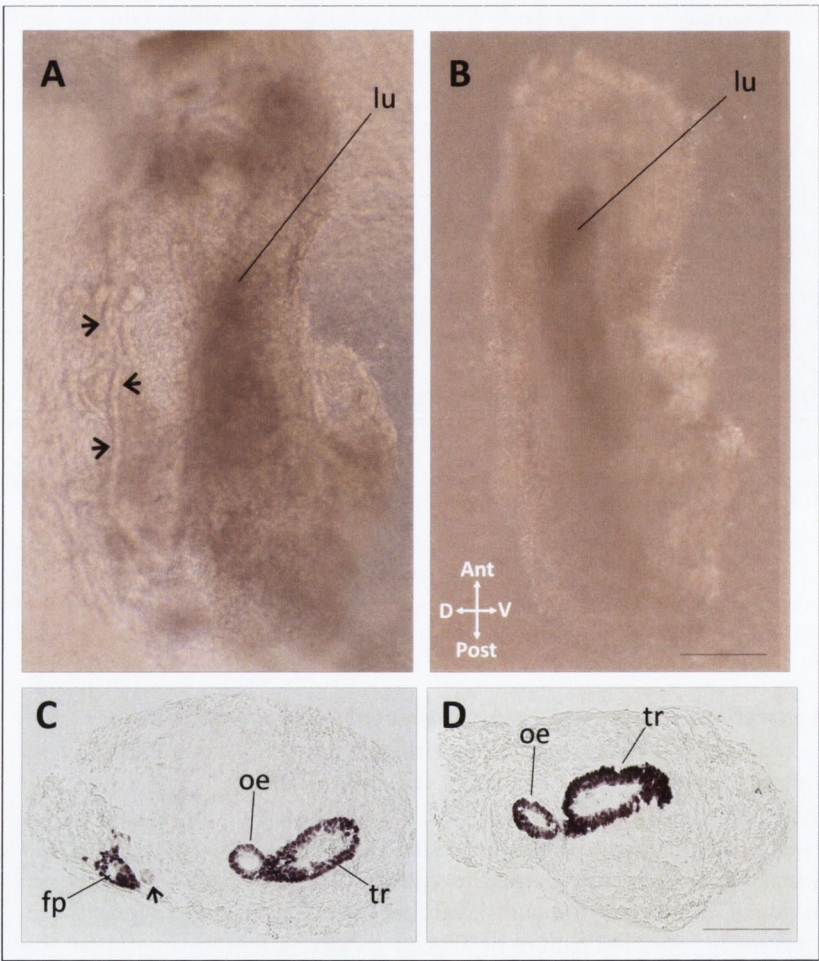


Figure 5.6 The effect of notochord removal on foregut development. Photographs in A and B show isolated foregut specimens embedded in collagen at 0 hours of culture and immunohistochemistry for Hnf3 β is shown in these specimens after 48 hours of culture at the level of foregut division. The foregut lumen (lu) can be appreciated in A and B. A and C (indicative of n=6) show the endogenous notochord maintained in culture (arrowheads) together with a small amount of neural floor plate tissue (fp) at the dorsal foregut endoderm. In B and D (indicative of n=10) the notochord has been removed. Normal foregut separation into oesophagus and trachea (as in C) has occurred in the absence of the notochord in D. Scale B = 100 μ m, D = 50 μ m.

In order to test the hypothesis that excess notochord tissue can induce foregut malformations, transplant of notochord obtained from somite matched littermate embryos onto the dorsal foregut endoderm was performed in explant culture. Specimens receiving notochord grafts had either their endogenous notochord in place ($n = 4$) (Figure 5.6 A and C) or removed ($n = 4$). Foregut morphology and molecular patterning in samples with additional notochord was compared to somite count matched isolated foreguts with or without their endogenous notochord in place. The notochord grafts contained a small amount of neural floor plate tissue and in samples receiving additional notochord, careful removal of the native neural floor plate allowed for clear differentiation between endogenous and transplanted notochord (Figure 5.6 F inset). We observed incorporation of the transplants into the foregut explant and growth of the transplant occurred in line with that of the entire explant. No disruption in morphological or molecular foregut properties were encountered under any of the transplant conditions (Figure 5.6 F and G). Transplanted notochord morphology was not different to native notochord following culture and maintained Hnf3b and Sox2 positivity (Figure 5.6 F and G) as present in normal in vivo embryos (Figure 5.6 E and H respectively). Transplanted notochord position in relation to the dorsal foregut wall was comparable to that of the native notochord however direct apposition to the dorsal foregut endoderm was not achieved (Figure 5.6 G). Complete removal of mesoderm in order to allow transplant positioning in direct contact with the foregut ventral wall was found to consistently result in foregut endoderm damage and therefore was not pursued. Therefore abnormal ventral positions of the notochord and the characteristic branching defects as seen in the AMM were not replicated entirely with this method.

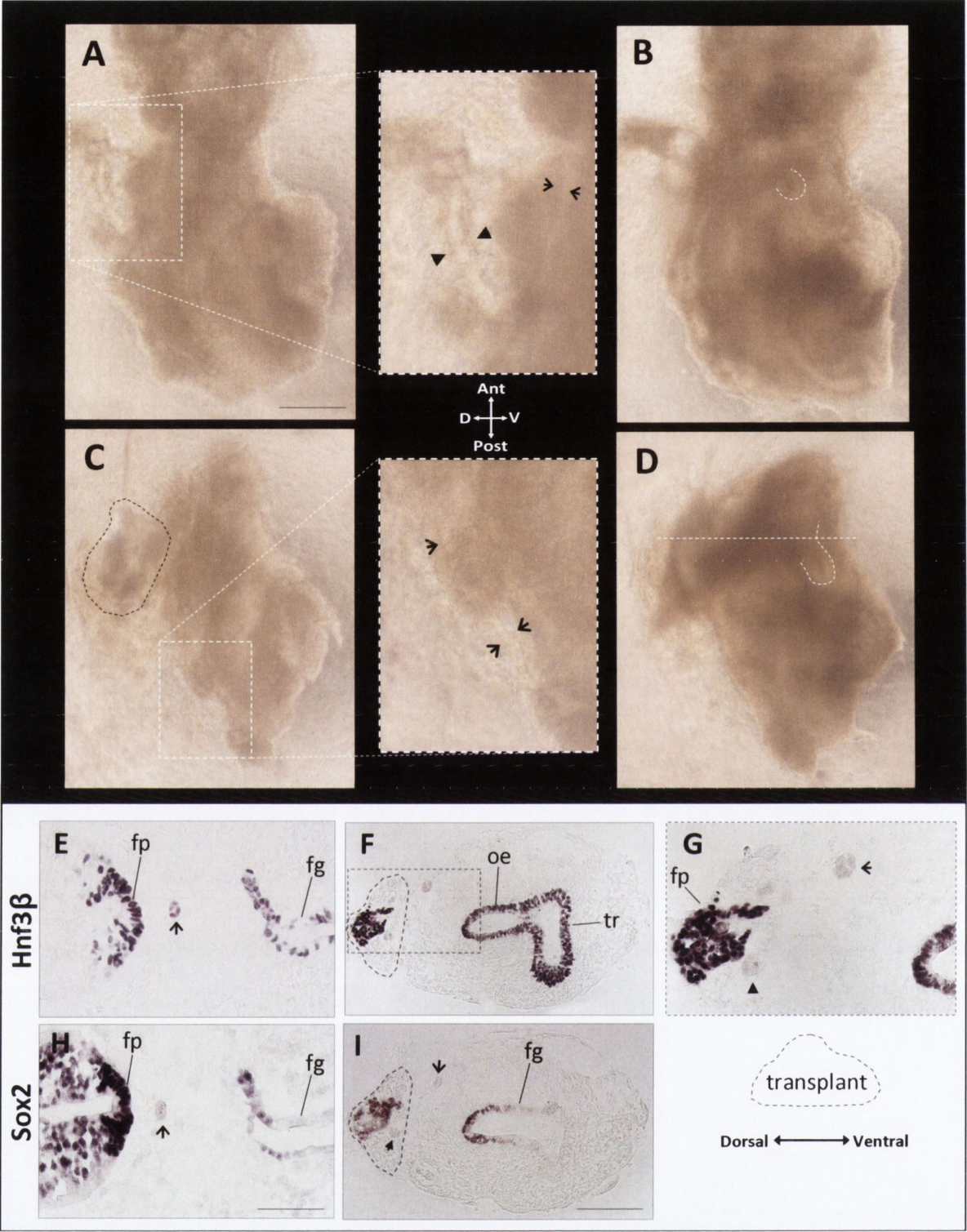


Figure 5.7 The effect of additional notochord transplant on foregut separation. (see figure legend facing).

Figure 5.7 The effect of additional notochord transplant on foregut separation.

Photographs A and C show isolated foregut specimens together with notochord transplants at the dorsal anterior foregut at 0 hours in culture. The same specimens are shown in B and D respectively at 48hrs of culture. Immunohistochemistry is shown for Hnf3 β or Sox2 (as indicated) in cryosections of normal embryos at E10.5 (E,H) and isolated foregut explants after 48 hours of culture together with notochord transplant (F, G, I). G is magnified from the boxed area shown in F. Plane of section for F and I (serial sections) is at the dividing foregut (fg) and is indicated in D with a white dashed line.

A and C boxed regions are magnified to show endogenous notochord (line arrowheads) and notochord transplants (block arrowheads). The notochord transplant is indicated by a black dashed shape in C, F and I. At 48 hours of culture lung buds can be seen to form in the region of the notochord transplant in B and D marked by the broken white guideline. Endogenous notochord and transplanted notochord maintained positivity for Hnf3b (F,G) and Sox2 (I) markers as seen in normal embryos (E,H) respectively. The foregut (fg) divided normally into oesophagus (oe) and trachea (tr) in the presence of additional notochord (F) and DV patterning was maintained as reflected in the dorsal restriction of Sox2 (I). Note a small amount of neural floor plate transplanted together with the notochord allowed clear specification of the transplant versus endogenous notochord which had its notochord removed. Scale bar in A=100 μ m and applies to photographs. Scale bar in H =30 μ m and is also for E, in I =50 μ m and is also for F.

5.4 Discussion

The work presented in this chapter characterises successful foregut culture in a new system for *in vitro* manipulation that was applied to investigate the impact of the notochord on foregut development. The absence of the notochord or presence of additional notochord at the dorsal foregut did not affect DV patterning or morphology. These results do not support the hypothesis that notochord signalling is required for normal foregut patterning or separation but the examination was limited to a precise developmental window, as discussed below. The establishment of isolated foregut 3D explant culture is an important achievement as this system permits *in vitro* foregut separation into distinct oesophagus and trachea over 48 hours and is amenable to various manipulations as shown in this and the subsequent chapter. A further achievement was the production of a morphological staging system of normal foregut development which was then used to assay foregut explants. Together these data show that foregut separation in 3D explant culture recapitulates the morphology and DV patterning of normal foregut separation in a reliable manner.

5.4.1 Isolated foregut 3D explant culture

During development, tissues sharing proximity exert reciprocal influences over each other to varying degrees. The ability to manipulate these physical relationships and also to introduce aberrant signalling is a powerful way to ask direct questions about the molecular control of morphogenesis. Signalling alterations but not physical manipulations can be achieved in expensive and often complex genetic models however they do not offer the wide range of applications that a single effective, simple and low cost *in vitro* approach does, such as the 3D culture system reported here. The application of *in vitro* techniques to the investigation of foregut development is not a new concept however I have established the first model of foregut culture to provide 3D tissue support to allow stable physical manipulations as well as the only model to my knowledge to successfully recapitulate oesophagus, trachea and lung bud development from undivided foregut.

Isolated foregut explants in 3D culture faithfully exhibit morphological features of normal foregut development and also correct molecular characteristics by maintaining strict dorso-ventral foregut domains of Sox2 and Nkx2.1 expression respectively. Reproducible foregut separation when cultured at E9 reflects the nature of the tissue explanted, in that all of the factors needed for successful foregut division are at that point

autonomous to the foregut and its immediately ventral mesoderm and exogenous influences are not required for the formation of oesophagus, trachea or lung buds or even for the onset of branching morphogenesis in the lung buds as described in later stage foregut explants. This contrasts somewhat with the widely held view that signalling from the cardiac mesoderm (by Fgf molecules) is required for lung bud formation and lung branching morphogenesis (as reviewed by Cardoso and Lu, 2006). As this tissue is absent in isolated foregut explant culture these molecules may exert sufficient influence on the foregut prior to E9 for lung budding to proceed or may also be present in the ventral foregut mesoderm maintained in explants.

The innovation of the collagen plastic ring construct resulted in marked improvement in explant outcomes as reflected by explant viability and the assessment of external morphological features. The plastic ring buoyancy together with a minimal collagen gel base and overlay provides a unique combination of stable 3D culture while allowing the explant to float just below air-medium interface. This most likely allows for improved oxygenation and waste exchange but may also mimic conditions *in vivo* more closely. As described in section 5.3.1 the spreading of gel on a solid substrate base creates biomechanical disturbances that impacted explant morphology. This was avoided by the introduction of plastic ring constructs. The further improvement in the outcome of explant culture, as reflected by consistent attainment of foregut separation in explants, on refining the dissection to include only foregut tissue was striking. Additional improvement in oxygenation and nutrient diffusion, lack of competition for available resources and amelioration of space restriction could all contribute to this effect.

To aid in explant assessment a detailed staging system was produced based on key features of the dividing foregut *in vivo* which may find wider applications in future. Detailed 3D reconstruction with surface render of OPT scans was utilised to generate anatomical representations of foregut development from E9 to E11. I note that the distinction of lung bud position relative to the foregut as ventro-lateral at E9.5 and entirely ventral at E10 has not previously been made (Kaufman and Bard, 1999). Foregut separation is widely reported to occur between E9 and E10 (Theiler, 1989a). These data add that foregut separation into clearly distinct oesophagus and trachea as characterised by the presence of intervening mesoderm between their endoderm walls is first detectable at E10.5, at which stage the stomach was previously described to be also morphologically distinct for the first time. Using this system, isolated explant foreguts were consistently found to achieve approximately 36 hours of development in a 48 hour culture period emphasising the success of the 3D culture. This staging assessment approach provides an

easily performed and convenient technique with clearly established outcomes in foregut development which can be used as a standard for comparison in future manipulations in explant culture as detailed in Chapter 6.

5.4.2 The notochord to foregut relationship

An abiding mystery in early embryonic development is the nature of the interaction that occurs between foregut and the notochord during foregut division. I have found that the presence of the notochord is not required for foregut division after E9 in the 3D culture system. Transplant of notochord to the dorsal foregut endoderm was performed to mimic the distinctive notochord abnormalities of Adriamycin treated mice to test the hypothesis that the effect of Adriamycin on the foregut is mediated by the notochord in this model. No morphological or molecular disturbances were found under any of the transplant conditions.

These results confirm that at E9 the notochord is not required for foregut division and also does not disrupt foregut molecular patterning or morphology when present in excess. The inability of the notochord to influence foregut separation at E9 does not preclude possible susceptibility of the foregut to altered notochord behaviour at earlier gestational windows. DV specification of foregut domains occurs prior to E8.5. Conditional Noggin knockout in the notochord and neural floor plate is required to occur prior to notochord delamination at E7.5 for the induction of an oesophageal atresia phenotype (Fausett et al., 2014). In the phenotypically similar AMM, treatment at E7 and E8 is required to induce notochord and foregut malformations (Dawrant et al., 2007a). Our model does not currently support foregut culture prior to the 15 somite stage (E8.5) as reported in the results. Other techniques of foregut culture such as in liquid medium or by using an alternative species such as chick may allow examination of the effect of notochord removal at earlier gestational stages.

These experiments have tested the influence of notochord transplants taken from stage matched normal embryos on the foregut. AMM notochord is both structurally and molecularly disrupted and may represent a highly aberrant entity with much altered signalling properties in comparison to the notochord of normal embryos (Hajduk et al., 2012). Nonetheless the nature of any impact the notochord may have on the foregut during normal development is not powerful enough at E9 to induce morphological or molecular consequences by any of the manipulations performed. During the course of these experiments several unusual behaviours of the notochord were encountered. This structure appears to not be easily amenable to manipulation in the early embryonic mouse and this

may account for scarcity of published data pertaining to *in vitro* techniques of notochord manipulation in the mouse. In the chick however the notochord has been amenable to these types of experiments (van Straaten and Hekking, 1991) and may provide a future solution to these difficulties .

A true limitation of the notochord transplantation experiment was the inability to position notochord directly adjacent to the dorsal foregut endoderm. Ultimately this manipulation would have required unacceptable damage to the dorsal foregut endoderm. While notochord has been shown to produce long range signalling via Shh (Fan and Tessier-Lavigne, 1994) the current experiments were conceived in order to mimic notochord branching in the AMM. Ultimately this was not satisfactorily recapitulated in explant culture and may be better tested in future in the application of localised signalling manipulations such as Shh protein as a proxy to the dorsal foregut. In the subsequent chapter the isolated foregut explant system is used together with the localised application of exogenous signalling, demonstrating that such an approach for the examination of the effect of known notochord derived signalling molecules on the foregut would be highly feasible in future work.

5.5 Conclusion

Isolated foregut in 3D explant culture faithfully recapitulates morphological and molecular facets of foregut development into oesophagus and trachea in a highly reproducible manner. Comparison of development in explants to normal foregut was possible following the production of a morphological staging system which offers a useful technique for foregut assessment in future investigations. The use of these approaches did not identify a response in the foregut to manipulations of the notochord at the examined gestational stage. The *in vitro* approach established by this work further offers a simple and adaptable system for manipulation of the foregut environment that can ask unique questions about the molecular control of foregut separation.

Chapter 6

**The treatment of isolated foreguts in
3D explant culture with Bmp4 and
Noggin protein soaked beads:
Bmp4 treated foreguts exhibit an
OA/TOF phenotype.**

6.1 Introduction

The data presented in Chapter 3 identify abnormal BMP pathway activity occurring in association with OA/TOF phenotype in Adriamycin treated mice. This was manifested by the ectopic localisation of phosphorylated SMAD protein in the dorsal foregut endoderm and notochord, and disturbed spatial distribution of foregut specific BMP target genes, together indicating BMP signal over-activity in AMM foregut. In contrast BMP ligand gene expression was not found to be disturbed in the foregut or adjacent notochord of the AMM for Bmp2, Bmp4 and Bmp7 (cf. Chapter 3) however the gene encoding the BMP antagonist Noggin was found to be strongly abnormally expressed at these sites (Hajduk, unpublished results). Accordingly an incomplete picture exists regarding the mechanism by which Adriamycin acts to induce BMP signalling disturbances. The precise nature of the association between BMP ligand and Noggin expression with abnormal oesophageal development requires further resolution.

Conflict across the wider literature is also apparent with regard to the role of BMP signalling in foregut development. BMP signal attenuation by Noggin specifically localised to the notochord and neural floor plate but not in fact to the foregut endoderm is required for proper foregut separation as shown by conditional Noggin gene knockout in mice (Fausett et al., 2014). This model demonstrates an OA/TOF like phenotype and notochord abnormalities in the absence of DV foregut patterning disruption, somewhat in contrast with the outcome of conditional Bmp4 gene inactivation in the anterior foregut endoderm of mice, generating tracheal agenesis together with altered foregut dorso-ventral patterning as reflected in abnormal ventral localisation of Sox2 (Li et al., 2008a). When it is considered that rescue of oesophageal abnormalities can be achieved in mice lacking Noggin by lowering Bmp4 or 7 gene dose it becomes evident that balance in BMP signalling is crucial for proper foregut separation. Important questions do remain however, such as whether BMP ligand over-activity, and in particular Bmp4, can directly induce oesophageal malformations and whether Noggin can act on the foregut to influence morphogenesis.

The successful establishment of an *in vitro* approach that can answer these direct types of questions is presented in Chapter 5. BMP ligand and Noggin proteins have been applied *in vitro* to induce both morphological and molecular response in an extensive range of target cell and tissue types and include experiments on foregut endoderm examining liver development (Rossi et al., 2001) and foregut derived lung growth (Weaver et al.,

2000). These assays have not been previously applied to the anterior foregut to examine oesophagus and trachea specification. The application of additional signals *in vitro* offers distinct advantages over other methods that allow similar investigations such as genetic models. Tissue culture offers a favourable cost profile and is often much simpler to develop and maintain. Culture techniques are particularly useful when important developmental patterning signals, such as BMPs, are of interest. For genetic mutants complex set up of temporo-spatial limits on gene inactivation is required to circumvent the often lethal early embryonic consequences of interfering with these genes at a whole organism level (Wang et al., 2014). Furthermore manipulating signals at a protein level can offer added clarity in interpretation of the target tissue response over genetic approaches by avoiding unwanted post translational modifications.

A particularly striking feature of BMP signalling abnormalities in the AMM is their focal nature, localising to a very discrete domain of the dorsal foregut endoderm (Chapter 3). 3D culture is amenable to the stable co-culture of precisely positioned materials together with the tissue of interest. The use of beads of various types, impregnated with pure recombinant proteins, for localised delivery of signalling molecules or antagonist is well established. Placement of recombinant BMP ligand or Noggin protein soaked Cibacron[®] blue agarose beads has been successfully used *in vitro* to demonstrate a morphological response in gut-like embryoid bodies (Torihashi et al., 2009), in neural tube following whole embryo culture (Ybot-Gonzalez et al., 2007) and in 3D neuron culture in collagen (Fantetti and Fekete, 2012). Affi-Gel is a commercially available product used for protein binding across a wide range of applications. This product contains a mesh of cross-linked agarose beads which are 75 to 150 μm in diameter. The basis of protein uptake and subsequent release from these beads is the coupled Cibacron[®] blue F3GA dye which can function as an ionic, hydrophobic, aromatic, or sterically active binding site (Subramanian, 1984).

In vitro models can be criticised in that they remove the target tissue from natural environs, however isolated foregut 3D explant culture is the first model to successfully recapitulate the generation of oesophagus from undivided foregut and faithfully maintains the morphological and molecular properties of foregut development *in vivo* (Chapter 5). This system lends itself to investigating the response of the foregut to the application of highly controlled signalling manipulations in terms of both anatomical and patterning disruptions. This presents a unique opportunity to examine the direct influence of BMP ligand and Noggin activity on oesophageal formation in isolated foregut culture.

The main objective of the work presented in this chapter was;

To test the hypothesis that focal disturbance of BMP activity at the foregut, such as that seen in the AMM, is sufficient to induce OA/TOF.

This was addressed by:

1. The analysis of the morphological impact of placement of Bmp4 and Noggin protein soaked beads at the dorsal foregut in isolated foregut 3D explant culture. I used Optical Projection Tomography together with Hnf3 β immunolocalisation as a marker for foregut endoderm in whole specimens for accurate anatomical 3D reconstruction.
2. The analysis of the molecular impact of Bmp4 and Noggin protein on foregut in culture using immunohistochemistry for Sox2 and Nkx2.1 as markers of dorso-ventral foregut patterning together with Hnf3 β in cryosections.
3. The in-depth examination of molecular patterning in Bmp4 treated foreguts using immunofluorescent double labelling for dorsal marker Sox2 and ventral marker Nkx2.1 combined with Confocal laser microscopy to allow high resolution imaging at the cellular level.
4. The comparison of morphology and molecular patterning in Bmp4 treated foreguts to known models of tracheo-oesophageal malformations, and in particular the AMM, to identify whether Bmp4 treatment induces a recognised OA/TOF phenotype.

6.2 Methods

6.2.1 Isolated foregut 3D explant culture with beads

Foreguts for explant were obtained from embryos with 15 to 30 somites as described in Section 2.8.2 and were embedded within collagen plastic ring constructs as described in Section 2.8.3. Affi-blue gel agarose beads (#153-7302 Bio-Rad) were washed in PBS 3 times for 30 minutes in a 1 ml tube at room temperature. Beads for subsequent incubation with Bmp4 protein were additionally washed in 4mM HCl with 0.1% bovine serum albumin for 1 hour at room temperature. Beads were incubated with either PBS for control, 0.1 µg/µl solution of Recombinant Mouse Noggin protein (# 719-NG-050 R&D Systems) and a 0.1 or 0.01 µg/µl solution of Recombinant Human Bmp4 protein (# 314-BP-010 R&D) overnight at 4°C and for at least 1 hour at 37°C prior to use. Beads were transferred by unpulled glass capillary (internal diameter 0.3mm) under mouth control onto the base of collagen in a plastic ring construct containing a foregut explant. Excess protein solution was removed using a fine pulled capillary under mouth control. Beads were positioned along the dorsum of the foregut explants using tungsten needles. The explants and beads were secured in place using a 10µl overlay drop of collagen, with additional positioning prior to complete collagen gelling using tungsten needles. Explants with added beads were cultured for 48 hours and harvested for further processing as described for foregut explants.

6.2.2 Overview of explant experiments and analysis.

Isolated foregut explants were cultured with beads soaked in PBS for control (n=13), Noggin (n=12) and Bmp4 at 0.1 µg/µl (n=21) and 0.01 µg/µl (n=4) over the course of 3 sets of experiments. All samples were morphologically assessed using Hnf3β immunolocalisation either in wholemount specimens (as described in Section 2.4) together with OPT scanning (as described in Section 2.5) or by immunohistochemistry on cryosections (as described in section 2.6). Cryosectioned samples were additionally assessed by immunohistochemistry for dorso-ventral patterning using serial sections so that matching foregut domains could be analysed with Sox2 as a dorsal marker and Nkx2.1 as a ventral marker. Immunodetection of Noggin and pSMAD was performed together with immunofluorescent labelling and captured using a compound fluorescent microscope again

as described in Section 2.6. Double-labelling for Sox2 and Nkx2.1 was also performed with immunofluorescent detection using the methods described in Section 2.6, and examined using Confocal laser microscopy (as described in section 2.7).

6.2.3 Individual explant assessment

Prior to foregut dissection for explant culture, each embryo was finely staged by counting of the number of somite pairs present. The initial somite counts were recorded for each foregut explant. All bead treated explant samples were photographed at the time of embedding and immediately following harvest from culture. Beads placed at the foregut were counted for each individual explant to give the total number of beads used and also the number placed at the anterior half of the foregut explant, directly adjacent to the site of foregut division. Each sample was allocated an individual identification which was maintained throughout culture, processing and analysis allowing comparison of morphological outcome with initial sample characteristics such as somite count or number of beads used. Explants were morphologically assessed on the basis of Hnf3b immunolocalisation as detailed in the preceding section. For control and Noggin treated samples morphological staging was performed as described in Section 5.2.2. For Bmp4 treated samples a grading of morphological severity was given to each sample of: no effect, mild, moderate or severe, based on the anatomical features that are detailed in the results (Section 6.3.3).

6.2.4 Statistical analysis

For Bmp4 treated foreguts individual sample bead counts, (as described in the preceding section), were averaged among explant groups based on grading of morphological defects (as described above and in Section 6.3.3). The mean number of total (T) and anterior (A) beads used were compared between groups by independent samples t-test using SPSS software with a significance level set at 0.05.

6.3 Results

6.3.1 *Morphological and molecular properties of isolated foregut explants were maintained when cultured with control PBS-soaked beads.*

Precise positioning of beads at the dorsal foregut was highly feasible in the 3D foregut explant culture model (Figure 6.1). On average 10 beads (range 3 to 20) were placed along the dorsum of the entire explant and 5 of these beads (range 1 to 7) were at the level of the anterior part of the foregut, where oesophagus and trachea subsequently arise. Post-culture preservation of collagen around the foregut throughout processing for the various analytic techniques allowed bead visualisation in the resulting data, most strikingly when 3D reconstruction was applied to OPT scanned samples as demonstrated in (Figure 6.1 C). It was noted however that antigen retrieval of sections prior to immunohistochemistry (for Nkx2.1) occasionally caused inadvertent bead section removal.

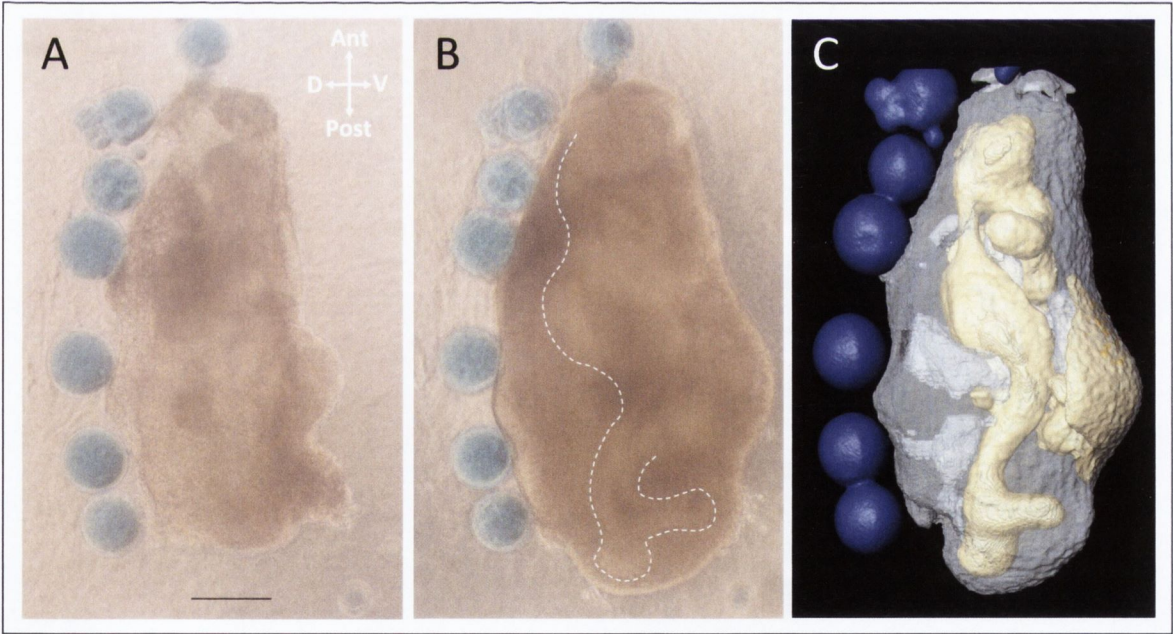


Figure 6.1: The treatment of isolated foreguts with beads in 3D explant culture. Beads (in blue) were placed at the dorsal foregut in culture at 0 hrs (A) and remained stably co-located with foreguts in culture at 48 hours (B). Beads were also maintained through processing for OPT scanning and could be visualised by surface render 3D reconstruction of OPT scans (C) where the immunolocalisation of Hnf3b (in yellow) revealed foregut morphology. Note that all images are of the same specimen by examining the shape and localisation of the beads in each. In B the endoderm (lighter) and lumen (darker) of the foregut and its derivate structures can be visualised as variations of tissue opacity within the explant tissue and are partially outlined by the white broken guideline. The shape of the foregut in C matches this outline exactly. Scale bar (in A) = 100 μ m

Morphological features and dorso-ventral patterning in control bead treated foreguts (Figure 6.2 B, E, H, and K) were consistent with non-treated foregut specimens (Figure 6.2 A, D, G and J). Furthermore control bead treated foreguts achieved comparable morphological staging following 48 hours of culture to non-treated foreguts. One control sample had a morphological variant of a single lung bud (as described for a small subset of non-treated foregut explants in Chapter 3) and so was not included in the staging analysis. Individual control bead treated samples are detailed in Table 6.1 including somite counts prior to culture and the resulting stage achieved in each sample.

Table 6.1: Morphological staging (TS) analysis of individual control PBS-soaked bead treated foregut explants reflecting initial somite count.

Sample	Somite count at time of explant	Stage (TS) achieved at 48 hrs culture
A	18	17
B	18	17
C	18	17
D	20	17
E	22	16
F	22	16
G	22	17
H	25	17
I	28	18
J	18	17
K	15	16
L	22	17
M	22	Single lung bud

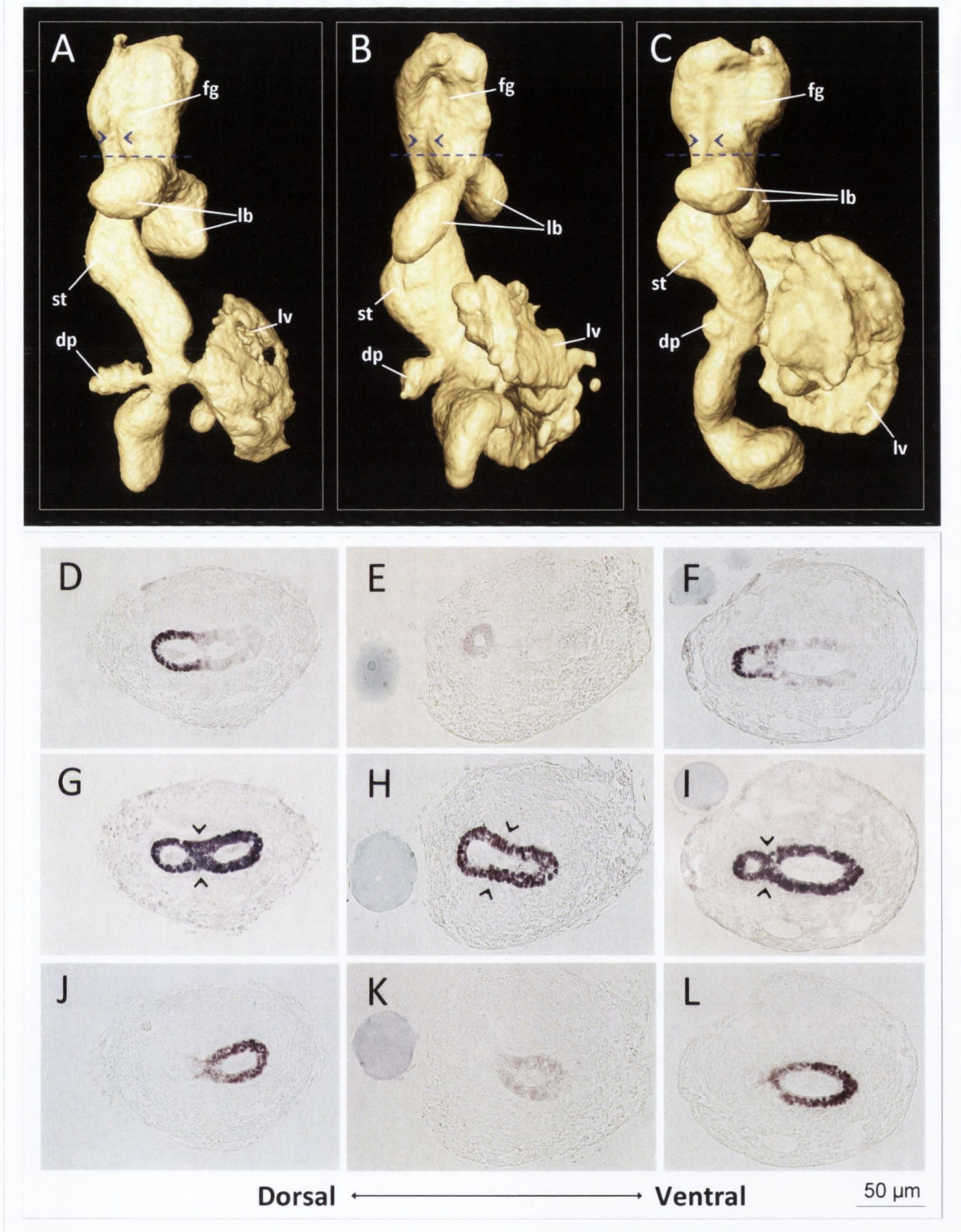


Figure 6.2: The morphological and molecular characteristics of foregut explants cultured under control and Noggin treatment conditions. Specimens treated with control PBS-soaked beads (B, E, H and K) and Noggin beads (C, F, I, L) compared to non treated specimens (A, D, G, J). A to C are lateral views of surface render 3D representations of Hnf3b wholemount immunolocalisation following OPT scanning showing lung bud (lb), stomach (st), dorsal pancreatic bud (dp) and liver (lv) formation from foregut (fg) across TS 16 staged explants. Blue arrowheads indicate an external foregut endoderm groove due to impending foregut separation into dorsal oesophagus and ventral trachea, also indicated by arrowheads in G to I. The level of cryosections assessed by immunohistochemistry (IHC) for Sox2 (D to F), Hnf3b (G to I) and Nkx2.1 (J to L) are given by the dashed lines in A to C. Positivity for Sox2 dorsal marker and Nkx2.1 ventral marker are maintained in the correct domains across all conditions. Note that beads occasional become detached during IHC (as in L) but that directly adjacent sections (as in I) demonstrate the presence of beads. Scale bar given applies to all sections.

6.3.2 *Exposure of foregut explants to Noggin-soaked beads in culture does not affect morphology or dorso-ventral patterning.*

The treatment of foreguts in culture with beads soaked in Noggin solution at 0.1 µg/µl did not impact foregut morphology or dorso-ventral patterning at the level of the dividing foregut (Figure 6.2 C, F, I and L). Dorso-ventral patterning of Sox2 and Nkx2.1 was maintained throughout the foregut, oesophagus and trachea in all Noggin treated samples assessed (n = 8) as shown in detail in Figure 6.3. Tracheo-oesophageal separation was visualised in surface rendered 3D reconstructions as a groove in the external foregut endoderm wall in control samples (Figure 6.2 A and B arrowheads) which was maintained in Noggin treated samples (Figure 6.2 C arrowheads). This feature could also be visualised by Hnf3β immunohistochemistry on cryosections in non-treated, control and Noggin treated samples (Figure 2 G to H arrowheads). Morphological staging of Noggin treated foreguts was comparable to control samples as detailed for each individual sample in Table 6.2 The presence of the single lung bud variant precluded two samples from staging analysis.

Table 6.2: **Theiler Staging (TS) analysis of Noggin-soaked bead treated foregut explants reflecting initial somite count of individual samples.**

Sample	Somite count at time of explant	Stage (TS) achieved at 48 hrs culture
A	18	Single lung bud
B	20	17
C	18	16
D	18	17
E	20	16
F	22	16
G	22	17
H	22	17
I	25	17
J	28	18
K	18	17
L	22	Single lung bud

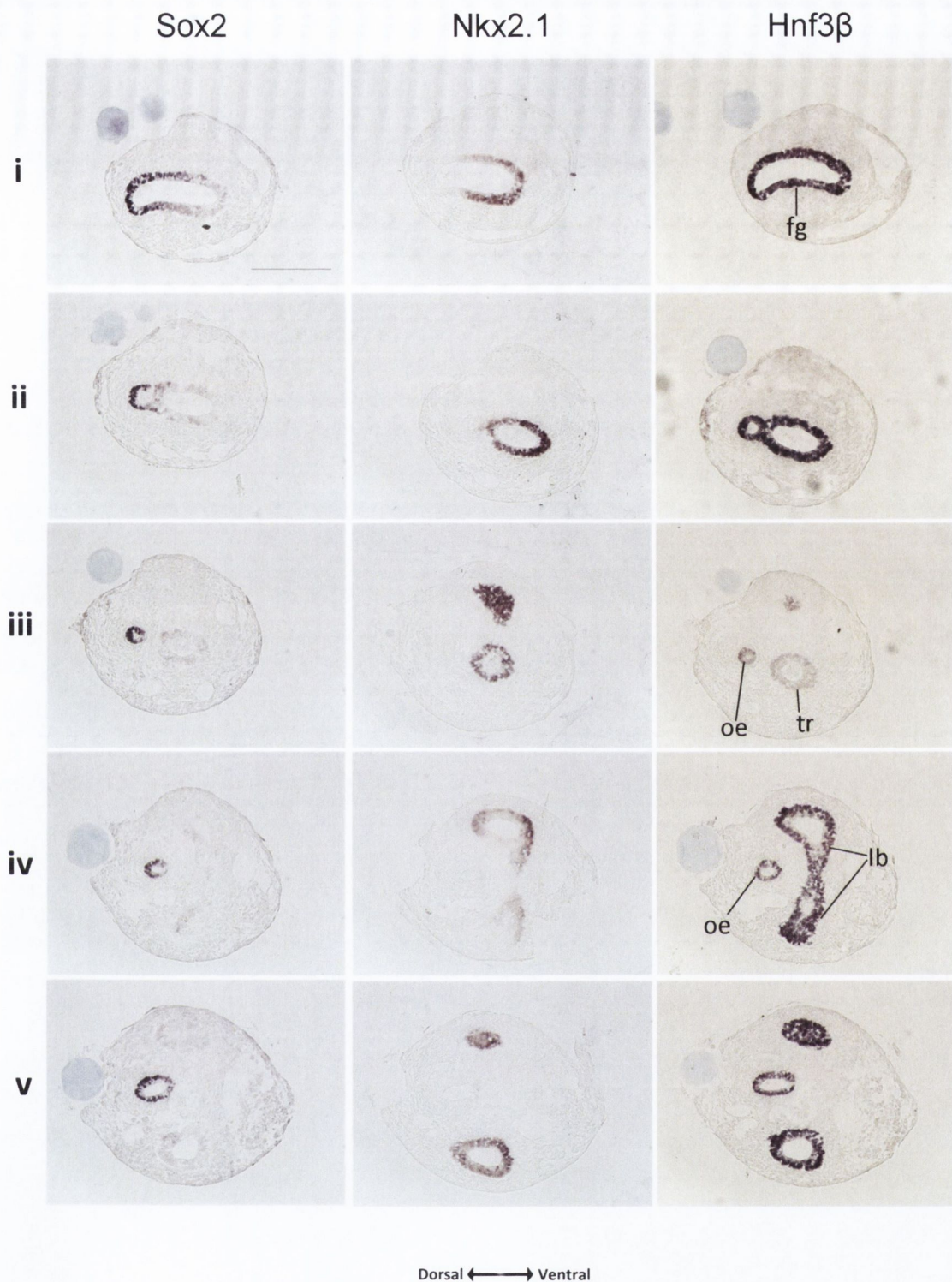


Figure 6.3 Molecular patterning in *Noggin* treated foregut explants. Immunohistochemistry for dorsal marker Sox2 in the left column, ventral marker Nkx2.1 in the middle column and Hnf3 β in the right column on directly adjacent sections for each row (i to v) revealed normal DV patterning of the dividing foregut (fg), oesophagus (oe), trachea (tr) and lung buds (lb) in foregut explants treated with *Noggin* soaked beads (blue) Note that beads occasional become detached during IHC (in Nkx2.1 column) but that directly adjacent sections (see corresponding shape and structures) demonstrate the presence of beads. Scale bar in i) = 100 μ m applies to all sections.

Immunolocalisation of Noggin and pSMAD was compared in Noggin treated (n=4 per marker), control (n=3) and non-treated (n=3) explants (Figure 6.4). In both non-treated and control samples Noggin was not prominent in either the foregut or the surrounding mesoderm. Intense Noggin protein localisation was detected in the Noggin-soaked beads compared to control PBS-soaked beads as expected (Figure 6.4 A and B). In the explant tissue, extracellular Noggin localisation was detected immediately adjacent to the bead (Fig 6.4 B inset highlighted by arrows). In the remainder of the explant including the foregut structures there were no appreciable differences in Noggin protein localisation compared to controls however IF detection is not directly quantitative and highly intense signal from beads may have precluded the identification of subtle alterations in Noggin signal.

In non-treated and control explants pSMAD was predominantly ventrally localised in the foregut endoderm and was also prominent in the trachea and lung buds (Figure 6.4 C and D) as has been previously described in normal embryos *in vivo* (cf. Section 3.3.1). Additional strong pSMAD localisation was noted at the explant surface, defining the outermost cells (Figure 6.4 C and D). Noggin bead treatment locally attenuated pSMAD signal at the outer surface of explants but was not seen to visibly alter the pattern of pSMAD in the foregut and its derivative structures (Figure 6.4 E). In order to increase Noggin protein delivery to explants a larger number of Noggin beads (up to 20) were used in samples without appreciable difference in outcome.

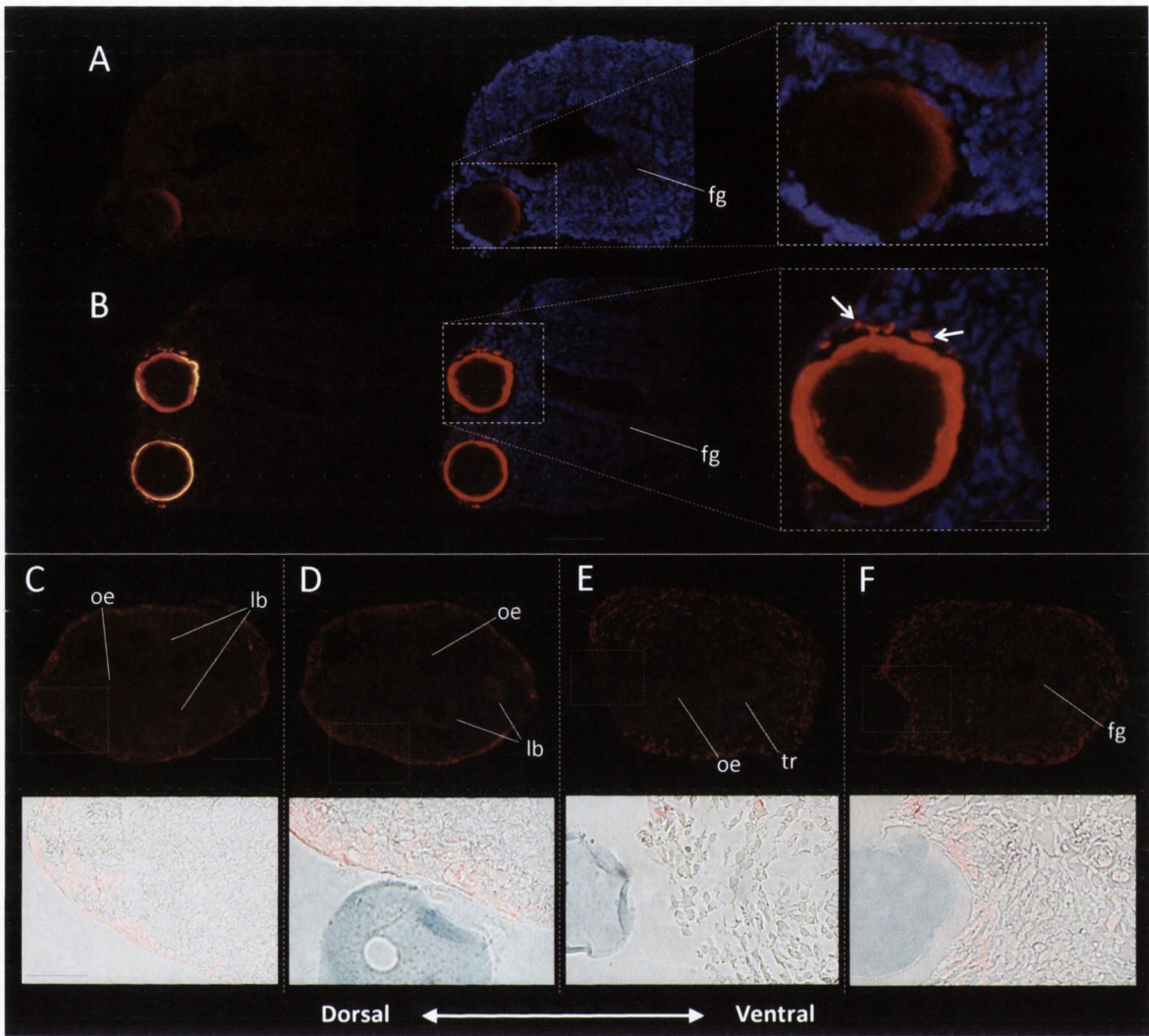


Figure 6.4 Immunofluorescent localisation of Noggin and pSMAD in foregut explants. Control PBS-soaked bead (A) and Noggin-soaked bead treated (B) foregut explants were analysed for Noggin (in red) localisation together with DAPI DNA/nuclear counterstain (in blue) revealing intense Noggin localisation in Noggin soaked beads (B) and additional Noggin signal in extra nuclear domains of the immediately bead adjacent explant tissue (inset highlighted by arrows). Noggin localisation in the foregut (fg) was not different to controls. Note the yellow areas in B represent highly intense signal that was not maintained in the red channel for merged image. Non-treated (C), Control bead (D), Noggin (E) and Bmp4 (E) treated explants were analysed for pSMAD (in red) immunolocalisation. Boxed regions in upper panels of C through F are shown in the lower panel merged with transmission images (visible light) to show bead placement (in blue). Noggin treated foregut (E) shows attenuated pSMAD signal compared to the other samples at the bead adjacent explant surface. In the foregut derived structures pSMAD distribution was more prominent in the lung buds (lb) and trachea (tr) under Noggin treated and control conditions. Scale bar in B and C upper panel = 50µm (also applies to A, D, E and F) and in B inset and C lower panel = 20µm (applies to all others).

6.3.3 *Bmp4 treatment of foreguts in culture induces foregut malformations consistent with an OA/TOF phenotype.*

Morphological abnormalities of foregut development broadly characterised by failure of oesophagus formation were consistently seen in response to Bmp4 treatment (0.1 µg/µl and 0.01 µg/µl) in foregut explants (Figure 6.5). The outcome of Bmp4 treatment was examined in foreguts taken from embryos with 15 through 30 somites (n=25) and was compared with control PBS soaked bead treatment in somite matched specimens. No control specimen or non-treated foregut explant specimen displayed any foregut morphological abnormalities except for a single lung bud variant in few specimens as previously described. Only 2 Bmp4 treated samples exhibited normal foregut morphology both of which were obtained from embryos explanted at 30 somite stage (Figure 6.5 F). All remaining specimens (n=23) had oesophageal atresia like abnormalities of foregut development.

Bmp4 treated foreguts frequently exhibited extreme narrowing (atresia) of undivided anterior foregut (14/23) (Figure 6.5 B and C arrowheads) or complete absence (agenesis) of the anterior foregut (5/23) (Fig 6.5 G to M). In specimens with complete anterior foregut agenesis it was confirmed that mechanical factors had not disrupted the integrity of the anterior foregut such as bead placement or injury during dissection (Fig 6.3 K to M). Lung bud morphology was abnormal in all affected specimens, ranging from misshapen and underdeveloped bilateral lung buds (Fig 6.3 B and C) to failure of appreciable lung bud development in association with a single tracheal bulge at the posterior extent of the anterior foregut (Figure 6.5 J and O). In specimens with complete anterior foregut agenesis this tracheal bulge persisted (Figure 6.3 E, F, H and K). In 2 samples there was failure of the anterior foregut to form any distinct features with a uniform appearance occurring along its length to the level of the stomach (Figure 6.3 D).

Typical stomach formation could be appreciated in all Bmp4 treated foreguts as well as successful pancreatic bud and liver formation (Figure 6.5 B to E). Abnormal communication occurred between the stomach and the trachea or lung buds in all 23 affected samples. This was evident in 21 specimens as a direct communication between the stomach upper pole and trachea or lung buds (Figure 6.3 B to D as highlighted by arrows). In 2 specimens abnormal communication occurred via a narrow connecting structure between the stomach and respiratory structures which distinctly resembled a tracheo-oesophageal fistula (Figure 6.5 E).

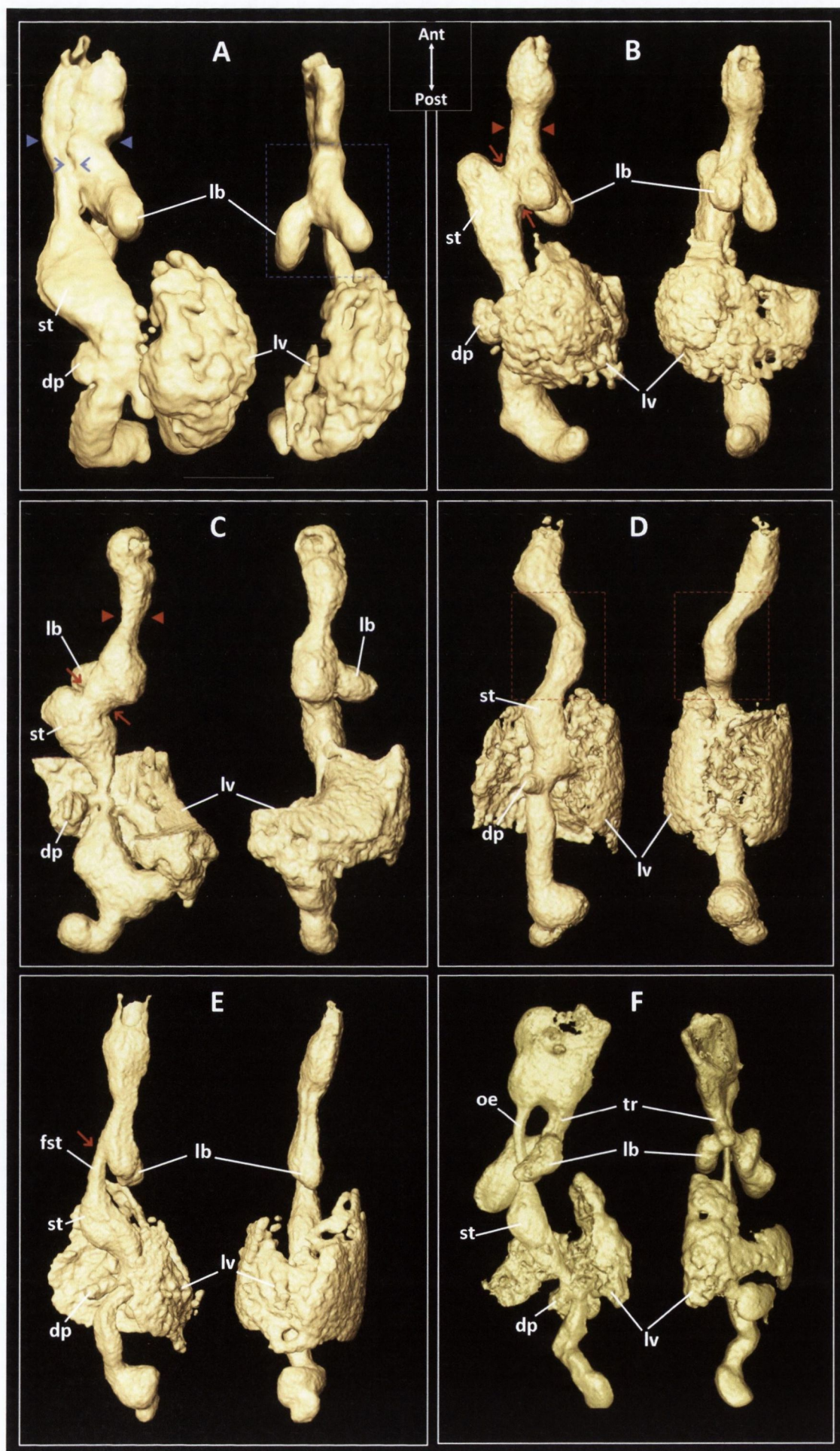


Figure 6.5 continued over

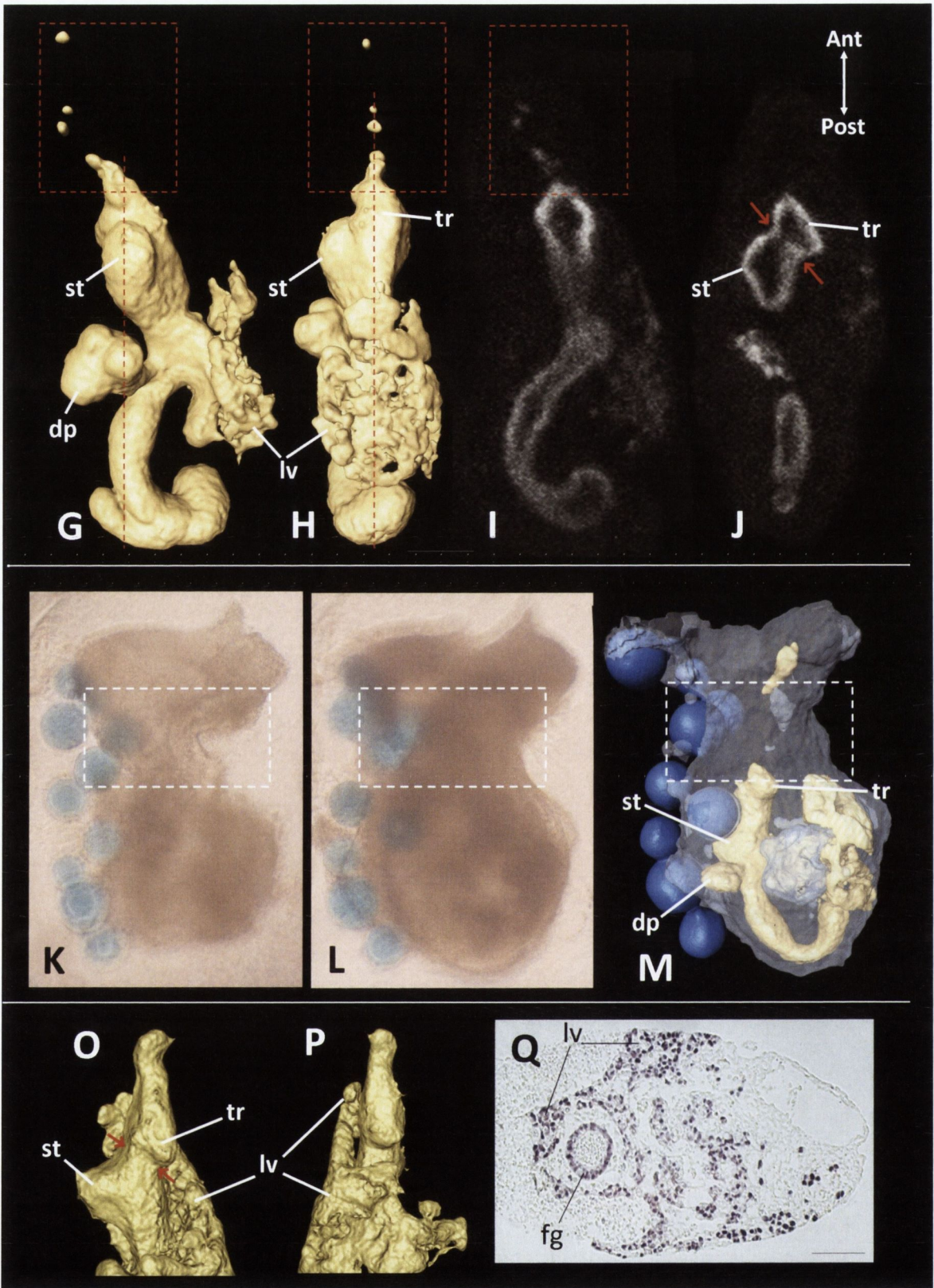


Figure 6.5: The morphological impact of Bmp4 treatment in isolated foregut explant culture. (see facing page for legend)

Figure 6.5: The morphological impact of Bmp4 treatment in isolated foregut explant culture.

3D reconstruction of Hnf3 β wholemount immunolocalisation in yellow (for surface render) or greyscale following OPT scan showing control (A) and Bmp4 treated specimens (all others). K and L show photomicrographs of explant in M at 0 and 48hrs and Q shows Hnf3 β immunohistochemistry on cryosection. K to M (same specimen) and C were treated with 0.01 μ g/ μ l of Bmp4, all others were treated with 0.1 μ g/ μ l. Note F is a 30 somite Bmp4 treated specimen that did not demonstrate morphological abnormalities. Planes for virtual sections I and J are given in H and G respectively.

All Bmp4 treated specimens failed to develop an oesophagus (oe). Further morphological abnormalities are depicted as follows: Anterior foregut atresia in B and C highlighted by solid red arrowheads, (compare to blue arrow heads in control A); Complete failure of foregut to specify into structures in D in red boxed area; Complete anterior foregut agenesis in G to I (same specimen) and M in the boxed areas showing remnants of Hnf3 β positive tissue in the anterior foregut region; Abnormal communication between the stomach (st) and trachea (tr) or lung buds (lb) highlighted by the converging red line arrows in B,C, J and O as a direct communication or via a fistula like structure (fst) as shown in E by a single arrow; Abnormal lung bud morphology in B,C and E or the presence of a tracheal bulge (tr) in H, J, M, and O; Extensive liver (lv) domains beyond the anterior limit of the stomach (upper red arrow) in O and encircling the foregut in Q; Absent external groove indicating foregut separation, highlighted by the blue arrowheads in control A (also cf. Figure 6.2).

Examples of no effect (F), mild (E), moderate (B,C and O) and severe (D, G to J, M and Q) grades of deformity are shown. Intact anterior foregut region is shown at 0 hrs (K) and 48 hours (L) of culture in a specimen with complete anterior foregut agenesis (M) (highlighted by the boxed regions). Stomach (st) and pancreas (dp) have formed in all specimens. Scale bar in Q = 20 μ m, for all others refer to A and H where scale bar = 100 μ m.

I graded the severity of the tracheo-oesophageal abnormalities detected following Bmp4 treatment on the basis of their morphological appearance into: No effect, Mild, Moderate and Severe. Mildly affected samples did not exhibit marked anterior foregut atresia and interestingly both of the samples with tracheo-oesophageal fistula like structures were included in this category (Figure 6.5 E). Moderately affected samples demonstrated anterior foregut atresia and a range of lung bud abnormalities as well as direct communication between the stomach and the trachea or lung buds (Figure 6.5 B and C). Severely affected samples had anterior foregut agenesis (Figure 6.5 G to J and M) or complete failure of the anterior foregut to differentiate into appreciable structures (Figure 6.5 D). The distribution of individual samples within this classification is summarised in Figure 6.6, which reflects a trend towards reduced severity of deformity as the initial gestational stage of the specimen (as described by the somite count) increases. However a single specimen at 28 somites does not fit with this trend and limited datasets for each stage as well as clustering of examples at both 18 and 22 somites precluded a conclusive observation.

A lower concentration of Bmp4 treatment (0.01 μ g/ μ l) was applied to four samples to determine whether the threshold of Bmp4 concentration required to induce foregut malformations could be determined (Figure 6.5 and Figure 6.6). Surprisingly all of these samples exhibited moderate (Figure 6.5 C) to severe (Figure 6.5 M) deformities.

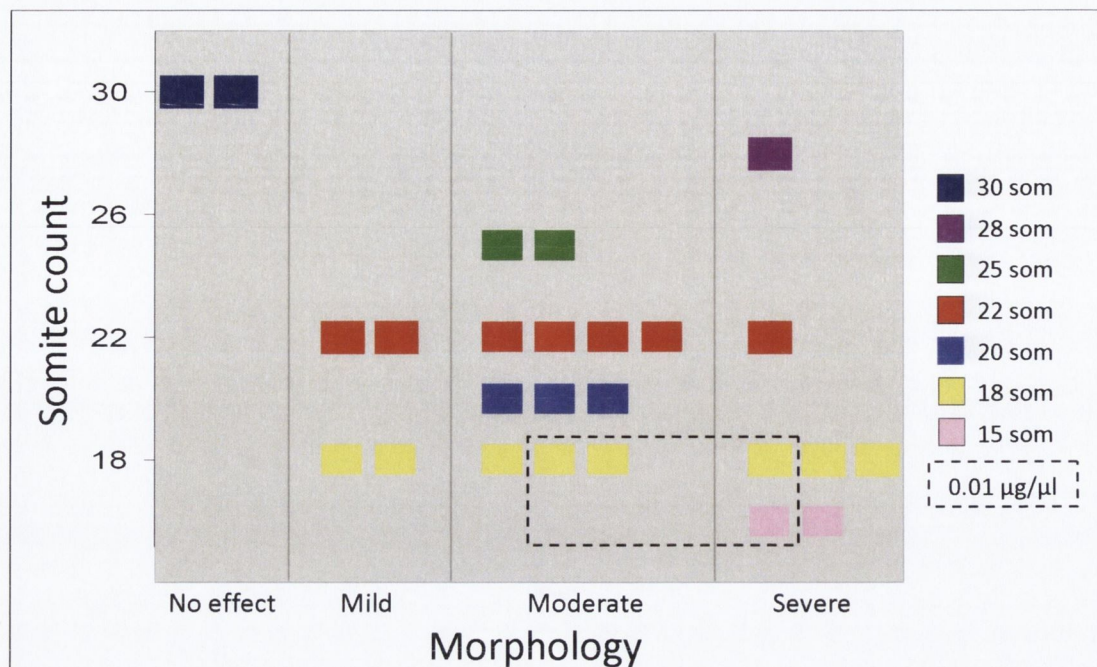


Figure 6.6: The relationship between severity of malformation induced by Bmp4 treatment and stage at treatment. Each box represents an individual explant specimen, colour coded for the initial somite count of the embryo from which the foregut was obtained, as indicated by key and on the Y axis. 4 samples were treated with a lower dose of Bmp4, all shown within the dashed box. Samples were graded according to severity of malformations, as shown on the X axis.

As a proxy for overall quantity of Bmp4 protein treatment applied to each explant, the numbers of anterior (A) and total (T) beads applied to each explant were compared between groups organised by morphological grading as follows; Mild (A, mean 3.75, range 2 to 6; T, mean 6.5, range 5 to 10), Moderate (A, mean 5.4, range 1 to 7; T, mean 11.5, range 3 to 20), or Severe (A, mean 5.6, range 5 to 6; T, mean 11.5, range 9 to 17). There were no statistically significant differences in the number of either anterior or total beads applied to specimens between groups with moderate or severe malformations. The low number of specimens in the mild group (n=4) precluded an appropriate comparison however a trend was noted towards fewer number beads applied in this group.

Extensive liver formation was observed in some specimens to abnormally encroach anterior and dorsal explant mesoderm domains (Figure 6.5 O to Q). This did not occur in control or non-treated foreguts. An excessive liver domain was defined, following morphological assessment of the entire range of foregut explants available, as extending anteriorly beyond the upper limit of the stomach (Figure 6.5 O and P) and/or dorsally beyond the limit of the foregut (Figure 6.5 Q). These large hepatic domains occurred in association with severe (4/7) (Figure 6.5 Q) and with moderate (4/12) (Figure 6.5 O and P) gradings of foregut malformations following Bmp4 treatment and also affected two specimens treated at the lower Bmp4 dose.

6.3.4 *Bmp4 treated foreguts in culture exhibit altered dorso-ventral patterning*

Under conditions of Bmp4 treatment, molecular patterning in the foregut, as reflected by Sox2 dorsal marker and Nkx2.1 ventral marker, exhibited disruption that was characterised by loss of Sox2 positive domains and extended Nkx2.1 domains in the abnormally undivided anterior foregut (Figure 6.7). Sox2 and Nkx2.1 are normally strictly localised to mutually exclusive domains of the anterior foregut endoderm during foregut division and these molecular signatures were maintained in isolated foregut 3D explant culture (cf. Section 5.3.4) as well as in foreguts cultured with control PBS-soaked beads (Fig 6.2 D, J, E and K). These strict domain boundaries in the foregut endoderm were perturbed by Bmp4 treatment in all samples analysed (n = 14).

In the most anterior region of the foregut Sox2 is not dorsally restricted, being expressed throughout the endoderm, and Nkx2.1 is absent in normal embryos *in vivo* as well as in non-treated and control PBS-soaked bead treated foregut explants (Figure 6.7 C). In Bmp4 treated specimens an abnormal domain of Nkx2.1 in the latero-ventral foregut endoderm was detected and appeared to co-locate with persistent Sox2 positivity in the most anterior region of the foregut (Figure 6.7 B i). At the level of foregut atresia in specimens with a moderate morphological severity, Sox2 and Nkx2.1 appeared to co-locate throughout the endoderm of the extremely narrowed foregut (Figure 6.7 B ii). Complete occlusion of the lumen was apparent in association with this highly abnormal pattern on examining these areas at high magnification (Figure 6.7 B iii).

In more posterior regions of the persistently undivided anterior foregut, complete or near complete ventralisation of the foregut was reflected by loss of Sox2 and gain of Nkx2.1 across the entire foregut endoderm in all specimens (Figure 6.7 B iv demonstrates near-complete ventralisation, note the complimentary weak Nkx2.1 localisation in a small region of the endoderm (lower left) and the only discernable Sox2 positivity). At the distal extent of the trachea or at the level of the lung buds a distinct dorsal domain of Sox2 positivity was re-established, highlighting the abnormal presence of tissue expressing dorsal foregut identity in the walls of the respiratory structures (Fig 6.7 B v arrowheads). This corresponds to the site of abnormal communication between the stomach and the respiratory structures observed by morphological assessment (Figure 6.7 B vi arrows, cf. Figure 6.5).

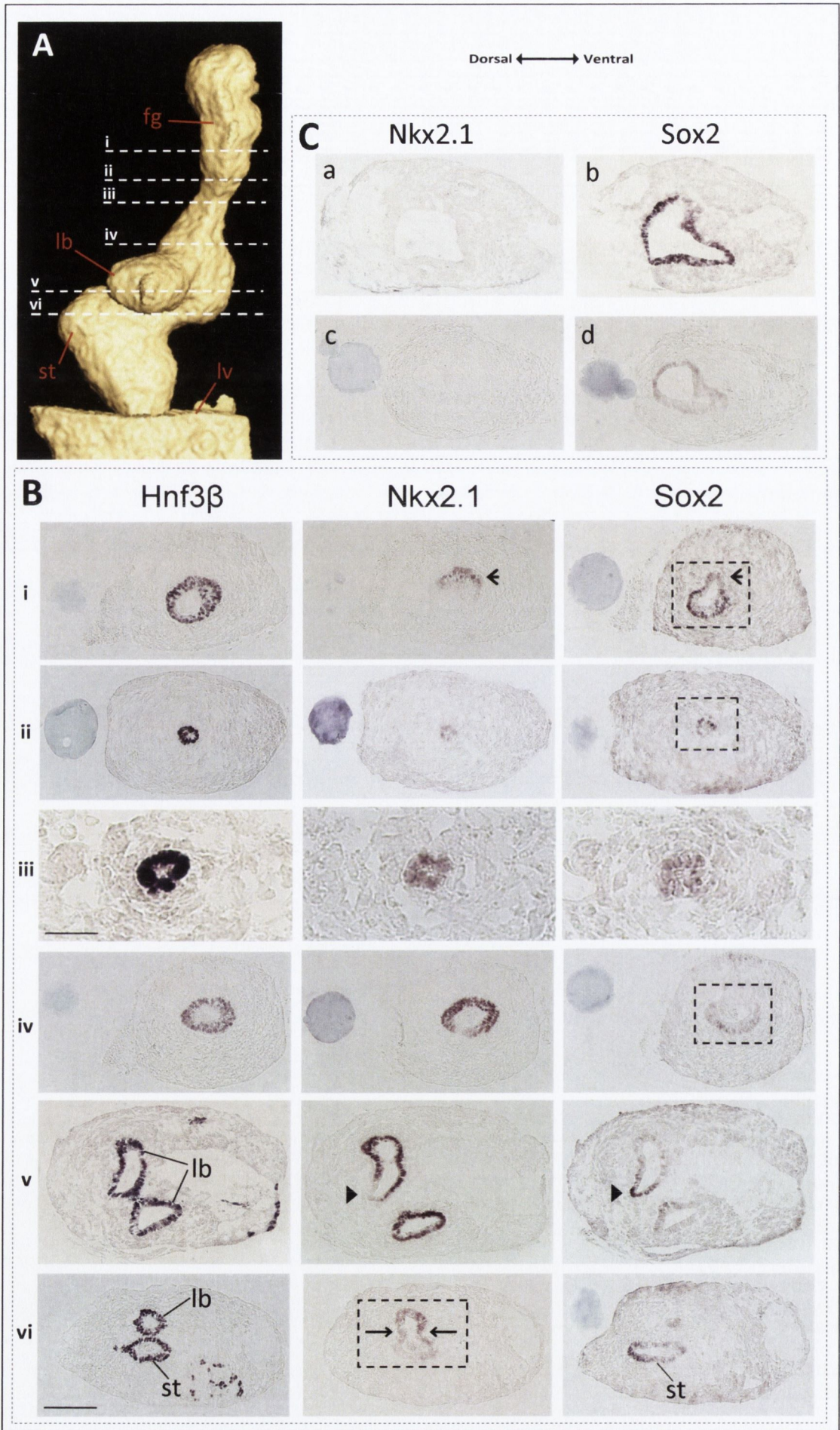


Figure 6.7: Dorso-ventral patterning in Bmp4 treated foreguts examined by immunohistochemistry.
(see over for figure legend)

Figure 6.7: Dorso-ventral patterning in Bmp4 treated foreguts examined by immunohistochemistry. **Panel A** shows a 3D reconstructed Bmp4 treated foregut displaying morphological abnormalities as previously described, note the liver (lv) surface has been cropped to aid visualisation. Roman numerals and dashed white lines give the level for the correspondingly named sections in Panel B (i to vi). **Panel C** shows IHC in sections of non-treated (a and b) and control bead treated (c and d) foreguts demonstrating no dorsal restriction of Sox2 and absence of Nkx2.1 in the most anterior part of the foregut at a level equivalent to i) in A and B. For comparison to the normal patterns at more posterior levels see Figure 3.4 (Chapter 3) for non-treated and Figure 6.2 for Control bead treated. In **Panel B**, at each level serial sections and Hnf3 β IHC were used to compare domains. Nkx2.1 and Sox2 appear to co-locate abnormally at the anterior most part of the foregut (i, highlighted by arrowhead) and at the level of foregut atresia (ii and iii). In B iii) the foregut lumen, within the stained cells for all markers, is occluded. In B iv) complete ventralisation of the foregut is indicated by loss of Sox2 and expanded Nkx2.1 domain. In B v) a re-established Sox2 domain highlighted by the solid arrowheads occurs where stomach (st) communicates with lung bud (lb). At level vi) this abnormal communication is highlighted by the converging arrows. Scale bar in vi) is 50 μ m and is comparable for all sections except in iii) scale bar is 10 μ m. Dashed boxes highlight areas examined in more detail by confocal microscopy (Figure 6.8).

6.3.5 *Altered intracellular localisation of Sox2 and Nkx2.1 visualised using Confocal microscopy.*

The highly unusual occurrence of Sox2 and Nkx2.1 co-location in regions of the foregut prompted analysis of these regions using double immunolocalisation of Sox2 and Nkx2.1 together with Confocal microscopy to determine in detail the precise cellular localisation of these transcription factors. Confocal laser microscopy confers the advantage of thin focal plane visualisation within a tissue section by using a pinhole that eliminates out of focus light and using laser excitation customised to the exact wavelength of fluorophores labelling the markers of interest resulting in image captured only at the desired plane. This produces high resolution images ideal for analysing protein localisation at a cellular and sub-cellular level, particularly when used together with markers such as DAPI, which specifically labels DNA indicating the nucleus. Using this methodology the domains of Sox2 and Nkx2.1 protein localisation were clearly visualised in foregut endoderm cells of Control PBS soaked bead (n=3) (Figure 6.8 C) and Bmp4 soaked bead (Figure 6.8 B) treated foregut explants (n=5).

Using directly adjacent sections to those already analysed by immunohistochemistry (see Figure 6.7 and Figure 6.8 A for boxed regions as guides) it was evident that in areas where colourmetric detection appeared to reveal co-location of Sox2 and Nkx2.1 in the foregut endoderm, Nkx2.1 was localised to the nucleus and Sox2 was in fact localised to the basal cell cytoplasm and basement membrane region (compare Figure 6.8 A i and ii to B i and ii). Extra-nuclear Sox2 protein localisation appeared to extend into the adjacent mesoderm when an abnormal domain of Nkx2.1 occurred in the most anterior region of foregut (Figure 6.8 B i, circled by dashed line). At this level occasional examples of Sox2 and Nkx2.1 intra-nuclear protein co-localisation could be seen within the abnormally Nkx2.1 positive domain (Figure 6.8 B i arrows). Moving posteriorly, at the level of foregut atresia in Bmp4 treated specimens, Sox2 was entirely absent from nuclei and localised only to the basal cell cytoplasm in association with abnormal Nkx2.1 nuclear localisation across the entire foregut endoderm (Figure 6.8 B ii). Further posterior to this level complete absence of Sox2 protein was apparent throughout the foregut endoderm and Nkx2.1 positivity persisted in all nuclei indicating complete ventralisation of the foregut (Figure 6.8 B iv). Consistent with results from the immunohistochemistry analysis (Figure 6.7 B v and vi), a dorsal Sox2 domain emerged at the level of the lung buds. Re-established intra-nuclear localisation of Sox2 transcription factor at this site confirmed the dorsal foregut nature of this tissue and together with the morphological results clearly denoted the presence of an abnormal stomach to respiratory tree communication (Figure 6.8 B vi).

Figure 6.8: Examination of dorso-ventral patterning in Bmp4 treated foreguts at a cellular level using Confocal microscopy.

In **Panel A** elements from Fig 6.7 are shown to demonstrate the level of the correspondingly named sections in Panel B. In A the boxed areas from cryosections assessed by IHC for Sox2 (i, ii and iv) and Nkx2.1 (vi) reflect regions examined in immediately adjacent sections by double immunofluorescent labelling together with DAPI DNA stain and Confocal microscopy in Panel B.

Panel C demonstrates Sox2 (red) and Nkx2.1 (green) patterning in the dividing foregut of a control bead treated foregut.

In **Panel B** i) and ii) Sox2 localisation to the basal foregut endoderm cell cytoplasm is highlighted by solid arrowheads and extension of Sox2 into the mesoderm is shown in the dashed circled. Occasional cells are noted to be positive for both Sox2 and Nkx2.1 at level i) as highlighted by the line arrows. Complete absence of nuclear Sox2 is shown at level ii) and iv). In iv) the stomach (st) connects abnormally to the lung buds (lb) and is reflected in a re-established domain of Sox2 localisation to the nucleus. Scale bars in C and vi) = 30 µm, in i) and iv) = 20 µm and ii) = 10 µm. Colours represent markers as indicated in figure.

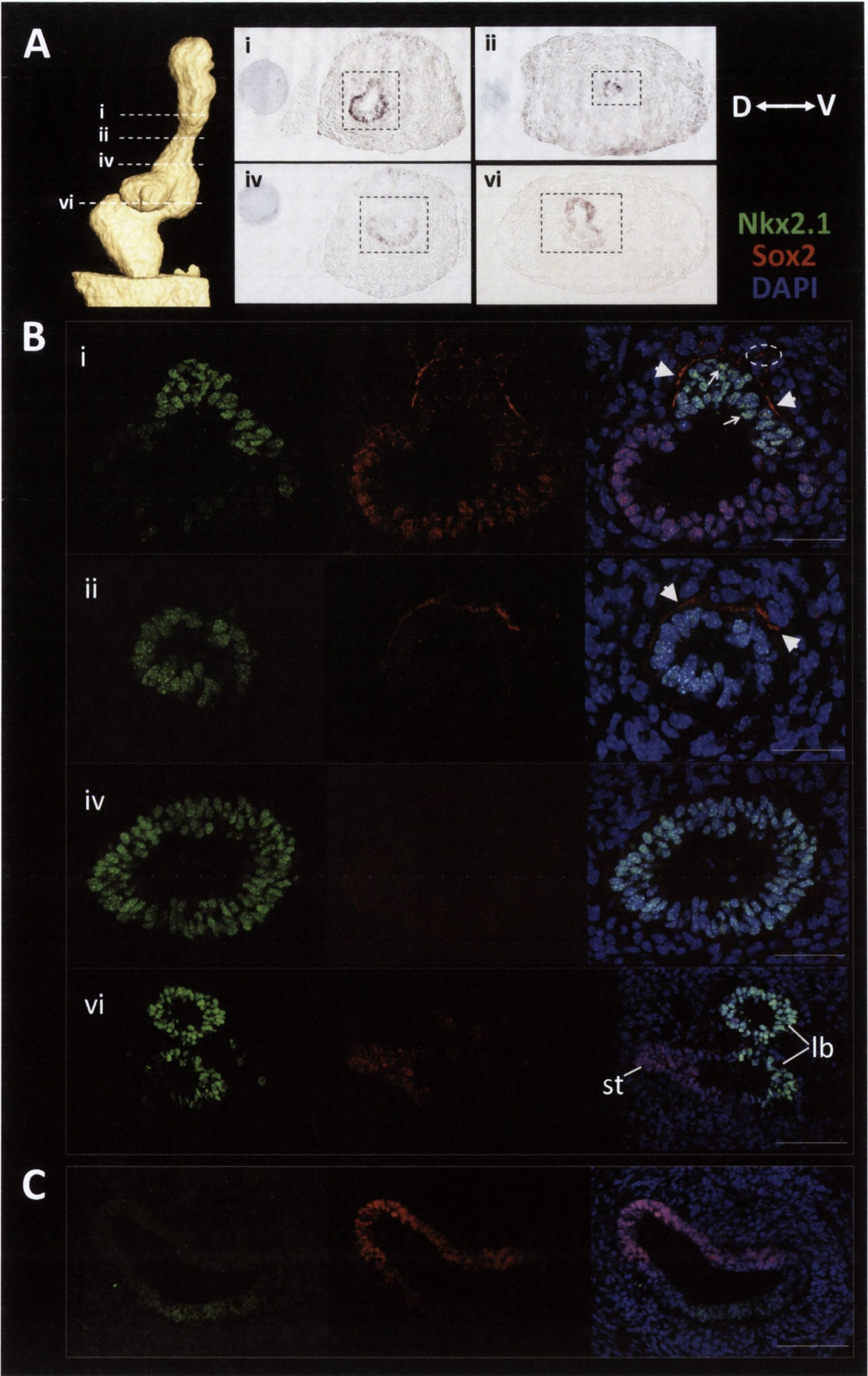


Figure 6.8: Examination of dorso-ventral patterning in Bmp4 treated foreguts at a cellular level using Confocal microscopy. (See figure legend facing page)

6.4 Discussion

The use of *in vitro* manipulation provides an effective system to directly assay the impact of applied signals on tissue in a highly controlled environment. In this chapter I have utilised 3D isolated foregut explant culture to further analyse the role of BMP signalling in the pathogenesis of OA/TOF in a manner that mimics the focal nature of ectopic BMP signalling disruption which I demonstrated in the AMM (cf. Chapter 3). These experiments have provided clear evidence of characteristic morphological and molecular disruptions in the foregut arising as a direct result of Bmp4 but not Noggin treatment. The anatomical disturbances observed in Bmp4 treated foreguts were consistent with OA/TOF deformities and in particular strongly correlated to the AMM phenotype. At a molecular level distinctive disruption in Sox2 and Nkx2.1 DV patterning of the foregut revealed that Bmp4 treatment causes abnormal ventralisation of the foregut, highlighting a key potential mechanism in the pathogenesis of OA/TOF.

6.4.1 *The role of Noggin in foregut malformations.*

Noggin protein treatment of foregut explants in culture did not induce morphological or molecular disturbances. The absence of foregut malformations in Noggin treated foreguts is surprising when it is considered that Noggin inhibits Bmp ligand activity in other tissues (as reviewed by Chen et al., 2004) and BMP signal attenuation results in tracheal agenesis when Bmp4 or Bmpr1a and b are conditionally inactivated (Domyan et al., 2011, Li et al., 2008b). The concentration of Noggin used in these experiments was comparable to experiments where a morphological response to Noggin bead treatment *in vitro* was demonstrated in the facial region (Ashique et al., 2002) however some authors used 10 fold more concentrated Noggin (Torihashi et al., 2009) but this was often when using a single bead treatment. To provide additional protein, the application of higher numbers of Noggin beads did not affect the outcome.

The ability of cells to respond to morphological cues may be subject to limits during development by requiring co-existing signals or by depending on exact gestational stage and cell differentiation state. In the Noggin knockout mouse, tempo-spatial gene inactivation was used to further resolve the requirement of Noggin for foregut separation in that model (Fausett et al., 2014). Those investigations revealed that isolated Noggin gene inactivation in the notochord and neural floor plate but not in the foregut endoderm results

in foregut malformations and the authors suggest that Noggin is required to control notochord derived BMP activity which then exerts influence on the foregut. Failure to induce foregut abnormalities by conditional Noggin inactivation in the foregut endoderm suggests that these cells may be incapable of responding directly to Noggin. In foregut explants I noted that Noggin treatment visibly attenuated BMP activity in adjacent explant mesoderm but not in the foregut, as reflected by pSMAD immunolocalisation, which further suggests inability of foregut endoderm to react to Noggin. Notochord was removed from Noggin treated foregut explants in order to specifically examine the response of the foregut to protein treatment. Future experiments may explore the response to Noggin treatment in foreguts cultured with their notochord to test if, as with Noggin inactivation, excess Noggin can also be mediated by the notochord to induce foregut abnormalities.

Fausett et al. (2014) further identified a gestational limit on the ability of Noggin knockout to induce foregut malformations prior to the onset of notochord delamination at E7.5, suggesting that direct notochord to foregut contact was required for loss of Noggin to increase BMP activity and induce foregut malformations. I have shown however that the foregut is capable of morphological response to excess BMP signal at E9, beyond the stage of notochord delamination from the foregut. BMP signalling throughout development is controlled by many other mechanisms which remain active when Noggin is absent and may compensate accordingly. The importance of notochord-to-foregut contact or gestational stage in the Noggin knockout model may reflect very subtle alterations in BMP dose from the notochord which may not be capable of influencing the foregut over distance.

While it is clear that lack of Noggin in the early embryo can result in foregut malformations it has not previously been examined whether the foregut is similarly vulnerable to excess Noggin at time points before E9. BMP signal under-activity does induce foregut deformity, illustrated by models with conditional inactivation of *Bmp4* and *Bmpr1* (Li et al., 2008b, Domyan et al., 2011), but in this work Noggin antagonism was not able to reproduce these effects in foreguts treated at E9. As 3D explant culture does not support foregut development at earlier stages, conditional Noggin gene over-activation could be used to identify any window of susceptibility not examined here. In the context of the available literature, this work supports the conclusion that abnormal Noggin gene expression in the AMM, as visualised at E10 and E11 gestational stages (Hajduk et al, unpublished results), is likely to be a consequence of deregulated BMP signalling rather than contribute meaningfully to morphological disruption. While this gene may regulate foregut separation by antagonising BMP activity at the notochord, Noggin is evidently not a potent morphogen when acting at the foregut at the examined gestational stage.

6.4.2 *Morphological disturbances in Bmp4 treated foreguts resemble those in Adriamycin treated mice.*

In Adriamycin treated models a range of foregut anomalies have been described that are strikingly similar to the human spectrum of OA/TOF birth defects (as reviewed by Mc Laughlin et al., 2013). Morphological abnormalities in Bmp4 treated foreguts strongly resemble those seen in AMM foreguts. In particular anterior foregut atresia or stenosis and abnormal communication of the posterior foregut or stomach to the respiratory structures in the absence of an oesophagus are characteristic of both the AMM and Bmp4 treated foregut in culture as further discussed in Chapter 7 (cf. Figure 7.2). In addition delay in lung bud development and abnormal lung bud morphology are well described in the AMM, a feature that is also apparent when foreguts in culture are exposed to additional Bmp4. Complete failure of anterior foregut specification into distinct structures in Bmp 4 treated foreguts correlates with a complete tracheo-oesophageal cleft phenotype in Adriamycin treated embryos as well as in humans. Complete foregut agenesis is the most severe foregut abnormality detected following Bmp4 treatment and has an immediate counterpart in the description of discontinuity of the foregut occurring in the AMM (Hajduk et al., 2011b).

Interestingly Bmp4 treatment did not discernibly impact formation of the stomach or pancreatic buds from the foregut. High concentrations of Bmp4 have previously been shown to restrict pancreatic growth in vitro (Dichmann et al., 2003). This was not evident in my results however the threshold for this effect may not have been reached. We did note extended liver domains in some Bmp4 treated samples that did not occur under control conditions. Bmp4 has previously been reported to promote hepatic development in vitro (Dong et al., 2007). The co-occurrence of severe foregut abnormalities with hepatic overgrowth may indicate that some affected specimens were subject to a higher dose of Bmp4 treatment however the bead count analysis and the recapitulation of these effects at a 10 fold reduction in Bmp4 dose do not support this suggestion. Liver domains appear inherently disparate when the entire range of isolated foregut explants is considered (across Chapter 5 and 6). Although extensive hepatic domains did not occur outside the Bmp4 treated group the spectrum of liver morphology suggests that hepatic growth depends on factors variably present in explants such as the adjacent mesoderm. The requirement of signalling from the mesoderm in liver development has previously been described (Rossi et al., 2001). In the isolated foregut model all non-foregut mesoderm and in particular the

heart is removed for improved morphological outcome (cf. Appendix isolated foregut protocol), possibly impacting on liver development.

6.4.3 Disturbed dorso-ventral patterning in Bmp4 treated foreguts draws parallels with other models of abnormal foregut development.

Molecular signalling disturbances arising in Bmp4 treated foreguts can be correlated to findings in Adriamycin treated mice as well as in other models of abnormal foregut development. The morphogenic significance of Sox2 expression in foregut development has been demonstrated in animal models and also in humans by the association of OA/TOF malformations with SOX2 gene mutations (Williamson et al., 2006). Loss of Sox2 gene expression in the dorsal foregut at the level of foregut abnormalities and particularly in areas of foregut stenosis illustrates loss of dorsal foregut identity in the AMM (Hajduk et al., 2011b) and corresponds to similar disruption at the protein level in the abnormally narrowed region of Bmp4 treated foreguts. However Nkx2.1 gene was normally expressed in the AMM foregut and in particular did not exhibit a dorsally expanded domain at sites of Sox2 loss. This contrasts with the observed ventralisation of the entire foregut following Bmp4 treatment in explant culture as reflected by dorsally expanded Nkx2.1 protein localisation together with Sox2 loss. Gene and protein product activity are not necessarily tempo-spatially synchronous and Nkx2.1 protein level may be influenced by post transcriptional modifications of Nkx2.1 gene activity not reflected by in situ hybridisation. This inconsistency will be important to investigate in future analyses.

In the Sox2 knockout mouse model however, complete ventralisation of the foregut as illustrated by Nkx2.1 expression in the entire endoderm does occur in association with failure of oesophageal formation (Que et al., 2007). Neatly juxtaposed to this, Nkx2.1 mutant embryos exhibit dorsalisation of the foregut illustrated by abnormal ventral expansion of Sox2 in association with failure of tracheal formation (Minoo et al., 1999). It has previously been shown that mice with double knockout for Bmpr1a and b also demonstrate tracheal agenesis together with ventral Sox2 expansion and Nkx2.1 loss and it has been suggested that attenuated BMP signal in this model is mediated by failure to inhibit Sox2 at the ventral foregut (Domyan et al., 2011). Bmp4 treatment in isolated foregut culture has provided valuable complimentary data to the Bmpr1a and b mutant model demonstrating loss of Sox2 together with failure of oesophageal formation under conditions of increased BMP signalling.

The extent of dorso-ventral signalling abnormalities induced by Bmp4 treatment in

foregut explants was restricted to the anterior foregut and in particular normal patterning of the stomach by Sox2 occurred despite the presence of directly adjacent treatment beads. Evidently the ability of Bmp4 to disrupt Sox2 expression and so re-specify tissue identity is limited to the anterior foregut in this model.

6.4.4 Foregut endoderm cell fate

Sox2 is a critical transcription factor regulating cell fate determination (Thomson et al., 2011). I have identified two highly unusual features of Sox2 protein localisation in the foregut endoderm following Bmp4 treatment in these results. The first of these was the occasional nuclear co-localisation of Sox2 and Nkx2.1 within abnormal domains of Nkx2.1 positivity in the foregut occurring anterior to the normal level of foregut division. To my knowledge this is the first description of co-location of these transcription factors which usually exhibit mutual incompatibility of localisation in the foregut and this may reflect highly disturbed cell fate determination under conditions of additional Bmp4 treatment. The second of these features was localisation of Sox2 in the basal cell cytoplasm in regions of the foregut which had abnormal nuclear presence of Nkx2.1 and also extension of extra-nuclear Sox2 positivity into the adjacent mesoderm. Localisation to cell cytoplasm is a previously reported molecular mechanism in the control of Sox2 activity, resulting from an acetylation step in post transcriptional modification of Sox2 and subsequent association with the nuclear export machinery in embryonic stem cells (Baltus et al., 2009). These abnormalities of Sox2 localisation are epitomised in Figure 6.8 B i), demonstrating abnormal presence of Nkx2.1 in the nucleus, co-existing with abnormal Sox2 in the cell cytoplasm and occasional co-localisation of both these transcription factors in the nucleus. These observations imply that a process of foregut endoderm cell fate identity reconfiguration from dorsal to ventral has been visualised in these data.

While Sox2 has been shown to be inhibited by Bmp4 in the control of endoderm cell fate determination (Teo et al., 2012), Nkx2.1 has not been shown to be directly regulated by BMP ligand signalling but instead to be promoted by Noggin in the induction of embryonic stem cells into lung progenitor cells (Ninomiya et al., 2013). The nature of cellular response to BMP signal varies widely however depending on tissue type (as reviewed by Wang et al., 2014). The mechanism by which abnormal Nkx2.1 domains are established in isolated foregut culture under Bmp4 treatment conditions may be by direct action of Bmp4 or mediated by loss of Sox2. The later mechanism is suggested by the comparable abnormalities exhibited between the foregut of Sox2 gene knockout in mice

(Que et al., 2007) and isolated foreguts treated with Bmp4 in culture. Conditional knockout of Bmp4 in anterior foregut endoderm of mice induced tracheal agenesis but no disruption of Nkx2.1 expression occurred (Li et al., 2008a) suggesting inability of Bmp4 to directly influence Nkx2.1 activity in the foregut. Induction of Nkx2.1 and respiratory identity in the anterior foregut endoderm has been widely reported to depend on FGF signalling from ventrally adjacent cardiac mesoderm (as reviewed by Cardoso and Lu, 2006). In isolated foregut explant culture cardiac mesoderm is removed and is not required for lung budding in non-treated and control bead treated specimens, although programmed respiratory identity can already be distinguished in ventral regions of the undivided anterior foregut at the stage of explant (E9). Under Bmp4 treatment conditions however gain of Nkx2.1 and respiratory identity across the entire foregut occurs in the absence of cardiac tissue, illustrating that Nkx2.1 is not exclusively induced by factors from the cardiac mesoderm.

The concept of a key gestational window within which correct patterning of the foregut must occur in order for proper foregut separation to follow has been established (as reviewed by Zorn and Wells, 2009a). Not previously reported is whether domains of the foregut in which tissue fate is designated, as judged by gene expression, exhibit plasticity in terms of possible cell fate re-specification during a subsequent period of development. Use of isolated foregut explant culture together with Bmp4 treatment on specimens staged at early E9 (15 somites) to E9.5 (30 somites) confirms that cell fate is not irreversibly determined but can be respecified from dorsal to ventral during these stages and suggests that abnormal expansion of ventral identity to the entire foregut endoderm in response to Bmp4 signalling may be a major pathological mechanism in OA/TOF.

The failure of Bmp treatment to induce a morphological response in foreguts obtained from 30 somite embryos together with the occurrence of moderate and severe malformations in 25 and 28 somite foreguts provides interesting additional insight into the nature of foregut cell fate determination. Although these data reflect few sample numbers, they suggest the existence of an abrupt cut off point in the ability of Bmp4 treatment to cause a morphological effect rather than a graded diminution of response over time. This was particularly remarkable when the contrast between Bmp4 treated 28 somite foregut and 30 somite foregut morphology was examined. When cultured under control conditions or with no treatment 28 and 30 somite foreguts all demonstrate highly similar morphology and staging (all TS18 following 48 hours). Further definition of the exact gestational time point at which foregut is no longer susceptible to the potent morphogenic effects of Bmp4 can only be confirmed by further experiments but is suggested by these data to occur approaching the 30 somite stage of development. It may follow that a specific

morphological event such as commencement of lung bud formation at 30 somites can define this cut off point.

6.5 Conclusion

The placement of Bmp4 and Noggin soaked beads at the dorsal foregut in 3D explant culture was designed to replicate ectopic localisation of pSmad and Noggin gene expression as described in the AMM. Focal Bmp4 but not Noggin treatment of foreguts *in vitro* induced foregut malformations consistently. In addition to failure of the oesophagus to develop, Bmp4 treatment resulted in morphological abnormalities including anterior foregut atresia or agenesis, abnormal lung bud morphology and direct communication between the stomach and the trachea or lung buds, together closely replicating an OA/TOF phenotype. In Bmp4 treated foreguts abnormal dorso-ventral patterning was manifested by complete ventralisation of the foregut endoderm in the region of expected foregut separation, demonstrated by diminished or lost Sox2 and extended Nkx2.1 domains. These results can confirm that focal administration of Bmp4 is sufficient to induce tracheo-oesophageal malformations in isolated foregut culture.

Chapter 7

Discussion

7.1 Summary of findings

The work laid out in this thesis examines key molecular and morphological features of foregut malformations, and in particular the role of the BMP signaling and the notochord in the pathogenesis of OA/TOF by addressing 3 broad objectives:

➤ *To test the hypothesis that BMP signalling is disturbed in the AMM and contributes to the pathogenesis of foregut malformations (Chapter 3 and 4)*

1. Novel use of wholemount immunolocalisation for pSMAD validated by simultaneous immunohistochemical techniques revealed DV restriction of BMP activity across the developing foregut, which exhibited disruption in the AMM, characterised by intense focal pSMAD localisation in the dorsal foregut endoderm adjacent to the level of notochord branching (Chapter 3).
2. A survey of array data and spatial gene expression archives identified candidate BMP downstream genes which were confirmed to have foregut specific expression profiles by in situ hybridisation and OPT scanning (Chapter 3).
3. Analysis of BMP downstream target genes in the AMM revealed abnormal foregut spatial expression profiles for 10 of the 15 genes selected for examination by using in situ hybridisation together with whole embryo OPT scanning and 3D reconstruction for the identification of AMM foregut and notochord defects (Chapter 3).
4. Extension of the gene expression analysis to include T-box signalling factors Tbx1 and 2 revealed highly specific localisation of Tbx1 in the developing oesophagus in normal embryos and abnormal gene expression in foregut of the AMM (Chapter 4).
5. Examination of the spatial gene expression profiles for Bmp2 and Bmp7 signalling ligands revealed no abnormalities of expression in the AMM foregut or notochord (Chapter 3)

- *To establish an in vitro system for foregut development and to examine a putative signalling relationship between the notochord and foregut during development by manipulating their physical relationship in vitro* (Chapter 5).
1. A newly established 3D culture system faithfully recapitulated separation of undivided foregut explants into oesophagus and trachea in terms of both morphological structure as detected by Hnf3b wholemount immunolocalisation and molecular patterning, as illustrated by correct localisation of markers Sox2 (dorsal) and Nkx2.1 (ventral).
 2. A morphological staging system was produced for the assessment of foregut development based on foregut anatomy in normal embryos at E9 through E11. 3D reconstruction of OPT scans for Hnf3 β immunolocalisation in whole Theiler staged embryos detected gestational stage specific features of foregut separation. The resulting morphological staging system could be used to assess progression of foregut development in a variety of models and experimental scenarios and was used here in particular for the analysis of foregut explants.
 3. Assessment of isolated foregut explants by Hnf3 β immunolocalisation and the morphological staging system revealed comparable morphological outcomes to foregut separation *in vivo*. Gain in development equivalent to approx 36 hours of that *in vivo* was consistently achieved by explants over a 48 hour culture period.
 4. Manipulation of the notochord in 3D explant culture revealed that the notochord is not required for normal foregut separation in isolated foregut explants from E9 embryos. Similarly the transplant of excess notochord to the dorsal anterior foregut did not result in morphological or molecular disturbance of foregut development.

- *To test the hypothesis that BMP signalling disruption can induce oesophageal malformations.*
5. The application of beads soaked in recombinant Noggin protein to the dorsal foregut in 3D explant culture did not impact molecular patterning, as illustrated by correct DV domains of markers Sox2 and Nkx2.1, morphology or gain in development compared to control PBS-soaked bead treatment or non treated foregut explants.
 6. The application of beads soaked in recombinant Bmp4 protein to the dorsal foregut in 3D explant culture resulted in a reproducible phenotype characterised by failure of oesophageal formation, atresia of the anterior foregut and abnormal communication between the stomach and the respiratory structures.
 7. Recombinant Bmp4 treatment of foreguts in 3D explant culture resulted in disrupted molecular patterning of the foregut endoderm as illustrated by loss of Sox2 from the dorsal foregut and abnormal extension of Nkx2.1 across the entire foregut, reflecting complete ventralisation of the undivided foregut.
 8. The morphological and molecular features in Bmp4 treated foreguts are comparable to known models of tracheo-oesophageal malformations, and in particular to the AMM, illustrating that Bmp4 treatment in foregut culture is sufficient to induce an OA/TOF phenotype.

7.2 The role of BMP signalling in the pathogenesis of OA/TOF.

In development BMP signalling provides cues for cell differentiation and proper organogenesis across a wide variety of tissue contexts. In this thesis specific focus was applied to the role of BMP signalling in normal foregut development and in particular oesophagus formation. BMP signalling is required for normal tracheal formation from the ventral foregut however a direct response of dorsal foregut morphogenesis to BMP ligands has not previously been proven. It has been shown however that BMP over activity resulting from loss of Noggin antagonism results in an OA/TOF phenotype and can be rescued by reduction of BMP ligand encoding gene dose. The Noggin knockout mouse model and the Adriamycin mouse model share remarkable phenotypic similarity highlighted by the presence of notochord branching in both models. Further characterisation of Noggin gene expression in the AMM revealed abnormalities of Noggin expression in the notochord and foregut, stressing the likely importance of balance in BMP signalling for normal foregut development.

I proposed to address the role of BMP signalling in foregut development and in the pathogenesis of OA/TOF using both *in vivo* (AMM) and *in vitro* (isolated 3D foregut explant culture) approaches. In the AMM the examination of BMP signalling was twofold, in the detection of patterns of BMP activity across the foregut by immunolocalisation of pSMAD and in the read-out of BMP signalling by analysis of foregut expression profiles for BMP downstream target genes.

A major output of this work was the novel identification of BMP downstream target genes specifically expressed in the foregut region as it separates to form oesophagus and trachea. The interrogation of freely available datasets resulted in the compilation of a list of candidate genes, borne out by subsequent *in situ* hybridisation experiments to have distinctive expression profiles in the developing foregut and furthermore to exhibit abnormalities of spatial expression in the AMM foregut. This analysis allowed the examination of BMP signalling at a functional level by assessing downstream readout but also, by observing spatial gene expression disturbances in the AMM, highlighted previously undescribed roles for these genes in foregut separation (which are described in more detail in Table 3.2 and also summarised in Figure 7.1). I found that the expression of *Id1*, *JunB* and *Prx2* were focally abnormal in the dorsal foregut endoderm wall at the level of notochord branching (Figure 7.1 C, H and F). These observations suggest highly localised over activity of BMP signalling at this site which is further illustrated by

abnormal pSMAD immunolocalisation and *Noggin* gene expression in these areas (Figure 7.1 A, B and E). Some BMP downstream target genes including *JunB*, *Msx1* and *Dpysl2* demonstrated gain of expression in the foregut adjacent to notochord branching together with loss of expression in slightly distant sites such as in the lung buds and the tracheal bifurcation. These observations may reflect AP differences in the response of foregut cells to BMP signals or a gradient response by these downstream genes to BMP signal. Interestingly only *Crabp2* gene exhibited abnormal expression in the notochord branches correlating with abnormal gene expression of *Noggin* in the AMM despite several other genes being expressed in the notochord of control embryos (cf. Table 3.2). This may reflect the highly specialised nature of the notochord cells and undoubtedly key differences in how endoderm and mesoderm cells respond to BMP signalling.

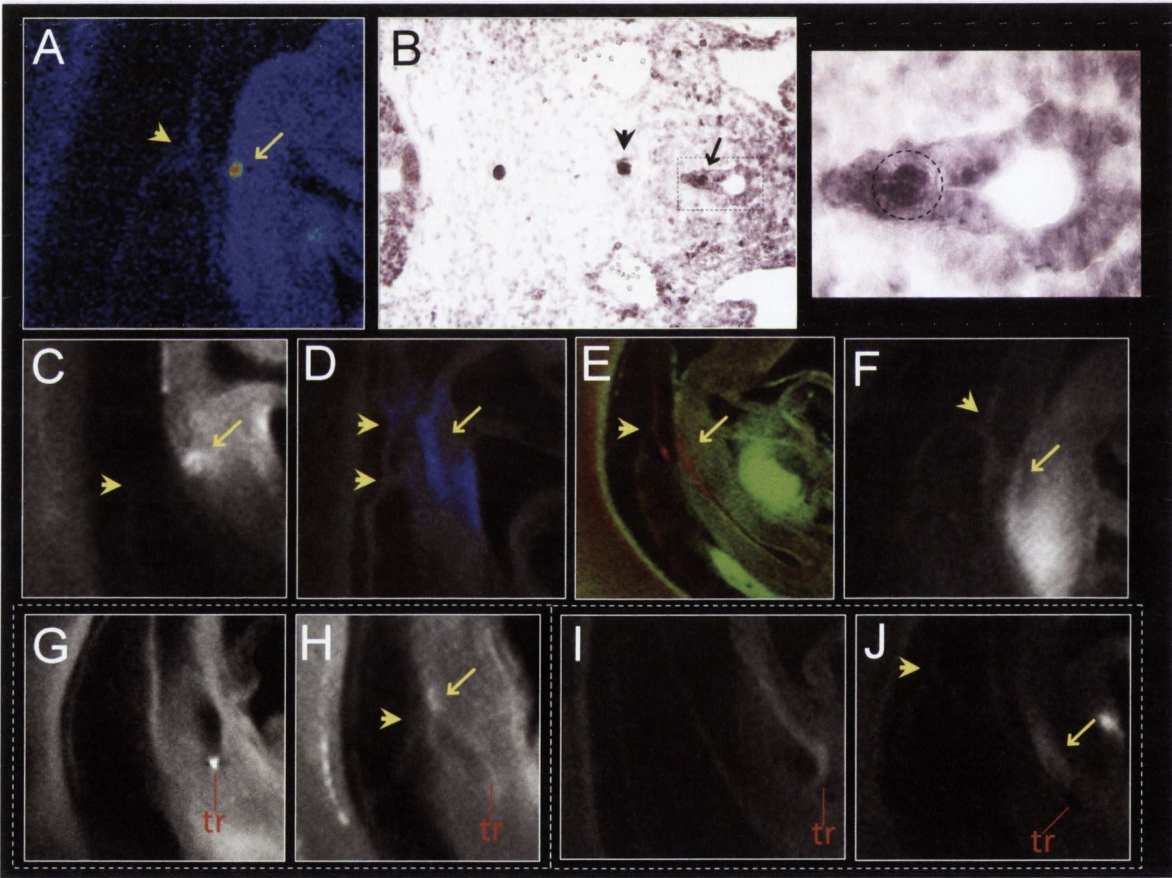


Figure 7.1 Composite results of BMP pathway examination in the AMM.

All images are of AMM except G and I which are controls. Immunolocalisation of pSMAD showing a heatmap on a virtual section of a whole embryo following 3D imaging (A) or on cryosection (B). In situ hybridisation (C through J) showing gene expression in greyscale intensity or pseudocoloured in blue or red. C is *Id1*, D is *Tbx1*, E is *Noggin*, F is *Prx1*, G and H are *JunB* and I and J are *Msx1*. Background in grey except for E (background in green). Notochord branching is indicated by arrowheads, abnormal signal in foregut is indicated by arrows. tr trachea. (cf. Chapter 3 and 4). Boxed region in B magnified to right.

The specific nature of the normal foregut expression profiles of many the BMP responsive genes, for example intense JunB expression at the tracheal bifurcation (Figure 7.1 G) or highly focal Tbx1 signal in the anterior oesophagus, suggests that these genes may contribute to the molecular control of foregut morphogenesis. Accordingly the observation of abnormal expression profiles in the AMM, such as loss of JunB at the tracheal bifurcation, may reveal new information regarding the pathogenesis of foregut malformations.

The cellular mechanisms which are prompted by molecular disturbance to result in faulty foregut separation are unclear. EMT is a fundamental mechanism for successful organogenesis. BMP activity is classically understood to drive cells towards mesenchymal cell identity in development (Richter et al., 2014). Several of the candidate genes selected for analysis in this work have been shown to be important effectors of BMP driven EMT response including JunB, Msx1 and Snail (Chen et al., 2008, Gervasi et al., 2012, Carver et al., 2001). At the tracheal bifurcation for example, distinctive expression profiles of JunB (Figure 7.1 G and H) and Msx1 (Figure 7.1 I and J) are disturbed in the AMM suggesting that BMP driven EMT is important at that site and is vulnerable to disruption. Disruptions in EMT could perhaps contribute to failure of separation of dorsal and ventral foregut structures, which can remain in abnormal communication at the tracheal bifurcation, embodied by a fistula in the AMM (Merei et al., 1997a). These data propose disrupted EMT as a possible mechanistic aetiology in OA/TOF by illustrating the focally abnormal behaviour of EMT related genes in the AMM foregut. It would be very interesting to examine Bmp4 treated foreguts for expression profiles of these genes to assess whether EMT can be similarly disrupted following direct BMP over-activation. A further interesting observation in BMP downstream target genes were shifts between endoderm and mesoderm expression profiles in several genes examined for example Msx1, Prx1 and Tbx1 (Figure 7.1 I and J), possibly reflecting altered cell fate at these sites.

For further examination of the role of EMT in foregut development, cell lineage tracing experiments, detecting markers of either endoderm or mesoderm could be used to tease out precise cellular behaviours at the site of foregut division and tracheal bifurcation. In this thesis I have effectively used Hnf3 β endoderm marker to demonstrate features of foregut development. Modern imaging capabilities permit live Confocal imaging of *in vitro* experiments, generating tempo-spatial data to elucidate cell and tissue behaviour in 3D. A GFP reporter mouse line could be established for an endoderm marker such as Hnf3 β and be used to generate isolated foreguts for explant culture. Real time Confocal imaging

of GFP expressing foregut endoderm in explant culture could be performed in whole specimens and down to a subcellular level. These powerful techniques could reveal a great deal regarding both the cellular and morphological sequence of events involved normal foregut separation and could additionally be used to examine how those events are perturbed in an *in vitro* model of OA/TOF, such as I have established by Bmp4 treatment of foreguts in culture. Expansion of knowledge on the mechanistic basis for foregut development has important clinical implications. In the future *in vitro* oesophagus generation may offer a possible treatment option in children whose oesophagus cannot function, either due to congenital malformation or injury. It is envisioned transplantation of *in vitro* derived tissues will be subject to strict regulation and require ways to establish and then monitor “normality” in these tissues. This may particularly require mechanisms to precisely control cell behaviours, such as EMT, in order to achieve success. The identification of foregut specific expression of genes which regulate EMT could provide avenues to both influence and assess EMT in the future.

Although Bmp ligand encoding genes 2, 4 and 7 expression profiles were not visibly altered in the AMM, the detection of BMP response elements by both pSMAD and target gene analysis strongly implicate BMP signalling over activity in the AMM foregut. The highly focal nature of the abnormal BMP response was illustrated by the analysis of pSMAD in high resolution. Ectopic pSMAD signalling confined to dorsal foregut endoderm was associated with abnormal cellular organisation, shape and patterning as exhibited by clusters of cells with intense localisation of pSMAD in extra-nuclear domains (Figure 7.2 A). The significance of these observations is unclear but may reflect altered cellular behaviours under the control of BMP signalling. Further molecular disturbances were observed at this site in several BMP responsive such as Id1 and also T-box genes (in this work) as well established foregut DV patterning genes Sox2 and Shh (Hajduk et al., 2011b).

DV patterning establishes regional identity for subsequent dorsal oesophagus and ventral trachea formation in the undivided foregut (as reviewed in Zorn and Wells, 2009b). Strict domains are maintained in which cell fate is specified and can be detected by markers as oesophageal, (indicated by Sox2) and respiratory in nature (as indicated by Nkx2.1). The link between BMP signalling and DV patterning of the foregut is an important but to date poorly explained association. I have shown that BMP response across

the foregut exhibits a DV pattern as illustrated by the immunolocalisation of pSMAD in a predominantly ventral pattern. In our Bmp ligand gene encoding analysis Bmp4 was predominantly ventral while Bmp2 and 7 did not exhibit DV restriction (cf. section 3.3.5), therefore Bmp4 may be the important ventral signal contributing to the pSMAD distribution. Bmp4 has been proposed to suppress Sox2 in the ventral foregut when acting under instructions from Shh to promote respiratory identity and allow lung formation (Domyan et al., 2011). Disruption of BMP patterning in terms of DV restriction occurs in the AMM as reflected by abnormal pSMAD localisation to the dorsal foregut endoderm. This site correspond to areas with loss of Sox2 and gain of Shh expression as described in previous work by our group (Hajduk et al., 2011b). Together these findings indicate loss of a dorsalising influence or, given the over-active BMP response, perhaps gain of a ventralising one in the AMM.

Altered DV patterning resulting from Bmp4 treatment of the foregut was detected in 3D explant culture and was also found to be characterised by loss of Sox2 from the foregut and additional expanded Nkx2.1 localisation was detected across the entire foregut (Figure 7.2 B). Although extended Nkx2.1 domains have not been found in the AMM (Ioannides et al., 2010, Hajduk et al., 2011b), other models of abnormal foregut development have reported ventralisation of the entire foregut endoderm illustrated by abnormal Nkx2.1 localisation. This molecular analysis in Bmp4 treated foreguts confirms that BMP signal can directly regulate DV patterning in the foregut.

Co-localisation of Sox2 and Nkx2.1 was detected in the DV patterning analysis of Bmp4 treated foreguts. This prompted a high resolution analysis of the cellular localisation of transcription factors Sox2 and Nkx2.1 revealing key features of altered cell fate identity response to BMP treatment. This was highlighted in the abnormal subcellular localisation of Sox2 observed by Confocal imaging. Regions of the anterior foregut displayed abnormal domains of Nkx2.1 localising to the nucleus, together with a highly unusual pattern of Sox2 localising outside the nucleus to the basal cell cytoplasm. These findings occurred along with occasional nuclei exhibiting co-localisation of Sox2 and Nkx2.1, also a highly unusual occurrence. As discussed in Chapter 6, it appears that active change in cell fate identity from dorsal to ventral in response to Bmp4 treatment has been visualised in this analysis (Figure 7.2 C). These data show that at E9 re-specification of foregut endoderm cell fate identity is possible and occurs in response to Bmp4. The resulting abnormal expansion of ventral identity to the entire foregut endoderm may be a major pathological mechanism in OA/TOF.

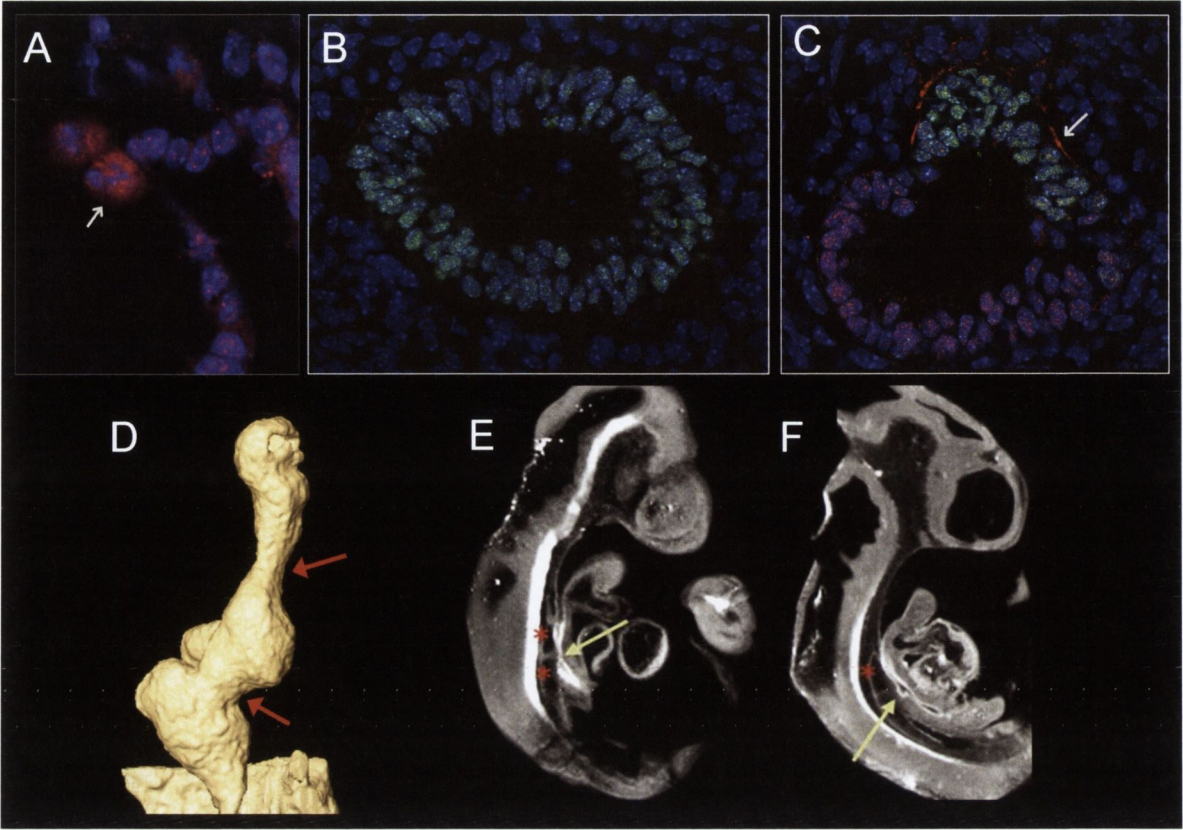


Figure 7.2 Comparison of molecular and morphological properties of the two model systems used in this study; Bmp4 treated foregut explants and the AMM. B, C and D are Bmp4 treated and A, E and F are AMM. A is a confocal image of pSMAD (red) and DAPI. B and C are confocal microscopy images of Sox2 (red), Nkx2.1 (green) and DAPI (DNA/nuclei). Arrows reflect sites of abnormal marker extra nuclear localisation for both pSMAD in A and Sox2 in C. OPT images following Hnf3b immunolocalisation highlight features of BMP4 treatment on explanted foreguts (D) and of Adriamycin treatment *in vivo* (E and F). The upper arrow in D indicates stenosis, similar to the phenotype indicated by the green arrow in E. The lower arrow in D indicates abnormal communication between the stomach and respiratory structures, similar to the phenotype indicated by the green arrow in F (cf. Chapter 3 for A, Chapter 6 for B,C,D and adapted from Hadjuk et al,2011 for E and F). Asterisk marks notochord branching.

Bmp4 treated foreguts reproduce many of the morphological features of AMM foregut abnormalities, as detailed in chapter 6 and summarised in Figure 7.2. In particular the failure of oesophageal development together with extreme narrowing of the anterior foregut and the existence of abnormal communication between the stomach and respiratory structures reflect an OA phenotype in both the AMM and Bmp4 treated foreguts (Figure 7.2 D to F). In a small number of older gestational stage foreguts, explanted at 30 somites, Bmp4 treatment did not induce a morphological response. This indicates the existence of an abrupt cut off point in the ability of the foregut to respond to the potent morphogenic effects of Bmp4. It would be interesting to extend analysis of older explants to establish a clear timeline of cell fate responses to Bmp4. If confirmed, this might be extrapolated to the human condition suggesting a window of susceptibility to OA//TOF. The precise

identification of the limits of this period of vulnerability may provide a clinically relevant time frame in which exogenous treatments may be applied. Likewise, identification of the time of onset of a period of vulnerability would be important and may be divined from further work on animal models such as the AMM and/or modified *in vitro* culture systems.

Importance of correct balance of BMP signal for foregut separation is highlighted in the results from both the AMM and Bmp4 treatment of foreguts. Further characterisations of BMP signalling in the foregut could be performed by molecular manipulations in foregut explants to examine interactions with other key developmental pathways.

Shh has been reported to be required upstream of Bmp ligands in foregut derived lung bud morphogenesis (Pepicelli et al., 1998) and is expressed across the foregut in a DV restriction pattern indicating the ventral foregut tissues. The role of abnormal Shh expression in the AMM notochord and foregut (Hajduk et al., 2011b) is uncertain given that the data presented in Chapter 6 clearly show that Bmp4 treatment is sufficient to induce structural and molecular foregut defects. The analysis of Shh localisation in Bmp4 treated explants could determine if Shh levels in the foregut are susceptible to Bmp4. Alternatively the morphological and molecular response of the foregut to Shh protein treatment, as described in more detail below, could explore whether BMP and SHH signalling are interdependent in foregut development.

Further characterisation of BMP activity in foregut morphogenesis could also be performed. The examination of the effect of other Bmp ligands and in particular Bmp7 would be of interest, as Bmp7 is expressed in foregut endoderm (Danesh et al., 2009) and Bmp7 gene dose, similar to Bmp4, can modulate the phenotype of the Noggin knockout mouse (Li et al., 2007). In order to examine the effect of BMP activity attenuation in the foregut, BMP antagonist Noggin was used to treat foregut explants but was not shown to have any effect. Other means of inhibiting BMP activity such as small molecule inhibitor Dorsomorphin could be alternatively applied in culture to both examine the effect of reduced BMP activity as well as to attempt rescue treatment in Bmp4 exposed specimens (Yu et al., 2008). This rescue approach could then be used to determine if the influence of other molecular players in foregut separation depend on BMP signalling over activity.

The failure of Noggin treatment to affect foregut separation is interesting given of the presence of foregut malformations in models with genetically reduced BMP activity (Li et al., 2008b). This may reflect temporo-spatial limits of foregut cell susceptibility to Noggin activity. A fascinating extrapolation of these findings may be that Noggin is not able to act directly on the foregut but has a special relationship instead with the notochord

which has not been tested in these data. In fact the *Noggin* gene has previously been shown to influence foregut morphogenesis at the notochord and not at the foregut using spatial limits of gene inactivation (Fausett et al., 2014).

7.3 The role of the notochord in the pathogenesis of OA/TOF

The nature of the relationship between the foregut and adjacent notochord during embryogenesis is an abiding mystery in developmental biology. In this thesis I have endeavoured to address this important question. Notochord defects are characteristic of both the AMM and *Noggin* knockout models of OA/TOF (Que et al., 2006, Possoegel et al., 1999). Notochord tissue remains abnormally ventral and in prolonged contact with the dorsal foregut endoderm, appearing to branch across the intervening mesoderm from the notochord rod (Hajduk et al., 2012). Notochord and foregut abnormalities also occur together in the Patch mutant mouse for *Pdgrf* gene (Snider et al., 2011). Accordingly notochord derived signalling may be involved in the pathogenesis of foregut malformations.

The notochord has been shown to signal to the foregut to allow morphogenesis of the pancreas (Kim et al., 1997) but despite the tantalising correspondence between ectopic notochord and foregut malformations in OA/TOF, it has not been directly shown to influence foregut separation into oesophagus and trachea. The potent ability of the notochord to provide DV patterning instructions and so alter the morphology of other adjacent tissues such as the neural tube and somites, has however been shown using notochord manipulation *in vitro* (van Straaten et al., 1985). The observed ventralisation of the lateral neural tube in the presence of a notochord transplant was subsequently discovered to be mediated by *Shh* signal from the notochord (Echelard et al., 1993). Foregut morphogenesis has been shown to be influenced by *Shh*, both when exogenous protein was applied, affecting pancreatic formation (Kim and Hebrok, 2001), and when the *Shh* gene was knocked out, resulting in tracheo-oesophageal malformations (Litington et al., 1998).

The requirement for DV patterning is a recurrent theme in organogenesis. In the foregut correct DV patterning is essential to designate tissue domains for subsequent dorsal oesophagus and ventral trachea formation as exemplified by the mutually exclusive domains of *Sox2* (dorsal) and *Nkx2.1* (ventral) maintained throughout the dividing anterior

foregut (as reviewed by Jacobs et al., 2012). Given the anatomical proximity of the notochord and the foregut, a suggestion naturally follows that powerful notochord signals could contribute to DV foregut patterning.

Altered DV patterning of the developing foregut has been shown to occur in models of OA/TOF. Markers such as Shh and Sox2 exhibit regionalised expression within the foregut illustrating DV tissue identity (Que et al., 2007, Sato et al., 2008). In the AMM disruption of Shh and Sox2 were seen to occur in areas of the foregut with directly adjacent notochord branches (Hajduk et al., 2011b). In this work I have shown that BMP response across the foregut endoderm also exhibits DV restriction as reflected by ventral pSMAD localisation (Chapter 3). Bmp4 has been shown to be a ventralising morphogen in other tissues (De Robertis and Kuroda, 2004) and is expressed in the ventral foregut mesoderm (Danesh et al., 2009) and so may contribute essentially to ventral prominence of pSMAD. I have found that this pattern of BMP activity is disrupted precisely adjacent to notochord branches in the AMM, by identifying highly focal areas of intense pSMAD signal as well as abnormal expression of foregut specific BMP target genes such as Id1 and JunB at these sites in the dorsal foregut endoderm (Figure 7.1). These results support the hypothesis that patterning disturbances in the AMM are induced by the abnormally adjacent and structurally deformed notochord, as well as suggesting that BMP signalling may be involved in mediating these effects. Further evidence of a role for BMP signalling from the notochord in foregut morphogenesis has been identified by conditional inactivation of BMP antagonist Noggin or conditional over activation of BMP receptor Bmpr1a limited to the notochord and neural floor plate (Fausett et al., 2014). These manipulations permitted excess BMP activity only in the notochord and neural floor plate but nonetheless resulted in foregut malformations in association with notochord branching. Similarly control of BMP activity by Noggin from the notochord has been shown to be important for the development of the neural tube and somites (McMahon et al., 1998).

In order to examine the role of the notochord in molecular control of foregut morphogenesis I performed notochord manipulation in foregut explant culture, which supports foregut separation *in vitro*, drawing inspiration from the classical developmental *in vitro* approaches used to prove that notochord signalling influences neural tube morphogenesis (van Straaten et al., 1985). These experiments were designed to test the hypothesis that notochord signalling contributes essentially to foregut separation. I found that removal of the notochord did not impact separation of the foregut when explanted at E9. Furthermore, in order to mimic the notochord branching defect seen in the AMM., additional notochord was transplanted to the dorsal aspect of the anterior foregut endoderm.

The foregut continued to divide normally in the presence of excess notochord and exhibited normal dorso-ventral patterning as illustrated by the persistence of Sox2 localisation in the dorsal foregut endoderm.

Although these results are subject to gestational limits that do not explore the relationship between the notochord and foregut prior to the 18 somite pair stage (E9), they show that foregut separation does not require the presence of the notochord from this stage onwards. It appears that foregut separation can proceed autonomously from this time. They further revealed that, while the notochord may signal to the foregut, the nature of these signals were not powerful enough from the excess notochord to induce disruption of foregut morphology or molecular patterning at E9. In particular the normal pattern of Sox2 in foreguts with additional notochord transplants contrasts to the abnormalities of Sox2 detected in the AMM at a similar gestational stage (E10 to E11) (Hajduk et al., 2011b). Despite close proximity, notochord defects may not be responsible for abnormalities of dorso-ventral patterning detected in the AMM.

Further experiments in foregut explants treated by recombinant proteins as detailed in Chapter 6 show that, while isolated E9 foregut is capable of forming oesophagus and trachea without the influence of adjacent structures, it is still vulnerable to exogenous signals. The dorso-ventral identity of foregut endoderm cells and the outcome of foregut separation were both susceptible to treatment with Bmp4 at this stage. Treatment with excess notochord tissue was not able to recapitulate these effects, drawing into question the purported association between altered BMP activity and notochord in the pathogenesis of foregut malformations. Together these data suggest that notochord branching may arise as an effect rather than acting as a source of abnormal signals that induce foregut malformations.

Notochord may however act on the foregut at earlier gestations and this could be the subject of future experiments. I have found that mouse notochord is difficult to manipulate at E9 in comparison to other tissues at that stage and also I have reported that the foregut explant culture system does not support foregut development prior to E9. In ovo or ex ovo chick embryonic culture may provide a more feasible system to test the response of the foregut to notochord manipulations at earlier embryonic stages. The notochord in chicks is widely reported to be amenable to ablation or further manipulations without sub-dissection and subsequent foregut development could be maintained in near-natural environs (van Straaten and Hekking, 1991).

The notochord in the AMM can be characterised by abnormalities of shape, position (in relation to the foregut) and molecular patterning that cannot be fully reproduced using

notochords obtained from normal embryos transplanted into culture with foreguts. Future experiments are envisioned to be possible involving the co-culture of notochord from AMM embryos with isolated foregut from normal embryos which may better mimic the conditions of Adriamycin treatment *in vivo*. However given the difficult nature of notochord transplantation in mouse embryos as previously alluded to, it would be more feasible and perhaps more direct to manipulate the molecular rather than the physical foregut environment to better understand the impact of notochord to foregut signalling. Shh is the immediately apparent choice for a factor that could act as a proxy for notochord tissue excess, especially given that Shh is over expressed in notochord branches in the AMM (Hajduk et al., 2011b).

Isolated 3D foregut explant culture provides a system that allows manipulation of the foregut signalling environment using diffusible factors and offers a range of effective outcome measures including the morphological staging system developed as reported in Chapter 5. The localised delivery of protein by bead to the foregut used in Chapter 6 was an effective method of signal manipulation and could in future be reprised using recombinant Shh protein. This may better mimic the nature of the highly abnormal notochord in the AMM and will also examine if Shh can directly influence morphogenesis in foregut separation.

Alternatively the direct treatment of isolated foregut explants with Adriamycin may reproduce the *in vivo* effects of this agent in culture. The tissue site where Adriamycin exerts its teratogenic effects remains unknown. *In vivo* treatment is administered at E7 and E8 (Dawrant et al., 2007a), at which gestational stages the notochord and foregut are intimately associated in terms of position and the molecular signalling relationship between these structures is unclear. It was shown that abnormal notochord derived BMP activity must occur prior to the stage of notochord delamination (E7.5) in order to induce foregut malformations in the Noggin knockout mouse by using temporal gene inactivation (Fausett et al., 2014). It remains to be determined whether Adriamycin acts broadly across embryonic tissues or requires the presence of the notochord or other developmentally important signalling centres to mediate its effects. The key question which could be addressed in future work is whether Adriamycin can directly act on the foregut to induce molecular and morphological disturbances. Explant culture, by allowing the disassociation of these structures, provides the means to examine this question. Although explant culture does not support foregut explants obtained from embryos prior to E8.5, foreguts from E9 are susceptible to morphogenic signals, as illustrated by the response to Bmp4 treatment reported on in Chapter 6. These experiments may elaborate the role of the notochord in the

AMM as either required in the induction of foregut malformations or as merely a co-existing anomaly.

7.4 A future approach for the systematic identification of molecular factors relevant to foregut development.

The characterisation of the molecular and cellular mechanisms by which normal foregut separation occurs remains incomplete. Often models of disease can offer insight regarding normal mechanisms of organogenesis when a specific facet can be targeted, which may then reflect a crucial role for that facet in the outcome following manipulation. This was demonstrated when the Apaf1 mutant mouse, which is genetically programmed to lack apoptosis, was found to exhibit normal foregut separation, discounting a prevailing theory that apoptosis is required for the foregut to separate normally (Ioannides et al., 2010). Other models such as the AMM provide an opportunity to broaden our understanding of normal foregut development as well as the pathogenesis of tracheo-oesophageal malformations. Although a small proportion of human OA cases results from various single gene mutations, a multi-genetic type of aetiology has been proposed following characterisation of the genetics of OA in humans (Brunner and van Bokhoven, 2005). The recapitulation of OA associated single gene defects in animal models, although highly important, does not offer new information about the aetiology of OA/TOF. Remarkable phenotypical correlation occurs between of the spectrum of deformities that occur in the AMM to the human VACTERL condition, which includes 20% of all cases of OA/TOF (as reviewed by Mc Laughlin et al., 2013). These broad effects suggest that Adriamycin treatment in mice may provide insight into which signals are crucial across many developing tissues and in particular the foregut, and could provide new information about the aetiology of OA/TOF to be pursued in humans.

In teratogenic models however, in accordance with a broad morphological response, extensive molecular disturbances may be found across many developmentally important pathways, the challenge being to separate out which are the important signals. I have established an effective method of investigating the morphological relevance of specific molecular signals in the foregut. The identification of abnormal BMP signalling in the AMM was followed by treatment of isolated foregut explants with Bmp4 and Noggin, ultimately leading to the conclusion that Bmp4 is a potent morphogen in foregut development. This combination of *in vivo* and *in vitro* approaches now provides a unique opportunity to identify which are the important molecular cues for foregut division, by the

detection of abnormal signals in the AMM, and subsequent characterisation of the role of these signals in a 3D culture system which supports oesophagus and trachea development from undivided foregut.

This might be performed on a much wider scale than reported in these initial experiments. The micro-dissection of the AMM foregut at E9 is feasible using the methodology described for isolation of foreguts for culture. At the level of the notochord branch, which is visible in micro dissected specimens (Figure 7.3) extensive molecular disturbances have been reported in the foregut in this work and previously by our group, suggesting that this site may be an ideal target zone for further molecular analysis. This tissue could be subjected to RNA sequencing in order to generate lists of differentially regulated genes following Adriamycin treatment compared to staged matched controls. Refinement of these lists by gene ontology and other analyses could identify candidates for investigation in isolated foregut explants at a protein signalling level. This approach provides a systematic methodology to identify and characterise signalling factors relevant to foregut development.

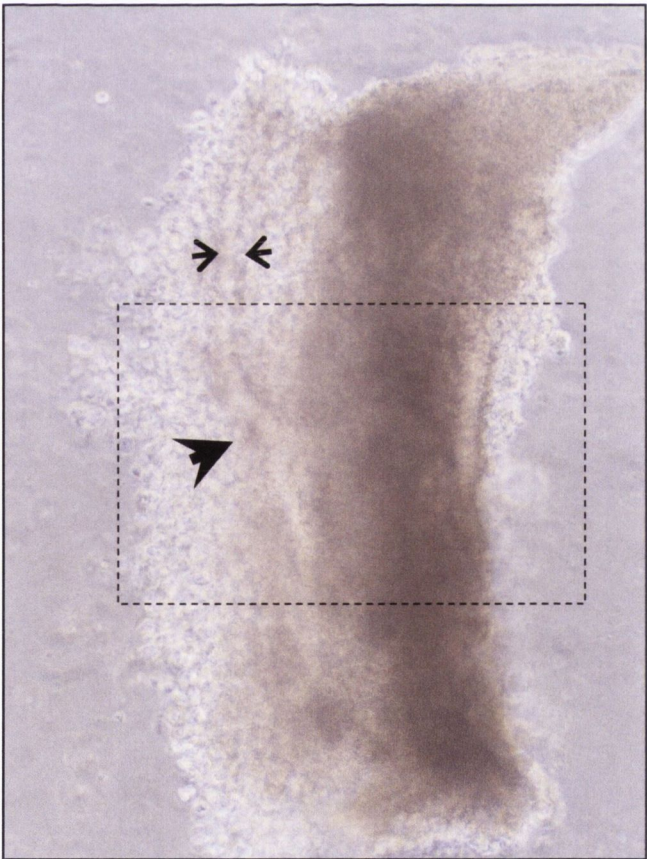


Figure 5.3 Micro-dissection of the AMM foregut at E9. Photomicrograph of foregut obtained from an E9 AMM embryo. The notochord is indicated by the converging arrows and the site of notochord branching by the arrowhead. The proposed region for further subdissection is boxed.

Another key output of this research is the establishment of methods by which the outcome of manipulations in explant culture can be assessed. In particular the production of a morphological grading system based on the anatomical properties of dividing foregut *in vitro* across E9 to E11 allowed a qualitative measure of normal development to be applied to foregut explant specimens which will also be useful to assess the outcome in future experiments and could be applied across a variety of experimental approaches to the investigation of foregut development. I also found that previously described techniques of surface render 3D reconstruction of OPT scans together with Hnf3b immunolocalisation in whole specimens as well as double immunohistochemistry for Sox2 and Nkx2.1 together with confocal microscopy were effective approaches to demonstrate morphological and molecular features of foregut division in explants.

7.5 Conclusion

The work presented in this thesis illustrates the effective use of complimentary *in vivo* and *in vitro* approaches to examine the molecular basis of foregut malformations. Findings in the well established Adriamycin mouse model implicated BMP signalling and notochord defects in the pathogenesis of OA, which were subsequently examined *in vitro* using physical and molecular manipulations in a newly established model which allows foregut separation in 3D explant culture. This combination of approaches led to the conclusion that overactive BMP signalling, and in particular Bmp4, can induce an OA/TOF phenotype and importantly that foregut endoderm cell re-specification from dorsal to ventral fate by molecular signals is an important pathogenic mechanism in the development of tracheo-oesophageal malformations.

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Appendix A

Protocol for isolation of foreguts

Foregut explants are prepared by microdissection of the entire and intact foregut from the trunk of Cd1 E9 embryos which have been accurately staged by somite counting. They consist of foregut endoderm and the well demarcated ventral foregut mesoderm together with a variable extent of the dorsal foregut mesoderm with or without related structures as required. The foregut explant extends anteriorly to just below the branchial arches and posteriorly to the lower limit of the upper limb bud/protrusion. Undivided foreguts can be shelled out of these early embryos by careful peeling away of loosely attached ventral and lateral ectoderm using fine tipped forceps (using the heart to stabilise the embryo during dissection) with additional blunt dissection using tungsten needles through the dorsal mesoderm tissue and the heart. The technique for foregut isolation is the culmination of 24 months of optimisation. A high level of competency in the general handling of E9 embryos was well established, as were finer dissection skills from experiments requiring the isolation of notochords as well as creating whole thorax explants, prior to the foregut isolation technique being devised. An intimate knowledge of E9 embryonic anatomy with particular familiarity with the foregut and its relationship to adjacent structures is a prerequisite for attempting these micro-dissections. It is hoped that this in-depth description of the technique will expedite the learning curve for future work. A staged dissection approach has been optimised as follows:

Materials

- Fine tip forceps (0.07x0.04 mm) x 2
- A bluntish tungsten needle with a curve approximating that of the dorsal embryo shape, in a flamed glass pipette holder was used. Not the micro tungstens (too sharp and not enough give).
- Instruments cleaned in 100% ethanol and air dried under hood prior.
- Forceps and tungsten needle need to be kept well clean while proceeding, I pinch and or wipe on the back of my glove (sterilised with ethanol at start).
- Medium (DMEM/F12 1:1), Fetal Bovine Serum
- 6cm plates for dissection

General comments/recommendations to start

- This description is for a right handed person. If left handed proceed exchanging left for right and vice versa at all stages or as desired.
- At all points herein the “embryo” refers to the tissue that is being maintained for eventual explant. The use of the term explant after removal of the dorsal mesoderm is arbitrary.
- Waste tissue should be removed from dish on an ongoing basis as can be (and have been) confused with eventual explant.
- Unless otherwise stated the embryo position is anterior (head) end pointing away from operator.
- Dissection time is 15 to 20mins per explant with practice (5 sessions minimum are predicted to be needed from a beginner skill level).
- No coffee before but plenty of water and jellies on hand
- Dissection dishes should be changed as needed for visibility. Ensure medium level completely covers all embryos. Inadequate volume causes the explant to move excessively when the medium is agitated by the instruments.
- Harvested E9 embryos are kept in a covered 6cm dish in medium *without* added FBS at room conditions while awaiting explant. These have also been maintained in the Co2 incubator at 37c while awaiting explantation but this softens the tissue causing increased difficulty in dissection without apparent improvement in viability.
- Dissections are performed in medium *with* FBS added for reduced tissue stickiness.
- Do not run the hood while dissecting (dries out everything). Have all surfaces sterilised with 100% ethanol and well dried by running hood for 1 hour before commencing dissection.
- Maintain good ergonomics while dissecting i.e. stay comfortable, optimise visualisation, rest elbows on surface etc. It is also highly useful to be ambidextrous if possible.
- The most important steps are summarised in bold within the text.

Technique

1. Dissect away all extra-embryonic tissues thoroughly. It is best to do this while harvesting for an accurate assessment of the available embryonic material.

2. **Somite counting:** For every explant somites are carefully counted for allocation to rounded somite groups as follows: 15 (15 to 17) (youngest stage feasible to dissect), 18 (18 and 19), 20 (20 and 21), 22 (22 to 24), 25 (25 to 27), 28 (28 and 29), 30 (oldest stage used). Explants are allocated to somite number labelled 3cm dishes following dissection and while awaiting embedding.
3. **Remove tail:** Keep embryo lying laterally with forceps holding head and firmly press down immediately below the upper limb bud against the bottom of the plate with the length of a curved tungsten needle until the tissue is cleanly cut (may require a bit of sawing motion while pressing down). Discard tail.
4. **Open the *entire* neural tube** in the dorsal midline using the tip of a tungsten needle while stabilising the embryo by holding the head.
5. Remove the head, again with tungsten needle pressure, just anterior to the otic vesicle and discard.
6. **Remove the left (embryos left) neural tube:** This is best done with a tungsten needle that is curved approximating the shape of the embryo dorsally. Hold onto the neural tube that is being removed with a fine tipped forceps (ectoderm gives better grip) and settle the curve of the needle against the ventral interior of the neural tube that has been split. Press down to cut the tissue, avoiding damage to the neural floor plate but also getting as much of the lateral neural tube flap along with the somites off as possible – this will aid in the later dissection. This is usually done in 2 stages (anterior first and then posterior to get good clearance. Be careful posteriorly as the foregut can be damaged. The posterior neural floor plate and notochord are quite distant from the foregut wall at this point and are often inadvertently removed here. This does not impact our area of interest.
7. For the subsequent stages it is important to have an anchoring point to stabilise the embryo. The heart will ultimately be discarded so it functions well in this role. The aorta is the most robust tissue for this but gradual damage will be inflicted requiring shifts in holding points to various areas in the heart as the dissection progresses.
8. Lay the embryo on its right side. Visualise the space between the lateral ectoderm and the foregut that can be accessed at the limb bud protrusion through the cut posterior end of the embryo. Use fine tipped forceps to hold embryo in place by the heart. Use the tip of a bluntish tungsten needle to **detach left lateral ectoderm**, starting at the dorsal aspect of the limb bud proceeding anteriorly along the embryo and with a left to right directionality of movement with the needle, **creating a flap** that exposes the underlying foregut and its dorsal mesoderm. In the most proximal

regions the lateral foregut wall can be easily damaged. A flap to just below the branchial arches will suffice and usually avoids damage.

9. **Remove this flap** by tungsten needle cut at its ventral attachment while pulling the free edge away from the embryo which can turn onto its ventral aspect to facilitate this. It is very helpful to use tension to aid in the neat removal of tissues. Pulling of the tissue that is to be removed with the forceps in the left hand, while restraining the explant with the tungsten needle which is positioned at the site of the intended cut with the right hand, will provide this tension while cutting.
10. Settle the embryo back onto its right side. The foregut shape is now visible. **Visualise the dorsal mesoderm** between the neural floor plate and the dorsal foregut wall. The notochord is visible in this space on careful inspection. The next steps depend on the intended plan for the notochord:
 - a. Maintain notochord with foregut. This is difficult. It can be attempted by dissecting between the notochord and the closely adjacent neural floor plate. However the only way to guarantee completely intact notochord and continue the dissection in a timely manner (referring to my own experience) is to keep the neural floor plate with the notochord in the explant.
 - b. Foregut culture without notochord. The notochord can be removed with the remaining neural tube by dissecting between it and the foregut as below. This part of the neural tube can be used for future notochord retrieval if desired and can be put to one side in medium. Our results to date indicate that the notochord presence does not impact on foregut division morphology or patterning at this stage therefore this is the preferred and most amenable approach for the majority of experiments.
11. **Remove the dorsal mesoderm and tissues:** Stabilise the embryo on its right side. Bluntly dissect in small increments using the tip of a bluntish tungsten needle from posterior to anterior along your selected track either between the foregut and notochord or between the notochord and neural floor plate or immediately dorsal to the neural floor plate, up to the posterior extent of the branchial arches. Remove as much of the right sided somites in this dissection as possible.
12. Make a transverse cut at the proximal extent of the dissection to this point just below the level of the branchial arches with tungsten needle pressure and discard the tissue.

13. **Create and remove a right sided ectoderm flap:** Flip the explant onto its left side with its anterior end now pointing towards you. As before a flap can be created between the foregut and the ectoderm (see and follow steps 8 and 9).
14. We are now left with foregut and heart. Any cardiac tissue left in the explant will beat progressively stronger with time and form a substantial cystic structure. The explanted foregut may be vulnerable to mechanical pressure which exceeds what it experiences in vivo due to the confined nature of the 3D culture. We have achieved better morphological results by **removing the heart**. All cardiac tissue should be grasped by a forceps and removed from the foregut by tungsten needle pressure. Any remaining tissues can be removed by careful piecemeal dissection to result in an isolated foregut explant as shown in picture.

General comments to finish

- The foregut should not be handled by forceps at any point in the dissection.
- Explant transfers are done (very gently – high attrition rate initially) using a medium pre-wetted borosilicate capillary lumen 0.86mm or similar bore (fresh one for each session) and mouth pipette. A dry run of transfer (i.e. up and down a few times) in the holding well will help avoid the explant sticking to the capillary when transferring for embedding. This is especially recommended in explants that have been in medium for a few hours.
- Dissected foreguts have been kept up to a maximum of 3 hours in medium (with added FBS) at room conditions (27°C) without impact on viability (but not assessed for longer than this). In general a successful dissection session by a single operator can produce 8 to 10 explants ready for embedding. Completing dissection before commencing embedding is highly recommended to allow clarity of thought and process and resulted in fewer errors.
- The remaining embryos (for Cd1 mice anywhere from 12 to 20 embryos per litter can be expected) can be staged by somite counting and stored as controls after 3 hours holding in medium at room conditions. Do not select out controls before completing required dissections in case of disaster or good stamina.
- I would not recommend more than one session of dissection per day. I would recommend scheduling explant dissections for 2 days in a row to have skills well honed and plan the most important experiments for the second day. More than 2 days in a row however does not refine skills any further and can induce fatigue and subsequently error.

Appendix B: Figures

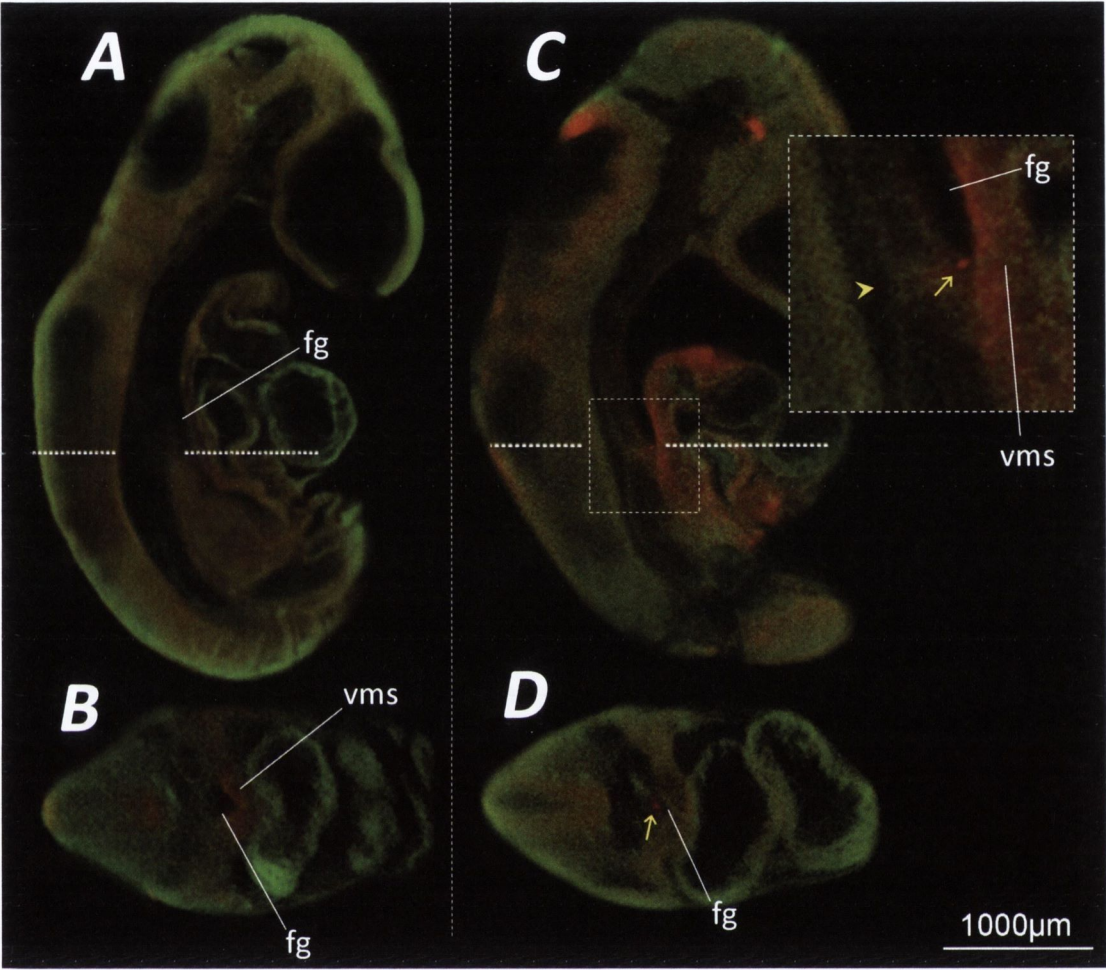


Figure 1: Expression of Id2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Id2 ISH in embryos at E9 in control (A,B) and Adriamycin treated (C,D). Dashed lines indicate planes of transverse and coronal section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows highlight abnormal gene expression. In control white arrows indicate normal notochord position. *vms* ventral mesoderm, *fg* foregut, Scale bar as shown.

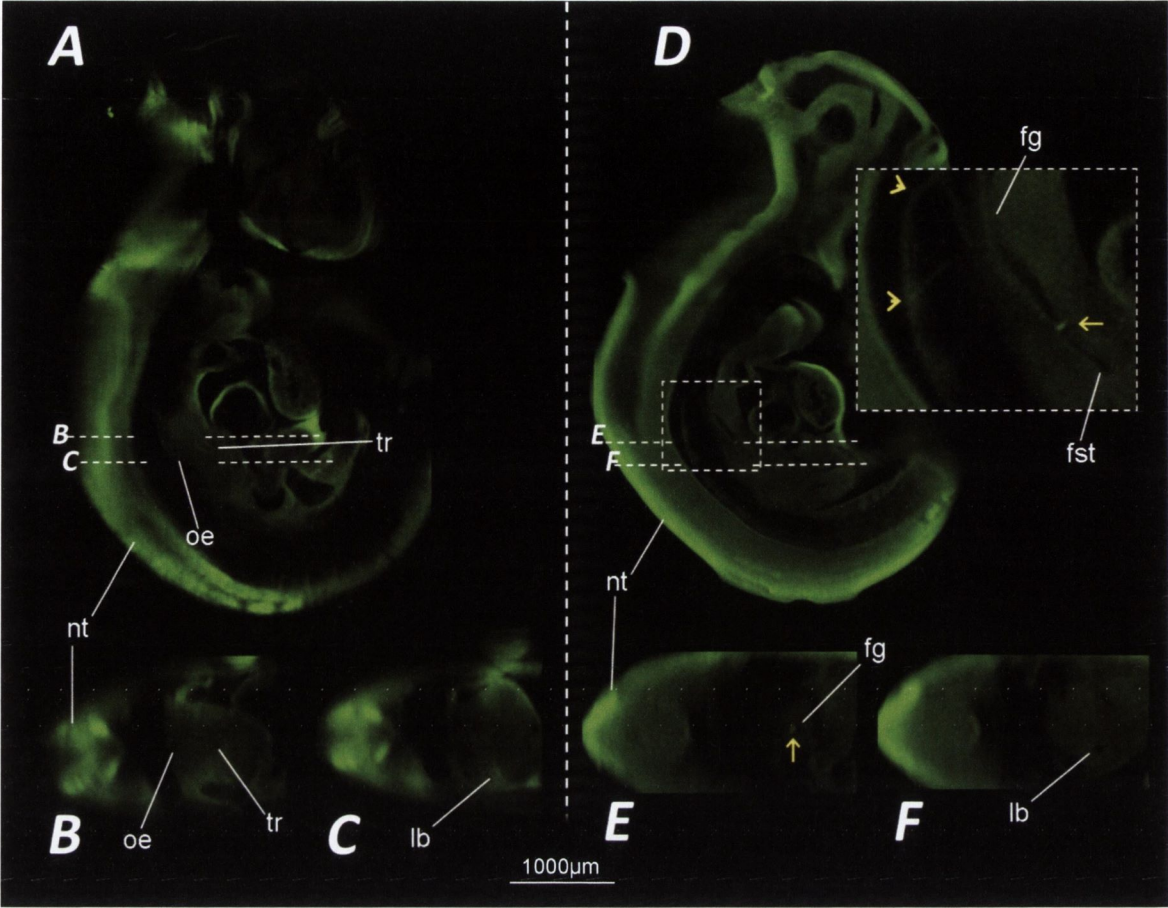


Figure 2: Expression of Dpysl2 in AMM embryos. Virtual sections of 3D reconstructed OPT scans of Dpysl2 ISH in embryos at E11 in control (A,B,C) and Adriamycin treated (D,E,F). Dashed lines indicate planes of transverse and coronal section from the main sagittal image, boxed region is magnified as shown. In AMM yellow arrowheads indicate notochord branching, yellow arrows highlight abnormal gene expression. *nt* neural tube, *tr* trachea, *oe* oesophagus, *lb* lung bud, *fst* fistula, Scale bar as shown.



Figure 3: Further morphological features of foregut explants. Hnf3b immunolocalisation, OPT scan and surface render in isolated foregut explants put into culture at 20 somites and for 48 hours in A, at 20 somites and for 72 hours in B and at 30 somites and for 48 hours in C. A has a morphological variant of a single lung bud (lb), note the lack of a liver (lv) domain. B and C are staged as TS18 (cf. Figure 5.3). B demonstrates kinking of the structures due to restriction of expansion space. Observe the shape of the lung buds in B and C showing evidence of the onset of branching morphogenesis. Scale bar = 100µm

