

**THE ROLE OF ABNORMAL
HAEMODYNAMICS AND CARDIAC
TROPONIN T IN CARIOGENESIS**



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Abstract

The heart is the first functioning organ to develop during embryogenesis to maintain the growing embryo with oxygen and nutrients. However, cardiogenesis is a complex and highly coordinated biological process, and any perturbation to this process can result in detrimental defects to the heart. Haemodynamics is known to play an important role in cardiac growth and vasculature remodelling. Congenital heart defects (CHDs) accounts for 0.4-1.3% of all live birth, whereas cardiomyopathy accounts for 8-11% of cardiovascular disease diagnoses detected in utero. Although the heart defects and cardiomyopathies are known to be attributed by genetic mutations, most cases have unknown etiology.

Hence, OFT-banding model was employed to alter the haemodynamic loading via pressure overloading. Upon alteration of haemodynamics, enlargement of the heart with a spectrum of cardiac anomalies were found (e.g ventricular septal defects, thickened epicardium and dysmorphic atrioventricular valves) upon morphological and stereological analysis. A study of global differential expression of OFT-banded hearts by RNA sequencing revealed a number of differentially expressed genes and they were associated with cardioprotection, metabolism, shear stress and valve development; further, a reduction of apoptosis was seen in these banded hearts as well.

One of the cardiac phenotypes seen upon OFT-banding, the abnormal primordial atrioventricular valve, was further characterized to provide an insight how the atrioventricular valve is affected upon alteration of haemodynamics. Aberrant expressions of extracellular matrix (ECM) genes such as *TBX20*, Aggrecan and Periostin alongside with the shear stress responsive genes (*KLF2* and *EDN1*) were found, and a decrease in apoptosis was seen. Moreover, dysregulation of ECM proteins

such as fibrillin-2, type III collagen and tenascin were further demonstrated in more mature primordial AV leaflets at HH35, with a concomitant decrease of ECM cross-linking enzyme, transglutaminase-2.

In addition, for many years sarcomeric proteins have been associated with a range of cardiomyopathies, but only in recent years they have been linked to congenital heart defects (CHDs). To date, cardiac troponin T (*TNNT2*) has been associated with cardiomyopathies but not with isolated CHDs. *TNNT2* encodes for cTnT regulatory proteins of the thin filament of the sarcomere and is vital for muscle contraction and force generation within cardiomyocytes. To investigate a role of *TNNT2* in the early developing heart, targeted manipulation of *TNNT2* was performed in embryonic chick to reduce the protein levels of cTNT (protein product of *TNNT2*) *in ovo* via translational block. Abnormal atrial septal growth, reduced ventricular trabeculation and ventricular diverticula were found upon *TNNT2* morpholino treatment. The abnormal phenotype observed in the *TNNT2* morpholino-treated groups was potentially suggested by differential expression of shear stress responsive gene, *NOS3* gene.

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Declaration

The RNA sequencing project was performed by Dr Sunir Malla, Dr Fei Sang from Deep Sequencing Department, School of Life Sciences, University of Nottingham and was in collaboration with Matthew Parnall (fellow Ph.D student). In *TNNT2* morpholino knockdown project, the total number of experimental and control embryos included for phenotypic analysis of 250µM and 500µM studies was based on the collaborative work with Jennifer England (former Ph.D student). In addition, the demonstration of *TNNT2* morpholino knockdown by western blotting performed by Jennifer England will be included and discussed in this thesis.

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Abbreviations:

ACTA1	Actin, alpha-1, skeletal muscle
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APES	3-aminopropyltriethoxysilane
AP	Anti-digoxigenin-alkaline phosphatase
ASD	Atrial septal defect
ATP	Adenosine triphosphate
AV	Atrioventricular
AVC	Atrioventricular canal
AVSD	Atrioventricular septal defect
ARVC	Arrhythmogenic right ventricular cardiomyopathy
BSA	Bovine serum albumin
CHD	Congenital heart defect
CCD	Congenital cardiac diverticulum
cDNA	Complementary deoxyribonucleic acid
DAB	3,3 diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCM	Dilated cardiomyopathy
DEPC	Diethylpyrocarbonate
DMP	Dorsal mesenchymal protrusion
DORV	Double outlet right ventricle
DSHB	Developmental society hybridoma bank
dNTPs	Deoxynucleotide mix
EC	Endocardial cushions

ECM	Extracellular matrix
EDN1	Endothelin-1
EMT	Epithelial-mesenchymal-transformation
EtBr	Ethidium bromide
EPDCs	Epicardium-derived cells
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBX2	Gastrulation brain homeobox 2
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
HH	Hamburger and Hamilton
HCM	Hypertrophic cardiomyopathy
IFT	Inflow tract
ISH	In situ hybridization
ifcSEM	Standard error of mean
IVS	Intraventricular septum
KLF2	Kruppel lung factor-2
LA	Left atria
LAL	Left atrial ligation
LB	Luria broth
LDHB	Lactate dehydrogenase
LGALS1	Galectin-1
LV	Left ventricle
LVNC	Left ventricular non-compaction cardiomyopathy

MAPQ	Mapping quality score
MABTw	Maleic acid buffer containing Tween-20
MHC	Myosin heavy chain
MMPs	Matrix metalloproteases
MV	Mitral valves
MYH7	β -myosin heavy chain
Nkx2.5	Homeobox protein 2.5
NPPB	Atrial natriuretic peptide
OFT	Outflow tract
PAA	Pharyngeal arch arteries
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Tween
PCA	Principal component analysis
PC1	Principal component 1
PC2	Principal component 2
PCR	End point polymerase chain reaction
PDCD4	Programmed cell death 4
PFA	Paraformaldehyde
PHF	Primary heart field
PLN	Phospholamban
PTA	Persistent truncus arteriosus
qPCR	Real time quantitative PCR
RA	Right atrium
RNA	Ribonucleic acid
RPKM	Reads Per Kilobase of transcript per Million mapped reads (RPKM)

RT	Room temperature
ROI	Region of interest
RV	Right ventricle
SC	Standard control
SSC	Saline sodium citrate buffer
SOC	Super optimal broth
S100A11	S100 Calcium Binding Protein A11 gene
TAE	1X-Tris-acetate-ethylenediaminetetraacetic acid
Tbx5	T-box 5
TBX20	T-Box 20
TdT	Terminal deoxynucleotidyl transferase
TIMPs	Tissue inhibitors of matrix and is metalloproteases
TGM2	Transglutaminase-2
TNNC2	Troponin C2, fast skeletal type
TNNT2-MO	TNNT2 morpholino
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
TOF	Tetralogy of fallot
TV	Tricuspid valves
VSD	Ventricular septal defect
VVL	Vitelline vein ligation

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1 CHAPTER 1 Introduction

1.1 Cardiac morphogenesis

The heart is a first organ to form in a developing embryo and it involves a complex of morphogenetic events. During the early stages of cardiogenesis, a linear heart tube forms which is followed by cardiac looping, ballooning and septation at specific regions to form a four-chambered heart (Manner, 2009, Contreras-Ramos et al., 2008, Abu-Issa and Kirby, 2007, Lamers and Moorman, 2002, Lindsey et al., 2014). This is followed by the development of cardiac valves and coronary vasculature as well as the conduction system to produce a fully functional heart (Christoffels and Moorman, 2009, Reese et al., 2002, Hutchins et al., 1988, Combs and Yutzey, 2009). Since it is an exquisitely sensitive process, any perturbation of these steps may result in congenital malformations.

In this section, a review on human (days) and chick (Hamburger and Hamilton (HH), as our research model) cardiogenesis of relevance to this thesis has been conducted to provide insights into how the heart forms in normal development and how it can go wrong giving rise to defects (Anderson et al., 2003, Moorman et al., 2003, Risebro and Riley, 2006, Hamburger and Hamilton, 1951). A table summarizing the major events on heart development is given in Table 1.1.

Human (days)	Chick (HH)	Important processes in heart development
21-22	9	Fusion of paired heart tubes
22-28	10-24	Cardiac looping
28	13-22	First definable endocardial cushions
28-41	14-24	Atrial septation
30-45	17-30	Ventricular septation and trabeculation Trabeculae appear in the primitive ventricle (HH17-18) Initiation of Ventricular septation (HH23) Complete ventricular septation (HH29/30)

Table 1.1 Parallel stages of heart development in chick and human. Human staging is based on days, and chick is Hamburger- Hamilton staging (HH). (Manner, 2009, Contreras-Ramos et al., 2008, Martinsen and Lohr, 2005, Abu-Issa and Kirby, 2007, Martinsen, 2005, Rutland et al., 2009, Lamers and Moorman, 2002, Lindsey et al., 2014).

1.1.1 Formation of primitive heart tube

A primitive heart tube is derived from the mesodermal cells located in the anterior part of the primitive streak on day 21-22 in human and at HH9 in the chick (Martinsen and Lohr, 2005, Abu-Issa and Kirby, 2007). Initially, cardiac progenitor cells (primary heart field) migrate alongside the midline in antero-lateral direction to the lateral plate mesoderm under the head folds and forms a crescent midline, with the secondary heart field cells situated medial and dorsal to primary heart field (Figure 1.1A) (Risebro and Riley, 2006). After residing in the cranio-lateral border of the primitive streak, the lateral plate mesoderm now splits into two layers which are somatic (outer mesoderm) and splanchnic (inner mesoderm). Cardiac progenitor cells can only be found in splanchnic mesoderm (Manasek, 1968). They become aggregated to form a pair of median endocardial heart tubes. At the same time, the folding of the splanchnic and somatic mesodermal layers ventrally and medially occurs from both sides and the fusion of both layers at the ventral midline results in the merging of the medial endocardial heart tubes to become a primitive heart tube (Moreno-Rodriguez et al., 2006). The linear tube (Figure 1.1B) is attached to the body wall by dorsal mesocardium and consists of three layers: outer myocardium and inner endocardium sandwich a thick extracellular matrix (ECM) layer called the cardiac jelly (Markwald et al., 1977, Anderson et al., 2006).

The primary heart field which forms the primitive heart tube, will give rise to the left ventricle (LV), atrioventricular canal (AVC), inflow tract (IFT) and part of the atria (Figure 1.1C) of the mature heart (Waldo et al., 2001, Martinsen, 2005, Jongbloed et al., 2012). Elongation of the heart tube is achieved by the addition of cells from the surrounding proliferating cells produced by secondary heart field (Sylva et al., 2014). The secondary heart field will give rise to the outflow tract (OFT) and right ventricle

(RV) (Figure 1.1C) of the mature heart (Henderson and Anderson, 2009, Abu-Issa and Kirby, 2007, Jongbloed et al., 2012). The heart tube starts off as peristaltic contraction and becomes sequential as the atrial contraction is followed by the ventricle.

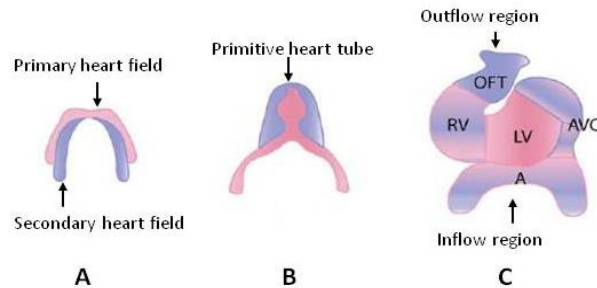


Figure 1.1 Formation of primary and secondary heart field in early stages of a developing heart. **A.** Primary heart field (shown in pink) is found in anterior lateral mesoderm and secondary heart field (shown in blue) is located dorsally and medially to the primary heart field. **B.** By human day 25, folding of the embryos results in the midline formation of the primitive heart tube from primary heart field. **C.** After cardiac looping, primitive ventricles are connected to the inflow region via the atrioventricular canal and atria and the outflow region via outflow tract. OFT, outflow tract; RV, right ventricle; LV, left ventricle; AVC, atrioventricular canal; A, atria. Adapted and modified from (Risebro and Riley, 2006).

1.1.2 Cardiac looping of primitive heart tube

Looping of the heart tube occurs between day 22-28 in human (Martinsen and Lohr, 2005). In chick, looping occurs in between HH10-HH24 in chick and it can be divided into C-shape (HH10-12) and S-shape looping (HH13-24) (Manner, 2009, Martinsen and Lohr, 2005).

Prior to looping, the primitive heart tube only consists of the primordia of the apical trabeculated regions of the future RV and LV (Manner, 2000). The addition of new segments of the primitive OFT, primordia of great arteries (truncus arteriosus), the primitive atria and the sinus venosus occurs during S-shape looping (Manner, 2000).

In early C-shape looping, the primitive heart tube elongates and it is followed by the torsion of the bending primitive ventricle around its cranio-caudal axis to cause a rightward C-shape looping by HH12 (Figure 1.2A-B) (Manner, 2000, Christoffels et al., 2000, Manner, 2009).

Transformation of C-shape looping into early S-shape looping occurs when the distance between the both ends of the primitive OFT and the primitive atria becomes shortened, and there is an increasing torsion of the bending ventricular segment. This moves the primitive atrium from its caudal position to the definitive cranial position in relation to the primitive ventricle (Figure 1.2B-C) (Manner, 2000, Manner, 2009).

Finally, the proximal OFT is shifted leftwards, with further expansion and differentiation of the chambers (Figure 1.2D) (Manner, 2000, Manner, 2009). The blood flow is transversed from the right and left horns of the sinus venosus into the primary atrium followed by the ventricle loop and the primitive OFT (arterial pole). The OFT, in turns, feeds the aortic arch arteries (Moorman et al., 2003).

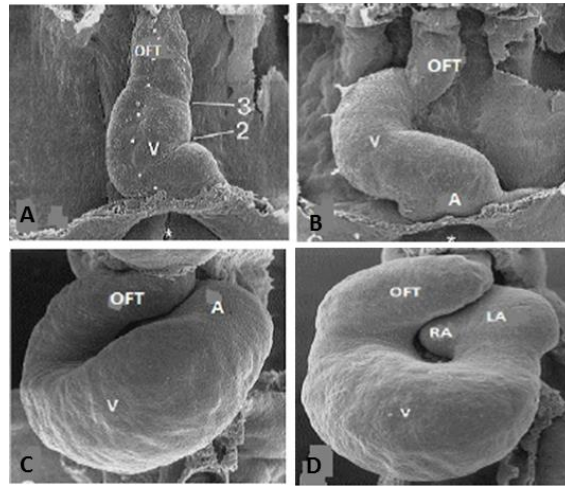


Figure 1.2 Cardiac looping. **A.** C-shaped looping is initiated at HH10-11, with numbers 2 and 3 indicating where looping is first discerned by the appearance of grooves. **B.** The heart undergoes ventral bending and rightward torsion at HH12. Primitive atrium is ventral to the primitive ventricle. **C.** The atrium is located cranial and dorsal to the ventricle during S-shape looping at HH18. **D.** Looping is complete at HH24. A, atria; LA, left half of the atria; RA, right half of the atria; OFT, outflow tract; V, ventricle. Adapted and modified from (Manner, 2000).

1.1.3 Endocardial cushion

At HH14 in the chick, when the looping process is still underway, a subset of endocardial cells receive signals from the myocardium to undergo endothelial-mesenchymal-transformation (EMT), where the endothelial cells lose intercellular adhesion and start invading and migrating into the underlying cardiac jelly (Barnett and Desgrosellier, 2003, Person et al., 2005, Butcher and Markwald, 2007). These cardiac jelly depositions are seen in between the myocardium and endocardium in the OFT and the AVC at HH16 and they are known as endocardial cushions (EC) (Figure 1.6) (Lindsey and Butcher, 2011). They contain highly proliferative, undifferentiated mesenchymal progenitor cells which are surrounded by hydrophilic ECM proteoglycans (Schroeder et al., 2003, Lincoln et al., 2004). They act as the primitive valves to ensure the unidirectional blood flow at the OFT (intercalated cushions, conotruncal cushions) and AVC (superior and inferior atrioventricular (AV) cushions, right and left lateral AV cushions) in the early heart development (see Figure 1.7A)

(Lin et al., 2012a). The heart valve development will be further discussed in section 1.1.9.

1.1.4 Chamber specification

The primitive heart tube consists of the outer myocardium and inner endocardium sandwiching ECM in between. The looping process then produces an S-shaped heart with outer and inner curvatures. The future chambers are now in the correct orientation and alignment for the development of the inflow and outflow regions and the great vessels of the heart (Martinsen, 2005). Primitive atria and ventricle are initially separated by the common AVC (Figure 1.3). Ongoing differentiation and proliferation of the outer curvatures, which is called secondary myocardium or working myocardium, produces ballooning in atrial appendages and ventricles of both sides of the primary atrium and ventricle respectively (Figure 1.3) (Christoffels et al., 2000). However, neither the AVC, OFT nor IFT differentiates, thus a primary phenotype on the inner curvature is maintained (Christoffels et al., 2000). Most of the atrial appendages will expand in parallel from the primary atrium and give rise to the left atrium (LA). Part of the atrial appendages together with the AVC form the future right atrium (RA) (30 days; HH14-18) (Horsthuis et al., 2009). Atrial and ventricular septation will be discussed further in section 1.1.5.1 and 1.1.5.2. The inflow tract and OFT connecting the atria and ventricle respectively will be described in section 1.1.6 and 1.1.7.

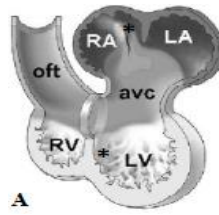


Figure 1.3 Formation of four chambered heart. Formation of four chambered heart at the end of looping and septation of atria and ventricle is still in progress (as indicated by asterisks) avc, atrioventricular canal; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; oft, outflow tract. Adapted and modified from (Postma et al., 2009, Lin et al., 2012a, Fijnvandraat et al., 2003).

1.1.5 Chamber septation

1.1.5.1 Atrial septation

Atrial septation also takes place to complete the division of both atria. Atrial septation occurs between 28 days (HH14/15) and 41 days (HH24) (Lamers and Moorman, 2002, Rutland et al., 2009, Hendrix and Morse, 1977). The crescent-shaped muscular septum primum develops from the dorso-cranial atrial wall, together with the mesenchymal cap on its leading edge, extends towards to fuse with the endocardial cushion in the AVC (Figure 1.4A) (Lamers and Moorman, 2002). A communication remains in between the right and left atria as the septum primum lengthens to fuse with the cushions (Figure 1.4A) (Lamers and Moorman, 2002). The mesenchymal cap fuses with dorsal mesenchymal protrusion or vestibular spine (a mass of extracardiac mesenchymal tissue that becomes muscularized and forms the base of atrial septum) (Anderson et al., 2003). Simultaneously, the septum primum undergoes apoptosis or perforations at the cranial part of the septum primum to form the foramen secundum (Figure 1.4B) (Lamers and Moorman, 2002). Another septum secundum grows alongside the septum primum to cover the foramen secundum and the upper part of the septum primum degenerates (Figure 1.4C). The opening between the two septums (the septum secundum and the inferior part of septum primum that had fused with the

EC) that forms by the 6th week is the foramen ovale (Figure 1.4D). This opening is closed by the valve of the foramen ovale (the lower or inferior part of the septum primum that had fused with the EC) after birth (Figure 1.4D) (Rojas et al., 2010, Anderson et al., 2003, Anderson et al., 1999).

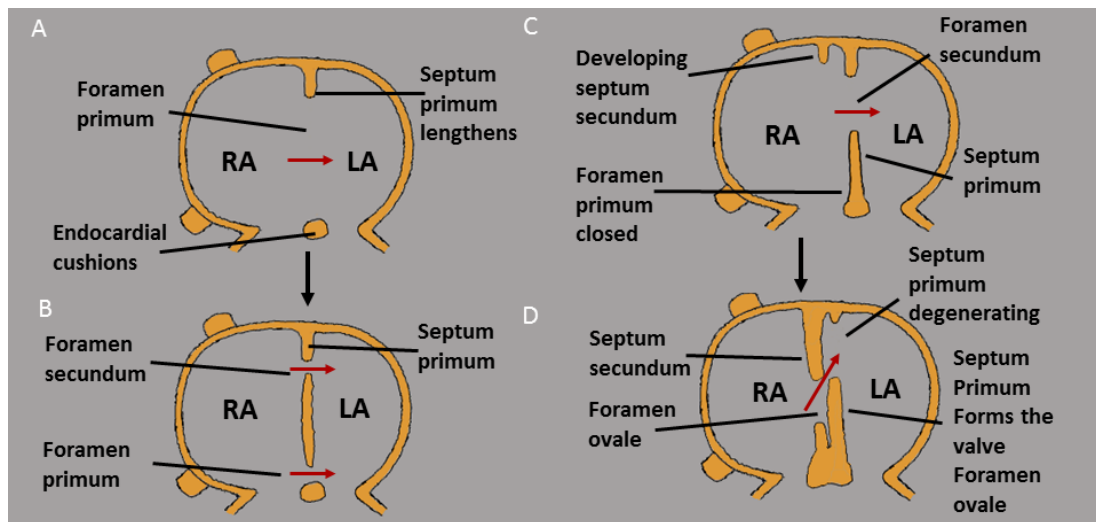


Figure 1.4 A schematic diagram describing atrial septation. A. Muscular septum primum develops from dorso-cranial atrial wall and lengthens to fuse with the endocardial cushions. A communication exists in between the left and right atria, the foramen primum. **B.** As the septum primum grows, perforations occur at the cranial part of the septum primum to form foramen secundum. **C.** Septum primum eventually fuses with the endocardial cushions to close the foramen primum, with the septum secundum developing next to the septum primum. **D.** Septum secundum lengthens to close the foramen secundum and septum primum forms the valve of foramen ovale to close the foramen ovale after birth. LA, left atrium; RA right atrium. Image obtained and modified from Dr. Siobhan Loughna.

1.1.5.2 Ventricular septation

As a result of the looping process, the ventricular loop which consists of both developing right and left ventricle is now moved to its definitive caudal position to the primitive atria. The AVC is supported by the inlet component of the ventricular loop whereas the OFT arises from the outlet component of the ventricular loop (Figure 1.3) (Moorman et al., 2003). At this stage, only the developing LV is communicating with developing LA (Figure 1.3). Thus, expansion of the AVC allows the communication in between the cavity of developing RA and developing RV to occur (Moorman et al., 2003, Henderson and Anderson, 2009).

Ventricular septation and trabeculation take place after cardiac looping in humans at day 30 (HH17) and complete by day 45 (HH30) (Contreras-Ramos et al., 2008, Martinsen, 2005). In response to signals emanating from the endocardium to myocardium, trabeculation begins to form from myocardium and appears like finger-like projections throughout the ventricular region by HH19-21 (Martinsen, 2005). They expand rapidly by proliferation via the addition of cells to the base of trabeculae instead of growing into the lumen. Spongy and trabeculated myocardium is thought to support the early contractile function of the ventricle. Trabeculations have an additional role of providing nutrients and oxygen to the embryonic myocardium for the development of coronary vascularization (Samsa et al., 2013).

Around the time of development of coronary circulation and septation, compact myocardium begins to form as a result of compaction and integration of the trabeculated myocardium into the ventricular wall (Samsa et al., 2013, Risebro and Riley, 2006). Compact myocardium which has higher proliferation now replaces the trabeculae to provide the contractile force to the embryonic heart (Sedmera and Thomas, 1996). It also contributes to the formation of papillary muscles (Moorman

and Lamers, 1994). Most importantly, compact myocardium plays a role in the formation of the interventricular septum (IVS). Coalescence of the trabeculae only forms the muscular portion of the IVS (Captur et al., 2016). This leaves an apical opening between both ventricles. Complete septation of both ventricles is accomplished by the fusion of the EC from the AVC with the proximal OFT, forming the membranous part of the IVS (Contreras-Ramos et al., 2008, Risebro and Riley, 2006).

1.1.6 The inflow tract

Initially, the systemic venous tributaries communicate with both sides of the primary atrium through the sinus venosus (an enlarged pouch that adjoins the heart and it received venous blood to be drained into primitive atrium) (Anderson et al., 2002). After atrial septation takes place, the systemic veins develop into the superior vena cava, inferior vena cava and the coronary sinus. As the atrial appendages are expanding, the pulmonary veins become incorporated and canalized in the developing LA (Moorman et al., 2003, Gittenberger-de Groot et al., 2005, Anderson et al., 2002). Subsequent muscularisation and bifurcation of the pulmonary veins results in the formation of the four pulmonary orifices in the LA (Sylva et al., 2014).

1.1.7 Outflow tract

The OFT is a single outflow vessel at the arterial pole that connects the aortic sac to the embryonic ventricle. It consists of proximal and distal parts delineated by a characteristic ‘dog-leg’ bend (Figure 1.5) (Webb et al., 2003, Moorman et al., 2003). Elongation of the OFT occurs via the addition of progenitor cells from the secondary heart field and this process is complete around HH21 in the chick and day 32 in human (Webb et al., 2003). Subsequently, the distal OFT is septated by the aorticopulmonary septum (Lin et al., 2012a). At the proximal OFT, right posterior and left anterior conal cushions are found and they are displaced by an invasion of cardiac myocytes (Lamers et al., 1995, Van Den Hoff et al., 1999). They fuse and form conal septum to divide the proximal OFT into outlet of left and right ventricles (Lin et al., 2012a, Webb et al., 2003).

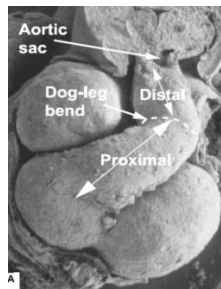


Figure 1.5 Outflow tract septation. A. Scanning electron micrograph of a ventral view of human embryo heart at day 36 in human. The unseptated outflow tract consists of proximal and distal components and is demarcated by a characteristic dog-leg bend (Webb et al., 2003).

1.1.8 Pharyngeal arches

The great arteries of the human heart are derived from the pharyngeal arch arteries (PAAs) (Johnson and Bamforth, 2016). Initially, the PAAs appear to be symmetrical and they undergo remodelling over a ~3-week period in embryonic development to form the asymmetric great arteries seen in adult, namely, the aorta, carotid and subclavian arteries (Johnson and Bamforth, 2016). The PAAs are derived from endothelial cell precursors and form the five pairs of vessels known as PAA 1-4 and 6 arranged in cranial-to-caudal sequence (Johnson and Bamforth, 2016, Bamforth et al., 2013). The first two pairs of PAA (1 and 2) regress early and contribute to the vascular network within the developing jaw (Johnson and Bamforth, 2016, Gupta et al., 2015). The asymmetric remodelling process that will result in the formation of the mature aorta, carotid and subclavian arteries involves PAA 3, 4 and 6. The third PAAs, on both the right and the left, give rise to the internal carotid arteries and part of the common carotid arteries (Johnson and Bamforth, 2016, Bamforth et al., 2013, Gupta et al., 2015). The fourth PAA persists on the right as the proximal part of subclavian artery, whilst on the left it contributes to part of the aortic arch between the origins of the left common carotid artery and the left subclavian artery (Johnson and Bamforth, 2016, Bamforth et al., 2013, Gupta et al., 2015). The right sixth PAA regresses, whilst the left sixth PAA becomes the arterial duct (ductus arteriosus) (Johnson and Bamforth, 2016, Bamforth et al., 2013, Gupta et al., 2015).

1.1.9 Heart valve development

As discussed in section 1.1.3, after EMT, the EC will be remodelled into valve leaflets by extension, condensation and delamination (Figure 1.6).

By HH19, the atrioventricular mesenchyme proliferates and migrates around the AVC. It thickens preferentially at the protrusions, forming left and right lateral cushions (Figure 1.7A) (Moorman et al., 2004). At HH25, the fusion and remodelling of superior and inferior atrioventricular cushion, while dividing the AVC, gives rise to the anterior mitral leaflet and the septal tricuspid leaflet on the left and right side of the heart respectively (Figure 1.7A-B) (de Lange et al., 2004). The left lateral cushion becomes the parietal mitral leaflets, whereas the right lateral cushion produces the parietal tricuspid valve leaflets (Figure 1.7B) (Lin et al., 2012a). For consistency, throughout this thesis, the atrioventricular valve leaflets that derive from the right and left lateral cushions will be referred to as the lateral leaflets. With regards to the OFT valve development, the aortic and pulmonic (semilunar valves) arise from the conotruncal and intercalated cushions at the OFT (Figure 1.7A) (Lin et al., 2012). Following fusion of the superior and inferior of the cushions, the valve primordia express more transcriptional factors such as TBX20 and ECM proteins such as aggrecan, versican and periostin associated with differentiation of the valve leaflets and supporting structures.

Beginning at HH26, the subendocardial portions of the atrioventricular cushions extend and elongate into the lumen by a combination of cell proliferation at the growing tip, apoptosis at the base of the cushion as well as matrix deposition (Hurle et al., 1980, Lincoln et al., 2006, de Lange et al., 2004). The portions of cushion contacting the myocardial layer of atrioventricular cushion differentiate and condense to form a thinner fibrous tissue (Butcher and Markwald, 2007). Fenestrations also

begin to develop in the myocardial layer of the atrioventricular cushions by HH30 in response to the expansion of the ventricular cavities and changes of haemodynamic loading. They coalesce and result in complete 'delamination' of the atrioventricular myocardial cushion from the ventricular wall (Butcher and Markwald, 2007). However, the lateral leaflets of the mitral and tricuspid valves are formed by protrusion and growth of a sheet of atrioventricular myocardium into the ventricular lumen rather than by delamination (Martinsen, 2005). The myocardial layer is subsequently removed by apoptosis (de Lange et al., 2004). Programmed cell death also occurs at the site of developing papillary muscle which creates mesenchymal tissue strands that develop into the chordae tendinae of the atrioventricular valves by HH34 (de Lange et al., 2004). Primordia valve leaflets secrete high amount of ECM proteins like collagens, proteoglycans and hyaluronan (Hinton et al., 2006, Lincoln and Yutzey, 2011, Tan et al., 2011b). Tensile strain from the chordae tendinae stimulates fibroblasts developing into a unique structure called fibropositors (de Vlaming et al., 2012). They promote secretion and alignment of the collagen fibrils (de Vlaming et al., 2012). Further, valve fibroblasts acquire a contractile phenotype which promotes ECM condensation and tissue compaction, resulting into highly organized stratified layers of valve leaflets (Figure 1.8) (de Vlaming et al., 2012, Butcher and Markwald, 2007).

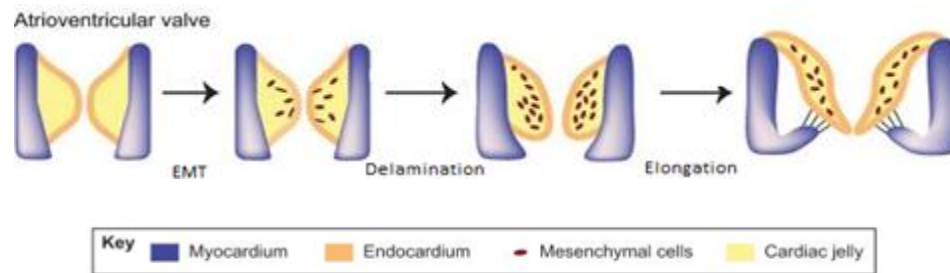


Figure 1.6 General stages of atrioventricular valve and semilunar valve development. Endocardial cells in the AV cushions and OFT cushions undergo endothelial-to-mesenchymal transformation (EMT) and generate mesenchymal cells that populate the cushions. The mesenchymal cushions of atrioventricular valve remodel by delamination. They then elongate themselves to form primitive valves that mature into thin leaflets. Adapted and modified from (Lin et al., 2012a).

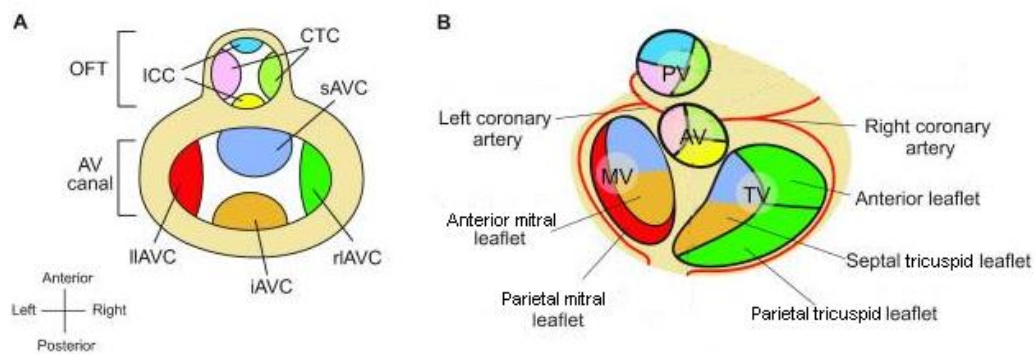


Figure 1.7 Endocardial cushions and the derived heart valve leaflets.

A. Schematic diagram of endocardial cushions in the atrioventricular canal (AVC) and the outflow tract (OFT). The cushions are color coded that correspond to the derived valve leaflets shown in (B). **B.** Schematic diagram of atrioventricular leaflets and semilunar valve leaflets derived from the corresponding cushions colour coded in (A). CTC, Conotruncal cushions; ICC, Intercalated cushions; sAVC, superior AV cushion; iAVC, inferior AV cushion; rLAVC, right lateral AV cushion; lLAVC, left lateral AV cushion. PV, pulmonary valve; AV, aortic valve; TV, tricuspid valve; MV, mitral valve. Adapted and modified from (Lin et al., 2012a).

1.1.10 Role of epicardium in heart valve development

The epicardium consists of mesothelial epithelial tissue that forms the outermost layer of the heart (Winter and Gittenberger-de Groot, 2007). During development, the epicardium-derived cells (EDPCs) differentiate into interstitial fibroblasts, smooth muscle cells of the coronary arteries and atrioventricular EC as mesenchymal cells (Winter and Gittenberger-de Groot, 2007). They also play a role in the development of the other structures such as compact myocardium and coronary vasculature (Winter and Gittenberger-de Groot, 2007, von Gise and Pu, 2012). EPDCs contribute to formation of the right and left lateral atrioventricular cushions, which develop into lateral leaflets in a mature heart (Figure 1.7). This is different from the anterior mitral leaflet and the septal tricuspid leaflet which are derived from the central EC (Figure 1.7) (Lockhart et al., 2014).

1.2 Mature heart valves

The heart valves, the atrioventricular and semilunar valves, are the structure with sufficient strength, elasticity and compressibility to ensure the unidirectional blood flow through the cardiac chambers and great vessels without obstruction or regurgitation. All four cardiac valves leaflets have a similar three distinct layers of ECM: the fibrosa, spongiosa and atrialis (in atrioventricular valves or the ventricularis in semilunar valves). The fibrosa layer is situated away from blood flow, the atrialis (Figure 1.8A) or ventricularis (Figure 1.8B) adjacent to blood flow and the spongiosa layer in between (Lincoln et al., 2006). The main ECM components in each layer and their putative roles in valve development are summarized in Table 1.2.

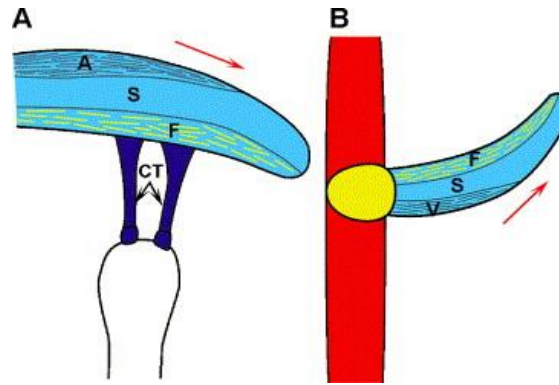


Figure 1.8 Schematic diagram of compartmentalization of ECM proteins in the mature atrioventricular (A) and semilunar (B) heart valves. The ECM proteins of both valves are arranged in parallel with blood flow (red arrow). The ECM proteins in atrialis (A) in atrioventricular valve and ventricularis (V) in semilunar valve are composed of elastin (gray lines). Spongiosa (S) layer is mainly constituted by proteoglycans (blue) and collagen fibers (yellow) are predominant in the fibrosa (F) layer. Chordae tendinae (CT) is the supporting apparatus. Adapted and modified from (Lincoln et al., 2006).

Location	Main ECM protein components	Putative role
Atrialis/ Ventricularis	Elastin: fibrillin-1, fibrillin-2, fibronectin	Extend and recoil in diastole and systole accordingly
Spongiosa	Proteoglycans : aggrecan, type II collagen, perlecan, versican	Shock absorber between ventricularis and fibrosa during valve closure
Fibrosa	Collagen type I, II, III, V, VI, XI, tenascin	Provide strength and stiffness during valve closure without prolapsing into atrium.

Table 1.2 Three stratified layers of cardiac valves and the main ECM protein components in each layer and their role in valvulogenesis. Adapted and modified from (Schoen, 2008).

1.3 Structure of a sarcomere in cardiac muscle

Muscle fibers are composed of tubular myofibrils with the myofibrils formed by repeating sections of sarcomeres, which appear as dark A and light I bands (Figure 1.9A) (Sherwood, 2007). The sarcomere is the basic contractile unit of striated muscle and is made of thick and thin filaments (Figure 1.9B) (Du et al., 2008). The sarcomere spans between two Z-discs to which the thin filaments (actin-based) anchor (Figure 1.9C) (England and Loughna, 2013). The myosin is interposed between the actin-containing thin filaments. Thick filaments are held in place by the central M-line in the sarcomere (England and Loughna, 2013). Titin extends in both directions from the M-line at its C-terminus along the length of the thick filaments to anchor both sides of the Z-discs at its N-terminus (Furst et al., 1988, Squire, 1997). Titin helps stabilization of the thick and thin filaments and acts as a mechanical spring in contraction (England and Loughna, 2013). The A band consists of a stacked set of thick filaments along with the portions of the thin filaments that overlap both ends of the thick filaments. The I band is made up of the remaining portion of the thin filaments that do not project into the A band (Figure 1.9C) (England and Loughna, 2013).

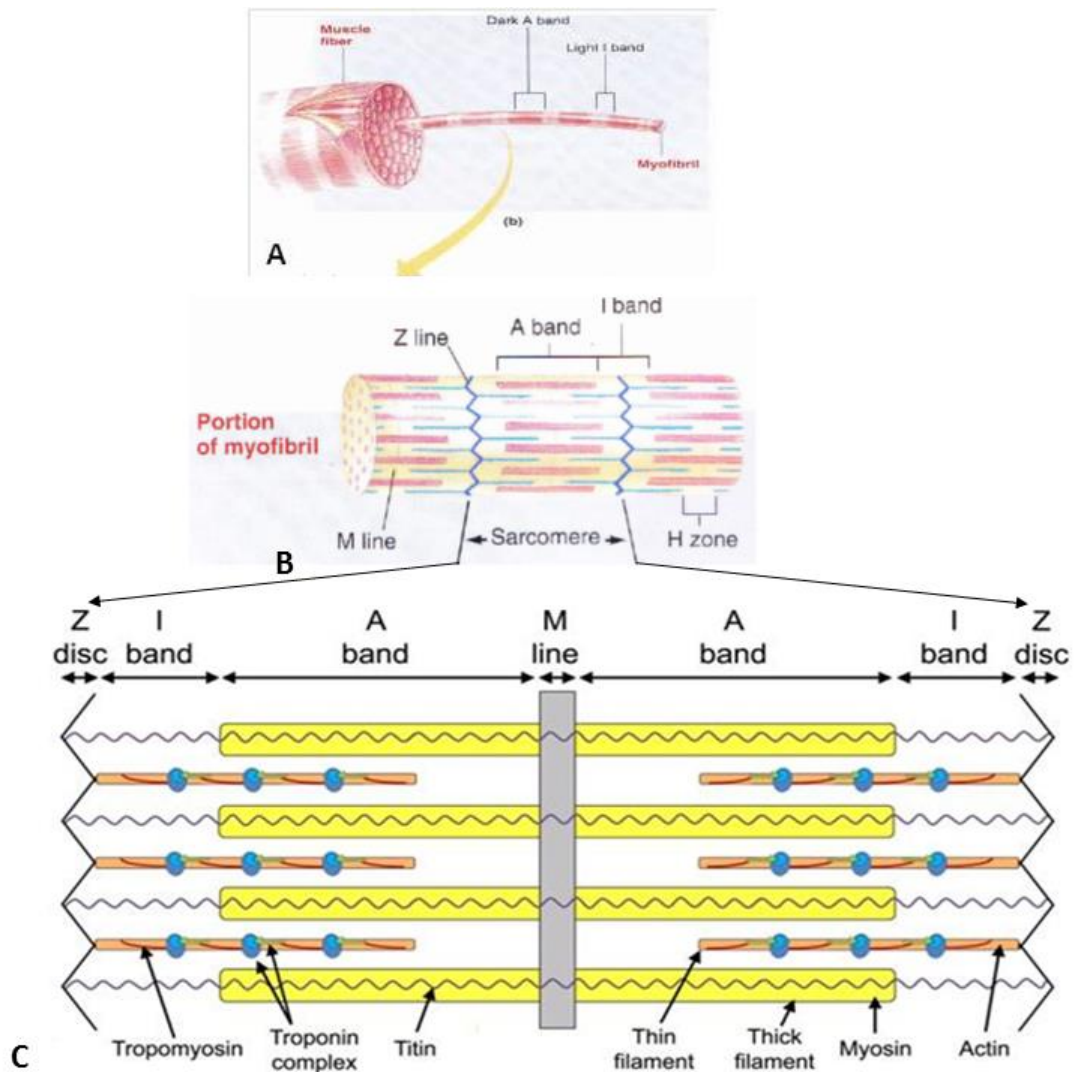


Figure 1.9 Structure of a myofibril and sarcomere. **A.** Schematic diagram showing a muscle fiber with an enlargement of a myofibril. **B.** The sarcomere is the basic contractile unit of striated muscle and is made of thick and thin filaments **C.** Simplified diagram of a sarcomere, which is bounded by two Z-discs joined end-to-end by a titin filament. Within the sarcomere, the A band comprises overlapping of thin and thick filament and I band contains only thin filament. Thick filaments which are made up of myosin filaments are held in place by central M line. Thin filaments are made up of actin and the troponin-tropomyosin complex. Adapted and modified from (England and Loughna, 2013).

1.4 Cardiac troponin T (cTnT) and its structure

The troponin complex consists of TnC, TnI and TnT. TnC is a Ca^{2+} -binding component, TnI is an inhibitory component of contractile interaction, and TnT binds the entire troponin complex to tropomyosin. TnI is strongly bound to actin-tropomyosin which holds the actin in position that inhibits the myosin-actin contractile interaction. On the binding of Ca^{2+} to TnC, TnI is pulled away from the actin-tropomyosin to TnC and the tropomyosin molecules shifts around the actin thin filament to expose the myosin-binding sites (Vinogradova et al., 2005, Frazier et al., 2011, Vibert et al., 1997). TnT integrates the regulatory function of the troponin complex. The inhibition by TnI was released Ca^{2+} -independently by the neutralizing action of TnC in the absence of TnT but was released Ca^{2+} -dependently in the presence of TnT (Ohtsuki and Morimoto, 2008).

The cardiac TnT (cTnT) gene, *TNNT2*, is located on the long arm of human chromosome 1 (1q32) and contains 17 exons. Alternative splicing occurs in the *TNNT2* gene which results in four different isoforms, where cTnT isoforms 1, 2, 4 are predominantly expressed in the human fetal heart, and isoform 3 is primarily expressed in the adult heart (Anderson et al., 1991).

cTNNT2 was initially in the chick in the precardiac fields at HH5-6 and in the heart tube region at the onset of cardiac differentiation around HH9 (Antin et al., 2002). The expression becomes restricted to the myocardium at HH12 (Antin et al., 2002).

TnT contains two domains which are T1 and T2 (Gomes et al., 2002, Palm et al., 2001). T1 (N-terminal region of TnT) consists of a rod-like 160 residues A-long tail that anchors the Tn complex to the thin filament by interacting with tropomyosin via hydrophobic and charge-charge interaction (Figure 1.10) (Palm et al., 2001). T2 (C-terminal region of TnT) consists of a globular head which interacts with TnC and TnI

in the troponin head complex in Ca^{2+} -dependent manner (Gomes et al., 2002, Vinogradova et al., 2005, Palm et al., 2001, Lu et al., 2013).

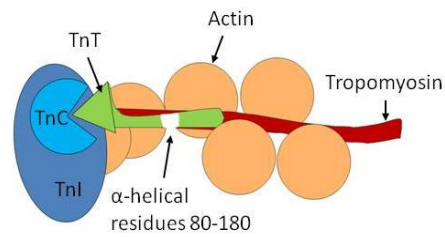


Figure 1.10 Simplified schematic to show the structure cardiac TnT and the interaction between the troponin complex and tropomyosin. The α -helical region in between residues of 80-180 facilitates the binding of the tail of troponin to tropomyosin and thus to actin. Adapted and modified from (England and Loughna, 2013).

1.5 Cardiac troponin T in the troponin complex in sarcomeric contraction

Muscle contraction is generated by the interaction of the myosin head and the actin filament powered by the ATP force and regulated by intracellular Ca^{2+} via the sliding filament mechanism. Myosin has two special sites; an actin-binding site and an ATPase site (Figure 1.11A) (Sherwood, 2007).

Prior to cross bridge formation, Ca^{2+} is unbound to the calcium-specific binding sites on troponin C (TnC). TnC is weakly bound to Troponin I (TnI) and Troponin T (TnT), and TnI is strongly bound to the actin filament (England and Loughna, 2013). Tropomyosin holds actin in a position that inhibits binding of myosin (England and Loughna, 2013). Upon muscle contraction, ADP and Pi are generated upon binding of ATP at the ATPase site on the myosin head (Figure 1.11Bi). The myosin globular head undergoes a conformational change that interacts with actin (Figure 1.11Bii). Ca^{2+} then binds to TnC inducing hydrophobic opening in the globular head of TnC (Figure 1.11Biii) (Vinogradova et al., 2005). The central linker of TnC stabilizes this opening to facilitate the binding of the switch segment of TnI to the hydrophobic opening and result in TnI being pulled away from the actin-tropomyosin to TnC (Vinogradova et al., 2005, Frazier et al., 2011). The tropomyosin molecules shift around the actin thin filament and expose the myosin-binding sites on a continuous increase of Ca^{2+} level (Figure 1.11Biii) (Vibert et al., 1997). Release of ADP and Pi causes a conformational change of the myosin head. This relays the internal strain to the lever arm of the myosin molecule allows the sliding filament to occur and thus muscle contraction (Figure 1.11Biv) (England et al., 2016). ADP dissociates and the myosin head detaches from actin and the cycle continues with more cross-bridges converting to force-generating

ADP and Pi (Figure 1.11Biv) (Uyeda et al., 1990, Maughan, 2005, Bagshaw and Trentham, 1973).

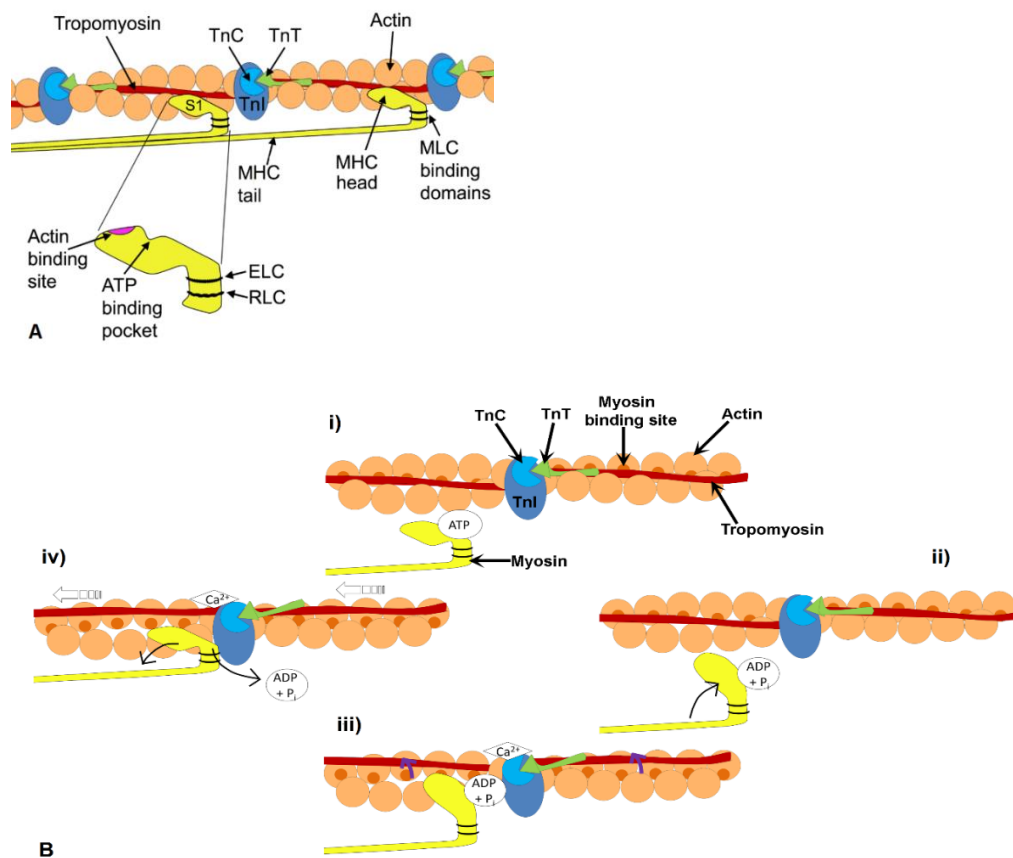


Figure 1.11 Schematic representation of cross bridge cycle in muscle contraction.

A. Simplified view of the relationship between the globular head of myosin heavy chain and thin filament and the magnification of the structure of the myosin head. The troponin complex (TnI, TnC and TnT), tropomyosin and actin are illustrated on thin filaments. A magnification of the globular head shows the myosin globular head that has two binding sites, the actin binding site and the ATP binding pocket for myosin-actin interaction in muscle contraction powered by ATP. The essential light chain (ELC) and regulatory light chain (RLC) are shown in this figure as well. **B.** Schematic illustration of the myosin cross-bridge cycle. **i)** ATP binds to ATP binding pocket on the myosin head. **ii)** ATP is hydrolyzed to ADP and phosphate allowing the myosin head to interact with actin. **iii)** Binding of Ca²⁺ to troponin C (TnC) results in conformational change in troponin complex and which allows the movement of tropomyosin around the actin filament (purple arrows). **iv)** Release of hydrolyzed nucleotides results the extension of myosin head (open arrows) for the sliding of actin thin filaments towards centre of sarcomere (green arrows). ATP quickly rebinds to the ATP-binding pocket on the myosin head to dissociate it from actin filament. The cycle is repeated. MHC, myosin heavy chain; MLC, myosin light chain; S1, subfragment-1; TnC, troponin C; TnI, troponin I; TnT, troponin T. Adapted and modified from (England and Loughna, 2013).

1.6 Haemodynamics in cardiac development

Haemodynamic force is an important epigenetic factor that modulates heart development by exerting strains and forces on the surrounding tissue. Pressure is the perpendicular force exerted by the blood flow to the vessel wall (Figure 1.12A) (Lindsey et al., 2014). The force exerted by pressure plays a role in valve leaflet elongation through tissue resorption on the inflow side of atrioventricular cushions and expansion on the outflow side (Buskohl et al., 2012). It is involved in the modulation of the proliferation and maturation of the cardiomyocytes (Sedmera et al., 1999, Tobita et al., 2002). Wall shear stress is the frictional force exerted by the flowing blood against the heart lumen wall and is involved in surface remodelling of the valve leaflet at its leading edge (Figure 1.12B) (Lindsey et al., 2014, Buskohl et al., 2012). It is important in triggering biological responses, endothelial cell organization and signalling (Hove, 2006, Van der Heiden et al., 2006). Stretch is the circumferential stress that occurs when the wall stretches in response to pressure (Figure 1.12C) (Lindsey et al., 2014).

At HH10, the heart tube starts off peristaltic contractions and the blood flow is slow and irregular (Yalcin et al., 2011). During HH17-23, the blood flow resembles a laminar profile at the AVC and OFT. The inner curvature of the heart is exposed to higher shear stress than the outer curvature, with the inner curvatures developing into EC whereas outer curvatures form ventricular trabeculae (Yalcin et al., 2011, Bharadwaj et al., 2012). The haemodynamic patterning then switches to a plug flow profile when the OFT and chamber septation are about to complete (HH23-30). The shear stress of the inflow surface of atrioventricular cushion is higher than the outer surface (Bharadwaj et al., 2012, Yalcin et al., 2011).

Endocardial cells at the AVC are exposed to constant shear stress but how these cells transduce the mechanotransduction stimulus remains elusive (Kalogirou et al., 2014). A number of potential candidates have been proposed to sense the mechanical stress on endothelial cells and they include cytoskeletal proteins, integrins, primary cilia and cell adhesion molecules (Kalogirou et al., 2014). In addition, gene expression in cardiac endothelial cells are also altered. The shear stress responsive genes include kruppel lung factor-2 (*KLF2*), endothelin-1 (*EDN1*) and nitric oxide synthase-3 (*NOS3*). *KLF2* and *NOS3* have overlapping expression patterns and are observed at higher shear stress area, e.g AVC, OFT and inner curvatures of the heart (Groenendijk et al., 2004). In contrast, *EDN1* expression is downregulated by high shear stress and is observed at the sites of coronary vessel formation (Groenendijk et al., 2004). It is well known that alteration of haemodynamics can cause a wide range of heart anomalies (Sedmera et al., 1999, Hogers et al., 1999).

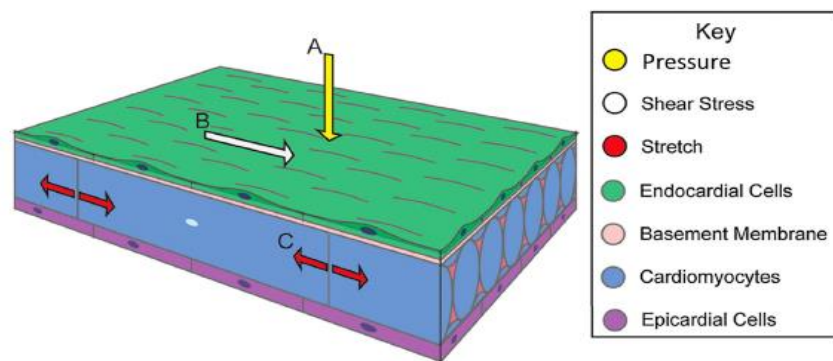


Figure 1.12 Biomechanical forces required for cardiac wall maturation during development. Biomechanical forces are pivotal for cardiac development. (A) Pressure is a force perpendicular to the vessel wall, (B) shear stress is the frictional force parallel to the vessel wall, and (C) circumferential stress that occurs when the walls stretch. Arrows indicate force vectors. Taken from (Lindsey et al., 2014).

1.7 Congenital Heart defects

Congenital heart defects (CHDs) are defined as the structural and functional defects of the heart and great vessels at birth that have a prevalence of disease of 8 per 1000 live births (Bernier et al., 2010, van der Linde et al., 2011). Worldwide, 1.35 million infants are born with CHDs each year and they are the main leading cause of death in infants under one year of age (van der Linde et al., 2011).

CHDs encompass three main categories: cyanotic defects, septation defects and left side obstruction defects (Bruneau, 2008). Cyanotic defects are caused by right-to-left shunts, bidirectional shunting and malposition of great arteries. Deoxygenated blood bypasses the lung and returns to the body without being oxygenated, resulting in mixing of deoxygenated and oxygenated blood and causing a 'blue' appearance in most babies (Sommer et al., 2008). Cyanotic defects include tetralogy of fallot (TOF), transposition of great arteries, tricuspid atresia, persistent truncus arteriosus (PTA) and double outlet right ventricle (Bruneau, 2008).

On the other hand, septation defects refer to left to right shunt lesions. In this instance, oxygenated blood returns to lung rather than the body (Sommer et al., 2008). They comprise atrial septal defects (ASDs), ventricular septal defects (VSDs) and atrioventricular septal defects (AVSDs) (Bruneau, 2008). Lastly, left side obstruction lesions include hypoplastic left heart syndrome, mitral stenosis and aortic stenosis and bicuspid aortic valve is classified as a separate entity (Bruneau, 2008).

The etiology of CHDs is diverse but genetic and environmental factors are likely to be the cause. Autosomal dominant, autosomal recessive and X-linked mutations are well recognized genetic causes of CHDs (Fahed et al., 2013). Other genetic causes include numerical or structural aberrations seen in chromosomal defects (e.g 22q11.2 deletion,

trisomy 21 and trisomy 18) and non-syndromic single gene defects (e.g cardiac transcriptional factors such as *TBX5*, *NKX2.5* and *GATA4*) (Fahed et al., 2013).

Recently, a number of sarcomeric protein mutations such as alpha cardiac actin 1 (Monserrat et al., 2007, Matsson et al., 2008), α -myosin heavy chain (α MHC) (Ching et al., 2005), β -myosin heavy chain (β MHC) (Postma et al., 2011) have been found to contribute to isolated CHDs.

Environmental factors that can predispose the risk of developing CHDs include teratogens (e.g retinoic acid and lithium), maternal exposures such as thalidomide, anti-seizure medications, diseases such as diabetes, systemic lupus erythematosus, as well as alterations of the haemodynamics (Huang et al., 2010, Fahed et al., 2013).

1.7.1 Atrial Septal Defects

ASDs are the third most common type of CHDs and occur in 56/1000 live births and are characterised by a defect of the interatrial septum dividing the LA and RA (Figure 1.13) (McCarthy et al., 2003, Hoffman and Kaplan, 2002). ASDs can be classified into three types of defects: patent foramen ovale, ostium secundum defect and ostium primum defect (Geva et al., 2014, Briggs et al., 2012).

Ostium secundum defect accounts for 80% ASD's and 10% of CHDs (Figure 1.13A,B) (Posch et al., 2010, Posch et al., 2011). It is characterized by the excessive apoptosis of the septum primum (Figure 1.13A) or the underdevelopment of septum secundum (Figure 1.13B), with the size of the lesions ranging from a few mm to 2-3 cm (Geva et al., 2014). Small lesions are usually asymptomatic. However, larger ostium secundum defects have pathological insults. Persistent left-to-right cardiac shunt leads to right atrial volume overload and hence pulmonary hypertension (Briggs et al., 2012).

Concomitant elevation of pulmonic resistance that increases the pressure on the right side of the heart leads to subsequent reversal right-to-left shunting. Thus, deoxygenated blood that is shunted from the right side of the heart is returned into the systemic circulation without being oxygenated, leading to cyanosis (Briggs et al., 2012, Engelfriet et al., 2007).

Patent foramen ovale is a hole in between the left and right atria chambers in the heart which normally closes after birth (Briggs et al., 2012, Silva, 2014). It is important during fetal development to direct the blood that has been oxygenated in the umbilical veins to be shunted from the right side of the heart to the LA (Briggs et al., 2012). This allows the blood to be pumped into the LV and the systemic circulation. Shortly after birth, the increased relative pressure in the LA and the establishment of pulmonic gas exchange, cause the septum primum to fuse with the septum secundum and thus the heart is fully partitioned into RA and LA (Briggs et al., 2012). Failure of closure of septum primum and septum secundum was found in 20%-34% newborn cases (Briggs et al., 2012, Silva, 2014).

Ostium primum defects, also called partial AVSD, account for 15% of ASDs and the defect is usually severe and is often accompanied by mitral valve (MV) and tricuspid valve (TV) defects (Balchum et al., 1956). Partial AVSD occurs when the defect occurs at the inferior part of the septum primum just above the atrioventricular valves (Figure 1.13C) (Jacobs et al., 2000). This can be due to the undergrowth of either the septum primum or EC which then allows interatrial communication to occur. In a complete AVSD, the defect occurs in the septum primum above the atrioventricular valves and also in the upper part (membranous part) of ventricular septum just below the atrioventricular valves (Jacobs et al., 2000). Interatrial and interventricular communications occur in this subtype of ASDs.

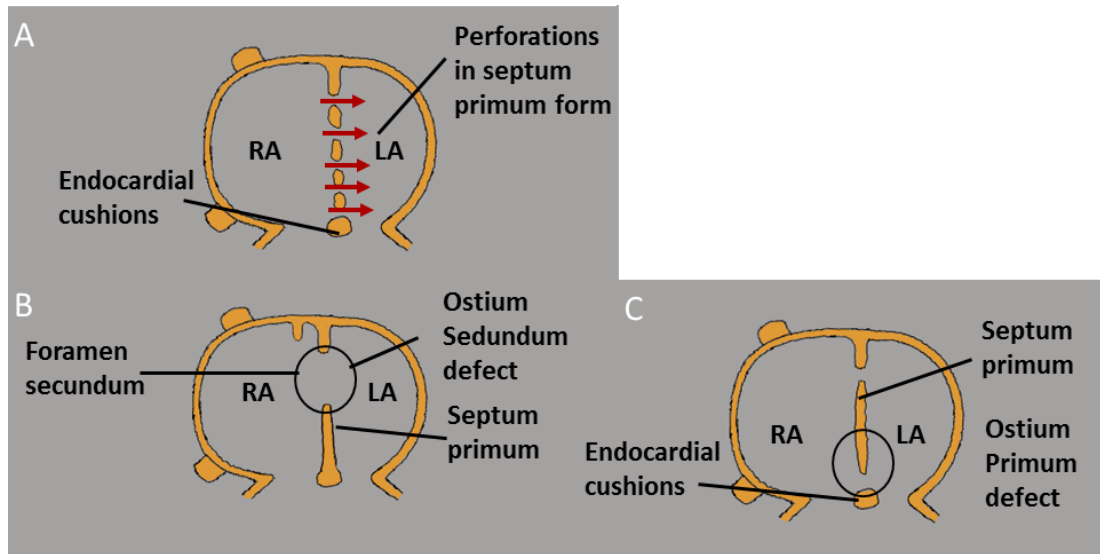


Figure 1.13 Atrial septal defects. Ostium secundum defect. Excessive apoptosis in the septum primum **(A)** or underdevelopment of septum secundum **(B)** leads to ostium secundum defect. Ostium primum defect. Underdevelopment of septum primum or endocardial cushion **(C)** fails to close the foramen primum resulting in ostium primum defect. Image obtained and modified from Dr. Siobhan Loughna.

1.7.2 Ventricular Septal Defects

Ventricular septal defects (VSDs) account for 40% of all CHDs and they appear as an isolated defect but also as an intrinsic component of other complex malformations such as TOF and transposition of great arteries (Driscoll, 2016b). VSDs can be classified according to their locations and can be mainly divided into four types: perimembranous VSD, muscular (trabecular) VSD, inlet VSD and outlet (infundibular) VSD (Driscoll, 2016b).

Perimembranous VSD is characterized by the incomplete closure of the membranous part of the septum or underdevelopment of the muscular component of the septum (Penny and Vick, 2011, Minette and Sahn, 2006). Anatomically, it is located in the OFT of the LV and immediately under the aortic valve (Driscoll, 2016b). In most cases, perimembranous VSD can extend into adjacent infundibular septum and opens

into inlet or outlet of the RV (Driscoll, 2016b). This defect can cause a confluent defect if they are big enough to open into all ventricles. Muscular (trabecular) VSD can be distinguished from perimembranous VSD by having underdeveloped muscular septum completely surrounded by the muscular rim (Minette and Sahn, 2006). It can be found at any part of the septum but involves no fibrous tissues (Minette and Sahn, 2006, Penny and Vick, 2011).

Outlet VSD is situated just below the aortic valve (Driscoll, 2016b). It occurs due to an inadequate septation of the infundibular septum that separates the right and left ventricular OFT (Minette and Sahn, 2006). Aortic valve is then prolapsed into right ventricular OFT, causing aortic regurgitation. Inlet VSDs are sitting posterior to the tricuspid and mitral valves and have no muscular rim between the defects and atrioventricular valve annulus (Minette and Sahn, 2006). They form as a result of failure of fusion of atrioventricular cushion with each other or with primitive atrial septum (Penny and Vick, 2011).

In most cases, VSDs will go undetected due to self-closure, which is more common in the muscular type of VSDs (Minette and Sahn, 2006). Clinical significance of VSDs are influenced by the size of the defects, the balance of the pressure in right and left side of the heart as well as the pulmonary resistance (Minette and Sahn, 2006). Shortly after birth, VSDs might not cause any functional limitations because the pressure balance in both right and left ventricles are still equal. However, a gradual increase in pressure difference between the ventricles leads to an increase in left-to-right shunting (Minette and Sahn, 2006). Hence, pulmonary hypertension that develops at the right side of the heart leads to Eisenmenger syndrome (a process in which a prolonged intracardiac left-to-right shunt caused by a combination of ASDs, VSDs and PTA. This

increases pulmonary hypertension and causes eventually reversal of cyanotic right-to-left shunt) (Minette and Sahn, 2006).

1.7.3 Persistent Truncus Arteriosus

Persistent truncus arteriosus (PTA) is a defect where a single great artery 'the truncus' emerging from the heart by means of a single semilunar valve, due to the incomplete division of the aorta and pulmonary trunk by a deficient aortopulmonary septum and the associated malformations are stenosis, interrupted aortic arch as well as OFT valve deficiency (Driscoll, 2016a). PTA is always accompanied by a VSD owing to the absent of the infundibular septum that fuses with the proximal OFT septum. Mixing of venous (deoxygenated) blood and arterial (oxygenated) blood is found in the ventricles as well as in the single truncus and they are redistributed to the systemic and pulmonary circulations (Driscoll, 2016a). The degree of cyanosis depends on the relative resistance of the pulmonic vascular and systemic vascular resistance, in addition to the presence and the severity of pulmonary stenosis (Driscoll, 2016a). In some cases, vascular resistance of pulmonary circulation is found lower than the systemic circulation (Lee, 2010). Increased blood flow to the pulmonic circulation results in the congestive heart failure (Lee, 2010).

1.7.4 Congenital valve defects

Congenital valve malformation has an estimated prevalence of disease of 2% of live births (Hoffman and Kaplan, 2002). The actual incidence is thought to be higher as most cases are subclinical and thus go undetected. Bicuspid aortic valve and mitral valve prolapse are the most common valve malformations (Hoffman and Kaplan, 2002).

As the name implies, bicuspid aortic valve is a type of congenital aortic valve anomaly with two unequal sized cusps rather than three cusps and has an estimated prevalence of 0.5%-2% (Siu and Silversides, 2010, Mordi and Tzemos, 2012). It is commonly associated with secondary valvular and aortic wall abnormalities such as calcific aortic valve disease and aortic dilation, respectively (Atkins and Sucosky, 2014). BAV is a heritable trait, with approximately 9% prevalence amongst first-degree relatives, and up to 24% in families with more than one affected family member and also had previously been associated with gene mutations such as *GATA5*, *NOTCH1*, *ACTA2* (Siu and Silversides, 2010, Mordi and Tzemos, 2012). Recent evidences have demonstrated that the BAV is morphologically stenotic and hence creates abnormal haemodynamics and uneven wall shear stress leading to leaflet calcification and aortic wall dilation (Padang et al., 2013, Atkins and Sucosky, 2014, Sucosky, 2014). BAV is not only a disorder of valvular development, but also coexists with other congenital defects such as ASDs, VSDs, patent ductus arteriosus and hypoplastic left heart syndrome (Siu and Silversides, 2010).

Mitral valve prolapse is characterized by abnormally thickened and floppy MV leaflets prolapsing into the LA during systole (Seguela et al., 2011). Myxomatous degeneration is known to be the main etiology of the prolapsing valvar leaflets (Seguela et al., 2011). In childhood, mitral valve prolapse represents as one of the components of more complex congenital connective tissue disorders, such as Marfan syndrome and Ehler-Danlos syndrome (Seguela et al., 2011).

1.7.5 Congenital ventricular diverticulum

Congenital cardiac diverticulum (CCD) is a rare localized protrusion of the endocardium and myocardium from the free wall of the heart (Wang et al., 2011, Dwivedi et al., 2012, Brachlow et al., 2002). Although they usually arise from the LV, many cases have also been presented with RV and both atrial diverticula (Binder et al., 2000, Ohlow et al., 2015). The prevalence of 0.04% in adult patients who underwent echocardiography was reported (Elshaer et al., 2015).

Two classification of CCD exists: the muscular type is always an embryological developmental defect and usually coexists with midline abdominal abnormalities and other CHDs, such as ASDs, VSDs and PTA (Di Sessa et al., 2006, Villegas Garcia et al., 2001, Romagnoli et al., 2015). A fibrous diverticulum is not associated with midline defects or other CHDs and is usually caused by coronary artery disease, myocardial infarction, or myocardial inflammatory disease (Ohlow, 2006, Pitol et al., 2005).

1.8 Cardiac troponin T in CHDs and CCD

TNNT2 knockdown had been performed in zebrafish and mouse. A significant reduction in TnI and TnC and tropomyosin leading to severe sarcomere defects were observed in zebrafish (Sehnert et al., 2002). Failure of assembly of sarcomeric thin filaments resulted in high lethality (around E10) in the homozygous cTnT^{-/-} mouse, whereas heterozygous cTnT^{+/-} mutants showed normal hearts (Nishii et al., 2008). An association between isolated CHDs and CCD in humans and the structural proteins cTnT mutation has not been reported to our knowledge.

1.9 Maladaptive Cardiac Remodelling

In response to the changes of neurohormonal stimuli or biomechanical stress (changes of blood flow and shear stress), the heart triggers a hypertrophic response which is a compensatory process to normalize the change in the wall stress.

Cardiac hypertrophy is defined as an increase in heart mass and it can be divided into physiological and pathological hypertrophies, depending on the type of stimuli. They can even further be sub-classified into concentric hypertrophy and eccentric hypertrophy (McMullen and Jennings, 2007). Concentric hypertrophy is induced by a pressure overload and involves the addition of the contractile proteins in parallel. This causes a relative increase of the myocytes in width rather than length and is seen by thickening of LV walls with mild dilatation in LV (Figure 1.14A,B) (McMullen and Jennings, 2007, Kehat and Molkentin, 2010). In contrast, eccentric hypertrophy occurs in response to volume overload. Series assembly of sarcomeric proteins causes the increase of the myocytes in length rather than width. Hence, thinning of LV wall with a larger degree of dilatation is observed (Figure 1.14A,B) (McMullen and Jennings, 2007, Kehat and Molkentin, 2010).

The hypertrophy is considered as 'physiological' when the stimuli are isotonic activities such as running, walking or swimming (McMullen and Jennings, 2007). This type of hypertrophy is found mostly in pregnant women and athletes. Isotonic activities cause the dilatation of the skeletal muscle and thus an increase of venous return and volume back to the heart (McMullen and Jennings, 2007). This results in volume overload and eccentric hypertrophy. Static exercise such as weight-lifting only develops muscle tension against resistance and it causes pressure overload and concentric hypertrophy (McMullen and Jennings, 2007).

In contrast, pathological concentric hypertrophy forms as a result from stimuli such as myocardial infarction, hypertension or genetic mutations (i.e. sarcomeric mutations) which causes pressure overloading. Valve disease such as aortic valve lesion or mitral valve regurgitation produces volume overload and forms eccentric hypertrophy (McMullen and Jennings, 2007).

As part of the remodelling process, the enlargement of myocyte size and the addition of the sarcomeric proteins are seen in both eccentric and concentric hypertrophies in order to normalize the wall stress and to preserve cardiac function (McMullen and Jennings, 2007). Pathological hypertrophy further involves the induction of the fetal gene programme, ECM remodelling as well as increased fibrosis (Kehat and Molkentin, 2010). In particular, fetal cardiac genes encoding for sarcomeric proteins, hormonal proteins (angiotensin converting enzyme, atrial natriuretic peptide) as well as proteins involved in glucose and lipid metabolism are activated (Lorell and Carabello, 2000). These phenotypes are not seen in physiological hypertrophy.

However, adaptive responses in pathological hypertrophy will eventually fail to sustain and thus decompensation occurs, which is marked by further chamber dilatation and impaired systolic and diastolic function (McMullen and Jennings, 2007). The cardiac output is reduced and this leads to the formation of CHDs and cardiomyopathies (McMullen and Jennings, 2007). In contrast, decompensation doesn't occur in physiological hypertrophy and hence the cardiac function is well preserved (McMullen and Jennings, 2007).

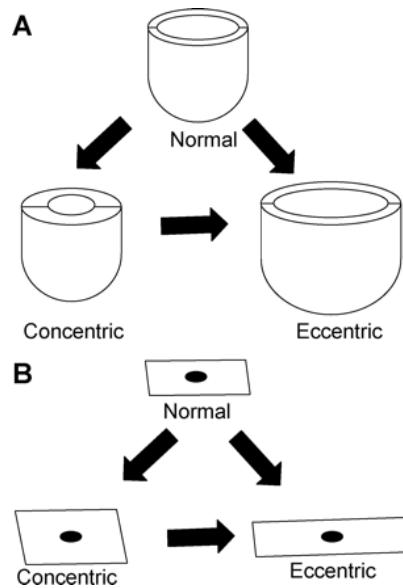


Figure 1.14 Macroscopic and microscopic view of hypertrophy.

A. Macroscopically, the heart can respond to stress by either concentric or eccentric hypertrophy. Concentric hypertrophy is associated with an increase of LV wall thickness without marked changes in chamber size. Eccentric hypertrophy is marked by the thinning of the LV wall with a larger degree of dilatation.

B. Microscopically, concentric hypertrophy is characterized by the addition of the contractile protein units in parallel which results increase in width, whereas eccentric hypertrophy is seen by an assembly of proteins units in series, and thus a greater relative increase of length of the myocytes. Normal heart can develop into both concentric and eccentric hypertrophies and the concentric hypertrophy can also turn into eccentric hypertrophy. Taken from (Kehat and Molkentin, 2010).

1.10 Cardiomyopathy

As discussed in section 1.9, cardiomyopathy develops when maladaptive responses of the heart occur. Cardiomyopathy is defined as a primary myocardium disease with impaired mechanical or electrical dysfunction that usually (but not invariably) exhibits inappropriate ventricular hypertrophy or dilatation (Maron et al., 2006). In addition, cardiomyopathies either are confined to the heart or are part of generalized systemic disorders that often lead to progressive heart failure (Maron et al., 2006).

Cardiomyopathies account for 8-11% of the cardiovascular diagnoses detected in utero and 3% newborns with cardiovascular malformations were identified (Pedra et al., 2002, Allan et al., 1985). Primary causes of fetal cardiomyopathy are mainly due to single gene disorders, such as autorecessive metabolic disorders, chromosome abnormalities and familial cardiomyopathy (Pedra et al., 2002). Cardiomyopathy can also be caused by sarcomeric mutations. The most common sarcomeric mutated genes are the β -MHC (Villard et al., 2005) and cardiac myosin binding protein C (Niimura et al., 1998, Oakley et al., 2004).

Cardiomyopathies are heart-specific or are part of generalized systemic disorders, resulting in heart failure or death. Cardiomyopathies are divided into dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, left ventricular non compaction cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy (Figure 1.15) (Harvey and Leinwand, 2011, McCartan et al., 2012, Morimoto, 2008, Towbin and Bowles, 2002).

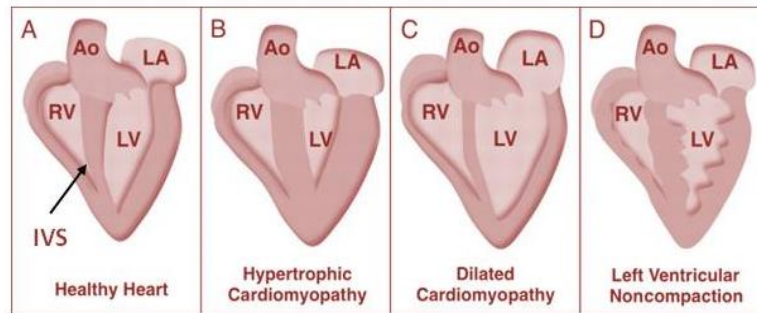


Figure 1.15 Clinical phenotypes of cardiomyopathies. **A.** A normal heart. **B.** HCM with enlarged heart and asymmetric hypertrophy of the IVS and anterior of LV wall. **C.** DCM with enlarged heart and thinning of ventricular walls. **D.** Spongy appearance of trabeculated left ventricular walls in NCCM. Ao, aorta; LA, left atrium; LV, left ventricle; RV, right ventricle; IVS, interventricular septum. Adapted from (Harvey and Leinwand, 2011).

1.10.1 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is the most common type of cardiomyopathy, inherited in an autosomal dominant manner with a prevalence of 1:500 in young people (Maron et al., 2006, Harvey and Leinwand, 2011, Towbin, 2014). HCM can be attributed by physiological or pathological hypertrophy, and the degree of severity can range from asymptomatic to sudden death (McKenna and Deanfield, 1984). HCM is characterized by an increased heart mass, asymmetrical IVS thickening and anterior part of LV wall, with no to mild dilatation of the ventricles (Figure 1.15B) (Maron et al., 2006). Histological hallmark of HCM reveals the enlargement of the myocyte size, myocyte disarray as well as interstitial fibrosis (Harvey and Leinwand, 2011). However, persistent HCM can lead to further dilatation of ventricular chambers and thinning of LV walls and hence heart failure.

To date, mutations identified in eight genes encoding sarcomeric proteins are associated with HCM, with both *MYH7* and *MYBPC3* contributing to 70% of all cases

and other genes include *MYH6*, *TNNT2*, *TNNI3*, *TNNC1* and *TPM1* (Maron et al., 2006, Maron and Maron, 2013, Teekakirikul et al., 2013, Parvari and Levitas, 2012).

1.10.2 Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is a common type of cardiomyopathy that occurs in 1 in 2500 individuals and accounts for 55% of cardiomyopathies in the US (Maron et al., 2006). It is the third most common cause of heart failure and heart transplantation is always required (Maron et al., 2006). Morphologically, it can be characterized by an enlargement of ventricles, thinning of ventricular walls and systolic dysfunction (Figure 1.15C) (Harvey and Leinwand, 2011, Towbin, 2014). DCM can be transmitted in an autosomal dominant or recessive manner, mitochondrial or X-linked inheritance, with the autosomal dominant inheritance being most common (Towbin, 2014, Maron et al., 2006, Harvey and Leinwand, 2011, Hershberger et al., 2010). In contrast to HCM, DCM is linked primarily with mutations in cytoskeletal proteins which are desmin, lamin and vinculin (Harvey and Leinwand, 2011, Towbin, 2014). DCM-causing sarcomeric protein mutations that have been reported are *MYH7*, *TNNT2*, *TNNI3*, *TNNC1* and *TPM1* (Parvari and Levitas, 2012, Teekakirikul et al., 2013, Cahill et al., 2013).

1.10.3 Restrictive cardiomyopathy

Restrictive cardiomyopathy (RCM) is the least common but the most lethal type of cardiomyopathy (Harvey and Leinwand, 2011). RCM is inherited in autosomal dominant pattern and is characterized by an impaired ventricular filling and reduced diastolic ventricular volume, with relatively normal ventricular wall thickness and systolic function (Teekakirikul et al., 2013, Cahill et al., 2013). RCM is found associated with HCM and sudden cardiac death is frequently reported in patients with RCM due to the high incidence of arrhythmias and heart block. Although mutations in cardiac troponin I are found to be the primary cause of RCM, other genes encoding sarcomeric proteins such as *TNNT2*, *TNNI3*, *MYH7*, *ACTC1*, *TPM1*, *MYH3* and *MYL* are also found in patients with RCM (Kaski et al., 2008, Karam et al., 2008, Caleshu et al., 2011, Mogensen et al., 2003).

1.10.4 Left ventricular non-compaction cardiomyopathy

Left ventricular non-compaction cardiomyopathy (LVNC) is an arrest in normal process of myocardial compaction in embryonic development (Towbin, 2014). As a result, two distinct layers form and they are the compacted outer myocardium layer and non-compacted inner spongy layer with deep ventricular trabeculations and intertrabecular recesses (Figure 1.15D) (Towbin, 2014). Patients with LVNC always have depressed systolic and diastolic dysfunction, with some of the conditions worsening to the development of arrhythmias and conduction block. LVNC also affects RV and both ventricles, but at much lower occurrences (Towbin, 2010). Familial LVNC is mainly contributed by autosomal dominant (70%) and X-linked inheritance (30%) (Towbin, 2014). Sarcomeres-encoding genes which are found

mutated in LVNC are *MYH7*, *MYBPC3*, *ACTC1*, *TNNT2* (Monserrat et al., 2007, Klaassen et al., 2008, Probst et al., 2011).

1.10.5 Arrhythmogenic right ventricular cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy (ARVC) occurs in 1:5000 in a general population and it mainly affects adults (Towbin, 2014, Basso et al., 2009). It accounts for 50% of the familial disease and is mainly contributed by autosomal dominant inheritance (Basso et al., 2009). ARVC is seen by progressive replacement of fibro-fatty tissues from the epicardium to the entire heart wall and it mainly affects RV. LV is only involved at a more severe stage of the disease (Basso et al., 2009, Towbin, 2014). Mutations in genes encoding desmosomal proteins are known to be responsible for ARVC (Harvey and Leinwand, 2011). Loss of desmosomal proteins integrity affects gap junction and sodium channel proteins, impeding a proper electrical impulse conduction and thereby promoting arrhythmias (Harvey and Leinwand, 2011). No sarcomeric proteins genes mutations have been reported so far.

1.11 Fetal cardiomyopathy and its clinical significance

Fetal cardiomyopathies are a very rare disease in fetuses and carry a poor outcome (Mongiovi et al., 2010). Fetal cardiomyopathy has a broad spectrum of aetiologies and is associated with substantial morbidity and mortality and can occur in isolation or is associated with other cardiac and non-cardiac malformations (Mongiovi et al., 2010). In a review of the outcomes of 193 fetuses with cardiac abnormalities detected by echocardiography, it was found that the structural heart defects were found in 55 (28%) and other non-structural abnormalities (DCM, HCM and isolated pericardial effusion) in 33 (17%) (Eronen, 1997). The prognosis was poor in fetuses with structural heart defects as 37 of 55 fetuses (67%) were found died in utero or postnatally, with a poor

outcome also found in fetuses with DCM in eight of 13 (62%) and with HCM in one of eight (13%) of cases (Eronen, 1997).

In another retrospective review of 61 consecutive fetal cases with a diagnosis of cardiomyopathy at a single center between 2000 and 2012, hypertrophic (HCM) or nonhypertrophic cardiomyopathy (NHCM) phenotype were identified in 21 fetuses and 40 fetuses respectively and they were showing overlapping clinical phenotypes (Weber et al., 2014). Both ventricles were hypertrophied or had normal wall thickness and impaired ventricular diastolic filling seen in RCM in adults occurred in all HCM and NHCM fetus (Weber et al., 2014). Systolic and diastolic dysfunction and significant atrioventricular valve regurgitation were implicated in most cases in fetal cardiomyopathies, with diastolic dysfunction imposing the highest mortality rate (Pedra et al., 2002).

In addition, marked LA and LV enlargement were found accompanied by the compression of the right heart in 14 fetuses and seven neonates with severe aortic stenosis and they occurred in conjunction with the abnormal MV and mitral regurgitation (Rogers et al., 2011, Vogel et al., 2011). Further, most of the fetuses displayed endocardial fibroelastosis in the LV (Rogers et al., 2011, Vogel et al., 2011). Fetal echocardiographic imaging is the best method currently available to diagnose congenital cardiac anomalies and fetal cardiomyopathies prenatally and the doppler mode is used to evaluate haemodynamic conditions in the fetal hearts (Zhang et al., 2016). However, perinatal morbidity and mortality are still significant, with most aetiologies remaining unknown despite massive efforts have been put to identify the causal diagnosis. Hence, DNA microarray and whole genome sequencing have been increasingly used to pinpoint the underlying mechanisms.

1.12 Cardiac troponin T in cardiomyopathies

cTnT has been suggested to play some functional roles in the troponin complex in the heart that undergoes remodelling (Sato et al., 2012). Re-expression of fetal isoforms of cTnT isoforms 1 and isoform 2 has been reported to respond differently to changes of Ca^{2+} concentrations within the cells and force generation in the sarcomere at both mRNA and protein levels in the failing heart (Anderson et al., 1995, Gomes et al., 2002).

Most mutations found in TnT are known to cause mild cardiac hypertrophy and remain subclinical, but in contrast they can also lead to sudden cardiac death (Lu et al., 2013). More than a hundred mutations of the troponin complex are found to be associated with HCM, DCM, LVNC and RCM, and over 50 mutations are attributed by *TNNT2* gene (Lu et al., 2013). cTnT mutations occur predominantly in the T1 region in patients with cardiomyopathies, but they result in two distinct forms of cardiomyopathy by altering Ca^{2+} sensitivity in cardiac muscle contraction in the opposite direction (Lu et al., 2013, Palm et al., 2001, Morimoto, 2008). Mutations in cTnT increased Ca^{2+} sensitivity by decreasing the inhibitory action of cTnI on the thin filament and they were found in patients with HCM and RCM, thus enhancing force generation (Lu et al., 2013). In contrast, mutations in cTnT also decreased the Ca^{2+} sensitivity by having cTnI inhibiting the thin filament more effectively in patients with DCM (Lu et al., 2013).

The heterozygous *Tnnt2*^{+/-} mouse showed a mild decrease in mRNA level and an unaltered protein level (Ahmad et al., 2008). In contrast, another study showed a decreased amount of protein (Nishii et al., 2008). However, they all exhibited a normal cardiac phenotype. On the other hand, most homozygous *Tnnt2*^{-/-} embryos lacked a heart beat and were not viable beyond E10.5, whereas surviving homozygous

Tnnt2^{-/-} mutants appeared to have an enlarged heart with looping and endocardial cushion defects (Nishii et al., 2008). However, normal cardiac looping was seen in another study (Ahmad et al., 2008).

Transgenic mice expressing <10% of the R92Q mutation showed a range of anomalies that resembled HCM phenotypes seen in humans (Tardiff et al., 1999). They included myofibril disarray, increased myocardial interstitial collagen and impaired sarcomeric relaxation (Tardiff et al., 1999). Overexpression of the *Tnnt2* gene by 30-92% resulted in smaller heart with diastolic dysfunction but enhanced systolic function (Tardiff et al., 1999). Altered mitochondria pathology, an increased number of mitochondria with increased lipid deposition and interstitial fibrosis were observed in these hearts (Tardiff et al., 1999). An increase in myofilament Ca²⁺ sensitivity impaired relaxation of the cardiac muscle (Morimoto et al., 2002). This caused a diastolic dysfunction which is believed to be a causal of high incidence of sudden cardiac death (Morimoto et al., 2002).

Mice with deletion DCM-causing mutation of Δ K210 were found to recapitulate some phenotypes seen in human with DCM (Morimoto et al., 2002). The phenotypes were seen as enlarged hearts and heart failure with a high incidence of sudden cardiac death (Du et al., 2007). The underlying mechanisms were suggested to be due to a decrease in myofilament Ca²⁺ sensitivity of force generation with a compensatory increase of peak amplitude of Ca²⁺ transient in cardiomyocytes. However, maximum force generation capability remained unaffected (Morimoto et al., 2002, Du et al., 2007).

1.13 Congenital heart defects and cardiomyopathy induced by haemodynamics intervention in embryonic animal model

Alteration of haemodynamics in an embryonic model can be achieved by performing surgical manipulations at different parts of the developing heart, resulting in defects resembling human CHDs (Midgett and Rugonyi, 2014). The three main surgical manipulated embryonic models described in the literature are vitelline vein ligation (VVL), left atrial ligation (LAL) and outflow tract banding (OFT-banding) (the embryonic animal model used in this project) (Figure 1.16) (Midgett and Rugonyi, 2014). VVL mimics placental infarction by reducing the venous inflow and thus haemodynamic load on the developing heart (Figure 1.16A,B) (Hogers et al., 1999). LAL reproduces hypoplastic left heart hypoplasia model via reduction of blood flow into LV and thus it successfully delays its development (Figure 1.16C,D) (Tobita and Keller, 2000). This leads to the compensation of the right side of the heart and thus the formation of the left heart hypoplasia. Lastly, OFT-banding mimics cardiomyopathy by increasing the haemodynamic load on the heart by constricting the diameter of the OFT and thus generating a high shear stress and high blood pressure condition in the heart (Figure 1.16E,F) (Sedmera et al., 1999). Although the resulting defects of distorted blood flow are known, the underlying mechanisms by which the haemodynamic forces lead to CHDs are yet to be determined. VVL and LAL models are beyond the scope of this thesis and OFT-banding model only will be further discussed. In this section, haemodynamic responses, together with the alteration of tissue properties and OFT-banding induced defects will be discussed.

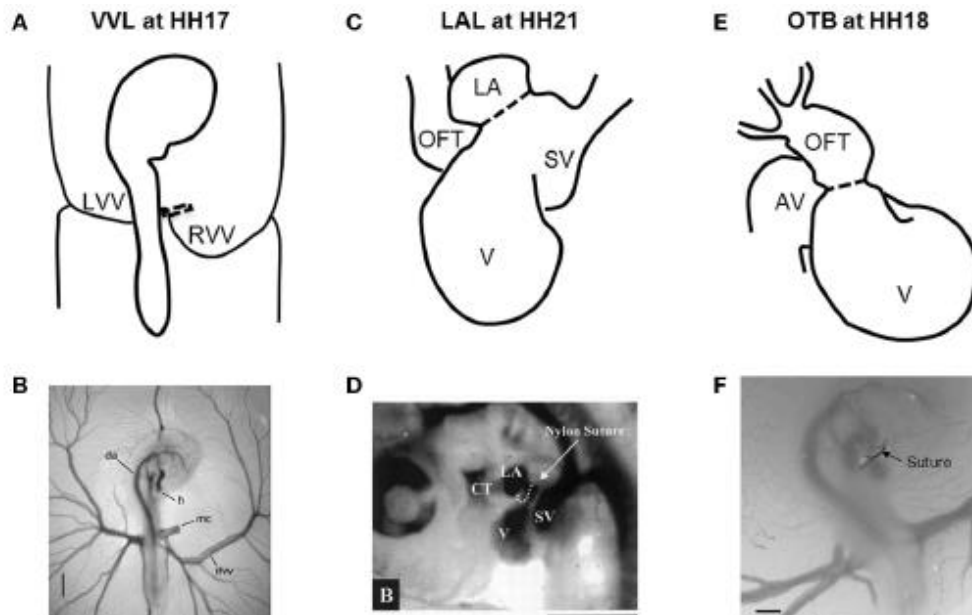


Figure 1.16 Schematic diagrams and experimental photos of three surgical intervention of haemodynamic alterations in chick embryos. (A,B). Right vitelline vein ligation (VVL) at HH17. The obstruction of venous inflow back to the heart is performed by clipping or ligation (see dotted line in A). **(C,D).** Left atrial ligation (LAL) at HH21. The dotted line in C represents where the suture is tied around the LA to reduce blood flow to the LV. **(E,F).** Outflow tract banding (OTB) at HH18. The dotted line in E shows where the suture is tied around the OFT to reduce the blood flow in the OFT and increases the blood pressure in the heart. AV, atrioventricular canal; CT, conotruncus, or outflow tract; DA, dorsal aorta; H, heart; LA, left atrium; LVV, left vitelline vein; MC, microclip; OFT, outflow tract; RLW, right lateral vitelline vein or RVV; RVV, right vitelline vein, SV, sinus venosus; V, primitive ventricle. Adapted from (Lindsey et al., 2014).

1.13.1.1 Outflow tract banding (our embryonic animal model in this project)

An overhand knot is tied to constrict the lumen of the OFT at HH18-HH21 (looping is underway and chamber is not fully septated); this reduces the blood flow in the OFT but increases the blood pressure in the whole heart (Figure 1.16E,F) (Sedmera et al., 1999, Shi et al., 2013). In this project, OFT-banding was performed only at HH21.

1.13.1.2 OFT-induced haemodynamic responses

After banding at HH21, embryos showed an acute increase of peak systolic by 35% and end diastolic ventricular pressure by 11% and stayed at sustained increase at all stages investigated (HH24, HH27, HH29, HH34) (Clark et al., 1989, Tobita et al., 2002). Increased regurgitation was seen in 75% of embryos in the OFT region whereas only in 12.5% embryos around the atrioventricular region (McQuinn et al., 2007). OFT velocity was significantly increased by 120% at HH24 and persisted until HH29 (McQuinn et al., 2007). Reduction of the OFT cushion volume and altered expression of OFT valve genes were reported after 1 hour of banding of the OFT in HH16-17 embryos (Menon et al., 2015). In addition, it had been reported that systolic and diastolic blood pressure in the ventricle, aortic sac and dorsal aorta were increased in a linear relationship with band tightness until it reached 40% band constriction (Shi et al., 2013). Despite a marked increase of ventricular pressure seen in OFT-banded embryos, heart rate and cardiac output/ejection fraction were not affected (Clark et al., 1989, McQuinn et al., 2007). This implies the function of the ventricle is still preserved.

1.13.1.3 OFT-banded induced tissue remodeling

Upon ligation of the OFT at HH21, an increased pressure load to the whole heart is imposed. To counteract this, the dilatation of the whole heart is marked by an increase of ventricular dimension and volume, giving rise to a spherical shape (Clark et al., 1989, Tobita et al., 2002, Buffinton et al., 2013). IVS is equidistant between RV and LV, which is different to a right-shifting of the IVS observed in uneven loading volume in LAL model (Rychter et al., 1979). Also, compact myocardium became thickened in the apical part of the heart, with a concomitant increase in ventricular lumen at HH29 (Clark et al., 1989, Tobita et al., 2002, Sedmera et al., 1999). However, the thickness of the compact myocardium, together with the overall wall thickness were significantly less than the controls at HH36 (Tomanek et al., 1999). Myocyte proliferation was significantly higher 48 hours after banding at HH21 in the OFT-banded hearts after banding at HH21 (Krejci et al., 2016). A concomitant increase of fibroblast growth factor 2 (FGF2) at mRNA and protein level were found and FGF2 has been reported to be involved in proliferation and morphogenesis (Krejci et al., 2016).

Dilatation of the heart is mainly attributed to the myocyte hyperplasia rather than hypertrophy (Clark et al., 1989). This is because the organelle proportion of myofibrils and mitochondria, DNA-to-protein ratio as well as the myocyte area were found similar in both the OFT-banded hearts and controls (Clark et al., 1989).

In normal development, the myofiber patterning was arranged in circumferential direction until HH27 and was changed to longitudinal orientation from HH27-HH36 but these changes became accelerated after banding at HH21 (Tobita et al., 2005). Banding of OFT at HH21 resulted in an increase stress-strain relations and myocardial stiffness at HH27-HH31 (Miller et al., 2003, Buffinton et al., 2013). However, an

increase of end-diastolic stress was reported in RV but both end-diastolic stress and strains were normalized in LV at HH27 after OFT-banding (Tobita et al., 2002).

The haemodynamically induced changes in myocardial architecture in the OFT-banded model could contribute to altered electrical pathway. Optical mapping was used to investigate the banded hearts and it was found that increased pressure loading resulted in precocious maturation of the ventricular conduction system (Hall et al., 2004, Reckova et al., 2003). At the molecular level, these changes were paralleled by upregulation of conduction system differentiation markers endothelin-converting enzyme 1 (ECE1) and connexin 40 (GJA4) (Hall et al., 2004).

1.13.1.4 OFT-banding induced cardiac defects

VSD is the most common defect seen in the OFT-banded model and it contributes to 76%-100% penetrance in manipulated embryos, compared to only 25% incidence observed in LAL embryos (Midgett and Rugonyi, 2014). Upon obstruction of the blood flow in the OFT at HH21, stunted growth in coronary arteries was seen in all three of the hearts analysed, with each heart giving different morphological phenotypes (Tomanek et al., 1999). Remodelling of right atrioventricular valve to bicuspid valve had also been reported in this model (Sedmera et al., 1999). In a recent study, it was found that the altered haemodynamics led to changes in OFT cushion volume and the dysregulation of a number of genes involved in EMT signalling (e.g type III transforming growth factor (*TGFβRIII*) and *TGFβ3*) and cell migration (e.g cadherin 11) (Menon et al., 2015).

A wide spectrum of pharyngeal arch anomalies (abnormal persistence and regression of any segments of the embryonic pharyngeal arch system) was also seen in 44%-100% of OFT-banded embryos, depending on the duration of outflow tract constriction

(Clark and Rosenquist, 1978). The malformations included the absence, interruption and tubular hypoplasia of the right transverse aortic arch in OFT-banded embryos at HH38-39 ($n=8$). Two of these embryos had further developed persistence of the left 4th aortic arch, with one of them formed the patency of the dorsal aorta and another one developed non-patent left arch and an anomalous vessel extending parallel to the left branchiocephalic artery (Clark and Rosenquist, 1978). All these embryos had associated ventricular septal defects (VSDs), with the exception of the embryo with tubular hypoplasia. In addition, the mean aortic root ratio (0.47) was significantly smaller in banded hearts when compared to controls (0.50), where $p<0.05$ (Clark and Rosenquist, 1978). Also, the right aortic arch ratio (0.17) was significantly smaller than controls (0.42), where $p<0.001$. The branchiocephalic artery and pulmonary artery ratio of the banded hearts were significantly larger than controls; $p<0.05$ (Clark and Rosenquist, 1978).

In addition, another 16 embryos were found to have isolated persistence of the left aortic arch at HH40 (Clark and Rosenquist, 1978). Bilateral aortic arches were joined to the dorsal aorta in one embryo, and the remaining 15 embryos displayed persistent left aortic arch coursed laterally to the left branchiocephalic artery. In these 16 embryos, three embryos had associated VSDs, two had right 4th aortic arch anomalies and one had VSD with persistent left aortic arch (Clark and Rosenquist, 1978).

1.14 Chick as our research model

Chick embryos have been employed systematically as a valuable research model in developmental biology for at least two millennia. Aristotle was the first to study development by opening chicken eggs at different time points and performing observations and dissections due to its ease of manipulation and accurate staging *in ovo* (Stern, 2005). It is known as one of the frequently employed model systems to study cardiac development and to understand the origin of the CHDs. First of all, chick genome sequences are highly conserved compared to humans (Wallis et al., 2004). Secondly, the stationary developing pattern at early stage of chick embryos make them easy accessible for genetic manipulation and haemodynamic intervention *in ovo*. Genetic manipulation by spatial and temporal gain and loss-of-function techniques (siRNAs and morpholinos) can be achieved by applying morpholino via pluronic gel directly on the chick embryonic heart (Rutland et al., 2011, Katahira and Nakamura, 2003). In addition, chick embryos are always employed to study normal haemodynamic pattern and altered haemodynamic condition upon surgical intervention (Midgett and Rugonyi, 2014, Midgett et al., 2015). Instantaneous change of blood flow and heart rate in chick upon haemodynamic intervention can be evaluated by ultrasound biomicroscopy and doppler optical coherence tomography (McQuinn et al., 2007).

Also, a specific stage of chick embryo can be easily acquired by incubating them for a specific amount of time (Hamburger and Hamilton, 1951). As eggs are self-sufficient, they can readily develop at 37°C and at 60% humidity without any artificial support media (Kain et al., 2014).

During development, the hearts in human and chick are very similar, as both develop from a linear heart tube to a four-chambered heart. However, there are some

morphological differences, in that septum secundum doesn't form in the chick during atrial septation. The septum primum will grow inferiorly from the cranio-dorsal wall of the atrium and closes within two days after hatching (Martinsen, 2005). With regards to the pharyngeal arch development, the right 4th arch persists as definitive right aortic arch but the left 4th aortic arch degenerates around days 6-7 in chick (Bellairs and Osmond, 2005, Johnson and Bamforth, 2016). In humans, the left 4th aortic arch is retained but the right 4th arch is lost (Bellairs and Osmond, 2005).

A disadvantage of using chick embryos is the knockdown techniques using clustered regularly interspaced short palindromic repeats (CRISPR) or transcription activator-like effector nucleases (TALEN) are not feasible in chick embryos to date.

1.15 Morpholinos as a tool of knockdown

Morpholinos are 25 bases of antisense oligonucleotides which bind to the complementary mRNA sequences by specific base-pair binding (Summerton, 2007). Morpholinos have hexameric morpholino rings replacing the normal occurring deoxyribose rings as a backbone. They are linked through the non-ionic phosphodiamidate intersubunit linkages instead of negatively-charged phosphodiester bonds between the RNA (Figure 1.17A,B) (Summerton, 2007). These structural changes result in high solubility in aqueous solution and higher stability in biological systems (Summerton and Weller, 1997, Hudziak et al., 1996). The highly unnatural backbone and its lack of charge properties confers no off target effects in morpholinos as they are not able to bind with extracellular, intracellular and cell surface proteins. Upon binding, they result in a steric blocking and inhibit the expression of its targeted mRNA (Summerton, 2007). Depending on the site of the morpholinos being designed, they can either prevent translation by targeting the 5' UTR through the firsts 25 bases

of coding sequence at the AUG start site in the cytosol or by impairing the transcription via modification of the pre-mRNA splicing at splice junctions or splice regulatory sites in the nucleus (Moulton and Jiang, 2009). Morpholinos differ from other antisense oligonucleotides such as phosphothioate-linked DNA (S-DNA) and short interfering RNA (siRNA); these oligonucleotides are dependent on the cellular RNase-H or the RNA induced silencing complex (RISC) to degrade the targeted RNA.

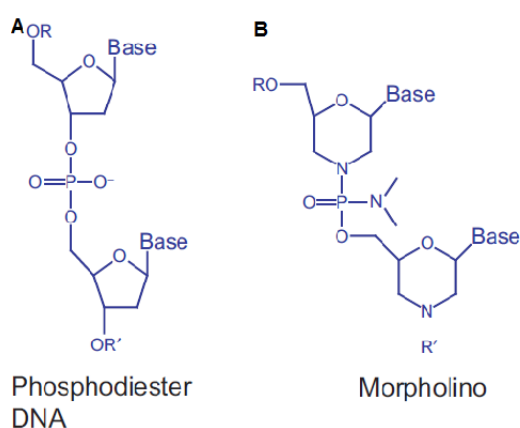


Figure 1.17 Structure of the phosphodiester DNA and morpholino. **A.** DNA is composed of 5-membered ring linked to negatively-charged phosphodiester bonds. **B.** Morpholinos contain a 6 membered morpholino ring and is attached with non-ionic phosphoramidate linkage which results in virtually no off-target effects and high stability in biological system. Adapted from (Corey and Abrams, 2001).

Aims and objectives of this study

Haemodynamics is an epigenetic factor that modulates the critical stages of heart development. Alteration of haemodynamics can be associated with CHDs and cardiomyopathies with unknown causes. Abnormal haemodynamic patterning causes instant changes of blood pressure and shear stress and hence triggers the endothelial cell reorganization and signalling by altering the endocardial cell expression of shear stress responsive genes (Groenendijk et al., 2004). As part of the compensatory processes, myofiber orientation adjusts to compensate the altered wall stresses and strains (Tobita et al., 2002, Tobita et al., 2005). Adaptive responses eventually fail to sustain and a spectrum of cardiac defects arise (Sedmera et al., 1999, Hogers et al., 1997). In addition, sarcomeric proteins have been associated with a range of cardiomyopathies but only have been linked with CHDs in recent years (E.g *MYH6*, *MYH7* and *ACTA1*). To date, cardiac troponin T (*TNNT2*) has been linked with cardiomyopathies but not isolated CHDs.

The OFT-banding model is a well-known haemodynamic intervention that leads to a wide range of CHDs. The altered tissue morphologies found in this model resemble the clinical features in human cardiomyopathies. This project comprises two major parts. Firstly, alteration of haemodynamics was achieved by surgical intervention, i.e, performing knot tying around the OFT in a HH21 chick embryonic heart *in ovo*. External and internal phenotypic analysis and stereological examination were performed to ascertain if any gross morphological abnormalities had occurred. RNA sequencing was performed to study altered global gene expression in OFT-banded hearts. The cellular effect and molecular effect of OFT-banding were particularly studied on developing atrioventricular valve in these hearts.

Secondly, to investigate the role of cardiac troponin T in a developing heart, antisense oligonucleotide morpholino was applied to downregulate the cardiac troponin T protein expression in the developing heart at the stage where the heart tube had just formed. External and internal phenotypic analysis were initially performed and followed by investigating the molecular effect of the morpholino treatment in the hearts.

The hypothesis of this thesis is:

H₀: Mechanical or genetic manipulation of the developing heart will not induce cardiomyopathy-like phenotype.

H₁: Mechanical or genetic manipulation of the developing heart will induce cardiomyopathy-like phenotype

2 CHAPTER 2: Materials and methods

2.1 Chick embryo techniques

2.1.1 Egg incubation and general care and conditions

Fertilized white leghorn chicken eggs (*Gallus gallus*; Henry Stewart, Lincolnshire, UK) were incubated at 38 °C with constant rotation in a humidified atmosphere until they reached the required age (Bellairs and Osmond, 2005). A small window was made on the blunt-end of the shell. 3-5 ml of albumin was carefully removed from the egg but avoiding the yolk and chorioallantoic membrane. This separated the embryo from the extra-embryonic membranes. The embryo was then localized and staged. Animal work was performed in accordance with national (UK home office) and institutional regulations and ethical guidelines. The Hamburger and Hamilton (HH) staging system was used to stage the developing chicks (Hamburger and Hamilton, 1992).

2.2 Morpholino knockdown

2.2.1 Morpholino design

An antisense oligonucleotide morpholinos targeting at the ATG translational start site was designed against *TNNT2* (ENSGALT00000000401; 5'-TGGCTATCTAGCAGAACGCAAGGCA-3'; GeneTools LLC, USA). Also, a GeneTools standard control (SC) morpholino designed to mutated human β -globin gene (5'-CCTCTTACCTCAGTTACAATTTATA-3'; GeneTools) was used. Morpholinos were 5'-fluorescein fluorescent tagged to detect morpholino uptake by the embryo. A BLASTN search revealed 100% homology of the morpholino nucleotides only to the *TNNT2* with E values close to zero, showing the morpholinos designed were highly specific. In addition, untreated controls (UT) were included, in which the

embryos underwent the same treatment as the experimental and SC groups but the morpholino/pluronic gel mix was not applied.

2.2.2 Morpholino application *in ovo*

Morpholino application was performed at HH10/11 (about 48 hours of incubation). Both experimental and control morpholinos were diluted in Hanks' Balanced Salt Solution (HBSS) and then in a 1 : 1 ratio with 30% F127 pluronic gel (BASF Corp., Germany) to give a final concentration of 250 μ M or 500 μ M for the morpholino and 15% for the pluronic gel. Morpholinos were kept on ice to prevent thermogelation of the pluronic gel. Morpholino/pluronic gel mixture was applied directly to the exposed chick heart. The eggs were then resealed using masking tape and were reincubated with no rotation until they reached HH19 (75-80 hours of development).

2.3 Qualitative assessment of morpholino uptake on HH19 embryos

At HH19, embryos were harvested *in ovo* by cutting around the chorioallantoic membrane and placed into a petri dish with ice cold phosphate buffered saline (PBS) (Thermo Scientific, UK). Chorioallantoic membrane was removed as it gave fluorescence owing to 5'-fluorescein morpholino application technique. Positive morpholino uptake was confirmed in each experimental and SC embryo by detection and qualitative assessment of the fluorescence intensity in the heart using a SV11 stereomicroscope (Zeiss, Germany).

2.4 Outflow tract banding

2.4.1 Optimization of outflow tract banding

For OFT-banding, embryos were incubated to HH21 (approximately 4 days 4 hours of development). A small window was made on the shell and the underlying inner shell membrane was removed to expose the embryo for staging. Any embryos that showed haemorrhage upon surgical manipulation or displayed obvious structural malformations and developmental retardation were excluded from the studies.

A swaged needle with 3/8 circle spatula advanced micro-point double needle attached to 10-0 suture with the length of 13mm (W1770 Ethilon, Johnson Johnson) was used. The needle body was grasped in the holders on its flattened area approximately one-third of its length away from the suture material. The needle was placed and passed in between the mid-portion of OFT and the primitive atrium in ventral-dorsal direction *in ovo* with slight pronation of the wrist. Force was applied in the natural curve of the curvature of the needle by rotating the wrist. When the needle emerged from the opposite site of the OFT, it was retrieved from the other end by grasping approximately one-third from needle tip and was pulled through following the natural arc of the needle. Suture was also be pushed through on the other end.

A loop was formed in the suture and the end of the suture was passed through the loop to make a first overhand knot. End of suture was passed through the loop again to make a secure double overhand knot. A completed knot was a snug surrounding the OFT. Excess suture on one end was trimmed but not to the extent of causing slippage of the knot. The window was then sealed with masking tape, and the eggs were reincubated with no rotation to HH29 (a further 3 days 4 hours) or HH35 (another 5 days 4 hours).

Three groups of embryos were studied: (i) unoperated group, in which no interventions were performed; (ii) sham group in which a 10-0 nylon suture was passed under the OFT but not tightened and was removed immediately; and (iii) outflow tract banding (OFT-banded) group, in which a 10-0 nylon suture was passed under the OFT and tied in a double knot around the OFT as described above. The OFT-banding procedure was performed using SV11 stereomicroscope (Zeiss, Germany).

2.5 Qualitative assessment of OFT-banded embryos at HH29 and HH35

To ensure constriction of the OFT of chick embryonic hearts had taken place after banding, all OFT-banded hearts were morphologically assessed upon harvesting. The OFT-banded hearts were only harvested when the suture was still attached with an indentation of the OFT and the nylon loop was frequently observed to be embedded in the OFT region as previously described (Figure 2.1) (Clark and Rosenquist, 1978, Sedmera et al., 1999).

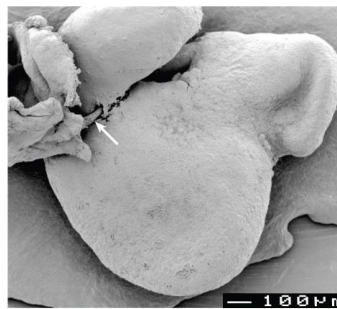


Figure 2.1 A scanning electron micrograph denotes an approximate position of a ligature (white arrow) placed around the OFT. Scale bar: 100µm. Adapted and modified from (Krejci et al., 2016).

2.6 Phenotypic analysis of morpholino-treated and OFT-banded embryos

2.6.1 Embryo harvesting, processing and sectioning

All embryos were externally examined upon harvesting. The whole embryo and the size and the shape of the heart were morphologically assessed. Any embryos showing structural deformities and developmental delay were excluded. For morpholino study, the embryos were harvested as a whole for HH19 [250µm study: control (untreated and sham) ($n=34$); TNNT2-MO-treated ($n=43$), 500µm study: control ($n=84$); TNNT2-MO-treated ($n=67$)]. For OFT-banding study, the embryonic hearts were dissected for HH26 [control (unoperated and sham) ($n=16$) and OFT-banded hearts ($n=16$)], HH29 [control ($n=97$) and OFT-banded hearts ($n=60$)] and HH35 [control ($n=35$) and OFT-banded hearts ($n=9$)]. They were then fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich, UK) for 1.5 hours at room temperature (RT) for HH19 morpholino-treated embryos and HH21 control embryos and overnight at 4 °C for HH25, HH26, HH29 and HH35 OFT-banded embryos. Embryos were washed in PBS and distilled water followed by graded ethanol series (70%, 90% and 100% ethanol). They were then cleared with xylene and were paraffin-embedded. HH19 and HH21 embryos, HH25, HH26, HH29 and HH35 hearts were all serially sectioned at 8µm in transverse orientation using a Leica DSC1 microtome (Leica, Germany). Sections were floated onto 42°C water bath and were then collected onto 3-aminopropyltriethoxysilane (APES)-treated glass slides. Positively-charged APES slides ensure firm adhesion of the sections to the slides. Sections were then left to dry out at 42°C on a hot plate overnight. Embryos with diverticula were serially sectioned at 6 µm sagittally, dewaxed and rehydrated in graded ethanol series and water, with

neighbouring sections placed on adjacent slides to allow for the analysis of different stains and these will be further described in section 2.6.3, 2.6.4 and 2.10.1.

2.6.2 Haemalum staining on morpholino-treated HH19 embryo

For internal phenotypic analysis, sections of morpholino-treated HH19 embryos [250µm study: controls ($n=15$) and TNNT2-morpholino treated embryos ($n=19$); 500µm study: controls ($n=38$) and TNNT2-morpholino treated embryos ($n=51$)] were deparaffinized in xylene, rehydrated in a graded ethanol series and were stained for nuclear stain Mayer's Haemalum (Fisher Scientific, UK) for a minute. They were then dehydrated in a graded ethanol series and cleared in xylene. Sections were finally mounted in DPX resinous mounting medium (Fisher Scientific, UK). Internal features such as primitive atrial septum, endocardial cushions, cardiac jelly and ventricular trabeculae were examined. Analysis was performed blinded using a Nikon Eclipse microscope (Nikon Instruments Inc., UK).

2.6.3 Alcian blue and haemalum staining on morpholino-treated HH19 hearts with diverticula and HH35 OFT-banded hearts

First set of serial sections of morpholino-treated HH19 embryos with diverticula ($n=2$ per group) were stained with Alcian blue (Sigma Aldrich, UK) for 15 minutes followed by the staining of Mayers haemalum. Alcian blue stains the glycosaminoglycans, which are the main components of extracellular matrix in developing embryos (Tuckett and Morriss-Kay, 1988). With morpholino-treated HH19 hearts with diverticula, primitive atrial septum, endocardial cushions and ventricular trabeculae were morphologically assessed as described in 2.6.2. The position of the diverticula and its communication in relation to both atrial and ventricular chambers were

morphologically examined as well. For internal phenotypic analysis of the sections of OFT-banded hearts harvested at different stages [i.e, HH26 ($n=5$ per group), HH29 (controls ($n=37$); OFT-banded ($n=31$)) and HH35 (controls ($n=8$); OFT-banded ($n=7$))] were stained with Alcian blue and haemalum. Analysis of internal structures such as atrial and ventricular myocardial wall, interatrial and interventricular septum, atrioventricular valves, ventricular trabeculae and epicardium were carried out. For phenotypic analysis at all stages, imaging was acquired with Axioplan microscope (Zeiss).

2.6.4 Masson trichrome staining on morpholino-treated HH19 hearts with diverticula and HH35 OFT-banded hearts

Second set of serial sections of HH19 hearts with diverticula ($n=2$ per group) and OFT-banded hearts at HH35 ($n=5$ per group) were subjected to Masson's Trichrome staining using Trichrome stain (Masson) kit (HT15, Sigma Aldrich, UK). Firstly, they were mordanted in Bouin's solution (Sigma Aldrich, UK) fixative for 1 hour at 56 °C and washed in running water until the sections went clear. Sections were then stained using nuclei stain Weigert's iron hematoxylin working solution for 8 min, Biebrich scarlet-acid fuchsin for muscle stain for 5 min, phosphotungstic-phosphomolybdic acid for 12 min, and aniline blue solution for collagen stain for 5 min. Sections were differentiated in 1% aqueous acetic acid for 5 min followed by dehydration and were finally mounted in DPX mounting resinous medium (Fisher Scientific, UK). Images were taken using an Axioplan microscope (Zeiss).

2.7 Systematic random sampling on HH29 OFT-banded hearts

For stereology, the stained slides of HH29 embryonic hearts were scanned on a whole slide scanner (Nanozoomer 2.0-HT, Hamamatsu, Japan) to acquire whole heart images at $\times 20$ magnification. Systematic random sampling was used to quantify the cardiac tissue proportions throughout the OFT-banded HH29 hearts from section 2.6.3. Two groups were analysed: sham ($n=6$) and OFT-banded hearts ($n=7$). A 96-point grid was placed over every fifth section throughout the heart, and the tissue type on each point was identified (4479 points counted for sham and 7868 points counted for OFT-banded hearts). The tissue region consisted of right (RA) and left atrium (LA), right (RV) and left ventricle (LV), dorsal mesenchymal protrusion, atrial septum, primordial atrioventricular (AV) region and epicardium. In addition, the tissue types counted included myocardial wall, extracellular matrix and lumen in the regions of RV and LV and myocardial wall and lumen for RA and LA. The proportion of the tissues was obtained by dividing the number of counts in that region to the total number of counts of the whole heart. The average tissue proportions of each group was calculated and tested for statistical significance. Numbers shown in results indicate average $\pm$ s.e.m.

2.8 Morphometric measurement of relative cushion area and valve length

The endocardial cushion area (μm^2) and the raw length primordial LAV septal leaflet (μm) were measured on every third section of HH29 hearts [control (unoperated and sham) and OFT-banded; $n=6$ for unoperated and sham respectively and $n=7$ for OFT-banded group] using nanozoomer viewing software (NDP.view2). The relative cushion area was determined by dividing the cushion area of OFT-banded hearts by that of control heart. Same procedure applied for the valve length.

2.9 Statistics

Pearson chi square test and levene's test for equality of variances followed by t-test for equality on SPSS V21 (SPSS, USA) were used; $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered to be significant.

2.10 Immunohistochemistry

2.10.1 Immunohistochemistry on morpholino-treated HH19 hearts

Fluorescence immunohistochemistry was performed on the third set of serial sections of HH11/19 cTnT morpholino-treated hearts with diverticula ($n=2$ per group). Antigen retrieval was performed (microwaving for 15 minutes in 10 mM Citric acid buffer pH 6.0). They were blocked in 5% pre-immune goat serum in 1% Bovine Serum Albumin (BSA)/PBS for 30 minutes at RT followed by the incubation of primary antibody A4.1025 (a marker for sarcomeric myosin heavy chain; 1 : 10; DSHB, USA) for an hour at RT. After washing (3X in PBS), sections were incubated with secondary antibody Cy2 (1 : 100; Jackson immunoresearch Inc, USA) for 30 minutes. Finally, nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; 1 : 1000; Sigma, D9542). Imaging was carried out on a LSM5 Exciter confocal microscope (Zeiss).

2.10.2 Immunohistochemistry on OFT-banded HH35 hearts and spatial expression profile studies of valve development of HH21, HH25, HH29 and HH35 hearts

HH35 OFT-banded hearts ($n=5$ per group) were isolated and wax-embedded as described in section 2.6.1. Antigen retrieval was performed by microwaving for 10 minutes in 10 mM sodium citrate, pH 6.0. Sections were then processed for immunofluorescence. They were blocked in 10% preimmune goat serum in 1% BSA/PBS for 2 hours at RT with primary antibody incubation overnight at 4 °C. After

washing (3X in PBS), the secondary antibody (Alexa 488, 1 : 100, Molecular Probes) was incubated for 1 hr at RT; finally nuclei were stained with DAPI (1 : 1000, Sigma : D9542). The following primary antibodies were used for immunostaining. They were JB3 (specific for fibrillin-2; 1 : 50; Developmental Studies Hybridoma Bank (DSHB), USA), M1-B4 (specific for tenascin; 1 : 50; DSHB, USA) and 3B2 (specific for type III collagen; 1 : 25; DSHB, USA).

To study the expression profile of the valve development, the HH21, HH25, HH29 and HH35 hearts ($n=3$ for each stage) were isolated and embedded in paraffin (section 2.6.1). Again, the antigen retrieval was performed using 10mM sodium citrate and the immunohistochemistry was repeated on these hearts using the same antibodies specific for fibrillin-2, tenascin and type III collagen as described above. Images were acquired using Leica DMIRE2 inverted microscope.

2.10.3 Quantification of fluorescence intensity

The fluorescent images of HH35 hearts were acquired on a Leica DMIRE2 inverted microscope, using a 10x air objective in the left AV region of first set of sister sections stained with fibrillin-2. The exposure times were similar for DAPI and FITC panels, with the images for hearts were acquired immediately after the images of shams. Mean of OFT-banded hearts intensities of intrinsic fluorescent signals of region of interest (ROI) were detected and calculated for quantification using MetaExpress software. Intensity of intrinsic signal intensities was reflected by grey values of all pixels within an ROI. Mean intensities were calculated across the valve region on sister sections of both OFT-banded hearts and sham of ($n=5$ for each group). A similar procedure was repeated on the second set of sister sections immunostained with type III collagen.

2.11 Western blot

Western blot was performed by Jennifer England (former PhD student) to demonstrate successful knockdown in the TNNT2-MO treated hearts. Individual hearts (TNNT2-MO-treated, SC and UT) were lysed in 60 μ L 10 mM Tris/EDTA buffer containing protease inhibitors. 15 μ L of 6 $\times$ loading buffer was added and samples were heated to 95 °C for 5 minutes. Samples were resolved by SDS-PAGE in a 4–12% precast gel (Bio-Rad, UK) along with Precision Plus Protein Dual Colour Standards (Bio-Rad). Gels were then transferred onto nitrocellulose membrane. Membranes were blocked in 10% bovine serum albumin (BSA) TBST for 1 hr at RT, followed by overnight incubation with CT3 anti-mouse antibody (1 : 10 dilution; DSHB) at 4 °C. As a loading control, blots were incubated with 1 : 2000 rabbit polyclonal GAPDH (Abcam, UK) for 1 hr at RT. Secondary antibodies were incubated for 1 hr at 37 °C at 1 : 10 000 dilutions (IRDye® 800CW Goat anti-rabbit and 680RD Goat anti-mouse secondary antibodies; LI-COR, USA). Antibody detection was performed using the Odyssey Infrared Imaging System (LI-COR). All immunoblots contained TNNT2-MO-treated, SC and UT samples, and the Western was carried out four times. Fluorescent intensities of the bands were gathered for statistical analysis.

2.12 Tissue collection and preparation for RNA work

HH29 embryos were harvested and placed in ice cold Diethylpyrocarbonate (DEPC)-PBS (Sigma Aldrich, UK) ($n=3$ for OFT-banded hearts and $n=3$ for sham). Decapitation was performed *in ovo* in accordance with institutional and UK Home Office guidelines. The hearts were dissected from the embryo using sterile autoclaved forceps and were then placed in RNase free micro-centrifuge tubes. The heart tissues were snap frozen in liquid nitrogen and stored at -80°C .

2.13 RNA extraction

HH29 heart tissues were homogenized in 1 ml of ice-cold monophasic lysis reagent, Tri-Reagent (Sigma Aldrich, UK) using 23G followed by 25G needles. The homogenates were incubated for 5 minutes at RT to permit complete dissociation of nucleoprotein complexes. 0.2 ml of 1-bromo-3-chloro-propane (Sigma Aldrich, UK) was added and vigorously mixed. The mixture was incubated for 3 minutes at RT and was centrifuged at $10,000 \times g$ for 15 minutes at 4°C to separate the mixture into two phases. The upper aqueous phase was carefully transferred to fresh 2 ml RNase-free tube, avoiding the contamination of the DNA in interphase and proteins in lower phase. For the precipitation of RNA from the aqueous phase to occur, pre-cooled reagents of 0.25 ml 2M sodium acetate, pH 4 and 0.7 ml 100% isopropanol (Fisher Scientific UK Ltd., UK) were added. They were gently mixed and stored at -20°C for 3 hours.

Precipitated RNA was collected by centrifugation at $17,500 \times g$ for 15 minutes at 4°C . The translucent white RNA pellet formed was then washed twice in 70% DEPC-ethanol and was resuspended in 87.5 μl DEPC-treated water at 62°C for 5 minutes. 10 μl RDD buffer was added followed by 2.5 μl DNase I (Qiagen, UK) into each RNA sample and they were incubated for 15 minutes at RT. 400ul of DEPC-ddH₂O was

added to bring the volume to 500 μ l and an equal volume (500 μ l) of phenol/chloroform/isoamyl alcohol, pH 4 (Amresco) was added for RNA extraction. The sample was vigorously mixed and was centrifuged at 11,000 x g for 10 minutes at 12 °C. Again, the upper aqueous phase was transferred to fresh RNase-free tubes and was brought up to a 500 μ l of DEPC-treated water. Removal of phenol was performed by purification with an equivalent volume of chloroform (Fisher Scientific, UK) and extraction by centrifugation at 11,000 x g for 10 minutes at RT. The upper aqueous phase was transferred to a fresh tube. Pure RNA was precipitated by the addition of 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volumes of absolute ethanol. The mixture was inverted for a couple of times and was incubated overnight at -20 °C. The precipitated RNA was collected by centrifugation at 17,500 x g for 15 minutes at 4 °C, washed twice with 70% DEPC-ethanol and was finally resuspended in 50ul DEPC-treated water. The concentration of RNA was measured by NanoDrop 2000c UV/IV spectrophotometer at 260 nm absorbance (Thermo Fisher Scientific). RNA samples were stored at -80 °C.

2.14 Quantitative and qualitative assessment of total RNA

RNA integrity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Total RNA with a RNA Integrity Number (RIN) above 8.0 were proceeded for globin depletion.

2.15 RNA-sequencing on OFT-banded hearts

2.15.1 Globin depletion

Successful globin depletion was optimized and performed by Matthew Parnall (PhD student). 1.5 µl of 10x oligo probes of six different globin genes : *HGB1*, *HBAA*, *HBAD*, *HBZ*, *HBE1/HBB* and *HBG2* were mixed with 3 µl of 5x hybridization buffer and 3 µg of RNA and were made up to a total volume 15 µl with nuclease free water on each RNA sample. The mixture was heated to 95 °C for 2 minutes and cooled down at 5 °C intervals for 50 sec each step and finally to 22 °C for 5 minutes for oligo hybridisation to occur. Hybridized RNA : DNA was degraded by RNase H digestion for 15 minutes at 37 °C. DNase I digestion was carried out for 10 minutes at 37 °C to degrade the DNA probes. Globin-depleted total RNA were quantified by Qubit Fluorometric assay (Thermo Fisher Scientific). Globin depleted RNA with RIN above 7.0 were taken forward for cDNA library preparation.

2.15.2 cDNA library preparation workflow for RNA sequencing.

cDNA library preparation was generated according to manufacturers' instructions of NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs Inc.(NEB) #E7420S/L; NEB, UK by Dr Sunir Malla from Deep Sequencing, School of Life Sciences. Only principal work flow of cDNA library preparation will be described (Section 2.15.2.1-2.15.2.3) (Figure 2.2).

2.15.2.1 mRNA isolation by poly-T oligonucleotides followed by fragmentation

A complete mRNA which contains a poly(A) tail was isolated from total RNA of each sample ($n=3$ of OFT-banded hearts and $n=3$ of shams) using poly-T oligonucleotides (Figure 2.2a). 15 μ l of NEBNext oligo d(T) 25 beads were washed by 100 μ l of 2X RNA binding buffer. 1 μ g of globin depleted RNA of each heart was diluted to 50 μ l and was added to the beads together with 50 μ l of 2X binding buffer. Reaction mixture was heated to 65 °C to denature the RNA and was cooled to 4 °C. The tubes were incubated at RT for 5 minutes to facilitate the binding of mRNA to the beads. They were then placed in the magnetic rack at RT for 2 minutes to separate the poly-A mRNA bound to the beads from the solution. Unbound RNA was washed off. 50 μ l of elution buffer was added and the tubes were heated to 80 °C for 2 minutes and decreased to 25 °C to elute poly-A mRNA from the beads. Re-binding of mRNA to the beads was repeated following by washing procedure. For fragmentation step, the purified mRNA was eluted from the beads in 15 μ l of the First Strand Synthesis Reaction Buffer (NEB, UK) and incubated at 94 °C for 15 minutes (Figure 2.2b) and Random Primer mix (2x) (NEB, UK) was added to hybridize with the fragmented RNA at random sites (Figure 2.2b).

2.15.2.2 First strand and second strand synthesis

Actinomycin D (0.2 μ g/ μ l) (NEB, UK), murine RNase inhibitor (NEB, UK) and Superscript® II Reverse transcriptase (NEB, UK) were added to the fragmented and primed mRNA. The mixture was heated to 70 °C and snapped cool on ice. The first single stranded cDNA was synthesized following thermal cycling conditions: 25 °C for 10 minutes, 42 °C for 50 minutes and 70 °C for 15 minutes (Figure 2.2c). 1 μ l cDNA was quantitated by Qubit assay kit and cDNA was diluted accordingly for real

time PCR reaction (Section 2.19). Second double stranded cDNA was synthesized by 10x second strand buffer with dUTP mix and second strand synthesis enzyme mix with 1 hour incubation at 16 °C (Figure 2.2d). Finally, the second double stranded cDNA was purified again using 1.8X Agencourt AMPure XP beads (Figure 2.2e).

2.15.2.3 End Repair/dA-tail of cDNA library and PCR library enrichment

Each double stranded cDNA library was treated with 10x End Repair Reaction Buffer and End Prep Enzyme Mix to generate blunt and 5'-phosphorylated dA-tailed ends with thermal cycling conditions based on manufacturers' instructions (Section 2.15.2) (Figure 2.2f). dA-tailed cDNA libraries were ligated to NEBNext Multiplex Adaptor which composed of a loop containing a U with complementary dT overhangs (NEB, UK) (Figure 2.2g). The second strand containing dUTP from the double stranded cDNA and the loop containing a U were removed by treatment with USER Enzyme (NEB, UK) to open up the loop and make the first strand available as a substrate for PCR (Figure 2.2h). After size selection, barcodes can be incorporated by use of the NEBNext index primers for multiplexing and cycling conditions during PCR library enrichment based on manufacturer's instructions (Section 2.15.2) (Figure 2.2i-k).

Finally, the size distribution of each enriched cDNA library was tested prior to sequencing using the Agilent Bioanalyzer (Agilent). Libraries consisted of fragments of 200bp to 500bp, with average size of around 290bp. Libraries were quantified using qPCR (Kapa biosystems, KK4824).

Barcoded libraries were sequenced according to manufacturers' instructions on eight tenths of a full high output NextSeq500 run to generate 2 x (75bp paired-end reads) on Illumina NextSeq 500 platform at Deep Sequencing department in School of Life Sciences, University of Nottingham.

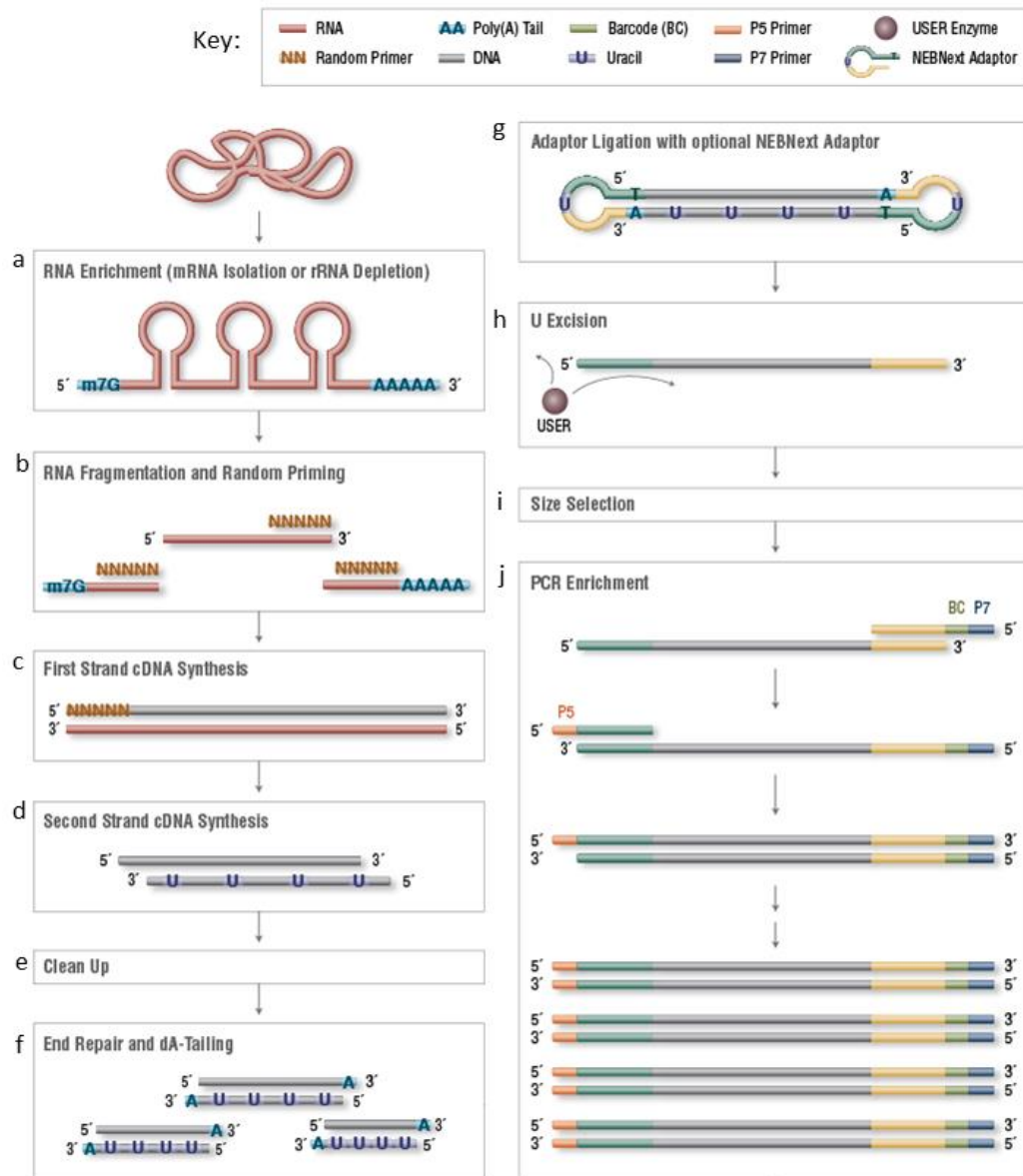


Figure 2.2 Ultra Directional RNA Library Preparation for Illumina workflow
Enrichment of mRNA which contains poly(A)tail was performed by doing mRNA isolation using poly-T oligonucleotides (a). Fragmentation of the mRNA was performed by incubation at elevated temperature followed by adding random primers to hybridize the fragmented RNA at random sites (b). First strand cDNA synthesis was performed following fragmentation and random priming (c). Second strand cDNA was generated and dUTPs was included to label the second strand and was cleaned up (d-e). Blunt and 5'-phosphorylated dA-tailed ends were generated to enable the single A overhang to ligate to the adaptors which contains a U-loop with complementary dT overhangs (f-g). The U in the center of the NEBNext Adaptor loop was removed by USER enzyme to open up the loop and the second strand was degraded by removal of Us as well (h). After size selection, the first strand was subjected to PCR amplification and barcodes were incorporated during PCR library enrichment(i-j). Adapted and modified from <https://www.neb.com/products/e7420-nebnext-ultra-directional-rna-library-prep-kit-for-illumina>.

2.15.3 Bioinformatic analysis on RNA sequencing data

Bioinformatics analysis of the RNAseq data was performed by Dr Fei Sang from Deep Sequencing, School of Life Sciences.

2.15.3.1 Trimming and mapping against reference genome

Bases and qualities output were firstly generated in sequencing output files (compressed FASTAQ files). Trimming was performed to filter reads for adapter and low quality bases. First, raw reads that aligned against adaptors sequences were filtered using Sythe (<https://github.com/vsbuffalo/scythe>). Reads with low sequencing score were then trimmed using Sickle (<https://github.com/najoshi/sickle>). The chicken reference genome was downloaded from UCSC database (<http://genome.ucsc.edu>) Nov. 2011 (ICGSC Gallus_gallus-4.0/galGal4). Reads were mapped to a galGal4 reference genome in the context of known gene exon coordinates by tophat mapping tool (<https://ccb.jhu.edu/software/tophat/index.shtml>) to generate sequence alignment for each sample in BAM format (the standard format of recording RNA sequencing alignment results). There are three ways of recording the read alignments against a reference genome. They are, 'Uniquely' mapped reads are reads that map to only a single location, and there are no significant hits to any other reference genome location. 'Primary' mapped reads record the best mapping position of each read, regardless of whether they map uniquely. Primary mapped reads are shown in the Bam files. 'All' mapped reads are when every single significant mapping position is recorded against the reference genome; therefore there can be a multiple recording for each gene.

2.15.3.2 Mapping Quality (MAPQ)

Both primary and unique mapped reads alignments ($47\text{-}90 \times 10^6$) were filtered based on their mapping quality score (MAPQ). MAPQ is a probabilistic metric of the accuracy of the read mapping. Low mapping quality implies low base quality of the read. This is attributed to wrong observed read sequence which then leads to wrong alignment. In addition, low sensitivity of the alignment algorithm and the reads that fall in repetitive regions of the reference genome result in low mapping quality. In our case, RNA sequencing is a paired-end sequencing. Read mapping in pairs is more likely to be correct. By definition, MAPQ 10 equates 1 in 10 chance of a mapped read being incorrectly placed, MAPQ 20 means 1 in 100 chance of a mapped read being incorrectly placed and MAPQ 30 equals to 1 in 1000 chance of a mapped read being incorrectly placed. In our case, MAPQ is MAPQ 20. 'Htseq-count' was used to determine the number of uniquely aligned reads per gene to generate the read counts per gene.

2.15.3.3 RPKM Expression Values

Reads Per Kilobase of transcript per Million mapped reads (RPKM) expression values are based on the RPKM calculation (Mortazavi et al., 2008). By definition, the RPKM is a normalized read count (stranded/sense reads) for a given gene by taking sample sequencing depth and gene length into account. The read count of the exon-space of a gene is normalised against the total number of mapped reads (with rRNA excluded, and reads not uniquely aligned and below MAPQ20 excluded) in that particular alignment file, and against the total length of the gene's exon-space.

2.15.3.4 Differential expression calculation

In order to identify that how biological replicates were similar within same group and were different between experimental groups, principal component analysis (PCA) was performed. It is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. Next, a differential gene expression package DESeq was employed for subsequent analysis (Anders and Huber, 2010). This program implements a single analytical approach. DESeq analyses the variance between them in order to create a better model of the expression values of individual genes within the group of replicates. DESeq was used to detect the differentially expressed genes for each comparison, a p-value < 0.05 was set as the DESeq threshold. Genes with average RPKM < 1 and ribosomal RNA RPKM were filtered out from the raw data for statistical analysis.

2.16 First strand synthesis for qPCR validation

In order to evaluate the results obtained from the RNA sequencing technology, real time PCR was performed on the original biological samples ($n=3$ for OFT-banded and $n=3$ for sham) used for RNA sequencing. They were essentially the technical replicates of the experiment.

Reverse transcription reactions were performed using SuperScript® II Reverse Transcriptase (Invitrogen, UK) following manufacturer's instructions. Each 10 μ l mixture was made by mixing 0.5 μ g total RNA, 0.25 μ l of 50 μ M random hexamer, 0.5 μ l of 10mM dNTPs and made up a total volume of 6 μ l of RNase free ddH₂O (Invitrogen, UK) in an RNase-free tube. The mixture was heated to 65 °C for 5 minutes and cooled on ice. 2 μ l of 5X reverse transcriptase buffer (Invitrogen, UK), 1 μ l of 0.1

mM 1,4-Dithiothreitol (DTT) (Invitrogen, UK) and 0.25 µl of RNase free ddH₂O (Invitrogen, UK) were added and mixed well. 0.25 µl of RNase OUT (Invitrogen, UK) was added and the mixture was incubated at 25 °C for 2 minutes. 0.5 µl superscript II enzyme (Invitrogen, UK) was added to the mixture to give a final total volume of 10 µl. The reaction was heated to 25 °C for 10 minutes and then to 42 °C for 50 minutes. The mixture was finally heated to 70 °C for 15 minutes to deactivate the enzyme and stored at -20 °C.

2.17 Primers design for real time PCR in RNA sequencing

Real time quantitative PCR (qPCR) primer pairs were designed using NCBI tool Primer BLAST with an annealing temperature at 62 °C and an amplicon size ranging in between 120-233bp for all genes of interest. All primers were designed with a spanning intron except *KLF2* (Table 2.1). Both forward and reverse primers were checked on OligoT analyzer for hairpin, homodimers and heterodimers for free Gibbs energy. Primers sets were then checked against BLAST for gene specificity.

2.18 End point polymerase chain reaction (PCR)

End point polymerase chain reaction (PCR) performed using Red Tag ® DNA polymerase (Sigma Aldrich). PCR reactions were conducted in 20 µl reaction mixtures comprising of 1X PCR buffer, 250 µM deoxynucleotide mix (dNTPs), 0.25 µM of each forward and reverse primer, 0.05U of DNA polymerase, 1 µl of cDNA made up to a 20 µl volume with ddH₂O. The PCR reaction was carried out using a T100 thermal cycler (Bio-Rad laboratories Inc., USA). The cycling programme commenced at 95 °C for 3 minutes to heat activate the DNA polymerase. This was followed by 34 cycles of 95 °C for 30 seconds, and annealing temperature at 62 °C for each gene designed (see

Table 2.1), 72 °C for 1 minute for the extension step. The cycling programme was ended by a single extension time 72 °C of 5 minutes.

2.19 Real time polymerase chain reaction (qPCR)

The real time PCR experiment was optimized by Matthew Parnall (PhD student). Each cDNA sample was diluted 4x with DNase-free water before the determination of dynamic range of sample concentrations for qPCR experiment. The starting amount of cDNA added and the dilution factor for the serial dilution varied were based on the expression level of each gene (Table 2.1). From the standard curve obtained, a concentration was chosen for relative quantitation that fell in the middle of the dynamic range. Efficiencies of all gene reactions were between 94.77 and 110%, with r^2 values > 0.98) (Table 2.2). PCR reaction was done with 96 well plates (MicroAmp; Applied Biosystems; Cat No. N801-0560) covered with optical adhesive films (Applied Biosystems; Cat No. 4313663). The instruments used was Applied Biosystems 7500 Fast Real-time PCR system under the following thermal cycling conditions: a single heat-denaturing step at 95 °C for 25 seconds, followed by 40 cycles of annealing and extension at 95 °C for 20 seconds and 62 °C for 1 minute. A melt curve analysis was performed from 50 °C to 95 °C, with no off-target products detected. All samples were run in duplicate within each qPCR experiment for the standard curve. Each 20 µl PCR reaction mixture consisted of 10 µl of iTaq™ universal SYBR® Green supermix (1X), 0.75 µl of each forward and reverse primer (375 nM) for genes and varied amount of diluted cDNA template based on individual gene. 0.5 µl of each forward and reverse primer (250 nM) was used in the qPCR reaction mixture for *KLF2*, Periostin and AggreCAN, *NOS3* (Table 2.1). The threshold cycle number for product detection (ΔC_T value) was used to calculate the relative gene

expression using *GAPDH* and *EEF1A1* as the reference gene using Pfaffl method and the fold change was calculated based on the equation shown as below (Pfaffl, 2001). Unpaired student t test assuming unequal variances was used to determine whether there is any statistical significance on delta CT (ΔC_T) values.

$$\text{Fold change} = \frac{(E_{\text{target}})^{\Delta C_{T, \text{target (control-sample)}}}}{(E_{\text{reference}})^{\Delta C_{T, \text{reference (control-sample)}}}}$$

where E (amplification efficiency) = $10^{-1/\text{slope}}$

qPCR was performed on the shear stress responsive (*KLF2* and *EDN1*) genes and the ECM markers characteristic of endocardial cushion genes (*TBX20*, Aggrecan and Periostin) on HH29 OFT-banded hearts ($n=5$ for OFT-banded hearts and $n=5$ for sham) and these results are described in chapter 3. qPCR was also carried out in HH19 TNNT2-morpholino treated hearts ($n=10$ TNNT2-morpholino treated hearts and $n=3$ for controls) on *NOS3* in chapter 4. In chapter 5, verification of genes for RNA sequencing was performed on *TNNC2*, *LDHB*, *TGM2*, *PDCD4* and *LGALS1* on HH29 OFT-banded hearts ($n=3$ for OFT-banded hearts and $n=3$ for sham). Table 2.2 lists down the R^2 and the efficiency of each individual gene.

Gene	Forward sequence/Reverse sequence	Primer concentration	Primer amount	cDNA amount	Size (bps)	Intron size (bps)
<i>TNNC2</i>	5'-GCATCTTCGACAAGAACGCTGA-3' 5'-CCCTTACTGCACACCCTCCAT-3'	1 in 4	0.75 µl	2 µl	130	2000
<i>LDHB</i>	5'-ATCCTCGGCAAGGGTCTTTGT-3' 5'-CCACAATCTTGGAGTTGGCTGT-3'	1 in 4	0.75 µl	2 µl	120	1500
<i>TGM2</i>	5'-CCAGCCCCACATGGAACAGA-3' 5'-CCACGCTGTCACCAGTCTCA-3'	1 in 3	0.75 µl	4 µl	128	1194
<i>PDCD4</i>	5'-GGCTACAAAGGCACTGTGGATTTC-3' 5'-CAGCAGGTCTCCAGAAAGCAAGTA-3'	1 in 3	0.75 µl	4 µl	195	2165
<i>LGALS1</i>	5'-GCACAGCAGCATCATGTCTTGTC-3' 5'-CACATCACCGTGAGCATCAAAGC-3'	1 in 3	0.75 µl	6 µl	184	1185
<i>KLF2</i>	5'-GCTTCTACCAGACAAACCCG-3' 5'-CAGGACTGGCCCATAACTGT-3'	1 in 2	0.5 µl	4 µl	233	NA
<i>TBX20</i>	5'-AGATATGCCTACCACCGCTCCT-3' 5'-TGATGTGGCCATGCTGATCCA-3'	1 in 3	0.75 µl	4 µl	178	5009
Aggrecan	5'-CGGCATCTGGACAAGAGACAGA-3' 5'-CTCCATTCAGACAGGGGCTTGA-3'	1 in 2	0.5 µl	4 µl	165	7533
Periostin	5'-AGAACCTGATCTCATGGCAACCA-3' 5'-GGGTTGTAAAACGTCAGTGGAATCT-3'	1 in 3	0.5 µl	4 µl	183	4300
<i>NOS3</i>	5'-ACCTACCAGAGCGAGAACGG-3' 5'-CTGCCAGAAGCTGCGGAAT-3'	1 in 6	0.5 µl	6 µl	198	200
<i>EEF1A1</i>	5'-GCTCTAACATGCCCTGGTTCAAG-3' 5'-TGGCTTCAGGACACCAGTTTC-3'	1 in 2	0.75 µl	2 µl	209	159
<i>GAPDH</i>	5'-AGACGGTGGATGGCCCCTCT-3' 5'-ACGGCAGGTCAGGTCAACA-3'	1 in 2	0.75 µl	2 µl	188	129

Table 2.1: A list of primers pairs for qPCR and their condition used for the standard curve. NA, not applicable.

Gene	R²	Efficiency (%)
<i>TNNC2</i>	0.997	105.40
<i>LDHB</i>	0.992	107.15
<i>PDCD4</i>	0.996	110.28
<i>LGALS1</i>	0.997	98.46
<i>TGM2</i>	0.998	110.10
<i>KLF2</i>	0.995	98.13
<i>EDN1</i>	0.996	94.20
<i>TBX20</i>	0.998	98.44
<i>NOS3</i>	0.996	97.66
Periostin	0.999	94.78
Aggrecan	0.990	96.69
<i>GAPDH</i>	0.999	98.90
<i>EEF1A1</i>	0.98	99.80

Table 2.2 The efficiencies and R² of qPCR primers

2.20 Reverse transcription reactions

Reverse transcription reactions were performed using SuperScript® III Reverse Transcriptase (Invitrogen, UK) following manufacturer's instructions. The mixture was made by mixing 200ng of Hexamer solution, 1 µg total RNA, 10mM dNTPs and 13 µl of ddH₂O in an RNase-free tube. The mixture was heated to 65 °C for 5 minutes and cooled on ice. 1X reverse transcriptase buffer, DTT, RNase OUT and 1 µl superscript III enzyme (200U) was added to the reverse transcription mixture to give a total volume of 20 µl. The reaction was heated to 25 °C for 5 minutes and then to 50 °C for 1h. The mixture was finally heated to 70 °C for 15 minutes to deactivate the enzyme and stored at -20 °C.

2.21 Primers design for riboprobe

The primers designed for riboprobe synthesis for the in situ hybridization (ISH) study are *KLF2*, *EDN1*, *TBX20*, versican, aggrecan, periostin and *GAPDH* with the amplicon size ranging in between 508bp-1001bp and the information of each genes detailed in Table 2.3. Each gene was amplified by end point polymerase chain reaction by 40 cycles as described in section 2.18 and was checked by gel electrophoresis for genomic contamination. They were proceeded for PCR cleaned up and were subcloned into pGEMT-easy vector (Promega, UK) followed by plasmid DNA preparation (Section 2.25) and linearization (Section 2.26) and riboprobe synthesis (Section 2.27) and purification (Section 2.28).

Gene name	Gene	Forward primer	Reverse primer	Size (bps)	Annealing temperature (°C)
<i>KLF2</i>	NM_001318423	5'-ATGGTGAATGACTGCCACAC-3'	5'-ATGTGCCGCTTCATGTGC-3'	515	62
<i>EDN1</i>	XM_418943.4	5'-GAAGTGAACGCCGCATCG-3'	5'-GCTTTTCCAGATGCTTTGCC-3'	528	62
<i>TBX20</i>	NM_204144.1	5' AGATATGCCTACCACCGCTC-3'	5'-ATGGTACCTTGGCATGTGGA-3'	783	62
Versican	NM_204787	5'-GCAACAAACAATACAGCCCC-3'	5'-CTCTCTCAGCCGTATCCCAG-3'	508	62
Aggrecan	NM_204955.2	5'-CTGCGTTCCTGAGATTAC-3'	5'-TTGCCAGGTCGATCTCAC-3'	963	62
Periostin	NM_001030541	5'-TAATGCTCTCCACCACCACA-3'	5'-TCTGCTGGCTTGATGATTTG -3'	1001	57.8
<i>GAPDH</i>	NM_204305.1	5'-GGGCTCATCTGAAGGGTGGTGCTA-3'	5'-GTGGGGGAGACAGAAGGGAACAGA-3'	810	62

Table 2.3 A list of primers pairs and annealing temperatures with amplicon size designed for riboprobe.

2.22 End point polymerase chain reaction (PCR)

End point polymerase chain reaction (PCR) performed as described in section 2.18 to test for primers designed for riboprobes. The cycling programme commenced at 95 °C for 3 minutes to heat activate the DNA polymerase (Sigma Aldrich, UK). This was followed by 34 cycles of 95 °C for 30 seconds, and annealing temperature at 62 °C for each gene designed except periostin, where the annealing temperature was at 57.8°C (see Table 2.3), 72 °C for 1 minute for the extension step. Cycling programme was ended by a single extension time 72 °C of 5 minutes.

2.23 Gel electrophoresis

1.5% agarose gel (Sigma Aldrich) was made with 1X-Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer and 0.1 µg/ml ethidium bromide (EtBr). 8 µl of amplified PCR product was loaded onto the gel, alongside with 1Kb plus ladder (Invitrogen) and run at 150V for 25 minutes. The gel was visualized and photographed under UV light using GeneGenius Bioimaging System (Syngene, UK).

2.24 PCR clean up

Amplified PCR products were purified with GenElute™ PCR Clean-Up Kit according to manufacturers' instructions (Sigma Aldrich). Filtered columns were first prepared by adding 0.5 ml of column preparation solution and they were then centrifuged at 12,000 x g for 1 min. 5 volumes of binding solution was added to the PCR products.

The mixture was transferred to column and centrifuged at 12,000 for 2 minutes. PCR products which now bound to the silica membrane in the column were washed with wash solution containing ethanol. DNA was eluted in 35 µl pre-heated 65 °C autoclaved milliQ ddH₂O. DNA concentration was checked by NanoDrop 2000c UV/IV spectrophotometer at 260 nm absorbance (Thermo Fisher Scientific) and was sent for sequencing analysis (DNA sequencing department, University of Nottingham).

2.25 Plasmid DNA preparation

2.25.1 Ligation and transformation

The PCR amplicons were ligated into the 1 µl of pGEM-T Easy vector (50ng/µl) (Promega, UK) at a either 3 : 1 or 1 : 3 ratio of PCR product : vector catalyzed by 1 µl of 100u T4 ligase (NEB) in 5 µl of 2X rapid ligation buffer and the mixture was made up to 10 µl as final volume with water and the mixture was left at 16 °C overnight. 2 µl ligation mix was added to 50 µl 5α-high efficiency competent E.coli cells (NEB, UK) and was gently mixed by flicking. It was placed on ice and incubated for 25-30 minutes. Cells were subjected to heat shocking for 2 minutes at exactly 42 °C in a water bath. There were immediately incubated on ice for 2 minutes. 950 µl super optimal broth (SOC) was added and the competent cell/DNA mixture was incubated for another 1 hour at 37 °C. 1 : 20 dilution of the mixture was made and 200 µl was used to plate out on the McConkey Agar containing 100 µg/ml ampicillin (in aseptic conditions) using a spreader. The plates were air-dried for few minutes with agar

facing down and were then incubated for 16-24 hours at 37 °C. Color screening was performed to detect transformed colonies.

2.25.2 Liquid culture inoculation and plasmid DNA isolation (midiprep)

A single white colony was picked from a plate and used to inoculate a 5 ml starter culture in Luria broth (LB) with 0.1mg/ml ampicillin. It was incubated overnight at 37 °C shaking. A volume of 0.625 ml starting culture was made up to 50 ml (1 in 80 dilution) with LB media and was grown at 37 °C shaking overnight. Plasmid DNA was extracted from 50 ml bacterial culture using the Plasmid Midi kit (Sigma Aldrich, UK). Cells were harvested by centrifugation at 5000 x g for 8 minutes. Pellet was firstly homogenised with resuspension solution followed by lysis solution with gentle inversion for 4-5 minutes. Neutralising solution was added to the mixture when the solution became clear and viscous. It was then pelleted down by centrifugation at 15,000 x g for 10 minutes. Clear lysate was transferred to column followed by centrifugation at 5000 x g for 2 minutes. The bound plasmid DNA were washed to remove contaminating bacterial components. They were finally eluted in ddH₂O.

2.26 Plasmid linearization by restriction digestion and sequencing

Linearization of plasmid DNA was performed with the reactions as follows: 15 µg of plasmid DNA, 7.5 µl NEB cut smart buffer, 1 µl specific restriction enzymes NcoII or PvuII and SpeI (NEB, UK) in 19 µl diluent and made up to 75 µl with DEPC-treated H₂O. Reaction was left at 37 °C for 1.5 hours. All genes were linearized with NcoII and SpeI except periostin, which was cut by restriction enzymes PvuII and SpeI (Table

2.4). Purification of the linearized plasmid DNA product was performed after the reaction. 425 µl of DEPC-treated H₂O was added to bring the volume up to 500 µl, with an equal volume of phenol/chloroform/isoamyl alcohol, pH 7 added (Amresco) for DNA extraction. The samples were intensely mixed and centrifuged at 11,000 x g for 10 minutes at RT. DNA was precipitated by an addition of 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volumes of absolute ethanol. The mixture was inverted for a couple of times and was incubated overnight at -20 °C. The precipitated DNA was collected by centrifugation at 17,500 x g for 15 minutes at 4 °C, washed twice with 70% DEPC-ethanol and finally suspended in 11 µl DEPC-treated H₂O. The concentration of DNA was determined by NanoDrop 2000c UV/IV spectrophotometer at 260 nm absorbance (Thermo Fisher Scientific).

2.27 Probe synthesis and digoxigenin labelling

Single stranded DIG-labelled RNA probes were synthesised using the DIG RNA Labelling Mix by in vitro transcription with SP6 or T7 RNA polymerases. Reactions were set up on ice as follows: 6 µl of 5x buffer, 3 µl of 100mM DTT, 1 µl of 40 U RNase inhibitor (Promega, UK), 3 µg linearized plasmid DNA, 3 µl 10x DIG RNA labelling mix (Roche Diagnostics Ltd, UK), 3 µl of (30 U-60 U) SP6 or T7 RNA polymerase (Promega, UK) and were made up to final volume of 33.6 µl of sterile RNase free water. Reactions were incubated at 37 °C for 2.5 hours. Table 2.4 lists all anti-sense and sense RNA probes used in this work.

Probe	Antisense: Enzyme/RNA polymerase	Sense: Enzyme/RNA polymerase
<i>KLF2</i>	SpeI/T7	NcoI/Sp6
<i>EDN1</i>	SpeI/T7	NcoI/Sp6
<i>TBX20</i>	SpeI/T7	NcoI/Sp6
Versican	NcoI/Sp6	SpeI/T7
Aggrecan	SpeI/T7	NcoI/Sp6
Periostin	PvuII/Sp6	SpeI/T7
<i>GAPDH</i>	NcoI/Sp6	SpeI/T7

Table 2.4 In situ probes used in this thesis

2.28 Probe purification and quantification

Riboprobes were purified using ProbeQuant G-50 micro columns (Illustra) according to manufacturer's instructions. Firstly, column preparation was performed by resuspension of the resin by vortexing, the cap was then loosened by one quarter turn, and the bottom closure was removed prior to the placement in the collection tube. The column was spinned for 1 minute at 735 x g. The column was placed in a new microcentrifuge tube. DIG labelling reactions from section 2.27 were made up to a volume of 50 µl of hybridization probe. The probe was carefully applied to the top centre of the resin, and was centrifuged at 735 x g for 2 minutes for elution. Probes were stored at -80 °C.

2.29 RNA gel

An aliquot of riboprobe (2.5 µl) and a positive control T7 (2.5 µl) were electrophoresed on a 1% agarose gel to check the size and integrity of the riboprobe synthesized. Reaction was set up as follows: 2.5 µl of riboprobe or T7, 7.5 µl denaturing buffer, 2.5 µl EtBr and 2 µl 6X Gel loading dye buffer (NEB, UK). The samples were put at 67 °C for 8 minutes and were loaded in a gel in pre-cooled 1X TAE running buffer at 70V for 1.5 hours. Riboprobes were made up to 50 µl with hybridization buffer shortly after synthesis and were stored at -80°C.

2.30 In situ hybridization

2.30.1 Embryo collection and preparation of HH26 and HH29 OFT-banded hearts for in situ hybridization on paraffin-embedded sections.

HH26 ($n=5$ per group) and HH29 embryonic hearts ($n=7$ for OFT-banded hearts and $n=9$ for controls) were dissected with autoclaved sterile forceps and were placed in ice-cold DEPC-treated PBS. They were fixed in 4% PFA at 4 °C overnight. Embryos were washed with DEPC-treated PBS with 0.1% Tween (PBST), followed by dehydration of a graded ethanol (EtOH) (Sigma Aldrich, UK) series made up of DEPC-ddH₂O (50% EtOH, 75% EtOH, 100% EtOH) and were cleared in xylene and embedded in paraffin. 8 µm serial sections of HH29 embryonic hearts were collected by RNase-free superfrost plus slides (Fisher Scientific) and were stored at 4 °C for maximum one month.

2.30.2 In situ hybridization on paraffin-embedded tissue sections

The paraffin sections prepared from section 2.30.1 were dewaxed and hydrated with a graded series of EtOH made up with DEPC-ddH₂O. Sections were equilibrated with DEPC-PBST. Tissue region of interest was demarcated with PAP hydrophobic pen (Zytomed Systems, Germany). Sections were treated with 0.02mg/ml Proteinase K (Sigma Aldrich, UK) in DEPC-PBST for 8 minutes at 37 °C. Proteinase K reaction was stopped by treating sections with 2mg/ml glycine (Fisher Scientific, UK) for 6 minutes at RT. Sections were then washed twice by DEPC-PBST for 3 minutes each. They were then refixed in 4% PFA (Sigma Aldrich, UK) for 12 minutes at RT followed by 5 minutes DEPC-PBST washing twice. Sections were treated with 0.25% acetic anhydride (Sigma Aldrich, UK) in 0.1M triethanolamine (Sigma Aldrich, UK) pH8.0 for 10 minutes and washed twice with DEPC-PBST.

Pre-hybridization mix was added to the sections and was spread across the sections by capillary action when the coverslip (Scientific Laboratories Supplies, UK) was applied. They were incubated in a humidified chamber for 2 hours at 67 °C in a hybridization oven (Biometra, V2). Excess pre-hybridization mix was drained off by placing slides vertically onto the tissues. Hybridization mix (please refer to appendices for recipe of hybridization mix) with specific antisense probe (4 µl-7 µl) was added onto sections with coverslip applied and sealed with vulcanizing rubber solution (Weldtite, UK). Slides were incubated for 16 hours at 67 °C in a humidified atmosphere.

Sections were subjected to three times of 10 minutes each wash in pre-warmed 50% formamide/2x saline sodium citrate buffer (SSC)/0.1% Tween-20 followed by 0.2x SSC/0.1% Tween-20 at 67 °C. Sections were equilibrated to MABTw (Maleic acid buffer containing Tween-20) by washing them twice for 8 minutes at RT. RNase treatment (100 µg/ml) (Sigma Aldrich, UK) was performed on sections to get rid of non-hybridized single stranded RNA probe for 15 minutes at 37 °C. Sections were washed off by MABTw. Sections were blocked with 2% Boehringer Blocking reagent™ (Roche) in MAB with 20% sheep serum (Sigma Aldrich, UK) for 1.5 hours at RT. The antisense RNA probe was labelled with an anti-digoxigenin-alkaline phosphatase (AP) Fab fragment (Roche) at 1 : 2000 in 1% Boehringer Blocking reagent and 2% sheep serum overnight at 4 °C. Excess antibody was drained off and washed with couple times of MABTw. Embryos were equilibrated with BCL buffer III containing 5mM levamisole (Sigma Aldrich) for three times 15 minutes each at RT. mRNA signal was detected by incubating sections in fresh BM purple AP substrate (Roche) to BCL buffer III in 1 : 1 ratio. The sections were left kept in dark for the colour development from 1 hour to 8 hours. To permanently stop the reaction, sections were washed in PBSTw 3 times for 5 minutes each time and were fixed in 4% PFA for 15 minutes at RT and finally mounted in 50% glycerol : 50% PBS sealed with nail varnish.

2.31 Apoptosis study on HH29 OFT-banded hearts

OFT-banded hearts were fixed in 10% neutral buffered formalin overnight at 4 °C ($n=5$ for OFT-banded hearts and $n=5$ for sham). They were dehydrated through a graded series of ethanol and embedded in wax. HH29 hearts were then sectioned at 5 μ M using DSC1 microtome (Leica), where every 5th section was collected on superfrost plus slides. Sections were then deparaffinized in xylene twice for 5 minutes each and rehydrated through a series of graded ethanol and distilled water. They were finally equilibrated in PBS.

2.32 Apoptosis study

Apoptosis was performed using ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit S7100 according to manufacturers' instructions (Millipore, USA). After rehydration, tissues were permeabilized with Proteinase K (20 μ g/ml) for 15 minutes at RT. They were washed with ddH₂O to stop the reaction. Endogenous peroxidase activity was quenched by immersing the slides in 3.0% hydrogen peroxide in PBS for 10 minutes at RT. Slides were incubated with equilibration buffer for 15 minutes. Without rinsing, working strength TdT enzyme was applied onto sections and they were incubated at 37 °C for 1 hour. Stop/wash buffer was applied onto each section to inactivate the enzyme for 10 minutes at RT. Slides were then washed with PBS followed by application of anti-digoxigenin conjugate with incubation in a humidified chamber for 30 minutes at RT. Again, sections were rinsed with PBS three times and 200 μ l 0.05% 3,3 diaminobenzidine (DAB) with 0.03% hydrogen peroxide in PBS was

applied onto sections. Colour development was carefully monitored under the microscope and was stopped by immersing the slides with ddH₂O. Non-apoptotic cells were counterstained with DAPI (1 : 500, Sigma Aldrich) for 15 minutes at RT. As a positive control, heart tissues were pre-treated with DNase I before incubation with equilibration buffer step. For the negative control, working strength TdT enzyme was substituted with PBS, with all other steps remaining identical.

2.33 Analysis of apoptosis

A total of 80 histological slides (each slide contains 25-30 tissue sections) were digitized with slide scanner Zeiss Axio Scan Z1 (Imaging & Cytometry, University of York). For each slide, a marker pen was used to draw every single tissue section on the slide. A preview was taken for automatic tissue detection. These selections were refined manually. In order to obtain high resolution images of the whole HH29 hearts, tissues were divided into multiple small tiles or images. They were photographed with brightfield imaging mode for brown (DAB reactive) apoptotic cells and fluorescent imaging mode with a 20x objective for non-apoptotic cells. Focus was applied on every third tile. Z-stacking was also activated to obtain a single sharp plane image. ZEN software (Carl Zeiss Microscopy) automatically produced aligned tiles as a single virtual histological slide in.czi format. To perform cell counting in the ventricle, a box was randomly placed on the left and right ventricular region of the heart and the tissues counted included ventricular compact myocardium and trabeculae. The base and the myocardial crest of the IVS as well as the primordial AV regions (i.e endocardial cushion, left AV septal and lateral leaflet and right AV septal and lateral leaflet) were

determined morphologically and demarcated using freehand drawing tool on Image J. The brown (DAB reactive) apoptotic cells were counted manually, whereas the total number of non-apoptotic cells in each section was counted using DAPI counterstain and the cell counting was performed by ImageJ software for automated cell imaging. Watershed was applied to separate the merged particles. The DAPI and DAB reactive cells were then added together to get a total cell count and the ratio of apoptotic cells to total cells obtained for each heart. Statistical unpaired student T-test was conducted as described in section 2.9.

3 CHAPTER 3: Investigating the effect of OFT-banding in the chick developing heart

Overview

The chick embryo is a valuable research tool to study the effect of abnormal blood flow on cardiac development. The outflow tract banding model is a well described altered haemodynamics model and a number of cardiac anomalies with altered tissue properties have been described in the literature (Clark et al., 1984, Sedmera et al., 1999, Tobita et al., 2002, Buffinton et al., 2013). Morphological analysis and the functional characteristics have partially been addressed previously, however, the quantitative information about three-dimensional (3D) features of tissues and the biochemical events in apoptosis and fibrosis are yet to be determined.

In addition, increased regurgitation and OFT valve anomalies in chick were seen in the OFT-banding model before (Clark et al., 1984, Menon et al., 2015, McQuinn et al., 2007, Shi et al., 2013). With regards to tricuspid valves, immature and abnormal right AV valves were found in morphological analysis (Hogers et al., 1997, Sedmera et al., 1999). Hence, our aim was to further characterize the effect of OFT-banding on the developing valves at the biochemical and molecular level.

The aims of these experiments were:

- To improve the OFT-banding model by doing a double overhand knot in the outflow tract of a developing chick heart at HH21.
- To determine if the OFT-banding results in abnormal gross morphology of the heart.

- To study how the three dimensional structures that are developing in the chick embryonic heart, such as the heart chambers, the ventricular trabeculae and the epicardium are affected upon OFT-banding.
- To study the cardiac fibrosis upon abnormal blood flow
- To assess functionally the consequence of the perturbation of blood flow on apoptosis in the interventricular septum and the ventricles.
- To determine the gross morphology of the primordia atrioventricular valve in outflow tract banded hearts.
- To investigate whether the primordial AV valve formation is affected by using extracellular matrix (ECM) markers characteristics of endocardial cushion.
- To investigate if the abnormal valve formation is possibly correlated with shear stress at HH29.
- To assess functionally the consequence of OFT-banding leading to an abnormal valve phenotype by the analysis of apoptosis.
- To perform an expression profile of the ECM proteins in primordial AV valve.
- To study the expression of ECM proteins in HH35 outflow tract banded hearts by qualitative and quantitative analysis.
- To investigate whether endocardial cushion was affected at HH26.

3.1 Improvement of the OFT-banding model by doing a double overhand knot around the outflow tract in chick

An OFT banding model in the chick embryonic hearts that has been previously established was employed for this study (Clark et al., 1984, Sedmera et al., 1999, Hall et al., 2004).

The embryo was first exposed via a small window cut in the shell. An incision of the inner shell membrane was then made to expose the chick heart. At HH21, a 10–0 nylon suture was passed around the OFT and tied in a single overhand knot snug against its wall (Sedmera et al., 1999). A similar approach was attempted in this project, with the intention of the suture growing into the ligature to develop a progressive constriction and the suture will still be attached with the OFT upon harvesting at HH29. However, in 90% of the OFT-banded hearts ($n=70$), the suture came undone. At HH29, the chick embryo is covered by a densely organized culture (Martinsen, 2005). When isolated through the window in the shell upon disruption of the vascular membrane, and the OFT was hardly seen and was often ripped off upon harvesting. This made it difficult to determine if the constriction has taken place in the OFT. The knots also became loosened in the remaining 10% of the banded hearts. Hence, in order to prevent the knot becoming undone and loosened, a slight modification was made by doing a double overhand knot at HH21 and also by harvesting the embryo from the bottom of the shell. This reduced the damage to the OFT during isolation. During banding at HH21, the end of the suture was passed through the loop that was formed in the first knot to produce a secure double overhand knot (arrow in Figure 3.1a). Upon harvesting, the egg was gently inverted with the sharp end facing up. A pointed

instrument was used to fracture and to pull the shell apart. The egg was then reverted to gently drop the embryo facing upward onto the petri dish with the vasculature still remaining intact. The double overhand knot was then examined for its attachment on the OFT on each individual banded hearts (arrow in Figure 3.1c) before and after isolation from the thoracic region and were compared with the controls (Figure 3.1b). In addition, the embryos with the banded hearts were examined and all appeared to be phenotypically normal.

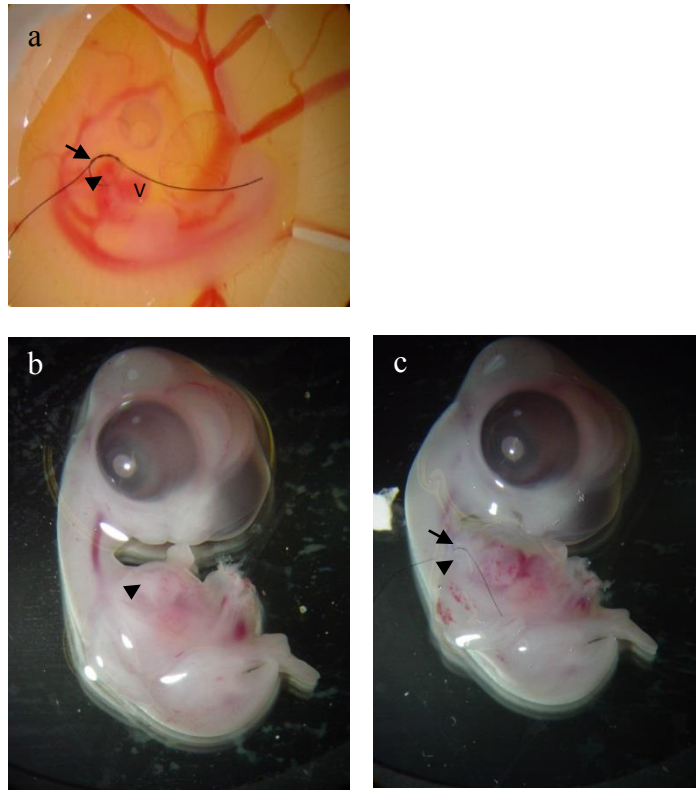


Figure 3.1 Improvement of the banding model was performed by doing a double knot around the outflow tract of a chick embryonic heart. A 10-0 suture was tied in a double overhand knot snug (denoted by an arrow) surrounding the outflow tract (arrowhead) of the chick embryonic heart at HH21 (a). Upon harvesting at HH29, the suture (arrow) was still attached with the outflow tract (arrowhead) in the banded embryo (c) in comparison to a control (b). LA, left atrium; OFT, outflow tract; V, ventricle. Scale bar for (b): 100 μ m.

3.2 Embryo grouping, survival of outflow tract banded hearts

OFT banding was performed at HH21 and hearts were harvested at three different stages, HH26, HH29 and HH35. The survival rates for the embryos harvested at these stages were examined to assess stability of the SHAM control. At HH21/26, the survival rates of the unoperated embryos ($n=15$), sham ($n=17$) were found to be 86.7% and 82.4% respectively ($\chi(1) = 0.112$, $p = 0.737$, no significant differences). Hence, sham were used as controls to be compared with OFT-banded hearts in the following studies as we have shown that there is no differences in survival rate in between two groups. As expected, the survival rate was significantly lower in the OFT-banded embryos, 42.5% ($n=40$) owing to the invasive nature of the banding experiment.

In HH21/29 study, the survival rate was 78% and 74.5% for unoperated ($n=82$) and sham ($n=102$), accordingly ($\chi(1) = 0.313$, $p = 0.576$., no significant differences). Thus, sham hearts were used as controls to be compared with OFT-banded hearts. As expected, the OFT-banded embryos ($n=212$) were found to have a lower survival rate as 36.3%.

Upon harvesting at HH35, the unoperated embryos ($n=30$) and sham ($n=28$) were reported to have a survival rate of 60% and 67.8% accordingly ($\chi(1) = 0.387$, $p = 0.534$., no significant differences). Again, sham hearts were used as controls to be compared with banded hearts. The OFT-banded embryos ($n=117$) had a high mortality rate, as there was only 11.1% embryos surviving at this stage. Any embryos showing developmental retardation were excluded from the study.

3.3 Outflow tract banded hearts are enlarged with abnormal shape in external analysis

At time of harvesting, all embryos were examined for developmental abnormalities. Upon isolation of the heart from the embryo, the external features such as the size and shape of the heart and the position of the OFT were particularly studied. The unoperated and sham hearts all appeared to be normal at all three stages examined (HH26 ($n=16$), HH29 ($n=97$) and HH35 ($n=35$); Table 3.1; Figure 3.2). Upon banding at HH21 and harvesting at HH26 (HH21/26), 31.2% of the OFT-banded embryos, the hearts appeared spherical ($n=16$) (Figure 3.2C). At this stage, the control hearts were still in the progress of acquiring the medial position of the OFT (Figure 3.2A,B). However, it could be seen that 94% of the banded hearts presented with a more lateral position of the OFT (Table 3.1; Figure 3.2C).

During harvesting at HH29 and HH35, the embryos presented more severe phenotypes with a higher rate. At these two stages, the OFT of the control hearts had shifted to the medial position due to the growth of both atria (Figure 3.2D,E and G,H for HH29 and HH35 accordingly). However, the OFT was still laterally positioned in the banded hearts, and this was found in 95% ($n=60$) and 89% ($n=9$) of the OFT-banded hearts at HH29 and HH35 correspondingly (Table 3.1; Figure 3.2F and I for HH29 and HH35 respectively).

At HH29 and HH35 (the stages where the ventricular septation is complete), the normal hearts had a pointed apex (arrow in Figure 3.2D,E and G,H for HH29 and HH35 respectively). However, a rounder apex was seen which made the OFT-banded hearts looked more spherical (55% at HH29 ($n=60$) and 89% at HH35 ($n=9$); Table

3.1; Figure 3.2F & I accordingly). Also, an irregular epicardial layer epicardium was seen in a subset of OFT-banded hearts (denoted by double arrowhead in Figure 3.2F and I; Table 3.1).

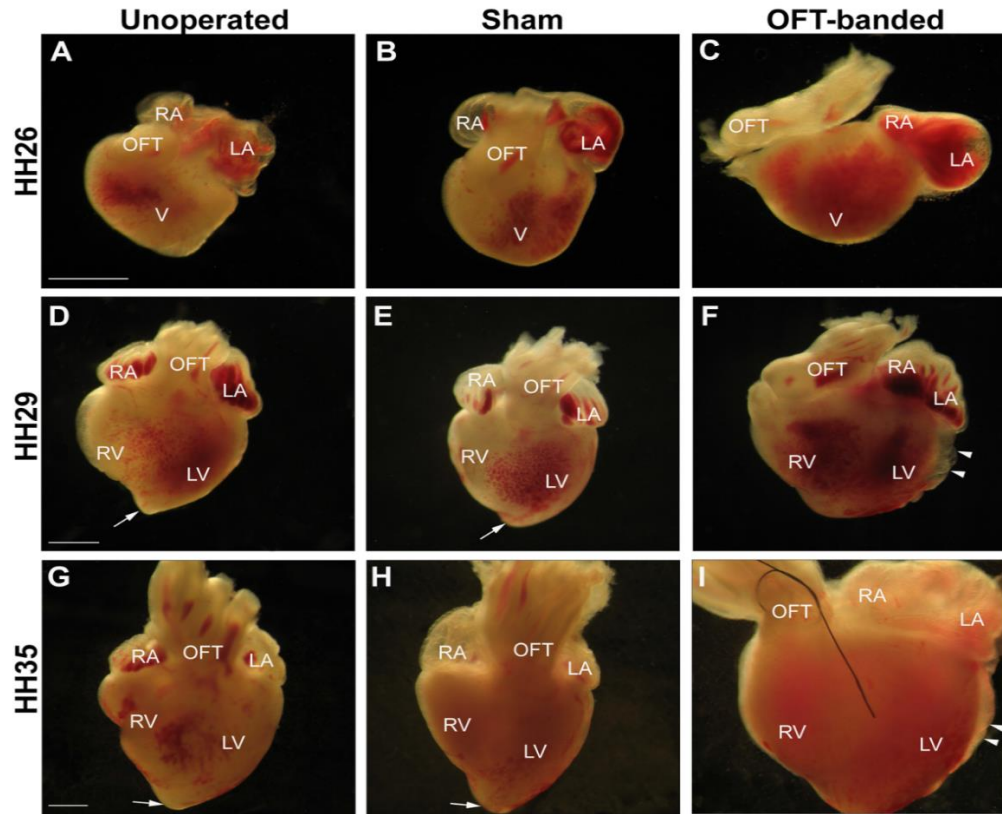


Figure 3.2 **OFT-banded hearts are enlarged with a rounded apex.** (A-C) At HH26, the controls (unoperated and sham) were still in the progress of acquiring the medial position of the OFT (A,B). However, it could be clearly seen that the outflow tract was laterally positioned at this stage in the banded hearts (C). (D-I) At HH29 and HH35, all controls displayed a normal external phenotype: the medial position of the outflow tract, an pointed apex (denoted by arrow) presented in both unoperated (D,G) and sham (E,H). However, the OFT-banded hearts exhibited a right-shifted position of the outflow tract with a rounded ventricular apex and the absence of the indentation at HH29 (F) and HH35 (I). In addition, a thickened epicardium was seen in a subset of OFT-banded hearts at both stages as well (double arrow in F & I). The suture was still attached with the OFT upon harvesting at all stages (c,f,i). LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle; V, ventricle. Scale bar: 1000 μ m

3.4 OFT-banding results in a wide spectrum of cardiac malformations

In order to determine whether the OFT-banding gives rise to cardiac anomalies, internal morphological assessment was carried out by analysing the serial histological sections of the OFT-banded hearts at different stages, e.g HH26, HH29 and HH35. A membranous type of VSD and a defective epicardium were revealed upon analysis in the banded hearts. In addition, OFT-banding also gave rise to a defective AV valve in the banded hearts and this will be further discussed in Chapter 4.

By HH29, the IVS formed by the merging and compaction of the ventricular trabeculae, and had grown superiorly to fuse with the endocardial cushion, which then divided the ventricular chamber into left and right ventricles. Upon banding, the membranous portion of the IVS that was derived from the endocardial cushion had failed to fuse with myocardial crest of the IVS. This gave rise to an opening (as indicated by an asterisk) which led to a communication of both left and right ventricles at HH29 ($n=26/31$) (Figure 3.3Ad; Table 3.1) and HH35 ($n=7/7$) (Figure 3.3Af; Table 3.1) in comparison to controls of corresponding stages (Table 3.1; Figure 3.3Ac,e). To further investigate on whether atrial septation had taken place properly, histological analysis was performed on the serial sections at HH26. A muscular septum (the primary atrial septum) had grown from the atrial roof and fused with the superior part of the endocardial cushion in all controls ($n=5$) (denoted by an arrow in Figure 3.3Aa; Table 3.1) and in the OFT-banded hearts ($n=5$) (Figure 3.3Ab; Table 3.1). This implied

that atrial septation had taken place properly. Normal atrial septum was observed at later stages of HH29 and HH35 (Table 3.1).

In addition, an impaired epicardial development was found in the OFT-banded embryos. Abnormal morphology and blebbing of the epicardial surface were seen in the OFT-banded hearts ($n=14/31$) (as indicated by an arrowhead) at HH29 (Figure 3.3Bd,f; Table 3.1) and HH35 ($n=3/7$) (Figure 3.3Bh,j; Table 3.1) in comparison to controls (Figure 3.3Bc,e and Figure 3.3Bg,i respectively). However, this phenotype was not seen in both control (Figure 3.3Ba; Table 3.1) and OFT-banded heart (Figure 3.3Bb; Table 3.1) at HH26. Pearson chi square analysis could not be applied when the expected number is less than five, where most control groups have a value of zero in our case (McDonald, 2009). Hence statistical analysis was not performed to test for differences for external and internal phenotypes between groups.

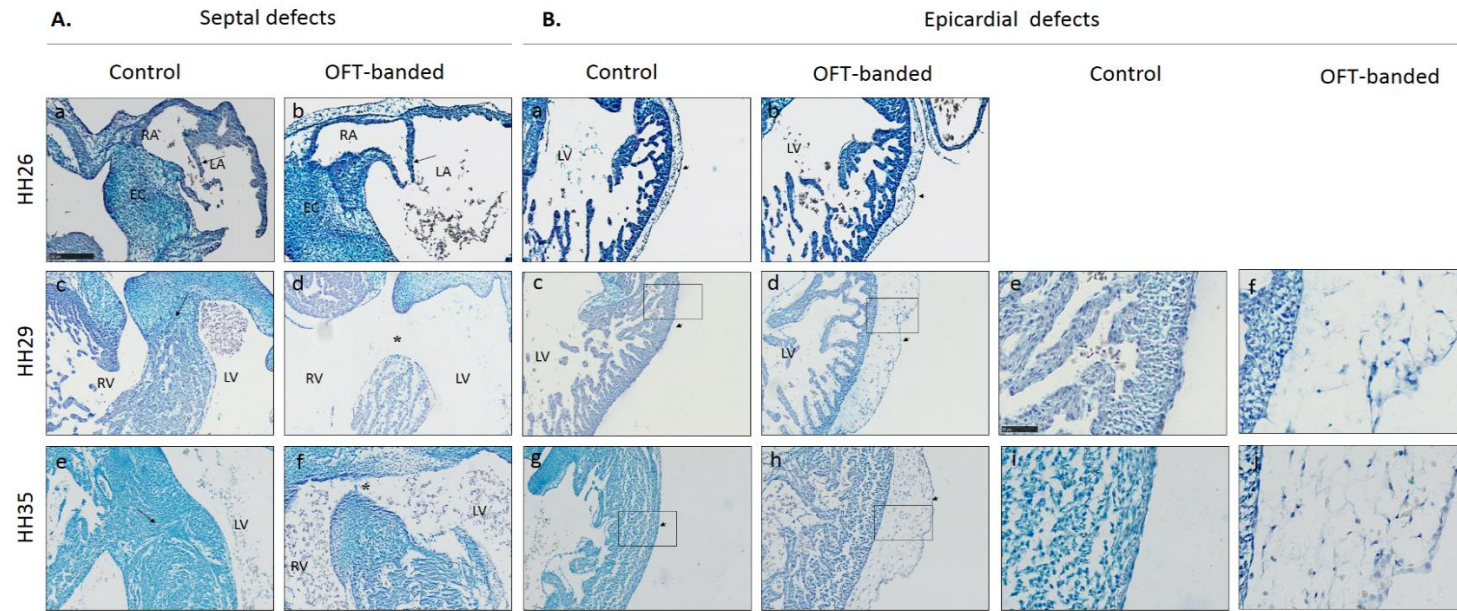


Figure 3.3 OFT-banding results in a spectrum of cardiac developmental defects.

A. OFT-banding results in the failure of ventricular septation but a normal atrial septation. Upon harvesting at HH26, the muscular atrial septum was fully fused with the endocardial cushion (denoted by an arrow) in both control (a) and OFT-banded embryos (b). At HH29 and HH35, the interventricular septum which grows in a superior direction has fused with the cushion in the control embryos (as indicated by an arrow) (c and e for HH29 and HH35 accordingly). However, this failed to occur in the OFT-banded hearts which led to a formation of an opening (asterisk) and thus a communication of both ventricles at both HH29 (d) and HH35 (f). **B.** A normal epicardial thin layer (denoted by an arrowhead) was seen in control at both HH29 (c) and HH35 (g) compared to a thickened epicardium with a blebbing appearance (arrowhead) in the OFT-banded hearts at these stages (d and h). However, this phenotype was not obvious at HH26, with a similar phenotype in control (a) and banded (b). e,f,i,j are the boxed areas in c,d,g,h respectively. EC, endocardial cushion; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. An asterisk (*) denotes a presence of ventricular septal defect. Scale bar: 250μm for Aa-f, Ba-d,g,h; 50μm for e,f,i,j.

Stage*	Embryo type □	Heart morphology				Total ext §	Septal defects				Epicardial defects		Total int §
		Spherical shape	Normal	Lateral position of OFT	Normal		ASD	Normal	VSD	Normal	Thickened epicardium	Normal	
HH21/26	Control OFT-banded	0	16	0	16	16	0	5	NA	NA	0	5	5
		5	11	15	1	16	0	5	NA	NA	0	5	5
HH21/29	Control OFT-banded	0	97	0	97	97	0	37	0	37	0	34	37
		33	27	57	3	60	0	31	26	5	14	17	31
HH21/35	Control OFT-banded	0	35	0	35	35	0	8	0	8	0	8	8
		8	1	8	1	9	0	7	7	0	3	4	7

Table 3.1 Summary of phenotypic analysis of OFT-banded chick embryonic hearts at HH26, HH29 and HH35.

Assessment of the size of the heart and the heart shape was based on the qualitative analysis of gross morphological features.

Qualitative assessment of the interatrial and interventricular septum and epicardium was carried out on serial histological sections.

*Stage of development when banding/harvesting performed.

□ Control embryos include unoperated and sham.

§ Total number of embryos analysed externally and internally.

ASD, atrial septal defect; ext, externally; int, internally; NA, not-analysed as the ventricular septation only completes at HH29 ; OFT, outflow tract; VSD, ventricular septal defect

3.5 Stereological analysis revealed thickened epicardium but normal proportions of other regions in the heart.

Stereology allows precise and accurate quantification of structural features within three-dimensional organs that relate in a meaningful way to integrated function. Due to low survival rate of OFT-banded embryos at HH29, sham and OFT-banded embryos were harvested from different batches ($n=6$ for sham, $n=7$ for OFT-banded hearts). Selection of OFT-banded embryos for stereology was based on the external morphological criteria (abnormal and spherical shape of OFT-banded embryos and lateral position of the OFT with knots embedded in the OFT) as well as the tissue integrity of the histological sections after processing. For stereological analysis, a 96-point grid was placed over every fourth $8\mu\text{m}$ section throughout the HH21/29 embryonic hearts. Each point at which the grid hit the tissue (myocardium, lumen, trabeculae and ECM) contributing to the regions (atria (contributed by RA and LA), ventricles (contributed by RV and LV) was counted. The individual point that the grid hit at the region of dorsal mesenchymal protrusion (DMP), atrial septum and epicardium was included as well. For the sham and OFT-banded hearts, a total points of 4437 and 7868 were counted for sham and OFT-banded accordingly. Once the counting was complete, the total number of counts per region was divided by the total number of counts for that heart, giving a percentage of the proportion that each region contributed to the heart.

When compared to the sham group, the OFT-banded hearts mainly showed no significant difference in most of the regions analysed ($P=0.354$, 0.196 , 0.3 and 0.637

for atria, ventricle, dorsal mesenchymal protrusion (DMP) and atrial septum respectively; Figure 3.4). However, the epicardium was found proportionally thicker in the OFT-banded hearts, where the sham presented $11.3 \pm 1.18\%$ and the OFT-banded hearts showed $18.15 \pm 2.02\%$; an increase of 60.1% was seen ($P=0.02$; Figure 3.4). This was in line with the thickened epicardium phenotype found in the internal morphological assessments. Lastly, the OFT-banded hearts were found 1.77 times (7868 points divided by 4437 points) larger than the controls approximately.

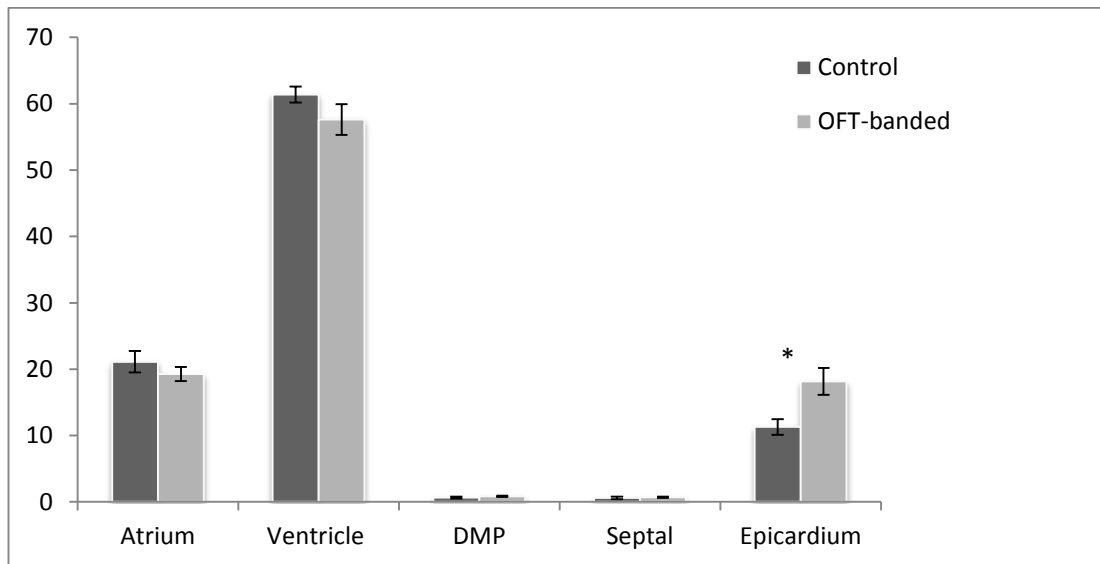


Figure 3.4 Stereological analysis revealed a thickened epicardium in the OFT-banded hearts. The proportion of atrium, ventricle, dorsal mesenchymal protrusion, atrial septum and epicardium contributing to each individual heart within each group was calculated using stereological methods ($n=6$ for controls and $n=7$ for OFT-banded hearts). No significant difference was seen between control and OFT-banded hearts in atrium, ventricle, dorsal mesenchymal protrusion and atrial septum, where $p>0.05$ in the regions analysed. However, the epicardium was found proportionally thicker in the OFT-banded hearts, $p<0.05$. Significant difference is indicated by an asterisk (*), when $p<0.05$. DMP, dorsal mesenchymal protrusion.

3.6 Stereology revealed enlarged OFT-banded hearts with changes in the chamber components

Stereological analysis was also conducted to look at each tissue type contributing to the region within the heart, i.e the percentage of the myocardium, lumen, trabeculae and ECM contributed to the right (RA) and left atrium (LA) and also to the right (RV) and left ventricle (LV) of the heart.

Upon analysis of the ventricular chambers, a significant reduction of the trabeculae was identified in both ventricular chambers of the OFT-banded hearts. Stereological analysis revealed the trabeculae of the RV in the sham heart presented with $10.66 \pm 0.36\%$ of the heart whereas the RV trabeculae in OFT-banded hearts accounted for $7.75 \pm 0.55\%$; a decrease of 27.3% was seen, $p < 0.001$ (Figure 3.5). The decrease of the trabeculae was more prominent in the LV upon banding. It was found that the trabeculae of the LV in the control was $13.30 \pm 1.65\%$, while the OFT-banded hearts presented only $8.53 \pm 0.37\%$ LV trabeculae of the heart; a decrease of 35.9%, $p < 0.01$ (Figure 3.5).

Consistent with this, a simultaneous increase of ventricular lumen was found in left side of the heart in the banded hearts. Stereology revealed that the LV lumen in both control and banded group were presented by $9.94 \pm 1.06\%$ and $14.27 \pm 1.40\%$ respectively, an increase of 43.6% was found, $p < 0.05$ (Figure 3.5).

Upon banding, a thinner myocardium was found in the LV but not in the RV. The myocardium in the LV in the control contributed to $9.41 \pm 1.05\%$ of the heart, whereas only $5.58 \pm 0.43\%$ of the LV myocardium was found contributing to the banded heart,

a decrease of 40.7%, $p < 0.01$ (Figure 3.5). However, there was virtually no change in the ventricular myocardium in RV of the heart upon banding, $6.39 \pm 0.86\%$ and $5.82 \pm 0.24\%$ were found in control and banded hearts respectively, $P = 0.55$; Figure 3.5. With regards to the myocardium of the atrial chambers, a reduction of 37.07% was identified in the myocardium of the LA in banded hearts, in which the percentage of the LA myocardium in both controls and the banded hearts were presented by $4.99 \pm 0.67\%$ and $3.14 \pm 0.24\%$ accordingly, $p < 0.01$ (Figure 3.5). However, the RA myocardium accounted for $5.16 \pm 0.94\%$ in the sham and $3.24 \pm 0.34\%$ in the OFT-banded embryos. However, the difference was not significant ($P = 0.06$).

A significant increase of the LA lumen by 47.4% was found in the banded hearts ($p < 0.01$), where the percentage contributing to the LA lumen of controls and banded hearts were presented by $4.9 \pm 0.45\%$ and $7.22 \pm 0.62\%$ accordingly (Figure 3.5). In contrast, stereology discovered no changes in the lumen of the RA, where $6.04 \pm 0.76\%$ and $5.68 \pm 0.74\%$ were identified in control and banded hearts correspondingly, $P = 0.7$. ECM was found to be unchanged in both the RV and LV in the controls and the banded hearts. However, the number of counts was extremely low (200 point counts were desired; 13 points and 14 points were only counted for controls and banded hearts respectively) (Mayhew, 1991). Thus, statistical analyses could not be completed for ECM.

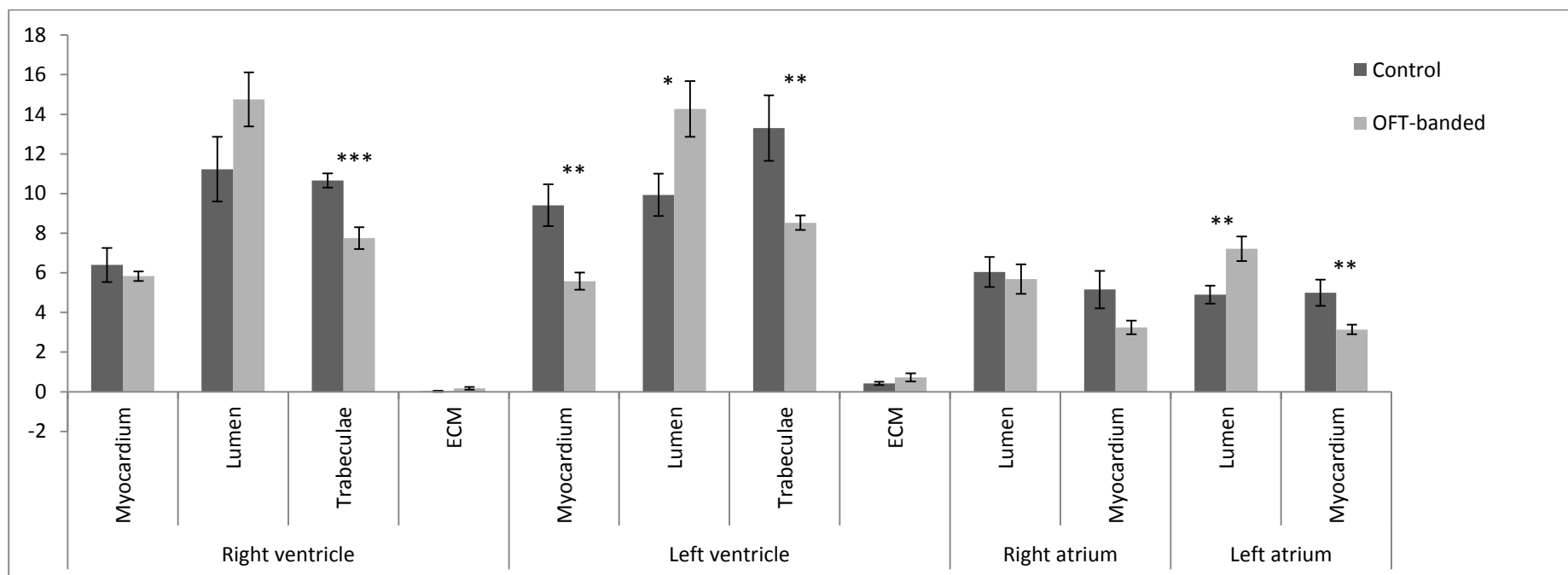


Figure 3.5 Stereological analysis of tissue types contributing to the left and right atrium and ventricles upon OFT-banding. Reduction of trabeculae was found in both right and left ventricle of the OFT-banded hearts, with an increase of ventricular lumen in left chamber. In addition, the myocardium was found thinner in the left ventricle but it appeared normal in the right ventricle. Also, banding led to an increase of the lumen and a decrease of myocardium in the left atrium. ECM, extracellular matrix. Significant differences are indicated by a single asterisk (*) when $P < 0.05$, double asterisks (**) when $P < 0.01$ and three asterisks (***) when $P < 0.001$. Error bars indicate s.e.m.

3.7 Outflow tract banding reveals no fibrosis

Cardiac fibrosis is one of the main features seen in the adult hypertrophied hearts in response to the pressure-overloading (Creemers and Pinto, 2011). To investigate if cardiac fibrosis presented within the pressured-overloaded embryonic hearts, Masson's Trichrome staining was performed ($n=5$ per group). The presence of black-blue staining indicates the deposition of collagen as seen in the chick skin as a positive control (Figure 3.6E). The degree of fibrosis was determined by a lack of black-blue staining upon qualitative analysis on serial sections of the banded hearts and controls. No fibrosis was observed in the myocardium of the atria (Figure 3.6C) and ventricle (Figure 3.6D) in comparison to control (Figure 3.6A,B).

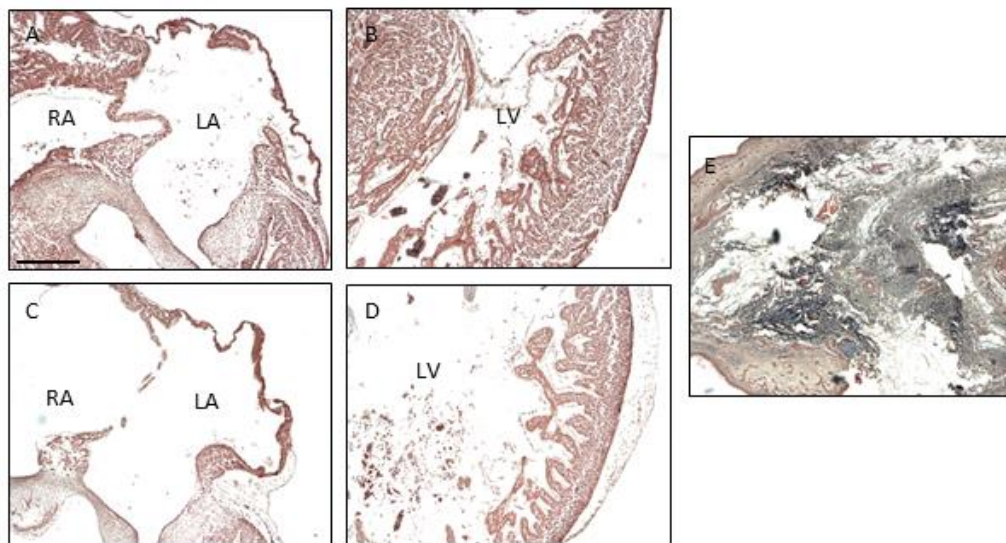


Figure 3.6 OFT-banding did not lead to cardiac fibrosis. Histological sections of HH35 chick hearts were examined for fibrosis by Masson trichrome staining. Deposition of collagen (black-blue staining) was not seen in the atria (C) and the ventricle of banded hearts. (D) compared to the atria (A) and ventricle (B) in the controls. Chick skin was used as a positive control (E). RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. Scale bar: 150 μ m.

3.8 Outflow tract banding results in decreased levels of apoptosis

Apoptosis was quantified in the chick heart by enzymatically labelling the free 3'-OH terminus of DNA with TdT. A total of 122,851 cells were identified as positive or negative for apoptosis in both RV and LV of controls and OFT-banded hearts ($n=5$ for each group). Cells positive for apoptosis were found scattered throughout the hearts and no clustering pattern was observed in both groups. A random field was selected from the RV and LV without overlapping with the IVS and the cell counting was performed at 25x magnification. The apoptotic cells counted in the ventricular region included the apoptotic cells in the compact myocardium and in the trabeculae. In the LV, the apoptosis accounted for $0.89 \pm 0.12\%$ of the control hearts but $0.5\% \pm 0.04\%$ for the OFT-banded hearts, a decrease of 43.8% of apoptotic level was seen with the difference being significant, $p < 0.05$ (arrow in Figure 3.7Aa'',b'', B). In the RV, $1.95 \pm 0.24\%$ cells were found apoptotic in controls and $0.85 \pm 0.11\%$ cells were undergoing apoptosis in the banded hearts. A significant decrease of apoptotic cells by 56.4% was detected in the OFT-banded hearts in this region as well, $p < 0.01$ (Figure 3.7Aa',b', B). In addition, the apoptotic level was studied in the IVS ($n=3$ for each group), where the analysis was performed in the base of the IVS and the myocardial crest of the IVS (1/3 length of the IVS). The IVS myocardial crest that forms the muscular portion of the IVS always fuses with the endocardial cushion to fully septate the ventricles. In the base of the IVS, no difference of the apoptosis was detected, $P=0.47$ (Figure 3.7Aa''',b''', B). However, a decrease level of the apoptosis was found in the crest of the IVS, where the apoptotic

cells accounted for $1.41 \pm 0.07\%$ in the controls but $0.81 \pm 0.12\%$ in the banded hearts, and the difference was significant, $p < 0.05$ (Figure 3.7Aa''''',b''''',B).

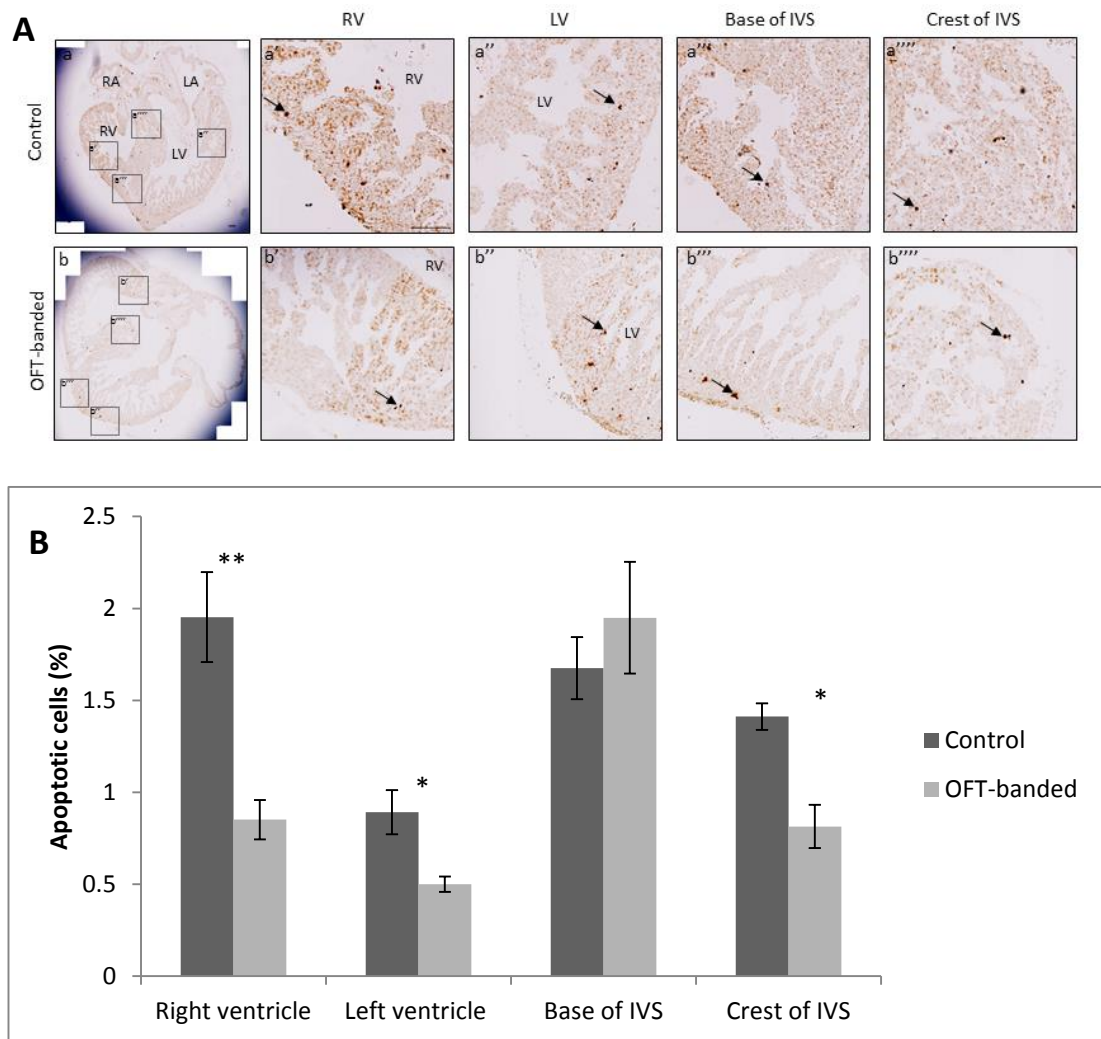


Figure 3.7 Apoptosis was decreased in both ventricles and in the myocardial crest of the interventricular septum in upon pressure-overloading in embryonic hearts. **A.** Apoptotic cells were identified and were found occurred in isolation in the compact myocardium and ventricular trabeculae of both right and left ventricles (as indicated by arrow in controls (a' and a'') and in OFT-banded hearts (b' and b'')). The apoptotic cells were also counted in the area of the base of interventricular septum and in the myocardial crest of the ventricular septum of both controls (a''' and a''') and banded (b''' and b''') accordingly. **B.** Apoptotic levels in both ventricles and the base and the myocardial crest of the ventricular septum of OFT-banded and controls hearts. A decrease of apoptotic cells was revealed in both of the right and left ventricle, with the $p < 0.01$ in the right ventricle and $p < 0.05$ in left ventricle. The level of apoptosis was not found significantly different in the base of the septum but a significant reduction was revealed in the crest, with $p < 0.05$. a and b are the control and OFT-banded hearts. Boxed areas in a and b are shown in higher magnification in a',a'',a''',a'''' and b,b'',b''',b''', respectively. IVS, interventricular septum; LV, left ventricle; RV; right ventricle. Scale bar: 100 μ m.

3.9 OFT-banding leads to the development of valve morphogenetic abnormalities upon histological assessment and stereology.

The effect of OFT-banding on the primordial atrioventricular (AV) valve region and its alignment with other septal components has yet to be determined. In order to investigate this, banding was performed in the OFT of the chick embryonic heart at HH21 (the stage where the EC is seen as a localized protrusion of cardiac jelly at the AVC and cardiac septation is still ongoing), with harvesting performed at HH26, HH29 and HH35.

In the histological sections at HH29 (when ventricular septation is complete), 61.2% of OFT-banded hearts ($n=31$) showed a defective atrioventricular AV valve (Table 3.2). The valve leaflets of the banded hearts appeared to be shorter and blunter (as indicated by an arrowhead in Figure 3.8Af) compared to the elongated valve leaflets seen in control hearts ($n=37$) (Figure 3.8Ad and e). At HH35, the primordial valve leaflets in the controls appeared to be more mature and lengthened for stratification to occur at the later stage of valve development (Figure 3.8Ag and h). Upon banding, 85.7% of OFT-banded hearts presented with a much more severe valvular phenotype at HH35 ($n=7$) (arrowhead in Figure 3.8Ai). Histologically, the valve leaflets in the banded hearts displayed a spectrum of phenotypes. The leaflets appeared to be either truncated (Figure 3.8Af) or presented with a nodular thickening at distal end of the valve leaflet (arrowhead in Figure 3.8Ai) (Table 3.2). It should be noted that the number of the banded hearts analysed at HH35 was much lower than the hearts analysed at HH29 due to a higher mortality rate. In the histological sections at HH26 (EMT is complete in the endocardial cushions and the atria septation has taken place), the endocardial cushion (EC) in the OFT-banded hearts retained a normal morphology (arrow in Figure 3.8Ac) compared to controls (Figure 3.8Aa and b). Also, it was found

that of all the hearts analysed which had dysmorphic AV valves also showed a ventricular septal defect (VSD) at HH29 (denoted by an asterisk in Figure 3.8Af) and HH35 (Figure 3.8Ai). In the control group, the AV cushion was fully fused with the interventricular septum (IVS) at HH29 (Figure 3.8Ad,e for HH29) and HH35 (please refer back to Figure 3.3Ae,f).

The stereological analysis was conducted in the AV valve region to investigate its area in relative to the whole heart at HH29. The AV valve region comprises the primordia AV septal/anterior and lateral/parietal leaflets in both right (tricuspid) and left (mitral) side of the heart (see Figure 1.7 in Chapter 1). Stereological analysis showed that the primordial AV valve region in the sham hearts ($n=6$) represented $4.97 \pm 0.47\%$, whilst the valve region in OFT-banded hearts accounted for $3.46 \pm 0.11\%$ ($n=7$), a decrease of 31.62% ($P=0.02$) (Figure 3.8Ba).

In addition, the relative valve length of the left AV leaflets was further examined at HH29. OFT-banding engendered shorter primordial left AV valve leaflets (as shown in Figure 3.8Af), which was 82% of the length of the controls ($p<0.001$) (Figure 3.8Bc). In line with the histological examination, the EC area was found smaller in HH29 OFT-banded hearts, which was 66% of the size of the controls when the relative EC area was calculated ($p<0.001$) (Figure 3.8Bb).

Alcian Blue staining was used to visualize the presence of glycoaminoglycans, the main component found in the endocardial cushion. Alcian Blue staining highlighted a slight reduction of glycoaminoglycans in the cushion of banded hearts ($n=4/13$) compared to controls at HH29 (red arrow in Figure 3.8f). The decrease of the staining was also observed in the primordial left AV leaflet at HH35 ($n=2/2$) (red arrow in Figure 3.8i). In addition, Masson's Trichrome staining was performed to investigate where there was any collagen deposition in defective valve primordia at HH35.

However, fibrosis was not detected in any of the primordial AV leaflets and EC in the OFT-banded hearts ($n=5$ for each group) (arrowhead in Figure 3.8Ca and b for controls and banded hearts respectively). Chick skin was used as a positive control (Figure 3.8Cc).

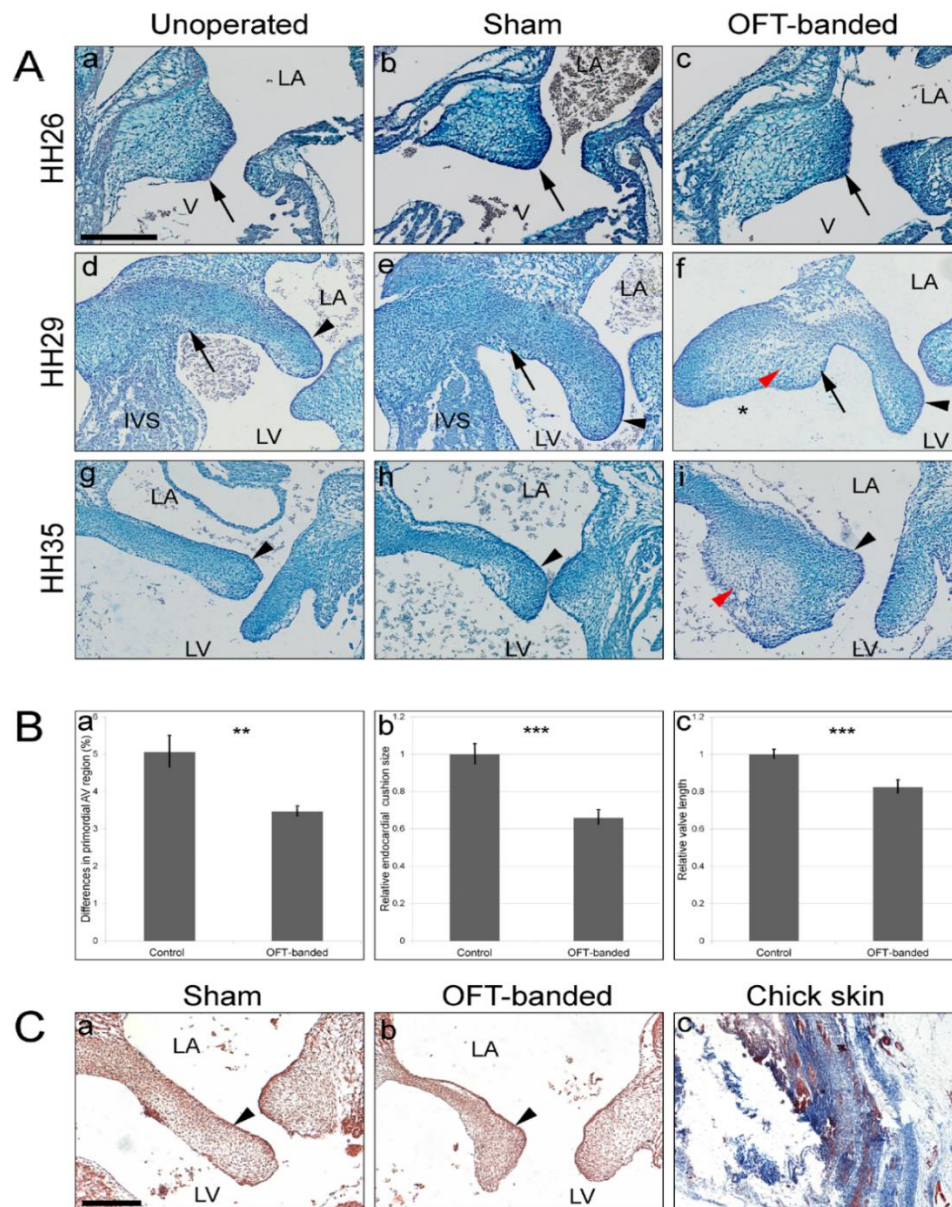


Figure 3.8 Defective atrioventricular cushion formation was seen in OFT-banded hearts. **A.** Transverse sections showing defective primordial atrioventricular (AV) valve regions of the OFT-banded hearts compared to controls (unoperated and sham) with a slight reduction of Alcian Blue staining in the banded hearts at HH29 (red arrow in f) and HH35 (red arrow in i) compared to controls (d-e for HH29) and (g-h for HH35). However, the valve morphology at HH26 appeared to be normal (a-c). Arrows indicate the endocardial cushions in the AV valve region, whereas arrowheads denote the primordial left AV valve septal leaflets. **B.** Stereology revealed the primordial AV region was proportionally smaller in the HH29 banded hearts (a). At HH29, relative endocardial cushion size was determined and it showed the AV cushion in the OFT-banded hearts was smaller than in the control (b). Relative valve length was also calculated and the valve leaflet in the OFT-banded hearts appeared to be shorter than controls in HH29 hearts (c). **C.** Histological analysis by Masson's Trichrome's staining revealed no fibrosis (determined due to lack of blue staining) in the primordial

left AV valve septal leaflets of the HH35 OFT-banded hearts, which would be indicated by the blue staining (arrowhead). Chick skin was used as a positive control for the Trichrome stain. Significant differences are indicated by double asterisks (**) when $P < 0.01$ and three asterisks (***) when $P < 0.001$. Error bars indicate s.e.m. An asterisk in A denotes the absence of the interventricular septum in the OFT-banded hearts. IVS, interventricular septum; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. Scale bar: 250 μ m for Aa-i; 80 μ m for Ca-c.

Stage*	Embryo type ^α	Dysmorphic AV valves	Normal AV valves	Total int [§]
HH21/26	Control	0	5	5
	OFT-banded	0	5	5
HH21/29	Control	0	37	37
	OFT-banded	19	12	31
HH21/35	Control	0	8	8
	OFT-banded	6	1	7

Table 3.2 Summary of dysmorphic primordial atrioventricular valve region of OFT-banded chick embryonic hearts at HH26, HH29 and HH35.

Assessment of the valve morphogenetic abnormalities of the primordia atrioventricular valve was based on the qualitative analysis of gross morphological features on serial histological sections.

*Stage of development when banding/harvesting performed.

^α Control embryos include unoperated and sham.

[§] Total numbers of embryos analysed internally.

AV, atrioventricular; Int, internally.

3.10 Altered expression of extracellular matrix genes was found in defective primordial atrioventricular valve at HH29 upon OFT-banding

The development of the abnormal primordial AV valve was further investigated at HH29 by ISH and qPCR. At this stage, the increased mesenchymal cell proliferation and matrix deposition of the central EC create the swellings to form the primordia valve leaflets. In order to examine if the abnormal primordia AV valve could be caused by potential altered shear stress, a few known shear-stress responsive genes (Kruppel lung factor-2 (*KLF2*) and endothelin-1 (*EDN1*)) were employed together with extracellular matrix (ECM) genes characteristic of valve primordia (*TBX20*, aggrecan and periostin) to examine their expression pattern on neighbouring sections throughout the heart in controls ($n=9$) and banded hearts ($n=7$) (Groenendijk et al., 2004, Shelton and Yutzey, 2007, Norris et al., 2004). *GAPDH* was used as an experimental control in this study (Figure 3.10Ae,e' and f,f'). Negative staining was seen in all the sense probes of the genes used in this study (refer to Appendices).

TBX20 was expressed intensely in the EC and in all four primordial AV valve leaflets and the myocardial wall of the atria and ventricles in the controls (Figure 3.9Aa,a'). In contrast, a marked reduction of *TBX20* expression was seen in the EC as well as in all four primordial valve leaflets in banded hearts (Figure 3.9Ab,b') ($n=5/7$) (Table 3.3). Despite the remarkable decrease seen, a distinct positive *TBX20* expression was apparent in the boundary of the EC subjacent to the atrial septum (denoted by an arrow in Figure 3.9Ab') and also delineated in the upper lining of the lateral leaflet of primordial left AV valve (indicated by an arrow in Figure 3.9Ab,b'). The mRNA level of *TBX20* was quantified by qPCR ($n=5$ for each group), demonstrating a 0.38-fold decrease ($p<0.001$) (denoted by three asterisks in Figure 3.9B).

Consistent with the literature, punctuate periostin mRNA expression was detected throughout the endocardium of both atria and ventricles as well as the ventricular trabeculae in controls (Figure 3.9Ac,c'). With the right and the left AV valve, expression was further restricted to the endocardial lining of the atrialis side of the primordial septal (arrow in Figure 3.9Ac,c') and lateral (arrowhead) leaflets. Additionally, abundant periostin mRNA was observed in the chordae tendinae of the left ventricular AV junction. However, periostin mRNA was not found differentially expressed in these regions in the banded hearts by ISH (Figure 3.9Ad,d') (Table 3.3). This was further confirmed by qPCR, with a non-significant upregulation of 0.1 ($P=0.24$) (Figure 3.9B).

At HH29, aggrecan was found localized in the core of the cushion area subjacent to the atrial septum in the controls (Figure 3.9Ae,e') but not in the posterior part of the EC (Figure 3.9Ah,h'). Similarly, aggrecan was also expressed in the core of the cushion in the banded hearts (Figure 3.9f,f'). However, an ectopic of aggrecan expression was found in five of the total banded hearts analysed ($n=7$) (Table 3.3), with the most intense expression was seen in the lateral part of the cushion of right AV(*) (Figure 3.9Ai,i'). An ectopic expression was also seen in the atrialis side of the primordia left AV valve leaflet (Figure 3.9Ag,g'). qPCR barely detected any changes of aggrecan mRNA in the banded hearts, where a non-significant upregulation of 0.12 fold change was found ($P=0.69$) (Figure 3.9B).

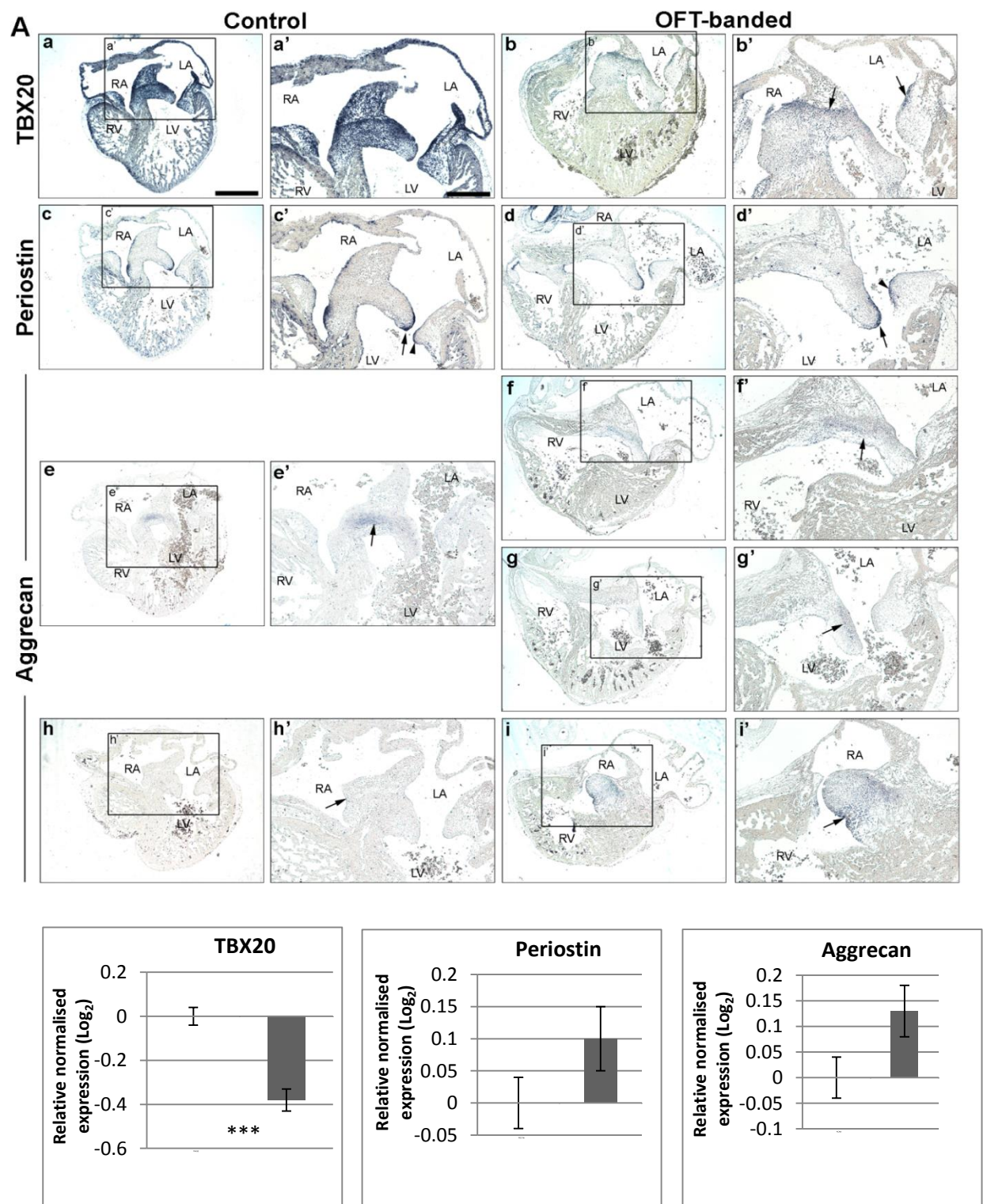


Figure 3.9 Expression of early extracellular matrix markers characteristic of valve primordia was found differentially expressed at HH29 upon OFT-banding.
A. (a-b') A marked reduction of expression of *TBX20* was observed in the entire endocardial cushion and the atrial and ventricular myocardial wall of the banded hearts (b,b') compared to control (a,a'). However, a distinct expression *TBX20* was seen in the boundary of the cushion (arrows in b') and in the upper lining of the left

atrioventricular (AV) valve (arrowhead in b'). **(c-d')** No differential expression of periostin was found, where the expression was predominantly detected in the endocardial lining of the left AV septal and lateral leaflets in both controls (c) and banded hearts (d) (denoted by an arrow and arrowhead for the septal and lateral leaflets in c' and d' respectively). **(e-i')** With aggrecan, it was expressed in the core of the endocardial cushion in control (e, arrow in e'). However, no aggrecan expression was detected in the posterior part of the cushion (h,h'). Similar expression was seen in the central cushion in the banded hearts (f, arrow in f') However, ectopic expression of aggrecan was also detected. A noticeable expression of aggrecan was seen in the atrialis side of the primordia left AV (g, arrow in g') and the lateral part of the cushion of right AV on the consecutive sections analysed (i,i'). Arrow indicates the most intense expression of aggrecan at the borders of AV cushion (i'). $n=9$ for control and $n=7$ for OFT-banded hearts. a', b', c', d', e', f', g', h' and i' are the boxed areas in a, b, c, d, e, f, g, h and i accordingly. **B.** Relative expression of early extracellular matrix genes characteristic of valve primordia was quantified by qPCR in the OFT-banded hearts at HH29. *TBX20* (a) was significantly downregulated in the banded hearts by 0.39 fold, where $p<0.001$ (denoted by three asterisks), when compared to controls ($n=5$ for each group). However, periostin (b) and aggrecan (c) mRNA levels were not significantly changed in between the two groups. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. Error bars indicate s.e.m. Scale bar: 300 μ m for Aa-i; 150 μ m for Aa'-i'.

3.11 Altered expression of shear stress responsive genes *KLF2* and *EDN1* in the OFT-banded heart at HH29

An investigation of whether the defective valve primordia seen in the banded hearts at HH29 occurs as a result of alteration of shear stress upon OFT-banding was performed by ISH on adjacent serial sections using *KLF2* and *EDN1* shear stress responsive genes. Intriguingly, *KLF2* mRNA showed a more intense and distinct expression at the endocardial lining of the atrialis side of the primordia left AV septal leaflets and lateral leaflets ($n=3/7$) (indicated by arrows in Figure 3.10Ab,b') compared to controls (Figure 3.10Aa,a') (Table 3.3). qPCR revealed a 0.23-fold increase in the expression of *KLF2* of the banded hearts compared to controls but their difference was not significant ($P=0.13$) (Figure 3.10B).

In normal hearts, mRNA expression of *EDN1* was detected in the lining of the outer curvature of the ventricular myocardium (Figure 3.10Ac,c') (Table 3.3). However, a decrease of expression of *EDN1* was seen in the banded hearts by ISH ($n=5/7$) (Figure 3.10Ad,d'). The expression of *EDN1* in the OFT-banded hearts was asymmetrical, with a normal expression seen in the RV compact myocardium (arrow in Figure 3.10Ad') but the expression was barely discernible in the LV ventricular myocardium (arrowhead in Figure 3.10Ad'). This was further confirmed by downregulation of 0.65 fold of *EDN1* mRNA level in qPCR ($n=5$; $p<0.001$) (denoted by three asterisks in Figure 3.10B). Lastly, the Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) gene was used as a positive and a loading control and the ubiquitous expression pattern was seen in both controls (Figure 3.10Ae,e') and OFT-banded hearts (Figure 3.10Af,f') (Table 3.3).

Taken together, a concomitant upregulation of *KLF2* and downregulation of *EDN1* suggested that OFT-banding resulted in an increase shear stress in the banded hearts and this is in concordance with the previous literature.

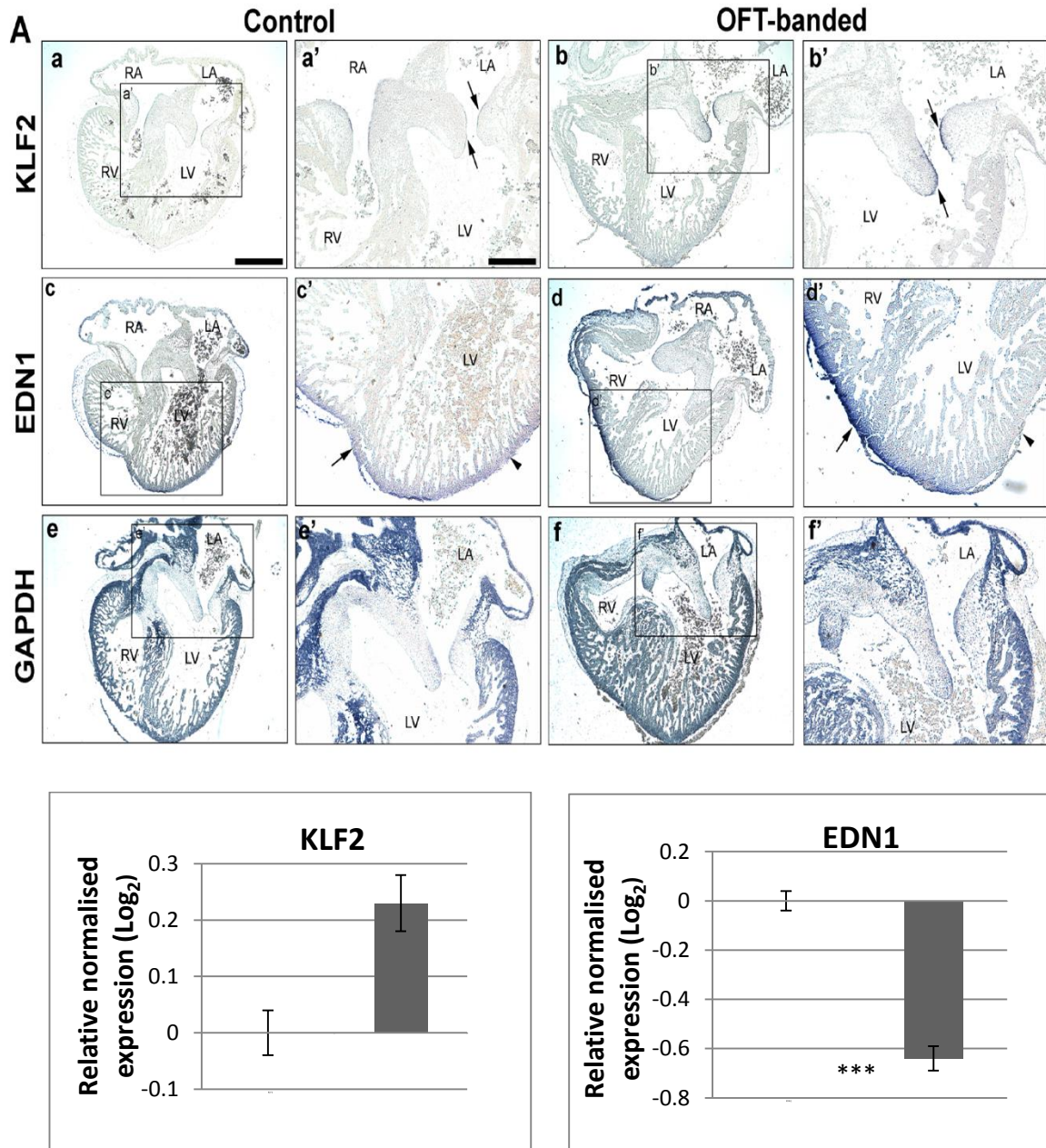


Figure 3.10 Increased shear stress was found in the OFT-banded hearts with valve morphogenetic abnormalities. A. An increase of the shear stress responsive genes, *KLF2* was detected in the endocardial lining of the primordial LAV of both septal and lateral leaflets in banded hearts (b) when compared to controls (a) (Arrows in a' and b' denote the *KLF2* expression of both leaflets in control and banded hearts accordingly). A noticeable decrease of *EDN1* was found in the compact myocardium of the outer curvature of the left ventricle (d, arrowhead in d') compared to the right ventricle (d, arrow in d') in the banded hearts. In contrast, *EDN1* was uniformly expressed in the compact myocardium of both ventricles (c, arrow and arrowhead in c'). *GAPDH* was used as an experimental control and it showed a ubiquitous expression pattern in both controls and banded hearts (e,e' and f,f'). *n*=9 for control and *n*=7 for OFT-banded hearts. a', b', c', d', e' and f' are the boxed areas in a, b, c, d, e and f accordingly. **B.** Relative of shear stress responsive genes was determined by

qPCR in the OFT-banded hearts at HH29. A slight upregulation of *KLF2* was found by 0.27 fold in the banded hearts compared to controls but statistical differences are not significant ($P=0.13$) ($n=5$ for each group). However, a downregulation of *EDN1* by 0.65 fold was revealed by qPCR in the OFT-banded hearts, where $p<0.001$ (denoted by three asterisks). RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. Error bars indicate s.e.m. Scale bar: 300 μ m for Aa-i; 150 μ m for Aa'-i'.

Gene	Embryo type □	Primordial AV valve		Total int §
		Differential expression	Normal	
Shear stress marker				
<i>KLF2</i>	Control	0	7	7
	OFT-banded	3 (↑)	4	7
<i>EDN1</i>	Control	0	4	4
	OFT-banded	5 (↓)	2	7
Primitive valve marker				
<i>TBX20</i>	Control	0	9	9
	OFT-banded	5 (↓)	2	7
Aggrecan	Control	0	9	9
	OFT-banded	5 (ectopic)	2	7
Periostin	Control	0	9	9
	OFT-banded	0	7	7
Experimental control				
<i>GAPDH</i>	Control	0	9	9
	OFT-banded	0	7	7

Table 3.3 Summary of differential expression of dysmorphic primordial atrioventricular valve region in OFT-banded chick embryonic heart at HH29.

Assessment of differential expression on primordia atrioventricular valve was based on qualitative analysis of gross morphological features on serial histological sections. The differential expression was seen as increased (↑) or decreased (↓) as well as ectopic expression.

□ Control embryos include unoperated and sham.

§ Total numbers of embryos analysed internally.

AV, atrioventricular valve; Int, internally.

3.12 OFT-banding results in defective valve primordia with decreased level of apoptosis.

Apoptosis was quantified in the chick heart by labelling the free 3'-OH terminus of DNA with free nucleotides catalysed by TdT enzyme. A total of 138581 cells and 86168 cells were identified and counted in sham ($n=5$) and OFT-banded hearts ($n=5$) respectively as positive cells for apoptosis. Cells stained positive for apoptosis appeared as dark brown as shown in Figure 3.11A. In this study, most of the apoptotic cells found in the EC region subjacent to the atrial septum in the controls appeared to be in a cluster (Figure 3.11Aa'), in contrast to the apoptotic bodies that occurred singly in the OFT-banded hearts (Figure 3.11Ab'). Otherwise, the apoptotic cells were found isolated and scattered in the primordial left (Figure 3.11Aa'',b'') and right AV valve leaflets (Figure 3.11Aa''',b''') throughout the heart in both sham and banded hearts. The primordia AV region was mainly divided into EC, left and right primordia AV region. The left and right primordial AV region was further divided into septal (denoted by arrow in Figure 3.11Aa''-a''', b''-b''') and lateral (arrowhead) leaflets. The most evident region that was undergoing apoptosis was in the EC, where $4.02 \pm 0.56\%$ cells were found in the control hearts (Figure 3.11Aa'). Intriguingly, only $1.69 \pm 0.37\%$ of cells were apoptotic identified in the OFT-banded hearts (Figure 3.11Ab') ($p < 0.01$; significant difference; Figure 3.11B). In primordial left AV septal leaflets, only $1.40 \pm 0.22\%$ of cells in banded hearts (arrow in Figure 3.11Ab'') were found undergoing apoptosis compared to $2.13 \pm 0.16\%$ in the controls (arrow in Figure 3.11Aa'') ($p < 0.05$; significant difference; Figure 3.11B). Further, the apoptotic cells in the right lateral leaflet were found significantly lower in the OFT-banded hearts (arrowhead in Figure 3.11Ab'''), with $1.22 \pm 0.22\%$ apoptotic cells detected in the banded hearts compared to $3.12 \pm 0.7\%$ of cells found in controls (arrowhead in Figure

3.11Aa''') ($p < 0.05$; Figure 3.11B). The apoptotic cells found in the primordia right septal leaflets showed a slight reduction of apoptotic cells in the banded hearts (arrow in Figure 3.11Ab'''), $2.04 \pm 0.46\%$ compared to $2.79 \pm 0.46\%$ of the apoptotic cells in the controls (arrow in Figure 3.11Aa'''). However, a significant difference was not found ($P = 0.28$). Lastly, there was virtually no difference in apoptotic level in the primordial left AV lateral leaflets in both controls (arrowhead in Figure 3.11Aa'') and banded hearts (arrowhead in Figure 3.11Ab''), with $1.21 \pm 0.26\%$ and $1.08 \pm 0.27\%$ of cells detected respectively ($P = 0.72$; no significant difference; Figure 3.11B). Taken all together, the total apoptotic cells identified in the primordial left and right AV region was found significantly lower in the banded hearts, with $1.53 \pm 0.26\%$ cells detected compared to $2.89 \pm 0.28\%$ cells identified in the controls ($p < 0.01$) (Figure 3.11B).

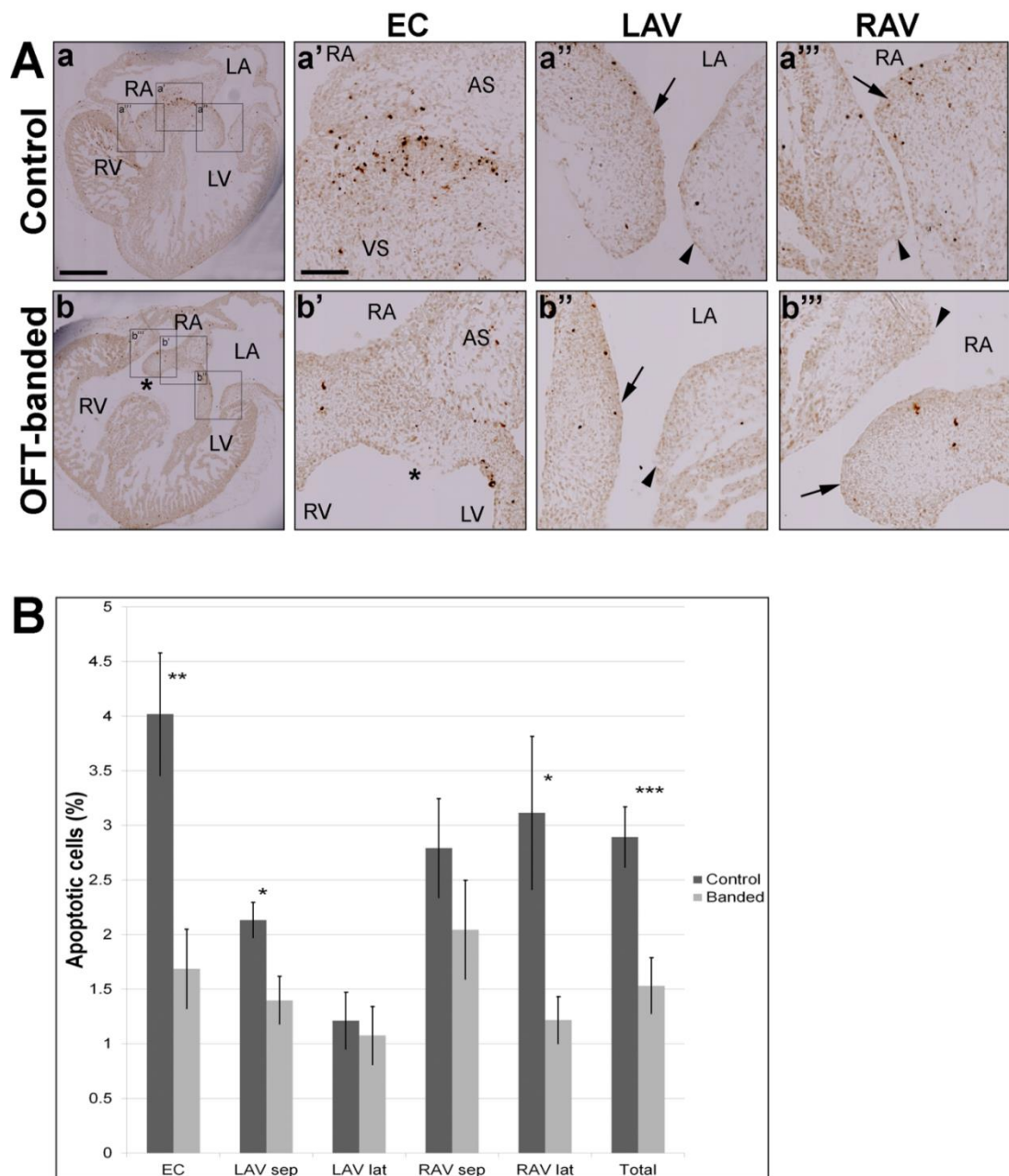


Figure 3.11. Apoptosis was decreased in the primordial atrioventricular valve region of OFT-banded hearts. A. Apoptotic cells were detected by TUNEL staining in control and OFT-banded hearts in the area of endocardial cushion, primordial left atrioventricular valve leaflet and primordial right AV valve leaflet (a-b). Clustering of apoptotic cells was identified in the endocardial cushion area (area between atrial septum [AS] and ventricular septum [VS] in a') in control, but with few apoptotic cells in the endocardial cushion area (between AS and asterisk in b') in the banded hearts. Otherwise, the apoptotic cells were found scattered in the primordial left AV (LAV) and right AV (RAV) septal (denoted by arrow) and lateral leaflets (arrowheads) in both banded (a'' and a''') and controls (b''' and b'''). Asterisk in b and b' denotes the ventricular septal defect in banded heart. **B.** Apoptotic levels in sham and banded hearts at HH29. A decrease of apoptotic cells was shown in all areas analysed in

primordial AV region, *** ($P<0.001$) ($n=5$ for each group). This was attributed by a significant decrease of apoptosis found in the endocardial cushion ** ($P<0.01$), left AV septal leaflet and right AV lateral leaflet * ($P<0.05$) in the banded hearts when compared to controls. However, no significant difference was found in the left AV lateral leaflet and right AV septal leaflet, where $P=0.73$ and $P=0.28$ accordingly. EC, endocardial cushion; LAV sep, left atrioventricular valve septal leaflet; LAV lat, left atrioventricular valve lateral leaflet; RAV sep, right atrioventricular valve septal leaflet; RAV lat, right atrioventricular valve lateral leaflet; arrow, lateral leaflet; arrowhead, septal leaflet. Scale bar: 500 μ m for Aa-b; 100 μ m for Aa'-a''', Bb'-b'''.

3.13 Expression pattern of the extracellular matrix proteins expressed in primordial AV at critical stages of valve development.

The expression of fibrillin-2 has partially been addressed within the literature across the developmental stages in the AV valve region in the chick, with gaps of knowledge still remaining (Wunsch et al., 1994). The expression of both type III collagen and tenascin have been studied in other species but are yet to be determined in chick (Imanaka-Yoshida et al., 2003, Peacock et al., 2008). Therefore, the antibodies of fibrillin-2, type III collagen and tenascin from DSHB were utilized in immunohistochemical analyses to study the spatial-temporal expression of these proteins at crucial stages of valve development (HH21, HH26, HH29 and HH36).

Of all the proteins, fibrillin-2 immunostaining was positive on all stages analysed. At HH21, distinct staining of fibrillin-2 was seen at the endocardial lining of the AVC (Figure 3.12a). At HH25, immunolocalisation of fibrillin-2 was restricted at the borders of the fused mesenchymalized EC (Figure 3.12b). However, positive staining was not observed in any parts of the developing heart with type III collagen (Figure 3.12f,g) and tenascin (Figure 3.12k,l) at HH21 and HH25 respectively. A more intense staining of fibrillin-2 was seen in the primordial left AV valve leaflet at HH29 (arrow in Figure 3.12c). Little to no staining of fibrillin-2 was observed in the left lateral leaflet (arrowhead Figure 3.12c). At HH36, significant immunolocalisation of fibrillin-2 was seen in a more restricted pattern in both septal and lateral leaflets, as well as in the chordae tendinae that is adjoined to the papillary muscles/part of the ventricular myocardium (Figure 3.12d). Additionally, a fairly strong immunostaining of fibrillin-2 was seen in the central endocardial cushion at this stage (arrowhead in Figure 3.12e). With type III collagen, its staining was seen to a lesser degree in the primordial left AV leaflet but was more centralized at the tip of the valve leaflet (Figure 3.12h).

However, stronger immunostaining of type III collagen was seen at HH36, where the staining extended from the septal leaflet to the chordae tendinae (Figure 3.12i). As seen in the fibrillin-2, type III collagen was also immunolocalised in the central EC at this stage (Figure 3.12j). Lastly, positive staining of tenascin was seen in the core cushion at HH29 (Figure 3.12m) and HH35 (Figure 3.12o). The staining of tenascin was found negative in the primordial left AV leaflet at HH35 (Figure 3.12n).

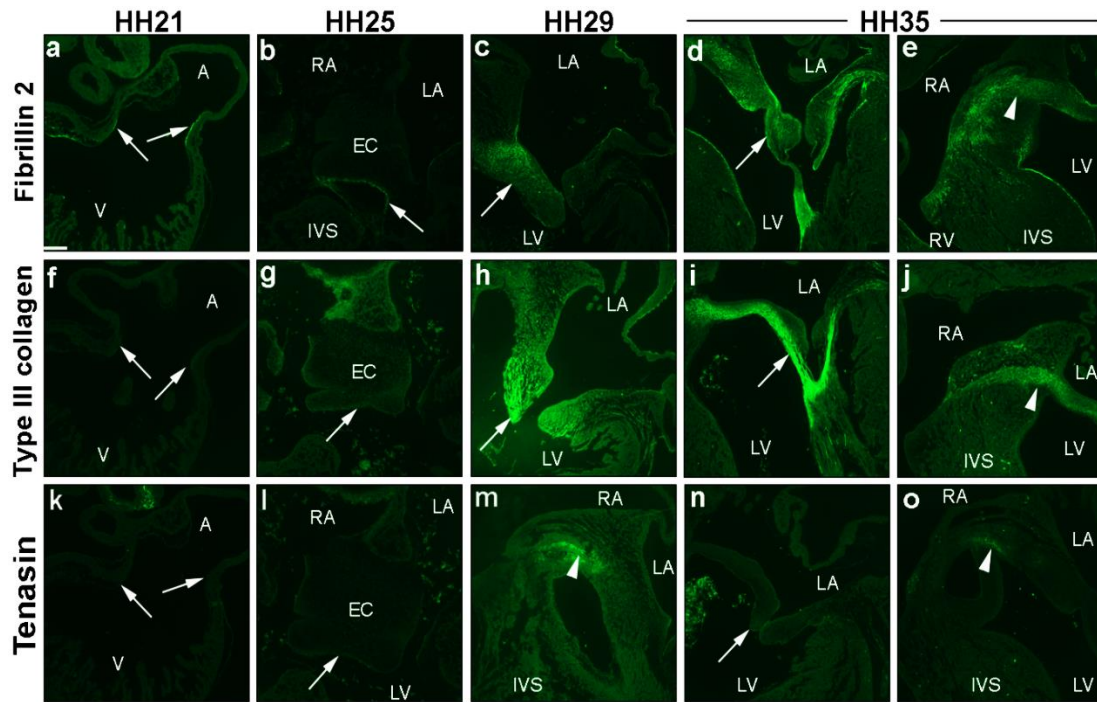


Figure 3.12 Spatial-temporal expression profile of fibrillin-2, type III collagen and tenascin at HH21, HH25, HH29 and HH36. Fibrillin-2 was predominantly expressed in the endocardium lining the atrioventricular canal at HH21 (a). However, positive immunostaining of both type III collagen and tenascin were not observed at the AVC (indicated by arrows in f,k respectively) or in any parts of the developing heart besides the pharyngeal arches (indicated by arrowhead). At HH25, again, only fibrillin-2 was positively delineated at the endocardial lining of the cushion mesenchyme (b). Otherwise, the staining of type III collagen and tenascin were found negative (arrow in g,l accordingly). At HH29, fibrillin-2 and type III collagen were seen positively in the primordial left AV valve leaflet (arrow in c,h accordingly). Tenascin was immunolocalized in the cushion at HH29 (arrowhead in m). At HH35, fibrillin-2 (d) and type III collagen (i) both exhibited a strong staining with zonal restriction in the primordial left AV valve leaflet, with the most intense staining seen in the chordae tendinae (arrow). However, the immunostaining of tenascin remained negative in the primordial left AV valve leaflet (n). A fairly strong and widespread of staining of fibrillin-2 (arrowhead in e) and type III collagen (arrowhead in j) were observed in the central cushion as well. The immunolocalisation of tenascin was not seen in the leaflet but only in the cushion (o). Scale bar: 120 μ m.

3.14 Decreased expression of ECM proteins in primordia AV in HH35 OFT-banded hearts.

Since the aberrant expressions of ECM genes of *TBX20*, Aggrecan and Periostin were seen in the valve primordia at HH29, immunohistochemistry was carried out to demonstrate whether the expression pattern of the valve proteins were affected progressively at later stages of valve development (HH35) on Fibrillin-2, type III collagen and tenascin. Fibrillin-2 and type III collagen were expressed strongly with zonal restriction in controls ($n=5$) (Figure 3.13Aa,c respectively). A noticeable decrease of immunostaining of fibrillin-2 ($n=3/5$) and type III collagen ($n=4/5$) was seen in the truncated primordial left AV valve leaflets in the OFT-banded hearts, with the staining appearing weaker and more diffuse (Figure 3.13Ab,d). Likewise, a significant reduction of type III collagen ($n=4/5$) was seen in the chordae tendinae in the banded hearts (Figure 3.13Af) when compared to controls (Figure 3.13Ae). Again, a positive staining was seen in the central endocardial cushion area subjacent to the atrial septum with zonal restriction with fibrillin-2 in controls (Figure 3.13Ba). However, the staining was decreased in the core cushion in three out of five banded hearts (arrow in Figure 3.13Bb). The positive immunolocalisation of type III collagen in central cushion was located a bit further away from the atrial septum in controls (arrow in Figure 3.13Bc). However, little to no staining of type III collagen was also seen in the OFT-banded hearts ($n=4/5$) (arrow in Figure 3.13Bd). Lastly, with tenascin, its staining was more centralized in the cushion of controls (Figure 3.13Be). Likewise, a similar decrease of tenascin was observed in the core cushion in all banded hearts ($n=5$) (arrow in Figure 3.13Bf). The fluorescent intensity was further quantified in the primordial left AV leaflets in fibrillin-2 and type III collagen (Figure 3.13Cc) ($n=5$ for each group). The average gray level of fibrillin-2 was found 50.48 ± 1.71 in the control

but 34.11 ± 8.02 in the banded hearts, a decrease 32.4% of fluorescent signal was seen. However, this reduction was not significant where $p > 0.05$. Similarly, the fluorescent intensity of type III collagen was found as 57.31 ± 6.39 in controls but it was 34.37 ± 3.92 in the banded hearts, with a reduction of 40% of fluorescence intensity found, and the difference was significant ($p < 0.05$). In addition, transglutaminase-2 (TGM2) was showing a downregulation by 0.74 fold at HH35 ($p < 0.01$) (Figure 3.13D) and this was previously shown as one of the differentially expressed gene in our RNA seq study (section 5.1).

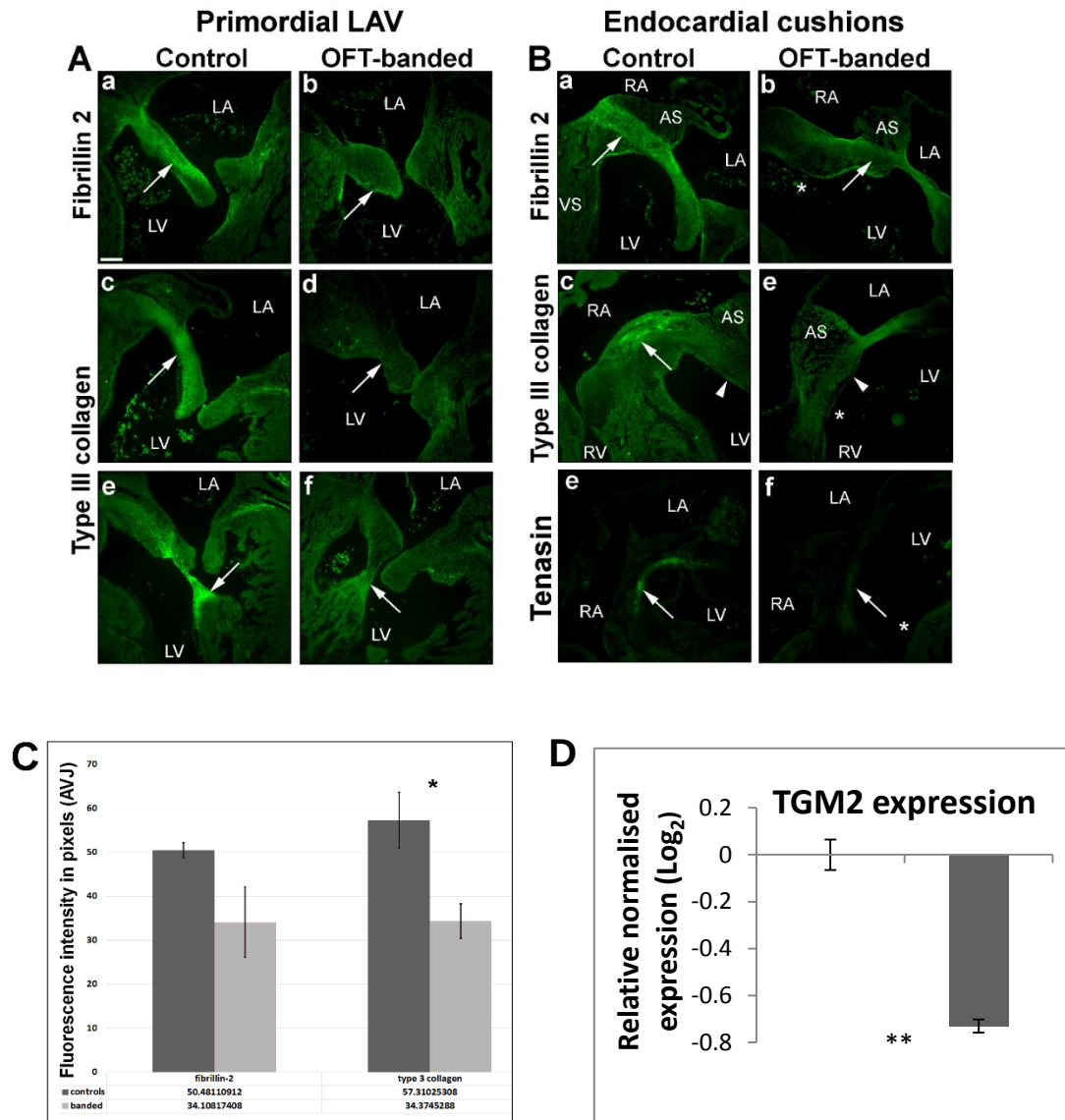


Figure 3.13 Decreased expression of different types of extracellular matrix markers characteristic of primordia left atrioventricular valve leaflet was found in the OFT-banded hearts. **A.** The representative immunohistochemistry result showed a decreased and a more diffuse staining of fibrillin-2 (arrow) (b) in the primordial left AV septal leaflet compared to controls (a). A similar reduction of type III collagen immunostaining was also seen in both leaflets (d) and the chordae tendinae (f) in OFT-banded hearts but not in controls (c and e). **B.** Positive immunostaining of fibrillin-2 (a), type III collagen (c) and tenascin (e) in the endocardial cushion (arrow) seen in the controls showed a reduction in the banded hearts (b,d and f respectively). An asterisk denotes a ventricular septal defect. **C.** The fluorescence signal intensity of the primordial left atrioventricular valve leaflet was semi-quantified using MetaExpress software, with a significant reduction of fluorescence intensity found in type III collagen, where $p < 0.05$ ($n = 5$). **D.** Relative expression of transglutaminase-2 (TGM2) determined by qPCR at HH35 was found downregulated by 0.74 fold, $p < 0.01$. AVJ; average gray level. Magnification bar in Aa 120 μ m; same for all panels in A&B

3.15 Determination of the stage to investigate the normal endocardial cushion development

At HH24, atrial septation is complete (Hendrix and Morse, 1977, Rutland et al., 2009). An inter-atrial septum is present and is fully extended and fused with the superior cushions obliterating the foramen primum, thus dividing the atria in two (Anderson et al., 2003). Also, the mesenchyme of superior and inferior AV cushions fuses at the AVC, dividing it into the left AV and right AV orifices (Lin et al., 2012a). In order to determine if OFT-banding affected the development of EC fusion at HH25, markers were employed to study the expression pattern in the EC, which were versican, *TBX20*, aggrecan and periostin (ECM proteins characteristic of endocardial cushion) by in situ hybridization (Henderson and Copp, 1998, Shelton and Yutzey, 2007, Norris et al., 2004).

At HH25, the most intense expression was seen in the region where the superior and the inferior AV cushions just fused together. Similar expression pattern was observed in versican, aggrecan and periostin (arrow in Figure 3.14a,e,g). *TBX20* was uniformly expressed in the entire EC (Figure 3.14c). In contrast, each individual marker showed a unique expression pattern at HH26. Versican was expressed in the dorsal mesenchymal protrusion (arrowhead in Figure 3.14b) and subatrial part of the central EC but not in the body of the atrial septum. *TBX20* was shown intensely in the entire EC (Figure 3.14d). Aggrecan was expressed to the lateral part of both sides of the central EC (arrows in Figure 3.14f) whereas periostin was strongly expressed in the endothelial lining of the central EC and in a subset of underlying mesenchymal cells (arrows in Figure 3.14h). Taken together, HH26 stage was chosen to study the effect of banding on the formation of the central EC due to a more distinctive expression pattern shown by the EC markers.

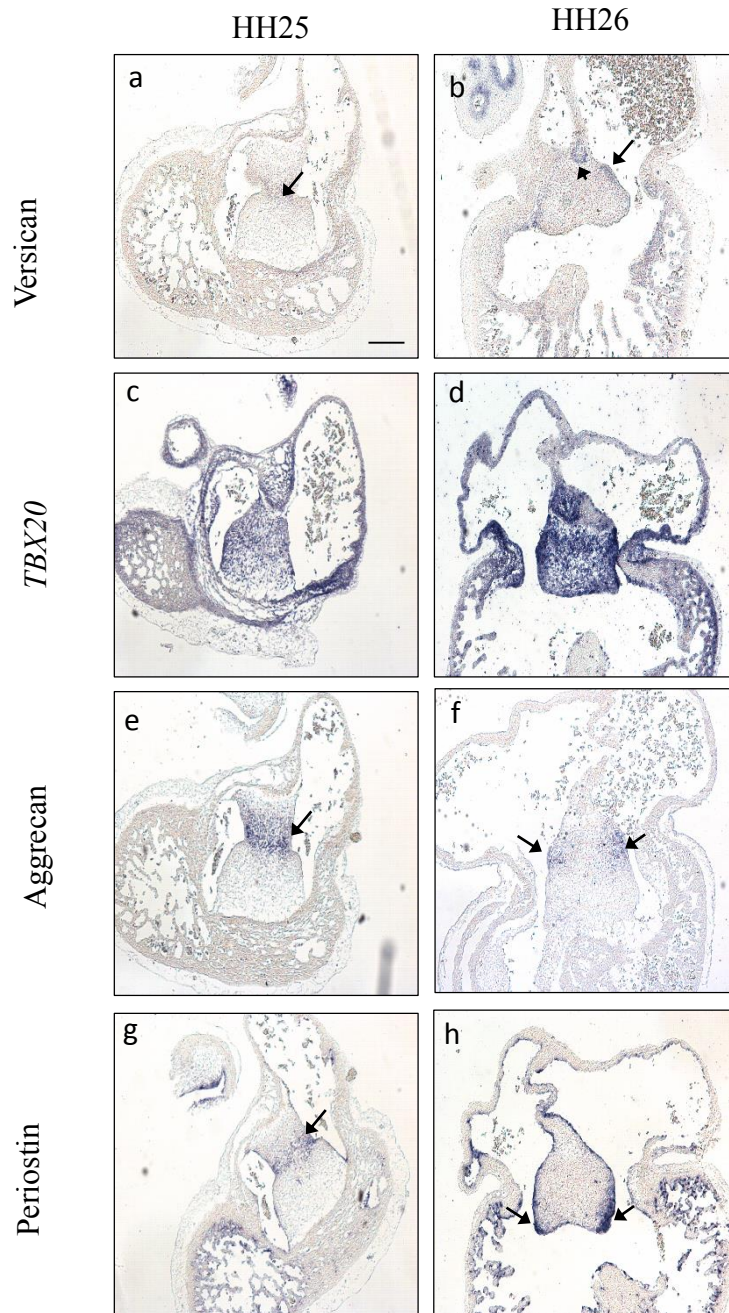


Figure 3.14 Study of expression pattern of different types of extracellular matrix genes characteristic of endocardial cushion by in situ hybridization at HH25 and HH26. At HH25, Similar expression pattern was found in versican (a), aggrecan (e) and periostin (g). The expression was restricted in the area where the superior endocardial cushion just fuses with the inferior cushion (arrow in a,e,g). In contrast, *TBX20* was expressed in the entire cushion region (c). However, at HH26, a more specific expression pattern of these genes was seen. Versican was predominantly expressed in the dorsal mesenchymal cap of the atrial septum that fuses with the cushion (arrowhead) and the subatrial part of the cushion (arrow) (b). *TBX20* was expressed intensely in the entire cushion at this stage (d). Aggrecan was expressed in the lateral part of the cushion adjacent to the atrial septum (arrows in f). Periostin was more restricted to the endocardial lining of the cushion, with more intense expression seen in both sides of the inferior part of the cushion (arrows in h). Scale bar: 150µm.

3.16 Endocardial cushion was morphologically normal in HH26 OFT-banded hearts with differential expression of *TBX20*

The studies described above suggested that the defective valve primordia at HH29 (section 3.9-3.10) and HH35 (section 3.14) might arise from a deformed endocardial cushion. In order to determine if the EC formation had occurred properly, RNA ISH was performed at HH26 on the neighbouring sections of the embryonic chick hearts to study the effect of banding on the formation of endocardial cushion at HH26 using versican, *TBX20*, aggrecan and periostin (ECM markers characteristic of EC).

In normal development, the superior and inferior AV cushions have already fused together by HH26 (Lindsey and Butcher, 2011). This forms a septation at the AVC that will join with the IVS and the dorsal mesenchymal cap of the primary atrial septum. As expected, it was seen that versican was strongly expressed in the mesenchymal cap of the atrial septum that fused with the superior and inferior AV cushions in both controls (arrow in Figure 3.15A,A') and OFT-banded hearts (Figure 3.15B,B') (Table 3.4) ($n=5$ for each group) to show normal atrial septation. Additionally, versican was also seen in the lateral part of the endocardial cushion and was excluded from the core region. No differential expression of versican was seen in the endocardial cushion in the banded hearts at this stage.

TBX20 was strongly expressed in the EC (asterisk) and part of the ventricular myocardium but was found to be absent in the interatrial septum (arrow in Figure 3.15C,C') of controls. Qualitative analysis revealed less intense *TBX20* expression on serial sections in two of the banded hearts analysed ($n=5$) (Figure 3.15E,E') (Table 3.4) compared to controls. Otherwise, all other banded hearts showed a normal *TBX20* expression in the cushion (Figure 3.15D,D') (Table 3.4).

At HH26, abundant periostin mRNA was detected in the endothelial lining of the cushion. In the AV cushions, a subset of mesenchymal cells immediately underlying the endothelium of the cushions showed the most intense expression for periostin. A marked gradient of decreased expression of periostin was observed in the mesenchymal cells located further away from the endothelium (indicated by an arrow in Figure 3.15F,F'). Differential expression of periostin was not observed in any OFT-banded hearts ($n=5$) (Figure 3.15G,G') (Table 3.4) compared to the controls.

In contrast to *TBX20*, aggrecan displayed a more specific expression pattern. It was more restricted to the lateral part of the left side of the endocardial cushion (arrow) (Figure 3.15H,H'). However, similar expression pattern was seen in the banded hearts (Figure 3.15I,I') (Table 3.4).

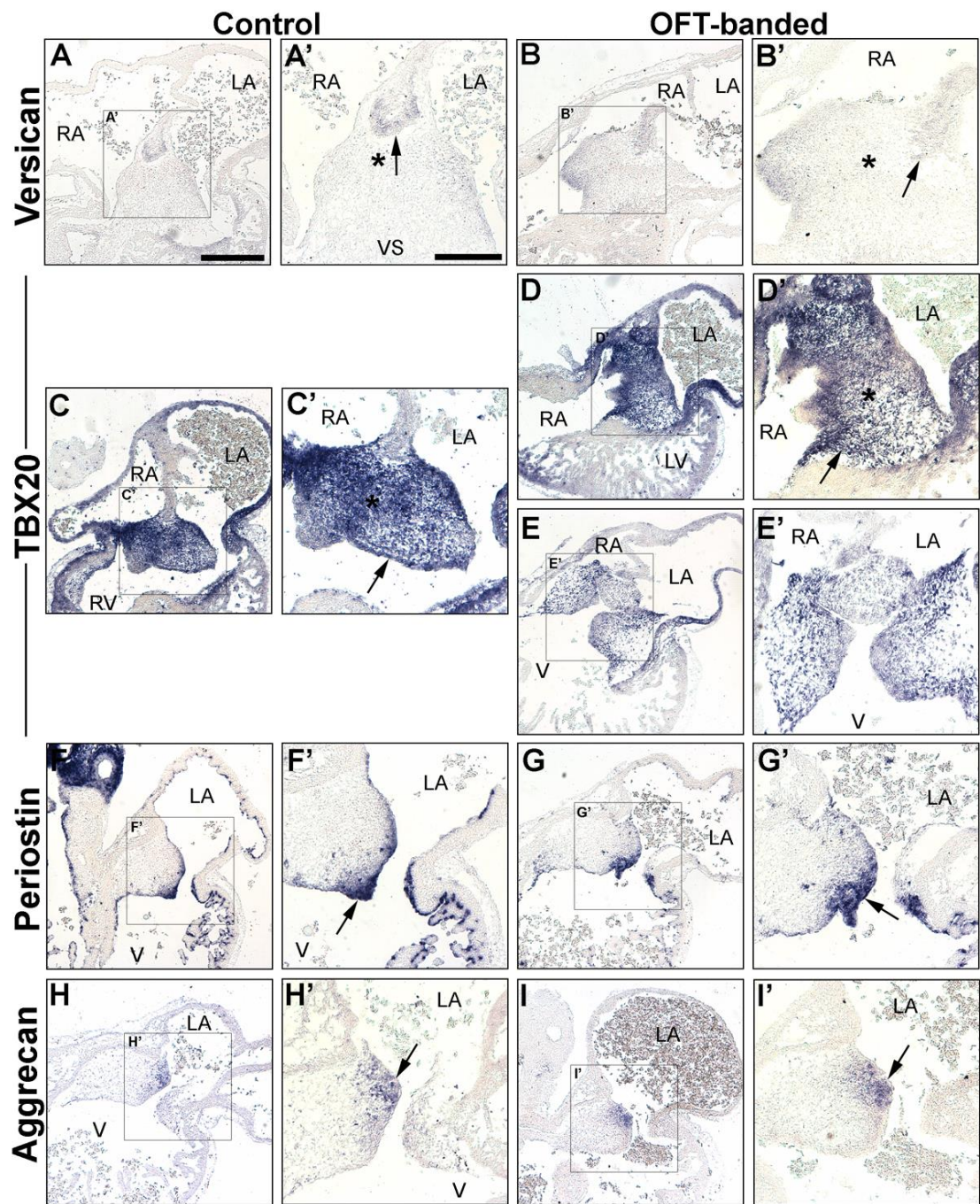


Figure 3.15. OFT-banding leads to a normal atrial septation with mild differential expression of early extracellular matrix markers characteristic of endocardial cushions at HH26. A-B': Normal atrial septation was shown by a positive expression of versican in the mesenchymal cap of the atrial septum (arrow) that fuses with the atrioventricular endocardial cushions (asterisk) in controls (A,A') and banded hearts (B,B'). **C-E':** *TBX20* expression was strong in the entire endocardial cushion area in controls (C,C') and in three of the banded hearts (D,D') (denoted by asterisk in C',D'). In contrast, decreased expression in the endocardial cushions in

two of the banded hearts (E,E') was seen compared to controls (C,C') (denoted by an asterisk) **F-G'**: Periostin was intensely expressed in the endocardial lining of the cushion in both controls (F,F') and banded hearts (G,G') (indicated by an arrow in F' and G') **H-I'**: Expression of aggrecan was seen in the subatrial region of the endocardial cushion in both control (H,H') and OFT-banded hearts (I,I') (denoted by arrows in H' and I'). *n*=5 hearts for both groups. A', B', C', D', E', F', G', H', and I' are the boxed areas in A, B, C, D, E, F, G, H and I respectively. RA, right atrium; LA, left atrium; V, ventricle. Scale bar: 150µm for A-I; 80µm for A'-I'.

Gene	Embryo type □	Primordial AV valve defects		Total int §
Atrial septum marker		Differential expression	Normal	
Versican	Control	0	5	5
	OFT-banded	0	5	5
Primitive valve marker				
<i>TBX20</i>	Control	0	5	5
	OFT-banded	2 (↓)	3	5
Periostin	Control	0	5	5
	OFT-banded	0	5	5
Aggrecan	Control	0	5	5
	OFT-banded	0	5	5

Table 3.4 Summary of differential expression of dysmorphic primordial atrioventricular valve region in OFT-banded chick embryonic heart at HH26.

Assessment of differential expression on primordia atrioventricular valve was based on qualitative analysis of gross morphological features on serial histological sections. The differential expression was seen as decreased (↓) expression.

□ Control embryos include unoperated and sham.

§ Total numbers of embryos analysed internally.

AV, atrioventricular; Int, internal.

3.17 Discussion

During embryonic development, the growth of the heart normally takes place by hyperplasia (increased cell number via proliferation) (Olson and Schneider, 2003, Aulakh, 2009). However, in the adult, the cardiomyocytes tend to grow through hypertrophy (increased cell size) under physiological conditions (Olson and Schneider, 2003, Park et al., 2011, Aulakh, 2009). Cardiac hypertrophy also occurs in response to pathological stimuli in an adult heart, which is a major component of many cardiac diseases (Park et al., 2011).

Cardiac hypertrophy can be an adaptive response to various stress conditions such as pressure-overload (Park et al., 2011, Kim et al., 2014). However, a hypertrophied heart under sustained pressure can lead to maladaptive remodelling and dilatation that progress to DCM and heart failure (Park et al., 2011, Kim et al., 2014). It is known that cardiac hypertrophy has been well documented at the level of cellular and molecular level in the pressure-overloaded induced hypertrophied model in experimental adult model as well as in patients (Song et al., 2012, Park et al., 2011). Other than embryonic chick, pressure-overloaded embryonic heart models have been described in neonatal and fetal lamb and guinea pig before (Bonnet et al., 1997, Saiki et al., 1997, Samson et al., 2000, Sedmera et al., 1999, Montgomery et al., 1998, Hall et al., 2004). In the fetal surgical study that was performed in guinea pigs, they were accessed by hysterotomy and their ascending aorta was constricted to 50% of its original diameter (Saiki et al., 1997). It was found that the left ventricular wall thickness increased significantly in the banded group and there was also increase of the percentage of the marker of proliferation Ki-67-positive cells in both ventricles but the apoptotic level had not changed (part of adaptation mechanism in other prenatal models) (Saiki et al., 1997). Another prenatal study model showed a slowed down

physiological ontogenic maturation of expression of the *SERCA-2a* gene (Bonnet et al., 1997). A similar study of this prenatal model, performed by Samson and colleagues and they found *SERCA-2a* mRNA levels decreased significantly to less than one third-of the sham group value. At the cellular level, transient hypertrophy of cardiomyocytes was found followed by hyperplasia and decreased proportion of binucleated cells (Samson et al., 2000). It is worth pointing out that these two studies were performed in fetal lambs on the preductal coarctation of the aorta rather than the ascending aorta itself and they were performed in a fully formed four chambered heart.

Therefore, the OFT-banding model, another pressure overloading embryonic model was employed to study the effect of pressure overloading on the developing heart.

This pressure-overloaded embryonic heart model has been previously described and has been proposed to give rise to cardiac defects that recapitulate clinical phenotypes observed in human with CHDs and cardiomyopathies (Midgett and Rugonyi, 2014, Sedmera et al., 1999). Although the blood pressure was not measured in our study, a significant increase of blood pressure in the ventricles of OFT-banded hearts has previously been published (Clark et al., 1989, Chivukula et al., 2016).

Using this model, many studies have addressed the altered fluid dynamic and the abnormal cardiac phenotypes that this pressure-overloaded embryonic heart model gives rise to (Sedmera et al., 1999, Chivukula et al., 2016, Shi et al., 2013). However, certain aspects of phenotypic characterization still remain to be determined. To address this deficit we had undertaken the RNA sequencing to provide a global understanding of the molecular underpinnings of the OFT-banded hearts (please refer to chapter 5) and also to study its functional consequences by performing apoptosis and fibrosis studies.

Upon banding, the OFT-banded hearts exhibited a more spherical shape and also a right-shifted position of the OFT when compared to control hearts (Figure 3.2C,F,I), both of which were the typical characteristics of pressure-overloaded embryonic hearts as previously described (Sedmera et al., 1999, Tobita et al., 2002, Hall et al., 2004). In addition, a subset of the banded hearts (14/17 of banded embryos at HH29 and 3/5 of banded hearts at HH35) was found to exhibit a novel cardiac phenotype, where a thickened epicardium with a blebbing appearance was seen. In keeping with the histological analysis, the epicardium was found proportionally thicker in the banded hearts when compared to the controls in stereological study. Stereology showed no differences in the total proportion of the atrium, ventricle, DMP and the atrial septal. Stereological analysis on DMP and atrial septal analysis was not possible owing to low count.

In addition, both ventricular chambers displayed a remarkable reduction of trabeculae. An increased lumen and a thinner myocardium were only found in the LV but not in RV. In addition, left atria displayed a thinner myocardium and an increased lumen. These morphological characteristics recapitulate the DCM phenotypes in humans (Harvey and Leinwand, 2011). The LA dilation has been postulated as a consequence of a more extensive LV damage and normally DCM patients with dilated ventricle accompanied by atrial dilation are associated with higher risk of morbidity (Modena et al., 1997). At HH29, a mild to moderate ventricular dilatation with concomitant thickening of compact myocardium (a resemblance of combination of DCM and HCM phenotype) was found in the OFT-banded heart performed by another group (Sedmera et al., 1999). At a more later stage of development (HH36), the thickness of the compact myocardium, together with the overall wall thickness were significantly less than the controls at HH36 (Tomanek et al., 1999). A potential explanation why a

thinner myocardium of both ventricles was seen in this study could be due to a more severe phenotype produced by the double knot of the OFT banding which caused the HCM phenotype of the banded hearts to evolve to DCM-like features. Interestingly, previous reports within the literature have shown that HCM can evolve to DCM and such patients are associated with higher sudden cardiac deaths (Hamada et al., 1992, Chang et al., 1998, Goto et al., 2013).

It has also been reported that the pressure overloaded embryonic model was associated with a range of cardiac malformations, such as pharyngeal arch anomalies, VSD, abnormal right AV valve morphology and stunted coronary arteries growth (Sedmera et al., 1999, Tomanek et al., 1999). Consistent with the literature, VSD was seen in our model as well. We also investigated the interatrial septum but it appeared normal. To further investigate the underlying mechanism of VSD, the apoptotic cells were quantified at the myocardial crest of the IVS. In normal cardiac development, a dramatic increase of apoptotic level was seen in both AV cushion and muscular crest of IVS for the fusion to occur in between HH28-HH30 (Cheng et al., 2002). In contrast, a decrease apoptosis level found in the muscular crest of the IVS of the banded heart might explain apoptosis did not occur properly for the ventricular septation to take place.

In addition, a defective epicardial development seen as thickened epicardium was revealed in both histological assessment and stereological analysis. The epicardium forms the outermost layer of the heart and the epicardium-derived cells differentiate into interstitial fibroblasts and smooth muscle cells of the coronary arteries to form the coronary vasculature (Winter and Gittenberger-de Groot, 2007, von Gise and Pu, 2012). Anomalies in the epicardium were known to give rise to the defective vascularization and impaired vasculogenesis and angiogenesis and the stunted

coronary growth had been reported in the banded hearts before (Roura et al., 2007, Pires-Gomes and Pérez-Pomares, 2013, Tomanek et al., 1999). Hence, it is possible that the abnormal epicardium is associated with coronary artery anomalies.

In the past, chronic pressure overload induced heart failure of the adult heart was associated with exacerbated cardiomyocyte apoptosis with myocyte loss in infant rabbit and adult rat experimental models (Minegishi et al., 2011, Choi et al., 2009, Teiger et al., 1996). Increased apoptosis which could have resulted from hypoxia, hypertrophy and heart failure are responsible for myocyte loss and thus myocardial dysfunction (Ding et al., 2000, Kajstura et al., 1995, Narula et al., 1996, Olivetti et al., 1996). In aortic-banded fetal guinea pigs, the left ventricular wall thickness increased significantly in the banded group and there was also increase percentage of proliferation cells (marked by Ki-67) in both ventricles but the apoptotic level has no changed (part of adaptation mechanism in other prenatal models) (Saiki et al., 1997).

In contrast a significant decrease in apoptosis was found in both RV and LV chambers of pressure-overloaded embryonic hearts in our study. This can be due to the potential cardioprotective role via the anti-apoptotic effect of a number of differentially expressed genes identified in the RNA sequencing and will be further discussed in chapter 5.

Cardiac fibrosis is one of the main pathological features seen in the pressure-overloaded hypertrophied adult hearts (Gulati et al., 2013, Ho et al., 2010). However, no evidence of fibrosis was seen in our pressure-overloaded embryonic model. The determinant of the degree of fibrosis was based on the presence of the collagen deposition, with the collagen mainly secreted by fibroblasts and the fibroblast is traditionally known to be mesenchymal origin (Souders et al., 2009, Chang et al.,

2014). The fact that fibrosis was not observed in our study at HH35 might be due to the abnormal loading shifts the balance of the mesenchymal cells to become non-fibroblasts, which might result in less collagen production (Norris et al., 2009). Similar study found no fibrosis stained by H&E and Alcian Blue in OFT-banded heart at a much later stage, day 12 (approximately HH39-40) (Pesevski and Sedmera, 2012). Interestingly, endocardial fibroelastosis (a condition where a diffuse thickening of the ventricular endocardium causing myocardial dysfunction and heart failure) was seen in the OFT-banded hearts at day 12, where a fine layer of fibrous tissue in subendocardium was detected by anti-periostin antibody and pan-collagen antibody (Pesevski and Sedmera, 2012).

Normal fluid flow in the heart is important in shaping the endocardial cushions (EC) into the developing cardiac valve leaflets (Hove et al., 2003, Vermot et al., 2009, Steed et al., 2016). Previous reports showed that any occlusion or alteration of haemodynamics will give rise to OFT valve anomalies in chick (Hogers et al., 1997, Menon et al., 2015, Clark et al., 1984). However, the effect of banding on the AV development was vaguely described in the previous literature, where the immature AV valves (vitelline vein model) and abnormal right AV valves (OFT-banding model) were found upon morphological analysis without any further investigation (Hogers et al., 1997, Sedmera et al., 1999). This highlights the need of further characterization of the endocardial cushion and hence more matures primordial AV valves at the biochemical and molecular level. The development of the chick right AV valve does not undergo remodelling as seen in humans, and hence chick AV valve is not composed of fibrous tissue but resembles more like a muscular flap (Yalcin et al., 2011). Left AV valves is composed of fibrous tissue and are similar to cardiac valves seen humans and hence is the main focus of investigation.

To delineate the effect of the OFT-banding on the development of the primordial AV valve and its alignment with other septal components, OFT-banding was performed at HH21 (chamber septation in progress, endocardial cushions initiated) with harvesting at HH26 (atrial septation complete, fusion of superior and inferior cushions), HH29 (fused cushions mature, primordial AV valve elongating and ventricular septation complete) and HH35 (AV undergoing remodelling).

Upon harvesting at HH26, gross morphological analysis revealed no structural abnormalities of atrial septum or the endocardial cushion. A normal endocardial cushion is comprised of four mesenchymal tissue: the superior and inferior EC, the mesenchymal cap and the dorsal mesenchymal protrusion and its normal formation and maturation is required to align with the primary atrial septum and the IVS for the chamber septation to occur (Snarr et al., 2007, Lin et al., 2012a). In order to determine if this is the case, the spatial expression of ECM gene characteristics of endocardial cushion such as *TBX20*, aggrecan, periostin and versican was performed in control and banded hearts at HH26. Versican is negatively expressed in the body of atrial septum but is strongly expressed in the mesenchymal cap of the primary atrial septum (Henderson and Copp, 1998). The dorsal mesenchymal protrusion normally fuses with the mesenchymal cap of the atrial septum and the AV cushions and thus results in the closure of the ostium primum (Briggs et al., 2013). Positive expression of versican was expressed in the mesenchymal cap in all banded hearts and controls analysed at HH26, confirming that normal atrial septation occurred. Further, aggrecan was seen in the subatrial region of the cushion in both banded hearts and control. Similarly, periostin expression was predominantly expressed in the endocardial lining of the cushion at HH26 and no differential expression was seen.

Interestingly, mild differential expression of *TBX20* was seen in 2/5 of the banded hearts analysed, with normal expression of periostin and aggrecan observed in the sister sections. The reason for this could be due to a more constricted OFT was generated upon banding at HH21 in two of the five banded hearts. It has been previously published that both periostin and aggrecan were found downregulated at the mRNA level by ISH in *Tbx20* knockdown (Cai et al., 2013). Hence, we postulate that the *TBX20* was primarily affected upon banding as it acts as upstream of both periostin and aggrecan.

Upon harvesting at HH29, where the fused cushions are undergoing maturation with the primordial AV valve elongating, defective AV valves were observed. First of all, histological analysis revealed 62% of OFT-banded hearts ($n=31$) had developed dysmorphic AV valves at HH29. The primordial AV valve leaflets appeared to be shorter and blunter compared to the elongated AV valve leaflets seen in the controls. In some of the banded hearts, a smaller cushion was seen and it failed to fuse with the IVS and thus resulted in a ventricular septal defect (VSD). In addition, it was found that 85.7% embryos presented a more severe but similar phenotype at HH35. However, the number of hearts analysed was low ($n=7$). The AV valve malformations have been reported in other surgically altered haemodynamic model, the vitelline vein ligation model (Hogers et al., 1997). About 8% of manipulated embryos showed morphologically distorted and developmentally immature AV valve (Hogers et al., 1997). In addition, OFT banding also led to a considerable decrease of OFT cushion volume with altered expression of a number of genes crucial for OFT valve development by qPCR (Menon et al., 2015).

The effect of OFT-banding on AV valve morphogenesis was quantified by stereological analysis; a significant decrease of 31.62% of primordial AV valve region

in the banded hearts was seen, where $p=0.02$. Morphometric analyses were carried out by measuring the cushion area and the primordial left AV valve septal leaflet length. The relative primordial left AV septal leaflet in the banded hearts presented only 82% of the length of the controls whereas the relative EC area showed a slightly more severe phenotype, which was 66% of the size of the controls, and $p<0.001$ was found in both studies.

The histological assessment also revealed that the staining of acidic glycosaminoglycan by Alcian Blue was slightly reduced in a few of the OFT-banded hearts at HH29. A previous report had shown that the matrix organization of embryonic heart glycoaminoglycans is required for the migration of cushion tissue mesenchyme (Markwald et al., 1978). Hence, a slight reduction of Alcian Blue seen in the banded hearts might be suggestive that the mesenchymal cell migration was impaired upon OFT-banding. Masson's Trichrome staining was also performed but no deposition of collagen was seen in the OFT-banded hearts at HH35.

To further investigate if the primordial AV valve region was abnormally formed upon banding, ISH was performed to look at the spatial expression of a number of ECM genes which are crucial for valve development. The mRNA level of these ECM genes was further quantified by qPCR. *TBX20* was intensely expressed in the entire EC as well as in all four primordial AV valve leaflets in controls, with a remarkable reduction seen in the OFT-banded hearts. Also, a significant downregulation by 0.37 fold was seen in *TBX20* by qPCR, in keeping with the ISH data. In addition to its expression in the endocardial cushion, *Tbx20* expression was also detected in the atrial and ventricular myocardial wall as previously published and has been associated with other developmental processes such as chamber specification and maturation (Yamagishi et al., 2004, Plageman and Yutzey, 2005). A prominent reduction of *TBX20* expression

in the atrial and ventricular myocardial wall might imply chamber maturation has not taken place properly. This was in accordance to the findings of the enlarged LA and the ventricles in the stereological study. However, the expression of these areas was not the focus of the study and therefore they were not examined.

TBX20 is known to be expressed in the avian EC and is important to promote EC cell proliferation, EC migration and ECM gene expression *in vitro* (Shelton and Yutzey, 2007, Shelton and Yutzey, 2008). Also, *Tbx20* plays a role in EC maturation and valve elongation by regulating the Wnt/ β -catenin signalling pathway (Cai et al., 2013). Upon knockdown of *Tbx20* in mice, severe defective elongation of mutant mitral and tricuspid valves (the left and right AV valves respectively) was found (Cai et al., 2013). In our study, we postulated that the OFT-banding led to the reduction of *TBX20* which engendered AV valve elongation defects. A previous report showed that Wnt/ β catenin signalling is disrupted upon *Tbx20* knockout and *Wnt7b*, *Wnt9b* and *Lef1* were significantly dysregulated (Cai et al., 2013). Wnt signalling is an important regulator of endocardial cushion maturation as well as valve leaflet stratification, homeostasis and pathogenesis (Cai et al., 2013, Alfieri et al., 2010).

Aggrecan was found localized in the core cushion subjacent to the atrial septum in both controls and banded hearts. In addition, an ectopic expression was found in the banded hearts, where the expression was shifted to the atrialis side of the primordia left AV valve leaflet. The most intense ectopic expression was also detected at the borders of the lateral part of the EC of the right AV. Although the right AV valve doesn't remodel human AV valve and hence is not a focus of this project, the fact that we have seen an ectopic expression of aggrecan upon banding is suggestive that the abnormal cushion was formed to give rise to the valve abnormalities. Aggrecan is one of the main chondroitin sulfate proteoglycan constituents of the spongiosa layer in

avian valves, and is required to withstand compressive forces against blood flow (Shelton and Yutzey, 2007). Aggrecan has been reported to have a role in valve cell differentiation (Shelton and Yutzey, 2008, Zanin et al., 1999). An ectopic expression of aggrecan seen in the dysmorphic AV valve in this study implies differentiation has not occurred properly.

Expression of periostin was restricted to the endocardial lining atrialis side of the primordial leaflets of the right and left AV valve as well as a subset of underlying mesenchyme. Additionally, abundance of periostin mRNA was observed in the chordae tendinae of the left ventricular AV junction (Norris et al., 2008). Periostin has been previously reported of its multifaceted role in the cell migration and its differentiation role of promoting mesenchymal cells into a fibroblastic lineage while repressing transformation into myocytes lineages (Shelton and Yutzey, 2008, Norris et al., 2009). Periostin is also able to interact with other ECM components such as collagen, tenascin and fibronectin as well as the cell surface integrins on the mesenchymal progenitor cells to initiate signalling related to migration and collagen crosslinking and compaction through Rho and PI3 kinases (Butcher et al., 2007, Norris et al., 2007, Kii et al., 2010). Interestingly, although periostin mRNA was not found differentially expressed in these regions in the banded hearts by both ISH and qPCR at HH29, a significant downregulation of periostin was found in qPCR at HH35, together with the decrease expression of type III collagen and tenascin in the immunohistochemistry. In contrast, an increase mRNA level of periostin was found in the OFT valve cushion of the banded hearts in another study (Menon et al 2015). The possible reason might be due to the alternative splicing that confers nine different transcript variants of periostin. Downregulation of mRNA level of one transcript

variants might lead to a compensatory upregulation of another transcripts variant of periostin upon banding.

Punctuate periostin mRNA expression was detected throughout the endocardium of the both atria and ventricles as well as the ventricular trabeculae in RNA ISH and the expression is consistent with the previous findings (Norris et al., 2004). However, the function of the periostin in the myocardium is yet to be determined. It might play a role in stabilizing the heart (Norris et al., 2004). Previous reports showed that the periostin may be involved in remodelling of the adult ventricle in response to stress responses such as myocardial infarction and hypertrophy (Stanton et al., 2000, Wang et al., 2003). Interestingly, an increase mRNA level of periostin was found in OFT cushion.

Periostin and aggrecan were found downregulated at the mRNA level by ISH RNA ISH in *Tbx20* knockdown (Cai et al., 2013). In our study, an ectopic expression of aggrecan was observed in the banded hearts instead of a downregulation at HH29. Also, periostin expression appeared to be normal in all banded hearts analysed. It is also worth noting that the alteration of *Tbx20* function by siRNA had no effect on the expression of periostin in endocardial cushion cells (Shelton and Yutzey, 2008). This might imply insufficient knockdown efficiency of *Tbx20* by siRNA to cause a downregulation of periostin. Likewise, in this study, a reduction of *TBX20* by 0.37 fold upon banding might not be sufficient to alter the expression of periostin.

The fluid flow and endocardial mechanotransduction are important in valve morphogenesis mediated by ECM synthesis (Hove et al., 2003, Vermot et al., 2009, Steed et al., 2016). As shear stress is positively correlated with the blood flow, it is conceivable that alteration of shear stress attributes to the development of these cardiac anomalies (Groenendijk et al., 2005). *KLF2* and *EDN1* were the shear stress responsive

genes chosen to investigate this. Both genes displayed mutual exclusive expression patterns *in vivo* during heart development (Groenendijk et al., 2004). *KLF2* is a member of the SP/XKLF family of transcription factors and is also known to be shear stress responsive (Philipsen and Suske, 1999, Dekker et al., 2002). It is been shown in the developing valves and is a reliable indicator of normal valve development (Vermot et al., 2009). *KLF2* is known to be upregulated by high shear stress and its expression is confined to the areas of high shear stress area such as the endothelium and endocardium at narrow parts of the developing heart such as the AVC (Groenendijk et al., 2004).

At HH29, positive *KLF2* expression was delineated in the areas of high shear stress which corresponded to the endocardial lining of the primordial left AV and lateral valve leaflet. Expression was more marked at the tip of both leaflets. An increased and extended *KLF2* expression was delineated in the endocardial lining of the primordial left AV and lateral valve leaflet in the OFT-banded hearts. An upregulation by 0.27 fold was seen in these banded hearts ($n=5/7$), however, the difference was not significant.

Endothelin-1 (*EDN1*) is a growth hormone involved in vasoconstriction and *EDN1* gene contains shear stress response elements in the promoter (Groenendijk et al., 2004). The expression of *EDN1* is not confined to the endothelium and endocardium and is developmentally regulated (Groenendijk et al., 2004). Around HH22, the expression of *EDN1* at atrioventricular canal became negative (Groenendijk et al., 2004). Further, the *EDN1* mRNA was observed in the compact myocardium corresponding to the sites of coronary vessel development from HH24 onwards (Groenendijk et al., 2004).

In OFT-banded hearts, a noticeable decrease of *EDN1* expression was observed in the LV compact myocardium when the heart was suggestive to be as a result of altered shear stress expression. The mRNA level of *EDN1* was also determined by qPCR with a significant downregulation by 0.65 fold ($p < 0.001$). Interestingly, increased shear stress responsive genes was found in our OFT-banding model (increased afterload/pressure-overloaded model), aberrant expression of these genes had also been reported in another altered haemodynamic model- venous clip model (increased preload model) and this led to the development of the cardiac anomalies such as ventricular septal defects, semilunar valve anomalies, and several types of pharyngeal arch artery abnormalities (Hogers et al., 1999, Groenendijk et al., 2005).

An increased phospho-smad2 (a downstream target of *KLF2*) protein expression was observed in the endothelial cells lining the EC after venous clipping at HH17 (Egorova et al., 2011). Knockdown of mechanotransduction protein *trpv4* attributed to the development of dysmorphic valve phenotype (Heckel et al., 2015). *trpv4* has been reported as an upstream of *klf2* in zebrafish (Heckel et al., 2015). A recent study had shown that the mechanical forces and valve morphogenesis are coupled via cellular arrangements mediated by extracellular matrix protein synthesis (Steed et al., 2016). *klf2a*^{-/-} mutants in zebrafish displayed an array of valvular phenotype and this was attributed by the cell disorganization at the AVC and impaired fibronectin synthesis (Steed et al., 2016). Upon OFT-banding, differential expression of *KLF2* and *EDN1* might contribute to the formation of abnormal valve primordia, with a concomitant aberrant expression of cell migration and differentiation markers, *TBX20* and aggrecan.

The dysmorphic primordial AV valve region was shown proportionally smaller in the banded hearts alongside with the aberrant expression of ECM genes. This phenotype

is also consistent with a decrease in apoptosis. During normal cardiac development, cushion mesenchyme is normally differentiated into myoblasts and fibroblasts of the connective tissues of the valves and thus secrete collagen (Abdelwahid et al., 2001, Abdelwahid et al., 2002, Kruithof et al., 2007). Clustering of apoptotic cells has been observed mostly to the differentiated part of the cushion, localized in the core EC subjacent to the atrial septum (Abdelwahid et al., 2001, Abdelwahid et al., 2002). A remarkable decrease of apoptotic cells was detected in a scattered pattern in the EC throughout the primordial AV valve region in banded hearts. This suggests that the differentiation has not taken place properly in the banding model. A significant decrease of apoptosis was also seen with primordial left AV septal leaflet and primordial right AV lateral leaflet. In addition, the overlapping and complementary expression pattern of the regulatory factors of *Bmp4*, *Bmp2* and *Msx2* and the apoptotic cells might suggest there is an inextricable link between them to promote the mesenchymal condensation and differentiation (Abdelwahid et al., 2002, Abdelwahid et al., 2001). However, the regulation of this pattern in the developing heart remains to be determined. In addition, the apoptotic cells were morphologically smaller in the OFT-banded hearts than in the controls.

Since the aberrant expressions of ECM genes of *TBX20*, Aggrecan and Periostin were seen in the valve primordia at HH29 in banded hearts, immunohistochemistry on ECM proteins fibrillin-2, type III collagen and tenascin was performed to determine if staining was affected in later stages of valve development (HH35).

According to the literature, at HH35, the primordial left AV leaflets still undergo the process of elongation (Tan et al., 2011b). In the HH35 OFT-banded hearts, they appeared to be more mature and elongated and showed a distinct and strong positive expression with zonal restriction in fibrillin-2 and type III collagen. However, weaker

and more diffuse expression of fibrillin-2 and type III collagen were seen in the truncated primordial left AV valve leaflets of the banded hearts. A more widespread of fibrillin-2 expression was seen compared to a more localized expression of type III collagen and tenascin in the central EC of the controls. However, little to no expression of these proteins was detected in the core cushion of the banded hearts. During post-EMT stages, the cushion mesenchyme normally differentiates into valve interstitial cells, the specialized valvular fibroblasts that express genes that encode fibrillary collagens, chondroitin sulfate proteoglycans as well as elastin which are associated with stratified ECM of the mature valves (Hinton and Yutzey, 2011, Hinton et al., 2006). The valve interstitial cells also normally undergo the transformation process that features of activated migration, proliferation and matrix synthesis (Liu et al., 2007).

Despite the primordial left AV valve leaflets are still undergoing elongation at this stage, the ECM proteins exhibited a specific expression pattern with zonal restriction for stratification to occur at later stage of valve development. Alteration of haemodynamic loading might causes the mesenchyme to fail to undergo differentiation into fibroblasts, which in turn fails to produce ECM proteins to form mature valve leaflets (Norris et al., 2009). In addition, ECM remodelling enzymes such as matrix metalloproteases (MMPs), tissue inhibitors of matrix and is metalloproteases (TIMPS) are expressed during valve maturation (Hinton and Yutzey, 2011). Disorganization or disrupted ECM integrity seen in the primordial valve leaflets in banded hearts might be due to disrupted activity of MMPs or low level of TIMPs but this is yet to be determined. Also, EDN1 and EDN3 are both involved in the synthesis of collagen but EDN1 predominantly affects the collagenase activity (Guarda et al., 1993). Hence, we postulate that the decrease of the type III collagen seen at HH35

might be due to the significant decrease of *EDN1* seen in the banded hearts at HH29 as previously mentioned. TGM2 was one of the statistically differentially expressed gene found in RNA sequencing (refer to Chapter 5 section 5.1; Table 5.2) and it plays an important role in cross-linking the ECM enzyme and also is involved in differentiation of osteoblast (Yin et al., 2012b). A downregulation of TGM2 at both HH29 and HH35 might contribute to a less degree of cross-linkage of ECMs and hence the decrease of ECM proteins of fibrillin-2, type III collagen and tenascin.

A normal endocardial cushion is comprised of four mesenchymal tissue: the superior and inferior EC, the mesenchymal cap and the dorsal mesenchymal protrusion (Snarr et al., 2007, Lin et al., 2012b). As atrial septation occurred normally in banded hearts by HH24, this suggests the endocardial cushions were normal at this stage. The same can be said for HH26 cushion, with periostin, aggrecan and *TBX20* (in two out of five hearts) as expected. The defective valve seen at the later stages of development at HH29 and HH35 in our studies together with VSDs suggested that the aberrant valvuloseptal mechanism was likely to occur in between HH26-HH35. In a normal valve development, from HH26 onwards, the main valve morphogenetic events involve migration of post-EMT endocardial cells into acellular cardiac jelly, proliferation of post-EMT cells and differentiation of the cushions into a fibroblastic phenotype (Butcher and Markwald, 2007, Inai et al., 2008). As previously mentioned, a slight reduction of Alcian Blue seen in the banded hearts at HH29 might be suggestive that the mesenchymal cell migration is affected. Future studies are needed to elucidate if these processes contributing to the defective valvular phenotype.

Taken altogether, we postulate the OFT-banding affects the post EMT endocardial cushion and hence primordial AV valve maturation and elongation

4 CHAPTER 4: Investigating the role of *TNNT2* in the developing heart in chick

Overview

Troponin T (cTNT) is a 30-35kDa protein that binds the troponin complex to tropomyosin and actin, serving as a scaffold and holding the thin filaments in contact (England et al., 2016). To date, *TNNT2* has not been linked to isolated CHDs, although its mutation has been highly associated with different types of cardiomyopathies before. Targeted manipulation of *Tnnt2* prior to heart development had been performed in the mouse and zebrafish. However, early embryonic lethality (around embryonic day 10.5) was found in the homozygous null mouse (*Tnnt2*^{-/-}), whereas the heterozygous *Tnnt2*^{+/-} has normal functional hearts (Nishii et al., 2008, Ahmad et al., 2008). A significant reduction in cTnI, cTnT and tropomyosin was observed leading to severe sarcomere defects when cTnT knockdown was performed in zebrafish (Sehnert et al., 2002). Hence, the chick embryo was employed to investigate the *in ovo* role of cTnT in septation and trabeculation at the stage where there is expression of sarcomeric proteins in the developing heart using targeted manipulation of morpholino.

The aims of these experiments were:

- To investigate a potential role for *TNNT2* in the developing chick heart by morpholino treatment *in ovo*. Gross morphological internal and external features of the atrial septum, ventricular trabecular and the endocardial cushion were studied upon *TNNT2* morpholino treatment.
- To determine if the novel cardiac diverticula found in *TNNT2* morpholino-treated hearts are of fibrous or muscular type.

- To investigate a potential cause of the abnormal atrial septum and reduced trabeculae in the TNNT-MO-treated hearts using a shear stress marker, *NOS3*.

4.1 Embryo grouping, survival and TNNT2 morpholino uptake

TNNT2 morpholino (TNNT2-MO) was used at two different concentrations which were 250 and 500 μ M and HH11/19 indicates the morpholino treatment at HH11, with harvesting performed at HH19. For the 250 μ M study, the survival rates for the untreated (UT) group ($n=22$), standard control (SC) group ($n=30$) and TNNT2-treated ($n=43$) embryos were 90.9%, 93.34% and 91.5% respectively with $P > 0.298$. The survival rates for the 500 μ M study for UT ($n=52$), SC ($n=56$) and TNNT2-MO-treated group ($n=106$) were 96.1%, 91.07% and 92.9% accordingly, where $P > 0.275$. Surviving embryos were then further examined for positive ‘morpholino uptake’ by the degree of fluorescence (Figure 4.1Aa-d). In the 250 μ M study, 43.8% of the SC and 58.1% of the TNNT2-MO-treated embryos were positive for the morpholino uptake ($P=0.448$). For the 500 μ M study, 63.6% of the SC and 73.6% of the TNNT2-MO-treated embryos were positive ($P=0.446$).

4.2 TNNT2-MO treatment results in the development of a diverticulum in the ventricular wall.

Once embryos were confirmed positive for morpholino uptake, they were assessed and photographed morphologically at low and high microscopic magnification. External features such as cardiac looping; atrial and ventricular positioning and size; the length and shape of the OFT were studied. In 250 μ M study, no external phenotype was observed in either the controls ($n=34$) or TNNT2-MO-treated ($n=43$) embryos (Table 4.1 for the summary of TNNT2-MO experiments). Similarly, for the 500 μ M study, all controls ($n=84$) (Figure 4.1Ba,a') and the majority of TNNT2-MO-treated embryos ($n=65/67$) appeared to have a normal external phenotype (Figure 4.1Bb,b'). However, two of the TNNT2-MO-treated hearts presented with a diverticulum on the heart wall. Both diverticula were located at the anterior part of the primitive ventricle with one present inferior to the OFT (diverticulum 1; Figure 4.1Bc,c') and another one situated inferior to the atrium (diverticulum 2; Figure 4.1Bd,d'). Based on the external morphological assessment, diverticulum 1 appeared to be a mild protrusion ventricular myocardial wall (Figure 4.1Bc,c'). Intriguingly, diverticulum 2 was seen as an extra chamber which was connected to the primitive ventricle via narrow communication and it displayed synchronized contractility with the ventricle (Figure 4.1Bd,d' and e,e').

4.3 Normal morphology and histology was found in the diverticulum of TNNT2-MO-treated embryonic hearts

In order to further investigate the ventricular diverticula, both hearts where this was observed were sectioned serially, with neighbouring sections collected on adjacent slides to allow for different histological analysis and immunohistochemical study (this will be discussed in section 4.4). The first set of these sections were Alcian Blue- and haemalum stained to determine the gross morphology. Diverticulum 1 appeared as a mild protuberance of the myocardium and endocardium situated just inferior to the OFT, on the anterior part of the right side of the primitive ventricle (Figure 4.1Bc,c'). However, the diverticulum 2 was seen to have a more complex structure compared to the first diverticulum. In Figure 4.1C, the gross morphology of the second diverticulum was compared with a control heart, with both hearts sectioned in a sagittal orientation. The control heart showed the structure of a normal heart progressing through the primitive ventricle and the atrium (Figure 4.1Ca-j). With diverticulum 2, it firstly appeared on the anterior part of the left side of the primitive ventricle inferior to the primitive atrium (arrow in Figure 4.1Ca'). As the sections progressed throughout the ventricular chamber, the first communication was seen between the ventricle and the diverticulum (arrow in Figure 4.1Cb'). This communication quickly closed (Figure 4.1Cc'-e'). Towards the end of the ventricular chamber, another communication appeared again when the atrioventricular canal and atrial chamber could be seen (Figure 4.1Cf'). Again, the communication closed (Figure 4.1Cg'-h') until the diverticulum reopened for the final time in the atrial chamber (Figure 4.1Ci'-j').

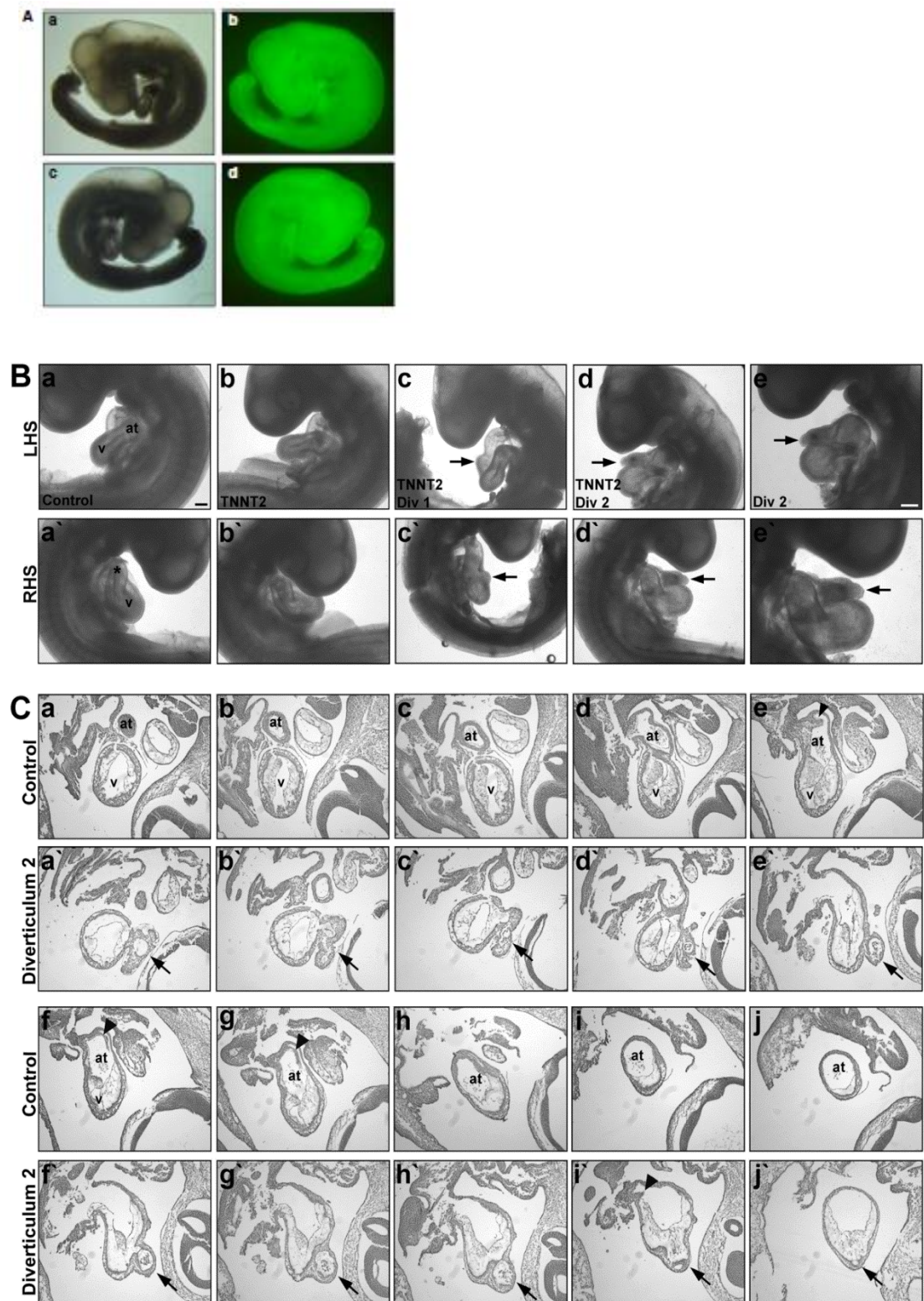


Figure 4.1 TNNT2-MO treatment results in the development of the diverticula from the ventricular myocardial wall. A. Embryos were treated with a TNNT2 or standard control fluorescein-tagged morpholino at HH11; only embryos showing a strong fluorescent signal were harvested at HH19 for analysis. Embryos were shown in fluorescence (b,d) in comparison to brightfield (a,c) on both left- (a,b) and right- (c,d) hand side. **B.** External morphological assessment revealed normal heart

morphology in most of the TNNT2-MO-treated embryos (b and b') when compared to the controls (a and a'). However, two embryos presented diverticula on the anterior part of the primitive ventricular wall (c and c' as Div 1 and d and d' as Div 2). Div 1 was seen as mild protuberance from the myocardial wall of the ventricle but Div 2 appears as an extra chamber. Div 2 is shown at higher magnification (e and e'). **C.** Internal analysis of gross morphology of embryo with Div 2 (a'-j') compared to control through serial sectioning across the heart sagittally (a-j). Three openings were present between the primitive ventricular chamber and the diverticulum intermittently (b', f' and i'). Arrowheads indicate the normal atrial septa growth in controls (f and g), which looks stunted in embryos with Div 2 (i'). * outflow tract; arrowhead, atrial septum; arrows, diverticulum; at, atrium; LHS, left-hand side of embryo; RHS, right-hand side of embryo; v, ventricle; Scale bars: 100µM.

Concentration of morpholino	Embryo type*	Internal heart structures							
		External heart shape			Atrial septum		Trabeculation		Total internal
		Presence of diverticulum	Normal	Total external	Reduced	Normal	Reduced	Normal	
500µM	Control	-	84	84	1	37	-	38	38
	TNNT2	2**	65	67	23	28	6	45	51
250µM	Control	-	34	34	-	15	-	15	15
	TNNT2	-	43	43	-	19	-	19	19

Table 4.1 Summary of phenotypic analysis after treatment with TNNT2 morpholino

Assessment of heart shape was based on qualitative analysis of gross morphological features.

Qualitative assessment of atrial septa development and the degree of ventricular trabeculation was evaluated from serial histological sections.

TNNT2 indicates the embryos treated with the TNNT2 2nd ATG start site morpholino.

Control includes standard control and untreated embryos.

** Both embryos with diverticula show reduced atrial septal size

4.4 Both diverticula were identified as muscular type in TNNT2-MO treated hearts

In order to investigate if the diverticula are of the muscular or fibrous type, Masson's Trichrome staining was performed on the second set of the serial sections to detect if there is any excess collagen deposition within the diverticula. No abnormal collagen deposition was detected in the diverticula as the diverticula were stained red but black/blue staining did not present at all. The initial histological staining indicated that the diverticula were of a non-fibrous phenotype (Figure 4.2Ab,b') in comparison with a control heart (Figure 4.2Aa,a'). The chick skin was used as a positive control (Figure 4.2Ac). To complement this data, anti-myosin antibody staining was performed on the third set of neighbouring sections to detect muscle fibres within the hearts with diverticula. When compared with a control heart (Figure 4.2Ba,a'), the muscle fibres appeared normal and are organized in mature sarcomeric assembly of myosin filament in the myocardium of all regions in both diverticula (Figure 4.2Bb,b' for diverticulum 1 and 4.2Bc,c' for diverticulum 2). This confirms the diverticula seen upon TNNT2-MO-treatment were of muscular type.

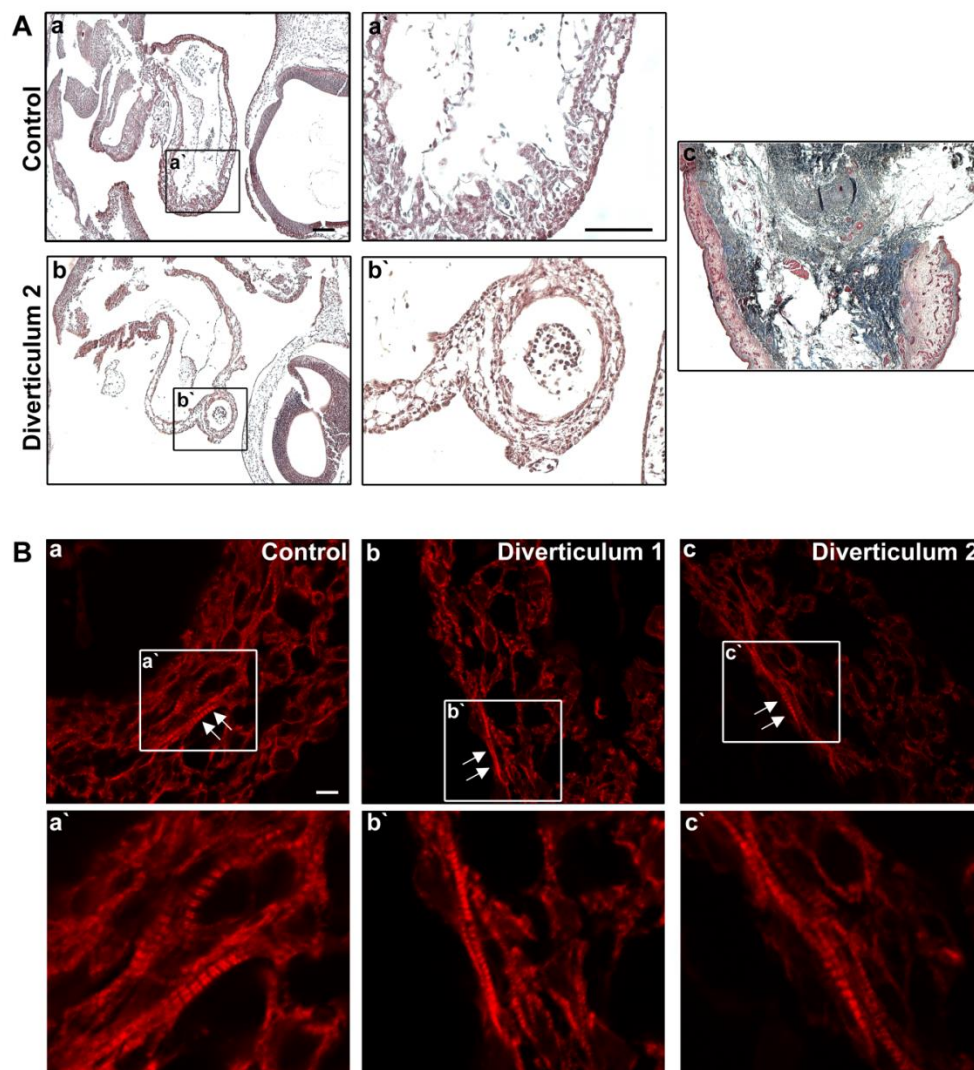


Figure 4.2 Histological and immunohistochemical analysis of the diverticula in the TNNT2-MO-treated embryos. **A.** Masson's Trichrome staining was used to detect if there is any collagen deposition due to fibrosis of the heart. However, the collagen deposition was not observed in the diverticulum which will be indicated by black/blue staining (b and b') when compared to a control heart (a and a'). Chick skin was used as a positive control (c). Scale bars: (a,a') 300 μ M. **B.** An anti-myosin heavy chain was used to detect the presence of cardiac muscle fibres within the diverticulum. When compared to the control (a), the diverticulum 1 and 2 both displayed a normal sarcomeric appearance, with the presence of mature and organized myofibrils in the myocardial wall (arrows; b and c). a',b' and c' images showing the sarcomeres at higher magnification from images a, b and c respectively. Scale bar: 80 μ M.

4.5 TNNT2-MO treatment results in abnormal atrial septa development

To determine whether the abnormal atrial septa had occurred upon TNNT2-MO treatment, HH11/19 embryos were serially sectioned in a transverse orientation to investigate the development of the atrial septum from the dorsocranial wall of the primitive atrium. In addition, the presence and the size of the endocardial cushions protruding into the lumen of the atrioventricular canal were also examined. In the 250 μ M study, atrial septation was found to have developed normally, and all embryos displayed a normal septum size in all controls ($n=15$) and TNNT2-MO-treated groups ($n=19$) (Table 4.1). In the 500 μ M study, the atrial septum was found normal in all but only one of the controls ($n=38$; Table 4.1). In the TNNT2-MO-treated hearts, the atrial septation was initiated but a stunted atrial septum growth was seen in 23 of all the TNNT2-MO hearts analysed ($n=51$; Table 4.1; Figure 4.3c,c'). Morphologically, the septum appeared small and resembled a knuckle-shaped outgrowth from the dorsocranial atrial wall (arrow in Figure 4.3c,c') compared to the controls (Figure 4.3a,a' and b,b'). This included the two TNNT2-MO-treated hearts with diverticula (see arrowhead in Figure 4.1Ci') compared with controls (arrowhead in Figure 4.1Cf' and g'). The endocardial cushion was examined in all controls and TNNT2-MO-treated hearts and no abnormalities were seen.

4.6 Abnormal trabeculation of the ventricular myocardium is affected in a subset of the hearts with stunted atrial septum.

Normal development of the ventricular myocardium as indicated by the degree of the trabeculation was examined morphologically on serial sections of the TNNT2-MO-treated hearts. In transverse orientation, the trabeculae all appeared as finger-like projections protruding into the ventricular lumen in both controls and the TNNT2-MO-treated group in 250 μ M study. Similarly, in the 500 μ M study, virtually all embryos displayed same degree of trabeculations as seen in controls. However, it was revealed that 6 out of 51 of the TNNT2-MO-treated hearts had a reduced trabeculae by qualitative analysis on consecutive sections throughout the heart (Table 4.1; Figure 4.3c,c'', indicated by arrow in c'') compared to controls (Figure 4.3a,a'' and b,b''). It is worth noting that all the hearts which displayed a stunted atrial septum growth also showed reduced trabeculation.

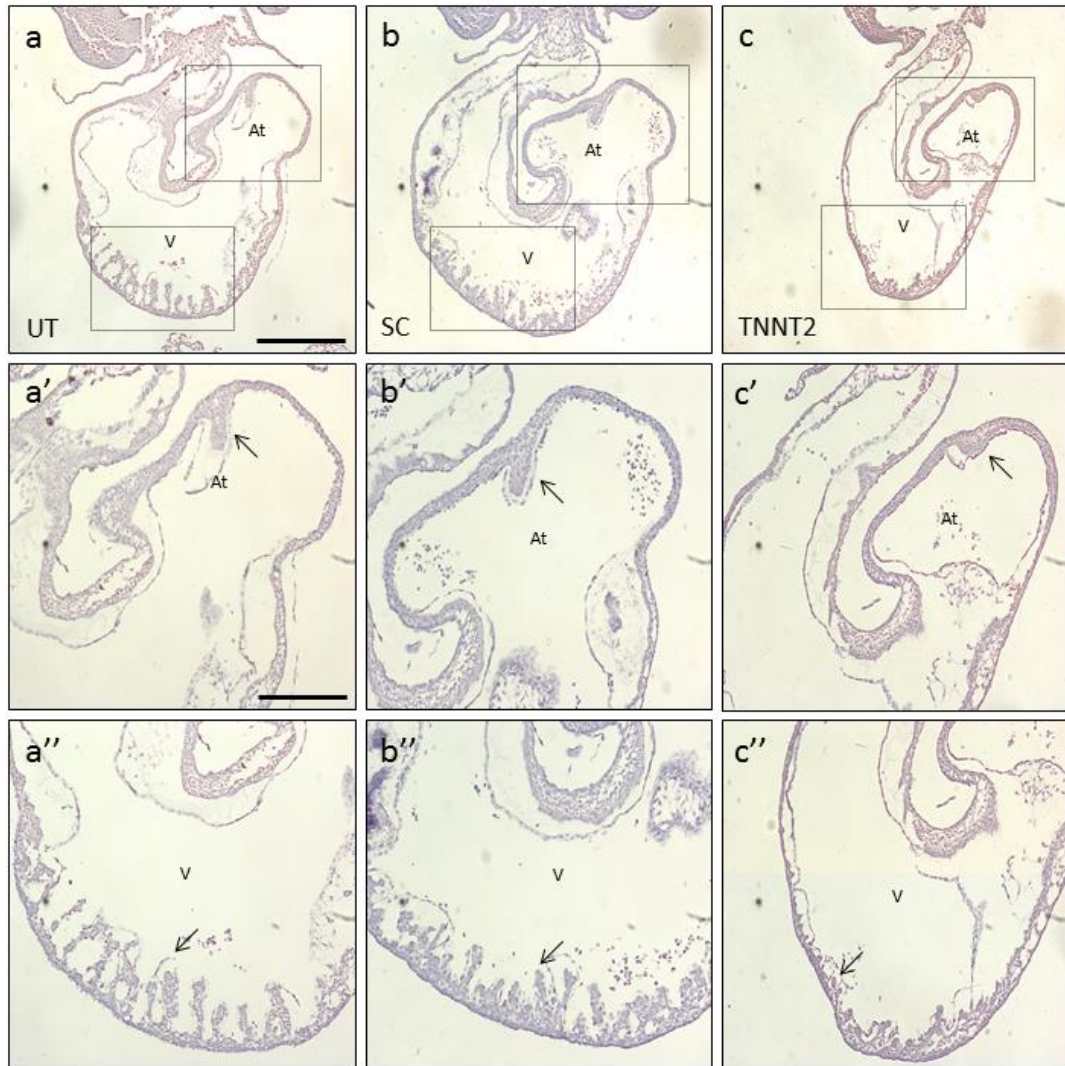
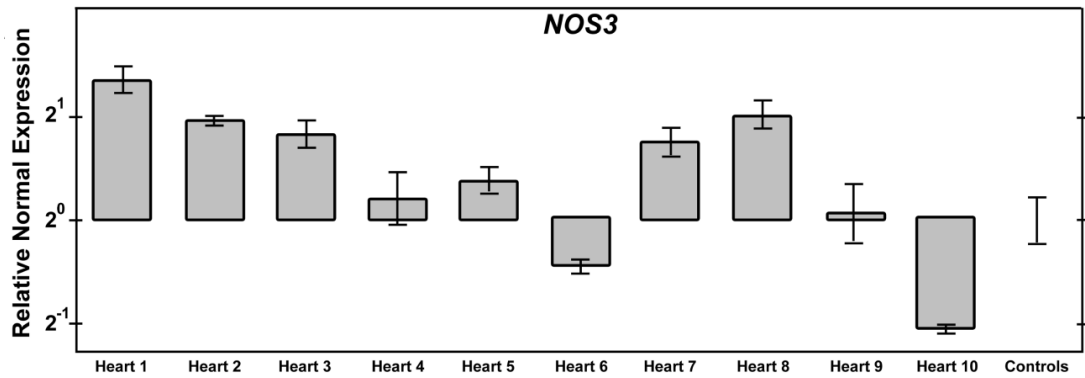


Figure 4.3 Abnormal atrial septation and trabecular formation were found in TNNT2-MO-treated embryos. Internal morphological assessment revealed normal structures, with initiation and growth of both atrial septum and ventricular trabeculae in both untreated (a,a',a'') and standard control (b,b',b''). Atrial septum initiation was seen in TNNT2-MO-treated embryos. However, they appeared comparatively smaller in size than controls (arrow in c' compared to control a' and b'). In the primitive ventricles, the phenotype seen was less severe. Only a subset of ventricles displayed reduced size and numbers of the trabeculae (arrow in c'') compared to both controls (arrows in a'' and b''). At, atrium; v, ventricle; SC, standard control; TNNT2, cardiac troponin T; UT, untreated. Scale bar: 150µm (a-c), 80µm (a'-c',a''-c'').

4.7 TNNT2-MO treatment alters the expression of a shear stress marker involved in atrial septation

Normal haemodynamics is inherently linked to embryonic cardiac development (Midgett and Rugonyi, 2014). Shear stress is the frictional haemodynamic force exerted by the blood flow to stimulate the mechanotransduction mechanisms that modulate the cardiac growth (Midgett and Rugonyi, 2014). *Nos3* is a shear stress-responsive gene and it is known to be essential for normal atrial septation (Groenendijk et al., 2004, Nadeau et al., 2010). Hence, in order to investigate the potential cause of the abnormal atrial septum and reduced ventricular trabeculae seen in TNNT2-MO-treated embryonic hearts, *NOS3* expression was quantitatively analysed by qPCR. qPCR was conducted on ten individual TNNT2-MO-treated embryonic hearts to evaluate a trend of *NOS3* expression. No significant difference in *NOS3* expression was found between the controls and thus they were pooled ($n=3$). Of the ten TNNT2-MO-treated hearts, *NOS3* expression was found increased in three of them (30%; hearts 1, 2 and 8; Figure 4.4A). An upregulation by 2.57-, 1.96- and 2.02-fold was identified in these hearts respectively. Interestingly, there was one heart (heart 10) that showed an inverse downregulation by 2.08-fold of *NOS3* expression (Figure 4.4A). Otherwise, qPCR revealed no change of *NOS3* expression in the remaining six hearts (60%; hearts 3, 4, 5, 6, 7 and 9) (Figure 4.4A). When all ten hearts were pooled for analysis, no significant differences was found, $p=0.23$ (Figure 4.4B).

A.



B.

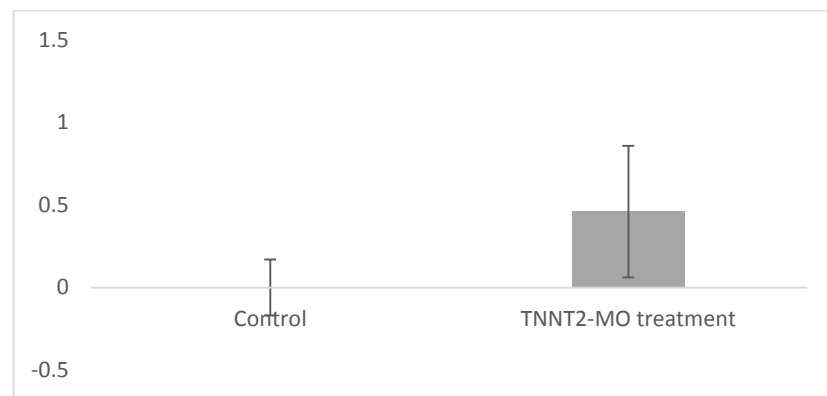


Figure 4.4 TNNT2-MO treatment results in the alteration of *NOS3* expression in the developing heart. A. A variable expression of *NOS3* was found in ten individual TNNT2-MO-treated embryonic hearts. *NOS3* expression was found increased in hearts 1, 2 and 8 when compared to the controls. However, heart 10 showed a converse decrease expression of *NOS3*. Otherwise, the expression in hearts 3, 4, 5, 6, 7 and 9 remained comparable to that in controls. **B.** No significant differences was found in between control and TNNT2-MO-treatment when all ten hearts were pooled for analysis ($p=0.23$).

4.8 TNNT2-MO treatment results in decreased cTnT in the heart

Western blot analysis was used to quantify the degree of protein ‘knockdown’ in individual hearts treated with *TNNT2* ATG start-site morpholino and this was carried out by Jennifer England (former PhD student). CT3 antibody detecting cTnT was normalized using GAPDH. No significant difference between the UT and SC groups ($n = 3$) was seen ($P = 0.233$) and these two groups were therefore pooled into one control group. When the control and *TNNT2*-treated hearts ($n = 5$) were compared, *TNNT2* was significantly decreased in the *TNNT2*-treated hearts and knockdown was achieved ($P = 0.016$; Figure 4.5 A,B).

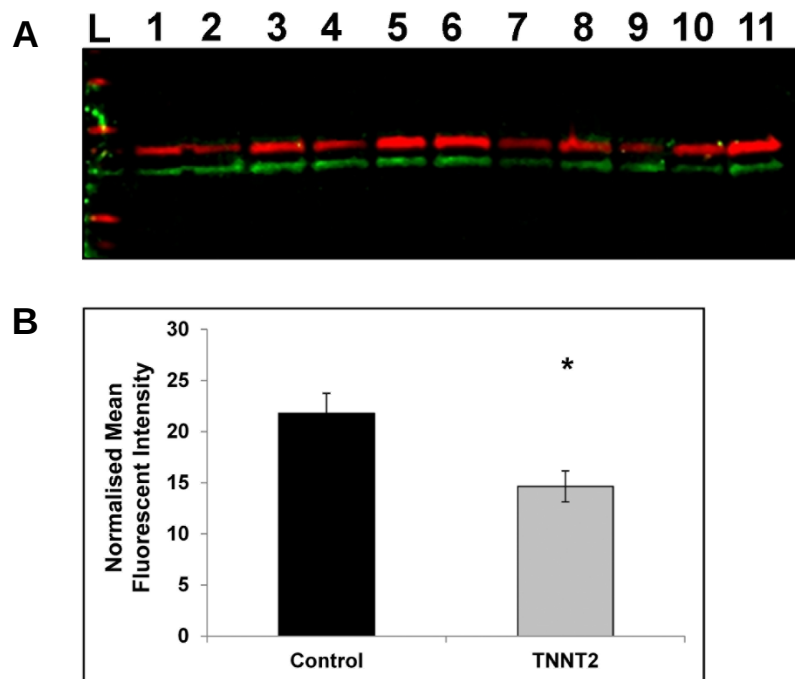


Figure 4.5 *TNNT2*-MO treatment results in a knockdown of cTnT. **A.** The lanes 1-3 are untreated, 4-6 have had standard control morpholino applied, and 7-11 are *TNNT2*-MO-treated. **B.** Western blot of showed a significant decrease of cTnT in *TNNT2*-MO-treated hearts at HH19 ($p = 0.016$) (indicated by an asterisk) when compared to controls.

4.9 Discussion

cTnT is one of the subunits that forms part of the troponin complex. The troponin complex binds to tropomyosin to regulate its movements around the actin filament (England and Loughna, 2013). Targeted manipulation of cTnT *in ovo* using morpholino resulted in the formation of diverticula from the myocardial wall of the primitive ventricle. Based on the literature, diverticula are embryological defects and can be defined as a localized protrusion of the ventricular free wall (Lowe et al., 1959, Ohlow, 2006). They usually arise from the left ventricle as a congenital left ventricle diverticulum and have been reported to occur at a frequency of approximately 0.05% in adult patients who underwent echocardiography (Elshaer et al., 2015). Many cases of both right and left atrial and right ventricular diverticula have been reported (Pitts and Potts, 1962, Binder et al., 2000, Di Sessa et al., 2006, Ohlow et al., 2015). Diverticula may or may not associated with other congenital heart defects (CHDs), such as atrial septal defects, ventricular septal defects, persistent ductus arteriosus coarctation of aorta and conduction anomalies (Villegas Garcia et al., 2001, Pitol et al., 2005, Di Sessa et al., 2006, Ohlow, 2006, Ohlow et al., 2015).

Diverticula contain all three myocardial layers (endocardium, myocardium and epicardium) of the ventricular wall and can be muscular or fibrous (Capasso et al., 2015). The muscular type tends not to rupture and always display a systolic contraction synchronous with the ventricular wall, whereas the fibrous type of diverticulum has no contractile function and tends to rupture (Shafter et al., 2016). Although ventricular diverticula can be asymptomatic, heart failure, systemic embolization, ventricular rupture and even sudden death can occur (Pitol et al., 2005). A previous report has shown that three patients with congenital ventricular diverticula have been associated

with hypertrophic cardiomyopathy (Maron et al., 1996). However, *TNNT2*-MO-treated hearts with diverticula both seem to display a normal size and did not appear enlarged in our study, despite *TNNT2* mutations have been associated with cardiomyopathy in the literature (Watkins et al., 1995).

One of the diverticula observed in our study (diverticulum 2; Figure 4.1Bd,d') displayed similar morphology as seen in humans; the localized protrusion of the diverticula of the ventricular and atrial wall and the synchronous contractility of the diverticula with the ventricle (Ohlow et al., 2015, Mori et al., 2010). Based on the phenotypic analysis, the 'outpouching' was connected to the primitive ventricle, atrioventricular canal and atrium through intermittent and narrow communications (Figure 4.1Cb',f' and i'), and had systolic contractility that was synchronous with the ventricle. Diverticulum 1 exhibited a different morphology in that it did not exist as an extra chamber but appeared more like a mild protuberance from the myocardial wall. Masson's Trichrome stain did not reveal fibrosis in the myocardium of the diverticula. Immunohistochemical staining with anti-myosin antibody showed normal sarcomeric assembly and structures in the myocardium, in keeping with the histological analysis. These studies indicate the diverticula presented here are of muscular type. In the literature, muscular type of cardiac diverticulum is commonly located on the inferior or anterior walls of the left ventricle at the apex and it has been reported that 56% of the LV diverticula was localised at the apex of the LV (Özdemir et al., 2007, Ohlow et al., 2015). However, in our case, the diverticulum 1 and 2 were located on the anterior part of the primitive ventricle inferior to the OFT and primitive atrium, respectively but not at the apex. To our knowledge, this is the first report linking the knockdown of sarcomeric proteins to cardiac diverticula. It is worth noting that the diverticula phenotype observed in this study was never present in any of the controls,

or in any other morpholino-mediated protein knockdown study targeting sarcomeric genes (Ching et al., 2005, Matsson et al., 2008, Rutland et al., 2009, Rutland et al., 2011). Even though the diverticula phenotype was rare ($n=2/67$) in our study, it was believed that the congenital left ventricular diverticulum was found in 0.04% of adult patients who underwent echocardiography (Elshaer et al., 2015). Interestingly, the co-existence of the muscular congenital cardiac diverticula with ASDs has been described in the clinical case reports (Walton-Shirley et al., 1992, Shahmohammadi et al., 2008, Ohlow et al., 2015); in this study, a stunted atrial septum growth was found in both of the hearts with diverticula.

Upon TNNT2-MO-treatment at 250 μ M at HH11, cardiac abnormalities were not found in any hearts analysed. However, internal morphological assessments of the TNNT2-MO-treated embryos at 500 μ M revealed an abnormal atrial septal growth in 45% ($n=23/51$) of treated hearts. The atrial septa were found present in all TNNT2-MO-treated hearts analysed but they appeared as a knuckle-shaped outgrowths from the dorsocranial atrial wall compared to the down-growing atrial septum in controls (Figure 4.3c,c'). This was also true for the reduced atrial septum seen in the hearts with the diverticula (see arrowhead in Figure 4.1Ci'). Despite the ventricular region of the cTnT knockdown hearts appeared normal in most embryos, a subset of the ventricular trabeculation appeared to be reduced (11.8% of embryos with a reduced atrial septum, Figure 4.3c,c'').

In the previous literature, targeted manipulation of *Tnnt2* prior to heart development had been performed in the mouse and zebrafish. The homozygous null mouse (*Tnnt2*^{-/-}) was not viable beyond (E)10.5 with no sarcomeric organization observed, whereas the heterozygous *Tnnt2*^{+/-} had normal functional hearts (Nishii et al., 2008, Ahmad et al., 2008). On the other hand, a significant reduction in cTnI, cTnC and

tropomyosin was observed leading to severe sarcomere defects (Sehnert et al., 2002), implying gene-specific cTnT knockdown was not achieved in the zebrafish. These preclude the analysis of the role of cTnT in cardiac development. In our study, upon TNNT2 MO-treatment at HH11 in chick, the size of the atrial septa and the number of ventricular trabeculae were reduced. To our knowledge, this is the first study associating *TNNT2* to abnormal atrial septal growth, reduced ventricular trabeculation and ventricular diverticula (England et al., 2016).

In addition, cTnT is thought to play a role in myofibrillogenesis (Fransen and Lemanski, 1989, Huang et al., 2009). Disorganized myofibrils which were seen in the Mexican axolotl mutant heart and the failure of formation of premyofibrils in zebrafish mutant have been reported in the literature (Fransen and Lemanski, 1989, Huang et al., 2009). In contrast, sarcomere assembly appeared normal upon TNNT2-MO-treatment, but a reduction of protein level by 32% was seen upon knockdown of cTnT protein. These two studies were performed by Jennifer England (former PhD colleague) (England et al., 2016). Here, we postulate that the possible reason of observing the reduced atrial septa and ventricular trabeculae might be due to a 32% cTnT protein reduction in TNNT2-MO treated hearts. In addition, the formation of cardiac diverticulum has been proposed that it is a developmental defect that potentially forms as a result of the structural muscle weakness of the heart (Lowe et al., 1959, Ohlow, 2006). Given that the sarcomeric assembly was normal in the diverticula of the TNNT2-MO-treated embryos, we here postulate that the reduction of cTnT protein leads to muscular weakness and hence the formation of the diverticulum.

During embryonic development, normal haemodynamics is essential for the development of the heart structures and associated vasculatures and is known to have a positive correlation with shear stress (Groenendijk et al., 2004). *NOS3* expression is

one of the shear stress responsive genes that was known to be expressed in the atrial septum and the ventricular trabeculation (Groenendijk et al., 2005), with the atrial septation being initiated approximately at HH14. In the *Nos3*^{-/-} mouse, a high incidence of septal defects (64% ASD; 11% VSDs) (Feng et al., 2002, Liu and Feng, 2012) was found. In addition, *Nos3* is known to interact with the transcriptional factor Tbx5, with Tbx5 expressed in endocardial cells to contribute to the formation of the atrial septum (Nadeau et al., 2010). *Nos3* is predicted to be the genetic modifier of Tbx5 in atrial septa formation based on the genetic mutants and *in vitro* studies (Nadeau et al., 2010). Hence, the expression of *NOS3* at the mRNA level was quantitated in this study in individual *TNNT2*-MO-treated hearts to see if there is any alteration in the shear stress. The expression of *NOS3* level in *TNNT2*-MO-treated hearts exhibits variability, with three hearts showing an increase but one heart showing a reduction instead when compared to controls. This suggested the hearts responded differently upon altered haemodynamic potentially caused by the muscle weakness upon *TNNT2*-MO-treatment.

Currently, over 100 mutations have been identified in troponin subunits associated with hypertrophic, dilated and restrictive cardiomyopathies, with over 50 of those found in cardiac troponin T (Lu et al., 2013). However, to date, *TNNT2* has not been linked to isolated CHDs. Our study is the first study to show that *TNNT2* might play a role in CHDs, in particular ASDs and congenital cardiac diverticula. Similar with cTnT, another two subunits of the troponin complex, which are cTnI and cTnC were also associated with different types of cardiomyopathies as described above but the mutations occurred at a lower occurrence when compared to cTnT (Lu et al., 2013). To our knowledge, both cTnI and cTnC have not been found associated with ASDs (Posch et al., 2011). So far, there was only one case study reporting about a de novo mutation

in *TNNI3* in an infantile case of VSD but this individual also had restrictive cardiomyopathy (Yang et al., 2010).

Taken together, upon *TNNT2*-MO-treatment, abnormal atrial septal growth, reduced trabeculation and ventricular diverticula were found. The reduction of cTnT protein and the alteration of *NOS3* expression were suggested to give rise to the abnormal gross morphology phenotypes observed in this study.

5 CHAPTER 5: Whole transcriptome analysis of the OFT-banded hearts using Deep Sequencing

Overview

OFT-banding model is an altered haemodynamics model known to give rise to a number of cardiac malformations as well as alteration of tissue properties (Sedmera et al., 1999, Buffinton et al., 2013, Clark et al., 1989). Although morphological analysis and the biochemical studies such as fibrosis and apoptosis studies had been described before, an accurate analysis of the global differential expression of OFT-banded hearts giving rise to the cardiac anomalies is essential. In this chapter, RNA-sequencing technology was employed to study the global differential gene expression in the OFT-banded hearts as it provides a more precise and sensitive method to map and quantify RNA transcripts compared with the analog data generated by microarray technology (Roy et al., 2011).

The aims of these experiments were:

- Investigate the global differential gene expression of OFT-banded hearts at HH29
- Validation of the RNA-sequencing results by real time PCR

5.1 Differential expression of genes was identified in OFT-banded hearts by RNA sequencing

In this thesis, RNA sequencing study was conducted by Dr Sunir Malla and Dr Fei Sang from Deep Sequencing, School of Life Sciences, University of Nottingham. The RNA-sequencing analysis was performed in collaboration with Matthew Parnall (PhD student). RNA-sequencing is a technique for sequencing the segments from a large number of cDNAs which enables a quantitative analysis of the transcriptome to be performed (Wang et al., 2009). RNA sequencing analysis involves aligning reads to a reference genome and then counting the reads that align uniquely and correctly to generate the counts per gene using htseq-count (Anders et al., 2013). RNA sequencing was undertaken on the sham and OFT-banded hearts at HH29 ($n=3$ for each group; ID for each individual heart, OFT-banded group: OFT_6, OFT_11, OFT_17; sham: SHAM_38, SHAM_42, SHAM_48).

HH29 was chosen as a stage of interest to investigate the differential gene expression due to a number of reasons. First of all, as discussed in chapter 3, the development of enlarged heart resembling DCM phenotype accompanied by a spectrum of cardiac anomalies such as VSDs, dysmorphic atrioventricular valves (AV) and thickened epicardium were found in the OFT-banded hearts at HH29. The epicardium was known to give rise to coronary artery formation and stunted coronary growth had previously been found in the HH36 OFT-banded hearts (Tomanek et al., 1999, Winter and Gittenberger-de Groot, 2007). With regards to the conduction system, a precocious maturation of ventricular conduction system was seen in the OFT-banding model at HH28 but how the alteration of haemodynamics impacts the atrial conduction system is yet to be determined (Hall et al., 2004, Reckova et al., 2003). At HH29, the pectinate muscles were said to be established in the definitive adult pattern and were involved

in the acceleration of the impulse propagation in atria (Sedmera et al., 2006). On the account of the finding of enlarged left atrium with thinner myocardial wall in the HH29 banded hearts in the stereological study, it can be postulated that the conduction pathway in the atria could be affected.

With regards to the valve development, from HH26 onwards, distal outgrowth and maturation of the ECs take place via migration and proliferation as well as expression of ECM proteins involved in maturation of the cushion mesenchyme and condensation of cushion tissue into fibrous leaflets takes place around HH30 and defective valves were found in our study (Inai et al., 2008, Yalcin et al., 2011). Hence, RNA sequencing was performed at HH29 to investigate the molecular underpinnings of the model.

In our study, 47-90x 10⁶ uniquely and correctly aligned sequence reads with the MAPQ above 20 were generated for all the samples and were mapped against the galGal4 reference sequence genome, using refSeq gene annotations from UCSC database (<http://genome.ucsc.edu>) Nov. 2011 (ICGSC Gallus_gallus-4.0/galGal4). Reads Per Kilobase of transcript per Million mapped reads) (RPKM) is a standard normalised read count of a gene taking the sample sequencing depth and gene length into account (Mortazavi et al., 2008). The read count of the exon-space of a gene is normalized against the total number of mapped reads (with the ribosomal RNA and the reads which were not uniquely aligned and were below MAPQ 20 excluded) in that particular alignment file and against the total length of the gene's exon-space. However, only 30 genes were found to be statistically significantly changed (increased or decreased) in the OFT-banded heart compared to sham (Table 5.1 and 5.2). GO enrichment analysis and pathway analysis were not performed as low number of genes were significantly differentially expressed in this study. Principal component analysis (PCA) plot was used to determine how the biological replicates were comparable

within the same group and were different between experimental groups by principal component 1 (PC1) and principal component 2 (PC2) (Figure 5.1). OFT_6 and SHAM_42 were both not considered as outliers as they both were separated at a similar distance compared to their biological replicates within their own group. The separation between the OFT-banded group and the control was by the PC2 and less so by PC1. This indicates only a modest number of differentially expressed genes and is in line with the small number of differentially expressed genes found in Table 5.1 and 5.2.

Table 5.1 and 5.2 summarises the genes which were differentially expressed with detailed information of gene ID, log₂ fold change, ifcSEM (standard error of mean) and p adjusted values. Some of the genes which were found significantly differentially expressed were pathological genes (*LDHB*, *TNNC2*) and were linked with cardio-protective (*PDCD4*, *S100A11*, *LGALS1*) and valve development (*TGM2*) (Table 5.1 and 5.2) (Olson et al., 2015, Liu et al., 2012b, Cheng et al., 2010, Inamoto et al., 2000, Nakatani et al., 1996, Sauls et al., 2012, Hamada et al., 1992, Wang et al., 2013). Dysregulation of other genes found were *GBX2* and *LBH* and they were suggested to attribute to the cardiac anomalies (Table 5.1 and 5.2) (Byrd and Meyers, 2005, Briegel et al., 2005).

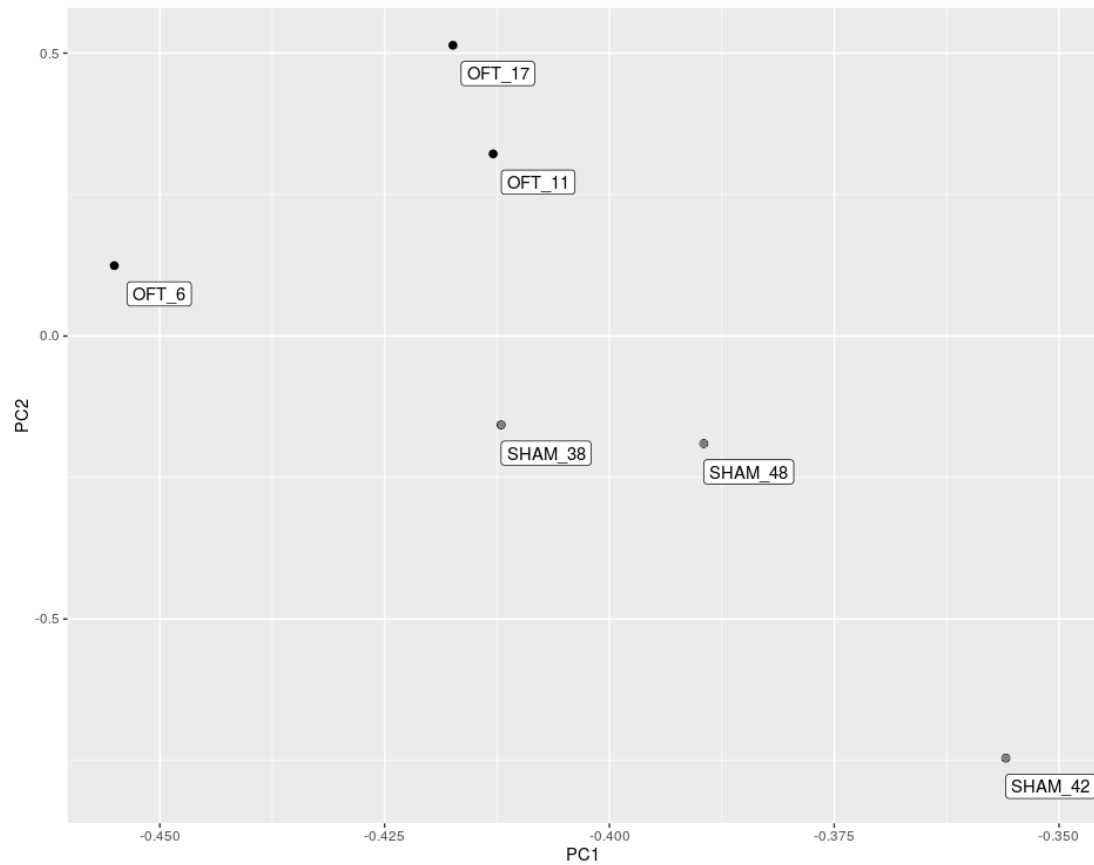


Figure 5.1 Principal component analysis (PCA) of OFT-banded hearts and controls.

The PCA plot was employed to evaluate the variances of the biological replicates within the same group and across the experimental groups on the OFT-banded group ($n=3$) and sham ($n=3$). OFT_6 and SHAM_42 were not considered as outliers as both of them were separated at a similar distance with another two biological replicates of their own groups. No cluster was found when OFT-banded group was compared against the sham. The separation between two groups was by the second principal component but less so with the first principal component. PC1, first principal component; PC2, second principal component.

GeneID	GeneName	log ₂ FoldChange	lfcSE	padj
NM_205068	<i>GBX2</i>	0.83	0.17	8.27E-04
NM_205450	<i>TNNC2</i>	0.75	0.16	1.34E-03
NM_001302201	<i>TIMM10</i>	0.72	0.13	2.98E-05
NM_001159315	<i>PVALB</i>	0.7	0.17	1.91E-02
NM_001097526	<i>MYL10</i>	0.67	0.18	4.37E-02
NM_204177	<i>LDHB</i>	0.64	0.13	1.23E-03
NM_205495	<i>LGALS1</i>	0.64	0.18	4.88E-02
NM_001031038	<i>LBH</i>	0.6	0.17	4.86E-02
NM_001079490	<i>PNO1</i>	0.59	0.13	3.62E-03
NM_001079501	<i>LLPH</i>	0.58	0.16	4.97E-02
NM_001130387	<i>IFNGR1</i>	0.55	0.15	4.46E-02
NM_001044632	<i>MYL1</i>	0.54	0.15	4.46E-02
NM_205166	<i>S100A11</i>	0.52	0.14	4.46E-02

Table 5.1 Significant differential expression of upregulated genes in the OFT-banding model. The genes which are highlighted in yellow are of interest to this thesis.

GeneID	GeneName	log ₂ FoldChange	lfcSE	padj
NM_001080873	<i>ENS-1</i>	-0.91	0.16	2.98E-05
NM_001081704	<i>HBE</i>	-0.8	0.18	5.64E-03
NM_001080874	<i>ERNI</i>	-0.76	0.15	4.79E-04
NM_205087	<i>ADORA2B</i>	-0.74	0.18	2.08E-02
NM_205160	<i>GLRX</i>	-0.71	0.18	3.33E-02
NM_001012588	<i>ITPK1</i>	-0.68	0.17	2.34E-02
NM_001004390	<i>HBG1</i>	-0.68	0.18	4.37E-02
NM_204818	<i>GSTA4</i>	-0.65	0.18	4.88E-02
NM_001044673	<i>H1F0</i>	-0.64	0.18	4.46E-02
NM_001100289	<i>CDH8</i>	-0.6	0.16	4.86E-02
NM_001031478	<i>RAP1GAP2</i>	-0.57	0.15	4.46E-02
NM_001177415	<i>DNAJC6</i>	-0.48	0.13	4.40E-02
NM_001030857	<i>ARFGAP1</i>	-0.47	0.13	4.46E-02
NM_204304	<i>PDCD4</i>	-0.41	0.11	3.52E-02
NM_205448	<i>TGM2</i>	-0.38	0.1	4.37E-02
NM_001030626	<i>PIAS2</i>	-0.36	0.1	4.37E-02

Table 5.2 Significant differential expression of downregulated genes in the OFT-banding model. The genes which are highlighted in yellow are of interest to this thesis.

5.2 Validation of differentially expressed genes by real time PCR

It was important to establish whether the RNA sequencing technique can reliably study the differential gene expression upon OFT-banding. Hence, the trends in the transcript abundance as measured by these RPKM values were validated by quantitative RT-PCR (qPCR) on a selection of genes of interest to determine whether the RNA sequencing results are reproducible on the same biological samples. The qPCR primers were all designed with an annealing temperature at 62°C (they worked at a range in between 60°C-64°C). Their amplification efficiency were determined before use (~98-110%) (refer Table 2.2 in chapter 2 for details).

A total of six genes of interest (*LDHB*, *TNNC2*, *PDCD4*, *LGALS1*, *TGM2*) were examined by quantitative PCR in order to determine whether the results of the RNA sequencing analysis were reproducible. Within the literature, *LDHB* and *TNNC2* are considered as pathological genes and *PDCD4* and *LGALS1* are associated with cardio-protective in myocardial ischemia and myocardial infarction (Liu et al., 2012b, Cheng et al., 2010, Seropian et al., 2013). *TGM2* are linked with shear stress and valve development respectively (Sauls et al., 2012, Heckel et al., 2015). The log₂ fold change generated by RNA-sequencing and qPCR for these six genes were summarised in Table 5.3.

The gel electrophoresis, standard curve and the melting curve analysis of representative genes, *LGALS1* and *PDCD4*, were detailed in Figure 5.2A-B. *GAPDH* and *EEF1A1* were both used as reference genes, with samples normalised according to the Pfaffl method (Pfaffl, 2001). First of all, qPCR revealed an upregulation of 0.65±0.11 and 0.62±0.05 fold change of *TNNC2* and *LDHB* in qPCR compared to controls ($p<0.05$; $n=3$ per group; Figure 5.3). *LGALS1* also showed an upregulation

of 0.38 ± 0.07 fold in the banded hearts in relation to controls ($p < 0.05$; Figure 5.3). In contrast, *TGM2* and *PDCD4* both displayed a downregulation of -0.53 ± 0.08 and -0.45 ± 0.05 fold in the banded hearts respectively, where $p < 0.05$ (Figure 5.3). *TNNC2* and *LDHB* both showed an upregulation of 0.65 ± 0.11 and 0.62 ± 0.05 fold change respectively by qPCR (Figure 5.4; Table 5.3). In RNA sequencing, a slight higher upregulation of 0.75 ± 0.16 was found in RNA sequencing with *TNNC2* but a similar fold change was identified with *LDHB*, where fold change equals to 0.64 ± 0.13 (Figure 5.4; Table 5.3). Both *TGM2* and *PDCD4* were found downregulated with the fold change by -0.38 ± 0.1 and -0.41 ± 0.11 fold change respectively in RNA sequencing (Figure 5.4; Table 5.3). Similar fold change was detected by qPCR, where a downregulation of -0.45 ± 0.05 was reported with *PDCD4* (Figure 5.4; Table 5.3). However, *TGM2* showed a slightly higher downregulation upon OFT-banding, with an -0.53 ± 0.08 fold change reported in the qPCR (Figure 5.4; Table 5.3). Lastly, with *LGALS1*, RNA seq detected a higher fold change which was 0.64 ± 0.18 upregulation whereas qPCR found only 0.38 ± 0.07 .

In all six genes analysed, the qPCR results demonstrated a similar fold change (upregulation or downregulation) of transcripts in the OFT-banded hearts as shown in the RNA sequencing. The Log_2 fold change of both RNA-seq and qPCR were tabulated in Table 5.3. Hence, the 'RNA sequencing methodology' successfully identified novel genes that are affected in the OFT-banded embryonic hearts.

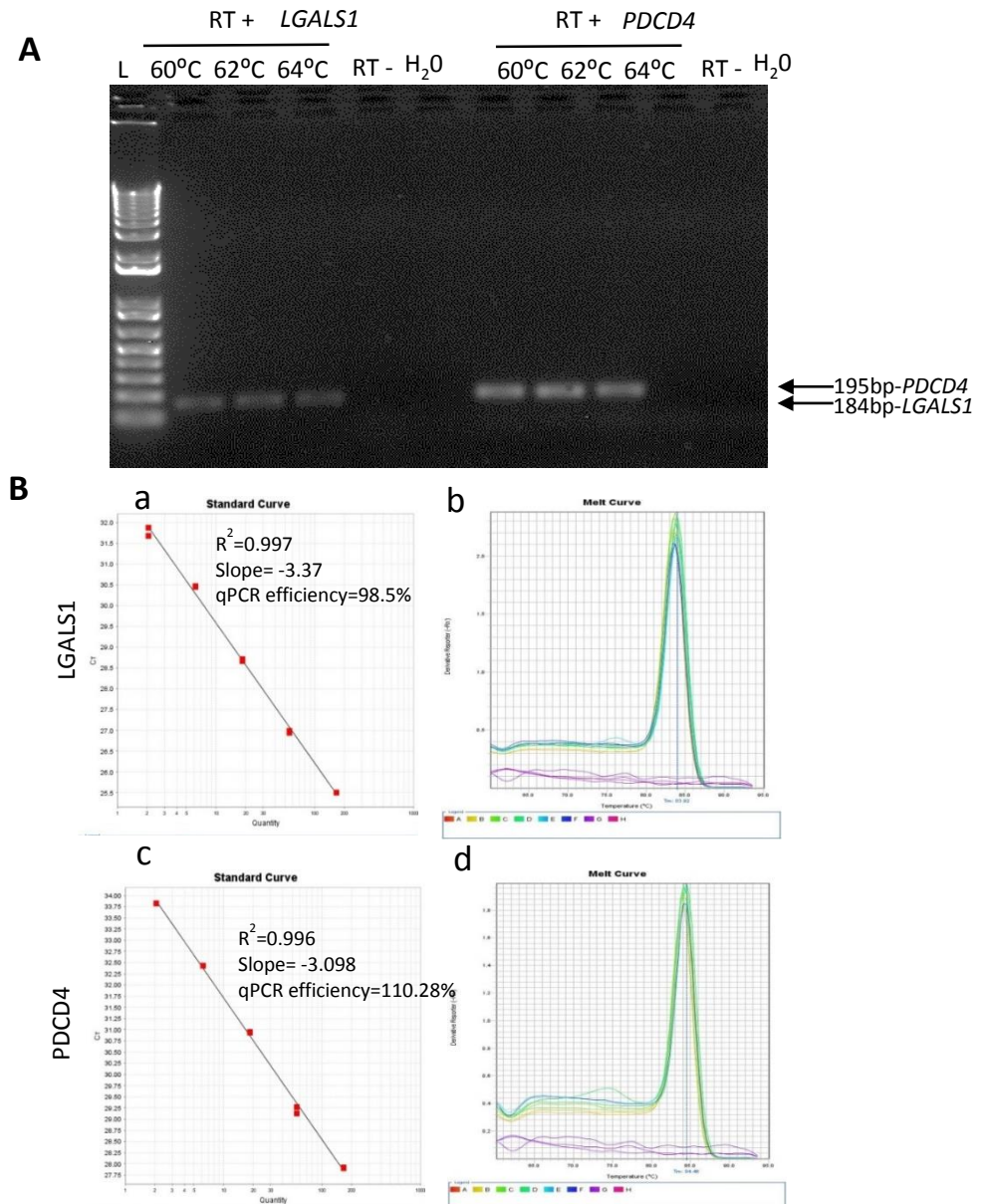


Figure 5.2 Representative results of optimized qPCR primers (*LGALS1* and *PDCD4*) for RNA sequencing verification. **A.** Gel electrophoresis of PCR products of *LGALS1* (184bp) and *PDCD4* (195bp) by end-point RT-PCR with the annealing temperature optimized at 60⁰ C, 62⁰ C and 64⁰ C on 1.5% agarose gel. **B.** Standard curve generated from 3-fold dilution of primers *LGALS1* (a) and *PDCD4* (c) using SYBR green amplified by qPCR. The means C_T values (y-axis) were plotted against the quantity of the samples (x-axis). The qPCR efficiency for *LGALS1* and *PDCD4* are 98.5% and 110.28% with a R^2 value of 0.997 and 0.996 respectively Melting curve analysis of *LGALS1* (b) and *PDCD4* (d) qPCR products. Y-axis represents the derivative reporter ($-\Delta R_n$) while x-axis represents the temperature ($^{\circ}$ C). The figure shows a melting temperature (T_m) of *LGALS1* as 83.92 $^{\circ}$ C and *PDCD4* as 84.5 $^{\circ}$ C and non-specific products and primer-dimers were not detected in negative controls. RT+, RT added; RT-, no RT added; H₂O, PCR control; bp, base pairs; L, 1Kb plus DNA ladder (Invitrogen).

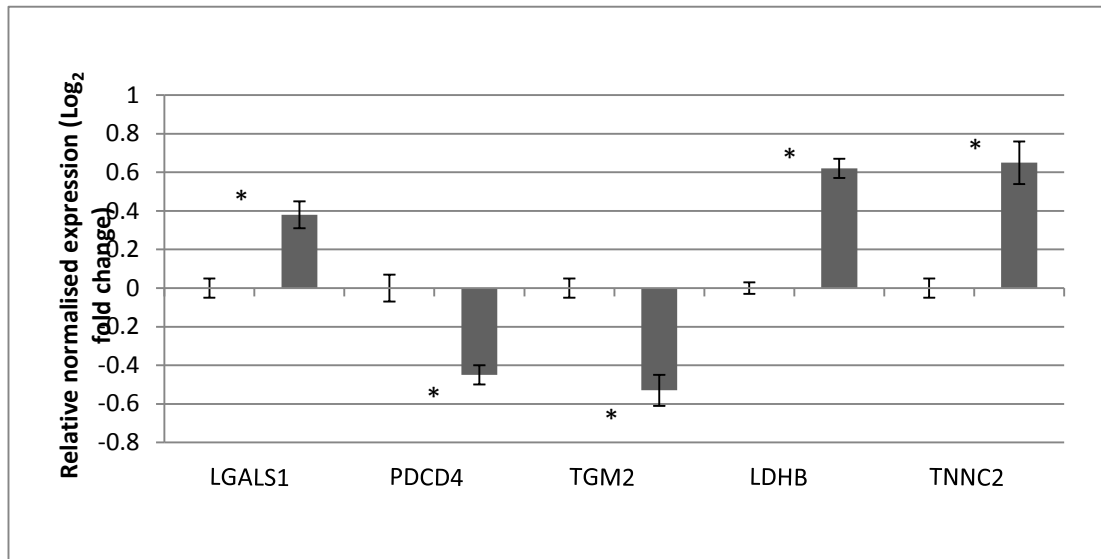


Figure 5.3 qPCR validation of the RNA-seq data. Log₂-fold change determined from the relative Ct values of five genes (*LDHB*, *TNNC2*, *LGALS1*, *TGM2* and *PDCD4*) compared to sham controls. Each gene was done in three replicates and the Ct values were averaged and were normalized against *GAPDH* and *EEF1A1*. $p < 0.05$ (indicated by an asterisk).

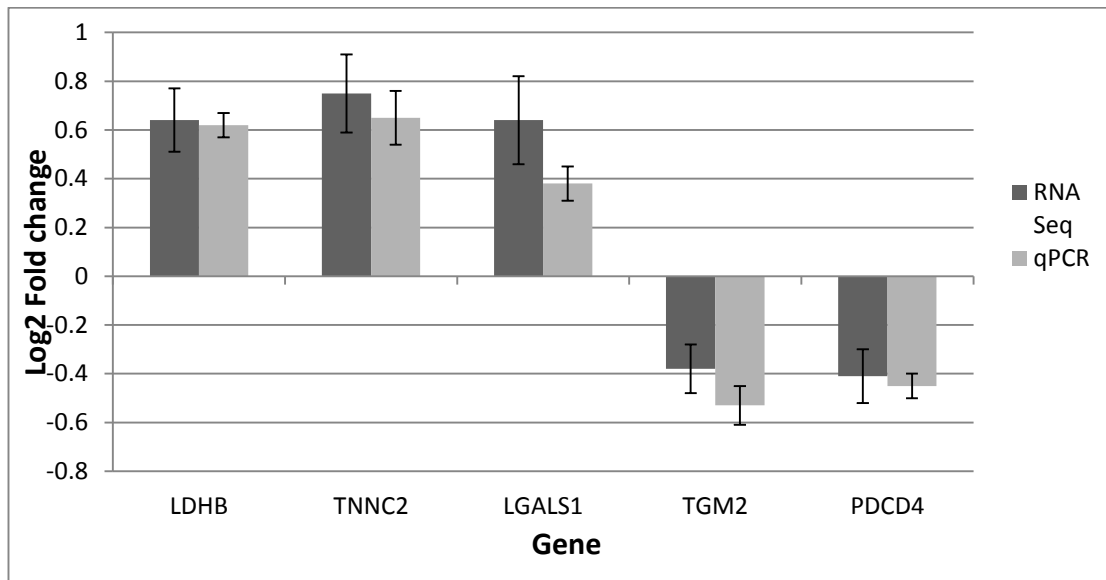


Figure 5.4 Comparison of the Log₂ fold change between qPCR results and the RNA-seq data. Log₂-fold change of the five genes (*LDHB*, *TNNC2*, *LGALS1*, *TGM2* and *PDCD4*) were compared to those detected by RNA-seq. *LDHB* and *TNNC2* both showed an upregulation with similar fold change. Similarly, *LGALS1*, *TGM2* and *PDCD4* displayed comparable fold change in both RNA sequencing and qPCR result as well.

Gene	Log ₂ fold change	
	RNA sequencing	qPCR
<i>LDHB</i>	0.64	0.62
<i>TNNC2</i>	0.75	0.65
<i>LGALS1</i>	0.64	0.38
<i>TGM2</i>	-0.38	-0.53
<i>PDCD4</i>	-0.41	-0.45

Table 5.3 The log₂ fold change of each gene validated by qPCR shows reproducibility of RNA sequencing result.

5.3 Discussion

In the whole transcriptome analysis of the OFT-banded hearts at HH29, a number of genes linked with shear stress, conduction system and cardiac valve development were found deregulated. Detailed expression profile and the functional studies of the genes which were significantly differentially expressed (Table 5.1 and 5.2) were opted out due to time constraints in my research and they will need to be elucidated in future studies.

In this section, only a subset of the genes which were potentially associated with cardiac phenotypes observed in chapter 3 will be further discussed. These genes are linked with cardioprotection (*PDCD4*, *S100A11*, *LGALS1*), glucose metabolism (*LDHB*), cytoskeletal organization (*TNNC2*) and valve development (*TGM2*) and they were verified by qPCR (except *GBX2* and *S100A11*).

Lactate dehydrogenase (*LDHB*) is one of the glucose metabolic enzymes found upregulated by 0.64 fold upon OFT-banding. *LDHB* is a subtype of lactate dehydrogenase that mainly exists in the myocardium and it could result in lactate reduction through the conversion of lactate to pyruvate (Tan et al., 2011a). Interestingly, LDH1 (the protein product of *LDHB*) was previously found to be upregulated in patients with HCM exhibiting DCM-like phenotypes (Hamada et al., 1992, Wang et al., 2013).

Troponin C2, fast skeletal type (*TNNC2*) expression was increased by 0.75 fold. Although *TNNC2* is known to be predominantly expressed in fast-twitch skeletal muscle fibers, it was found upregulated in vasoactive intestinal peptide knockout mice associated with the cardiomyopathy phenotype (Szema et al., 2013). Other genes linked with sarcomeric organization were found, e.g myosin light chain family genes, i.e *MYL1* and *MYL10* (personal communication, Matthew Parnall).

Gastrulation brain homeobox 2 gene (*GBX2*) was increased by 0.83 fold in the study. However, it was found that *Gbx2* null mutant embryos exhibit wide range of abnormalities: double outlet right ventricle (DORV), perimembranous VSDs and abnormal endothelial cell differentiation (Byrd and Meyers, 2005). In normal development, *Gbx2* was expressed predominantly in the pharyngeal surface ectodermal layer, although some staining could be detected in the pharyngeal endodermal layer in E8.5 mouse embryo (Calmont et al., 2009). Later in development, *Gbx2* expression was detected in the region where PAAs 3 to 6 are going to form at E9.25 and the expression was then restricted to the pharyngeal endoderm at E9.5 (Calmont et al., 2009). Future studies need to be elucidated to examine the expression pattern in the heart.

Limb bud and heart development gene (*LBH*) was upregulated by 0.6 fold change. The transgenic overexpression of *Lbh* in mice results in an impaired cardiac septation, pulmonary OFT valvulogenesis, ventricular enlargement with or without bilateral atrial dilation (Briegel et al., 2005). *Lbh* acts as a transcriptional cofactor and is highly conserved among different species (Briegel et al., 2005, Conen et al., 2009). It had been shown that overexpression of *LBH* in cultured mammalian cells represses the synergistic activation of the key transcriptional factors, *Tbx5* and *Nkx2.5*. This in turn led to repression of their downstream common target gene, *Anf* (*Nppa*) (Briegel et al., 2005). *Tbx5* and *Nkx2.5* are both known to be crucial genes for heart development (Horb and Thomsen, 1999, Mori and Bruneau, 2004, Akazawa and Komuro, 2005). The functional assays of mutant *TBX5* and *NKX2.5* were associated with significantly decreased transcriptional activity in DCM patients (Zhang et al., 2015, Yuan et al., 2015). Upregulation of *LBH* upon banding might lead to the repression of *TBX5* and *NKX2.5* and thus the development of enlarged heart resembling DCM phenotype. Despite the fold changes of both *TBX5* and *NKX2.5* levels showed no alteration at

HH29 (based on our RNA seq dataset), it can be speculated that the small fold change in the transcriptional cofactor *LBH* gene could have an inhibitory effect on *TBX5* and *NKX2.5* activities which led to a ‘knock-on’ effect on their downstream genes.

Programmed cell death 4 (*PDCD4*) is often upregulated in apoptosis and is a target of microRNA-21 (miR-21) and miR-499-5p in myocardial infarction and myocardial ischemic injuries (Jia et al., 2016, Cheng et al., 2010). miR-21 is known to link with multiple cardioprotective cell signaling pathways, exerting effects on *PDCD4*, Akt, and PTEN (Olson et al., 2015). Previously, miR-21 was reported to have a protective effect on ischemic preconditioning (endogenous defensive mechanisms that protect the heart against ischaemic damages) and on myocardial infarction by reducing cardiac cell apoptosis via downregulation of *PDCD4* (Liu et al., 2012b, Cheng et al., 2010). Overexpression of miR-499-5p also protected the cardiomyocytes against apoptosis induced by acute myocardial infarction by downregulating *PDCD4* (Li et al., 2016). In the current study, although the expression level of miR-21 and miR-499-5p expression were not measured, the finding of downregulation of *PDCD4* expression with a concomitant decrease of apoptosis suggested the embryonic hearts were undergoing an intrinsic cardio-protective mechanism in response to pressure overloading.

Another attractive gene candidate identified in the RNA-seq was S100 Calcium Binding Protein A11 gene (*S100A11*) by upregulation of 0.52 fold change. In previous reports, increased S100A11 protein level was found upon chronic stimulation of β -adrenergic stimulation of rat hearts and its role was suggested to be cardioprotective (Inamoto et al., 2000, Nakatani et al., 1996). The overexpression of S100A11 inhibited the apoptosis in the human uterine smooth muscle tumour cells and the suppression of S100A11 led to apoptosis which suggest its anti-apoptotic role (Kanamori et al., 2004).

Based on the preliminary findings, it is conceivable that S100A11 is potentially cardioprotective via its antiapoptotic role.

With Galectin-1 (*LGALS1*), a 0.64-fold increase was detected by RNA sequencing. Galectin-1 is an evolutionarily conserved β -galactoside-binding lectin and it plays a crucial role in the control of inflammation and neovascularization (Camby et al., 2006, Liu and Rabinovich, 2010, Thijssen et al., 2006). It was identified as a main component of the contractile apparatus of the cardiomyocytes and it shows a colocalization with the sarcomeric actin on the I band (please refer to Figure 1.9 of section 1.3 in chapter 1) (Dias-Baruffi et al., 2010). Our RNA sequencing result on Galectin-1 corresponded with the previous study, where it was previously found increased substantially in the heart at early stage of myocardial ischemia and acute myocardial infarction (Seropian et al., 2013, Al-Salam and Hashmi, 2014). Further, *Lgals1*^{-/-} mice showed a greater LV enlargement and reduction of systolic function with enhanced inflammatory response which led to an impaired ventricular remodelling after acute myocardial infarction (Seropian et al., 2013). The exogenous administration of galectin-1 attenuated the cardiac damage by preventing LV enlargement and restoring systolic function (Seropian et al., 2013). These results highlighted the cardio-protective role of endogenous galectin-1 normal homeostasis and post infarction remodelling. In our study, an upregulation of galectin-1 suggested it might exert the similar role in response to pressure overloading. This lectin was localized mainly in cardiomyocytes and inflammatory infiltrates in peri-infarct areas (Seropian et al., 2013).

A few shear stress related genes were also identified in the RNA seq. Flow induced *miR21* governs the valve development by enhancing endothelial cell migration via downregulation of *pdc4* (Banjo et al., 2013). Consistent with this, a decrease of 0.41 fold change of *PDCD4* was identified in our sequencing study. Lastly, Transglutaminase-2 (TGM2) was one of the genes found associated with valve

disease (myxomatous valve disease) (Sauls et al., 2012). During development, TGM2 helps cross-linking of the matrix proteins and TGF β activity and leads to the increased deposition and stability of ECM (Van Herck et al., 2010). TGM2 was required for the molecular interactions between filamin-A, serotonin in the valve interstitial cells to organize ECM efficiently for normal valve development (Sauls et al., 2012). A recent study showed the TGM2 expression pattern might define a border of differentiation (de Vlaming et al., 2012). TGM2 was devoid of its expression in the cushion but was found positively expressed in the adjacent myocardium in prior to cushion fusion. It was later found positively expressed in almost all the interstitial cells following cushion fusion (HH27) and maturation (HH35). TGM2 activity was also found to regulate osteoblast differentiation (Yin et al., 2012b). Hence, the downregulation of *TGM2* as shown in the RNA-seq might suggest differentiation of the cushion mesenchyme have not taken place properly and this might attributed to the defective valve primordial seen in HH29 and HH35.

Increased level of apoptosis with myocyte loss had previously been found in pressure overload-induced heart failure in infant rabbit and adult rat experimental models (Minegishi et al., 2011, Choi et al., 2009, Teiger et al., 1996). Downregulation of survival pathway (ERK1/2, Akt, GSK3beta) and activation of caspase enzymes with a concomitant enhanced cardiomyocyte apoptosis were implicated as critical mechanisms mediating from compensated hypertrophy to heart failure (Moorjani et al., 2006, Li et al., 2009). Increased apoptosis is responsible for myocyte loss and myocardial dysfunction could occur as a result of hypoxia, hypertrophy and heart failure (Ding et al., 2000, Kajstura et al., 1995, Narula et al., 1996, Olivetti et al., 1996). Increased level of proliferation but the apoptotic level remained unchanged were seen in the aortic-banded fetal guinea pigs (Saiki et al., 1997). In contrast a significant decrease in apoptosis was found in both RV and LV chambers of pressure-overloaded

embryonic hearts in our study. This can be due to the potential cardioprotective role via its anti-apoptotic effect by upregulation of *S100A11*, *PDCD4* and *LGALS1* in our RNA sequencing data as described above.

In light of our findings, the pressure overloading of the OFT-banded hearts led to the development of the abnormal cardiac phenotypes that resemble DCM-like features with a number of genes found differentially expressed were associated with cardioprotective, metabolism, cytoskeletal organization, shear stress and valve development. A reduction of apoptosis level seen in the ventricles of the banded hearts might be part of the cardio-protective mechanism that indirectly attempts to normalize the pathological insults to the fetal heart.

6 CHAPTER 6 General Discussion

Cardiomyopathies account for 8% to 11% of the cardiovascular diagnoses detected *in utero* (Pedra et al., 2002). Cardiomyopathies were diagnosed in 3% of live births with cardiovascular disease (Pedra et al., 2002). Most of the fetal cardiomyopathies showed overlapping features in between HCM and RCM between different types of cardiomyopathies (Weber et al., 2014).

In an adult, sustained LV pressure overload in response to the pathological insult such as aortic stenosis will lead to the pathological hypertrophy, reactivation of a fetal gene program and maladaptive molecular alterations that initiate the transition to the heart failure (Vandenwijngaert et al., 2013). In fetuses and neonates, severe fetal valvar aortic stenosis that occurred in conjunction with the abnormal MV and mitral regurgitation with marked LA and LV enlargement were found and compression of the right heart in all fetuses ($n=14$) and neonates ($n=7$), and most of the fetuses displayed endocardial fibroelastosis in the LV (Rogers et al., 2011, Vogel et al., 2011). Although most fetuses were successfully live born after pre-natal cardiac intervention (i.e aortic valvuloplasty), the survival rate was poor, nine out of 11 live born patients died at a median age of 6 days (Vogel et al., 2011). Also, the constellation of these anomalies often results in poor prognosis during fetal development.

In a review of the structural abnormalities and non-structural abnormalities detected by echocardiography in fetuses between 16 and 41 gestational weeks, structural heart defects were found in 55 (28%), and other non-structural abnormalities (DCM,

HCM, aneurysm of the foramen ovale, isolated pericardial effusion) were detected in 33 (17%) of 193 fetuses (Eronen, 1997). The survival rate of the fetuses with structural and non-structural were 75% and 73% accordingly (Eronen, 1997). In the prenatal diagnosis of the VSDs in 171 fetuses, it was revealed that 69% were diagnosed with a perimembranous VSD and 31% with a muscular defect in 171 fetuses and surgical intervention was required for the closure of the defect (Mosimann et al., 2014).

The severe clinical phenotypes of the structural and non-structural cardiac abnormalities and the poor survival rate of the fetuses and neonates highlighted the necessities of finding the possible underlying mechanisms attributing to these phenotypes. The pressure-overloaded embryonic heart models in neonatal and fetal lamb and guinea pig (as described in section 3.17) were used before (Saiki et al., 1997, Samson et al., 2000). They are not ideal models to study the effects of altered haemodynamics on cardiac development because they are very difficult to image and manipulate inside their mother's womb.

On that account, pressure overloaded embryonic chick model was employed in our study due to the easy access for surgical manipulations to alter blood flow and its similar development into a four-chambered heart as seen in human embryos. In addition, the acute changes in haemodynamics after surgical intervention to alter blood flow can be monitored and measured in chick using in vivo imaging techniques (such as optical coherence tomography and ultrasound biomicroscopy) (McQuinn et al., 2007, Liu et al., 2012a). In spite of measurements of haemodynamics responses

have not been performed, there is a body of evidence in the literature describing the OFT-banding induced haemodynamics responses (section 1.13.1.1).

To date, OFT-banding model is a well-known altered haemodynamics model in chick and a wide spectrum of severe cardiac malformations, such as pharyngeal arch anomalies, VSDs and double outlet right ventricle accompanied by increased stress-strain relations and myocardial stiffness were found in this model (Sedmera et al., 1999, Buffinton et al., 2013, Miller et al., 2003, Tomanek et al., 1999). In our study, defects such as VSDs were identified in the histological analysis but pharyngeal arch anomalies and double outlet right ventricles were not investigated.

For the first time, we found a thickened epicardium in most of the banded hearts analysed at HH29 and HH35; this phenotype has not been reported in the previous reports of the OFT-banded model, or in fact in any other altered haemodynamics models (e.g LAL and VVL). The epicardium is known to contribute to the formation of the coronary arteries (Perez-Pomares and de la Pompa, 2011). Stunted coronary arteries had been reported in this OFT-banded model before (Tomanek et al., 1999). However, the number of hearts analysed were low ($n=3$), with each of the banded hearts giving a distinct phenotype (Tomanek et al., 1999). The ongoing studies of the effect of altered haemodynamics on the epicardium formation and hence coronary vessel development are currently being undertaken by another PhD student (Perdios Chrysostomos).

The OFT-banding induced haemodynamic responses have been documented in the literature. Upon pressure overloading, instantaneous increase of the systolic and diastolic blood pressure in the ventricles were found at all stages examined (HH24,

HH27, HH29 and HH34) but the heart rate and cardiac output were normal (Clark et al., 1989, Tobita et al., 2002, McQuinn et al., 2007). In addition, pressure overloading was associated with an increased regurgitation around the OFT and AV valve area in the HH27 banded hearts (McQuinn et al., 2007). In our study, increase of the shear stress responsive genes as indicated by the upregulation of *KLF2* and downregulation of *EDN1* suggested the banded hearts were pressure-overloaded.

Interestingly, a few genes that have been found deregulated in the RNA sequencing, *PDCD4* (downregulated), *S100A11* and *LGALS1* (upregulated), and they were suggested of their cardio-protective roles previously via their anti-apoptotic and anti-inflammatory roles (Nakatani et al., 1996, Inamoto et al., 2000, Cheng et al., 2010, Liu et al., 2012b, Seropian et al., 2013). These molecular findings are in accordance with the observation of a decrease in apoptosis in the ventricles in our study. Decreased apoptosis has been suggested as an intrinsic protective/defensive mechanism going on in the fetal heart (Tao et al., 2015).

Of all the genes which were significantly differentially expressed in the banded hearts in this study, although only minor gene expression changes (e.g small fold change) were identified, distinct phenotypic differences were seen.

For the first time, we showed that a number of genes which have previously been shown as cardio-protective genes, in the myocardial ischemia and myocardial infarction models, were significantly differentially expressed in the pressure-overloaded embryonic heart (Cheng et al., 2010, Liu et al., 2012b). This is crucial as all these processes lead to the heart failure. It can be speculated that the decrease apoptosis and deregulation of these cardio-protective genes as part of the

innate/intrinsic protective mechanisms of the embryonic fetal hearts to prevent or reverse the pathological phenotypes at an early stage to circumvent the subsequent development of the heart failure.

To our knowledge, the detailed expression pattern of the potential cardio-protective genes detected in the RNA-seq remains elusive. Future studies should involve the study of the spatial temporal expression profile of these cardioprotective genes in the banded hearts by ISH or immunohistochemistry. Cardiac hypertrophy is seen in adult heart upon pressure overloading. Fetal heart reacts to pressure overloading by cardiac hyperplasia, which is different to adult hearts, in which they respond to pressure overloading by cardiac hypertrophy (Saiki et al., 1997, Samson et al., 2000). In the OFT-banding model, myocyte proliferation was found increased in the compact myocardium in the LV 48 hours after banding (Krejci et al., 2016). However, the proliferation has not been investigated in other regions of the heart and hence is worth pursuing in future. In this thesis, enlargement of the heart with different cardiac anomalies (e.g VSD and thickened epicardium) were found upon alteration of haemodynamics via pressure overloading. Moreover, defective AV valves were also observed and they were hence characterized further.

Fluid flow is crucial for valve development and valve morphogenetic abnormalities can occur as a result of abnormal haemodynamic changes or as a secondary effect of pressure overloading the heart as previously mentioned in fetuses with aortic stenosis (Hogers et al., 1997, Menon et al., 2015, Vogel et al., 2011, Yazawa et al., 1983). Failure of valvular development upon abnormal blood flow has been suggested to cause fetal wastage (Leiva et al., 1999). In our study, dysmorphic AV primordia were

seen upon altered haemodynamics. Aberrant expression of ECM genes such as *TBX20* and aggrecan alongside with shear stress responsive genes like *KLF2* and *EDN1* at HH29 were also observed in this HH29 banded hearts, accompanied by a significant reduction in apoptosis. Decrease ECM proteins such as fibrillin-2, type III collagen and tenascin were further demonstrated in more mature primordial AV leaflets at HH35, with a concomitant decrease of ECM cross-linking enzyme, transglutaminase-2 at its mRNA level. Besides playing a role in the ECM cross-linking processes, TGM2 is known to be involved in inducing the differentiation of osteoblasts (Van Herck et al., 2010, Yin et al., 2012a).

Therefore, we here postulate that the current findings of the dysregulation of the *TBX20*, aggrecan and TGM2 and a concomitant decrease in apoptosis suggested the differentiation of the endocardial cushion into valvular interstitial cells has not occurred properly, which in turn causes less secretion of the ECM proteins such as fibrillin-2, type III collagen and tenascin by the valvular interstitial cells. Another possible mechanism could be an increase in the oxidative stress that is put upon pressure overloaded heart was because of the enhanced activity of MMP-2/9, which in turn leads to collagen and fibrillin degradation (Sternlicht and Werb, 2001, Henderson et al., 2007). Future studies are needed to demonstrate if there is a reduction of TGM2 protein level in the banded hearts by western blotting. In addition, it would be desirable to determine if there is enhanced activity of MMP2 and MMP9 which might attribute to the decrease of ECM proteins in the primordial valves at HH35.

During normal EC development, most of the mesenchymal cells of the endocardial cushion tissue had a normal ultrastructure, but some had features typical of apoptosis i.e., heterochromatinization and chromatin margination, nuclear disintegration, shrinking of the cell with membrane blebbing and cytoplasmic condensation (Abdelwahid et al., 2002). Phagocytosis of the apoptotic bodies and fragments by neighbouring normal mesenchymal, endothelial or macrophage cells was a common feature in the developing cushion (Abdelwahid et al., 2002). It will be interesting to look into the features of the apoptotic cells in the banded hearts using transmission electron microscopy as these cells were found morphologically smaller by morphological analysis in the AV valve region in the banded hearts when compared to controls.

Surgical manipulation is one of the main methods that have been widely used to study haemodynamic conditions in the heart, with the chick an ideal model due to its easy accessibility and manipulation (Midgett and Rugonyi, 2014). Also, alteration of haemodynamics at different parts of the heart (LAL, VVL) that led to different types of CHDs that resembles the clinical phenotypes in human with CHDs makes it an invaluable technique to understand the origin of the CHDs (Midgett and Rugonyi, 2014). OFT-banding was employed as it resembles the pressure-overloaded embryonic heart as seen in the aortic stenosis and severe cardiac malformations such as abnormal MV with mitral regurgitation, dilatation of LA and LV and compression of right side of the heart found in human fetuses and neonates (Rogers et al., 2011, Vogel et al., 2011). The only pitfall with this technique was the constriction of the lumen upon pressure overloading often results in the eventual decrease of the cardiac

output and hence embryonic death. This limits further investigation of the effect of altered haemodynamics on the later stage of cardiac development. The double overhand knot that was performed in our study was thought to be more invasive to the embryos compared to the single knot that was performed by other groups in the literature, since only 10% of banded hearts were surviving until HH35 (Sedmera et al., 1999). The studies of the aberrant cardiac morphogenesis upon alteration of haemodynamics are traditionally performed through a window of the eggshell (Clark et al., 1984, Sedmera et al., 1999, Hall et al., 2004). This technique limits optical access due to window size and the embryo at HH23-24 are bigger and they always sink down the egg yolk, which made the surgical manipulation impossible. An *ex-ovo* chicken embryo culture system has been developed and was suitable for the microsurgical applications (Yalcin et al., 2010). Basically, the eggs were cracked onto a hammock created by a polyurethane membrane (cling wrap) affixed circumferentially to a plastic cup partially filled with sterile water. The embryos cultured in this way were reported to survive for at least 14 days without any morphogenic defect or developmental delay (Yalcin et al., 2010). With this *ex-ovo* chick embryo culture system, banding can be performed around HH23-25 and this might results in a better mid-term survival. Therefore, the effect of the altered haemodynamics condition can be studied at a later stage of development.

Another part of the thesis was studying the role of *TNNT2* in CHDs by morpholino treatment. Using antisense oligonucleotide morpholino technology, targeted manipulation of *TNNT2* gene was conducted *in ovo* using pluronic gel as a transporter. Aberrant formation of muscular diverticulum, abnormal atrial septation and ventricle trabeculation were found within *TNNT2*-morpholino treated hearts and

possibly be linked with the haemodynamic changes as indicated by the altered *NOS3* expression (England et al., 2016). Alpha and embryonic MHC knockdown studies have been carried out in the lab in a temporal and stage-specific manner, with knockdown performed prior to atrial septum initiation (HH12), as the septum is in the process of initiating (HH14) and the septum is growing (HH16) and harvested at HH19 (Rutland et al., 2009, Rutland et al., 2011). With embryonic MHC knockdown, the septa were found abnormal in 60 out of 61 embryos when compared to controls (Rutland et al., 2011). The septum looked either small in size or resembled a knuckle-shaped outgrowth from the dorsocranial atrial wall. Also, ventricular trabeculation was found severely reduced and this phenotype presented at a high penetrance in the embryonic MHC knockdown embryos ($n=60/61$) (Rutland et al., 2011). Similarly, it was noted that septum was absent in 11 knockdown embryos ($n=32$), where 18 of them showed a smaller septum in alpha MHC knockdown studies (Rutland et al., 2009). However, in contrast, the trabeculae were investigated but they showed consistent features as seen in the control hearts (Rutland et al., 2009). In our cTnT knockdown studies, 45% of *TNNT2*-MO hearts showed a small and knuckle-shaped like outgrowth from the atrial myocardium ($n=23/51$). This penetrance was based upon the morphological analysis of two targeted morpholinos designed against target *TNNT2* gene; the work was in collaboration with Jennifer England (former PhD student) (England et al., 2016). Only six out of 51 *TNNT2*-MO treated embryos were showing reduced trabeculation. Interestingly, cardiac diverticula was observed upon cTnT MO treatment but not in alpha- or embryonic MHC.

Future study of the altered embryonic haemodynamic conditions after cTnT morpholino treatment in the chick on the spatial and temporal distributions of stresses

and strains using *in vivo* imaging techniques (such as optical coherence tomography and ultrasound biomicroscopy) would provide us insights into the effects that reduced cTnT has on these processes (McQuinn et al., 2007, Rugonyi et al., 2008, Liu et al., 2012a)

In the previous literature, association of *MYH6*, *MYH7* and *ACTC1* have been linked to CHDs, where *MYH7* has been linked to VSD, while *MYH6* and *ACTC1* mutations have been found in patients with ASDs (Ching et al., 2005, Budde et al., 2007, Matsson et al., 2008). The atrial septum and ventricular trabecular phenotype were observed after knockdown of cTnT in the chick heart. In addition, the muscular diverticula identified in the morpholino-treated hearts suggested a novel role for mutations of structural sarcomeric proteins in the pathogenesis of congenital cardiac diverticula. Hence, *TNNT2* is a gene worthy of screening for those patients with a CHD, particularly atrial septal defects and ventricular diverticula. Morpholino has been widely used as a gene knockdown tool in xenopus, zebrafish and chick (Draper et al., 2001, Heasman et al., 2000, Nutt et al., 2001, Rutland et al., 2009). With xenopus and zebrafish, one of the main advantages was that microinjecting of morpholinos at the one cell stage of development in xenopus and zebrafish allows the morpholinos to be passed on subsequent daughter cells after cell division of the blastula. In this instance, nearly 100% knockdown of the targeted gene with high efficiency can be achieved as they are targeting manipulation prior to protein pools being produced (Sehnert et al., 2002, Nutt et al., 2001). However, this results in either early embryonic lethals or a spectrum of severe cardiac defects which precludes the investigation of the role that a protein plays in cardiogenic processes, such as atrial septation and ventricular trabeculation (Nishii et al., 2008, Sehnert et al., 2002). Also,

the xenopus and zebrafish models only develop a three and two chambered heart, respectively, making it impossible to examine the role of *TNNT2* in atrial and/or ventricular septation compared to the four chambered heart formed in the chick (Warkman and Krieg, 2007, Lieschke and Currie, 2007).

Despite a 100% degree of knockdown that has been achieved in the zebrafish was not possible in the chick as the morpholino application was only performed after the stage of the heart tube formation, successful protein knockdowns by 82.6% and 63% had been demonstrated for eMYH and α MYH respectively in our lab (Rutland et al., 2009, Rutland et al., 2011). Although a lower degree of cTnT knockdown (32.8%) was demonstrated in our cTnT study, this degree of knockdown still produced abnormal atrial septal and mild ventricular trabeculation phenotypes in the developing chick heart (England et al., 2016).

However, in recent years, the specificity of morpholino oligonucleotides has come under scrutiny. A comparison was done between morpholino treated and nuclease induced mutant embryos. 80% of the observed phenotypes in the morphants failed to recapitulate the phenotypes present in the mutant embryos due to the potential of the high level of non-specific effects from the morpholino (Kok et al., 2015). In addition, it was recommended that the newly described morpholino-induced phenotypes (morphants) should be compared with a genetic mutant prior to describing it as a specific phenotype (Kok et al., 2015).

In the chick, the morpholino was delivered to heart via pluronic gel. Pluronic gel is a cheap and effective way of internalising morpholino into developing embryonic tissue. However, it was impossible to quantify the amount of morpholinos become internalised and bound to its specific target. The morpholinos used in our study were

fluorescently-tagged and their location can be visualised in the embryo. It was conceivable that the off-target effects might arise from the unbound morpholinos. (Eisen and Smith, 2008). To circumvent this, two targeted morpholinos were designed against target *TNNT2* gene with careful bioinformatics analysis performed to reduce the chance of non-specific binding, together with a successful demonstration of cTnT protein knockdown (England et al., 2016).

In conclusion, despite non-specific effects of morpholino have been reported, there were still nearly 300 morphant phenotypes consistent with those observed in mutant embryos (Kok et al., 2015). Therefore, many advantages still remain for the use of morpholinos when comprehensive controls are used (Eisen and Smith, 2008, Blum et al., 2015).

Conclusions

Alterations in haemodynamics that developed during in utero are believed to be one of the causes of CHDs and fetal cardiomyopathies as they can exacerbate during postnatal life. Despite surgical interventions such as balloon aortic or pulmonary valvuloplasty were successfully performed to normalize the haemodynamics and hence prevented irreversible changes, they often resulted in poor survival rate of the fetuses and neonates. Hence, this highlights the need of the basics and the translational research into the mechanisms underlying the CHDs and fetal cardiomyopathies.

In this study, we showed that alteration of haemodynamics via pressure-overloading resulted in the enlargement of the heart with a spectrum of cardiac anomalies (VSDs, thickened epicardium and dysmorphic AV valves). A number of genes associated with cardio-protection, metabolism, shear stress and valve development were found differentially expressed. Deformed AV valves were further characterized by aberrant expression of ECM genes characteristic of valve primordia with a decrease of apoptosis.

Apart from abnormal haemodynamics, sarcomeric proteins have been associated with a range of cardiomyopathies and only in recent years they are known to be involved in congenital heart defects (CHDs). *TNNT2* has not been linked to CHDs but historically associated with cardiomyopathies. However, upon *TNNT2* morpholino treatment in the chick, abnormal atrial septal growth, mild reduction of ventricular trabeculation and ventricular diverticula were found. Altered expression of shear stress *NOS3* gene suggests that a reduction in cTnT in the developing heart affects important molecular signals required for normal development, and in turn results in the abnormal cardiac

phenotypes found in this study. Our current findings merit further investigation such as performing full gene sequencing of *TNNT2* in patients with CHDs, particularly atrial septal defects and cardiac diverticula.

Overall, H_0 is rejected in this thesis. Hence, the mechanical or genetic manipulation of the developing heart will induce cardiomyopathy-like phenotype.

7 References

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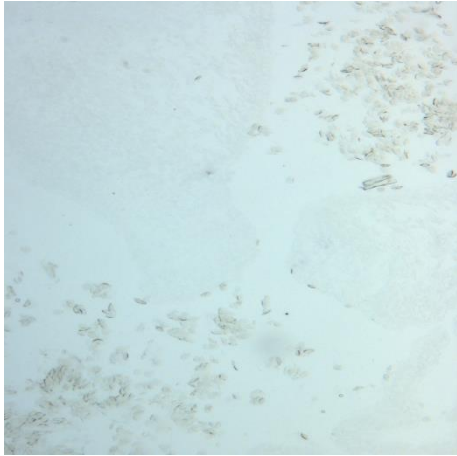
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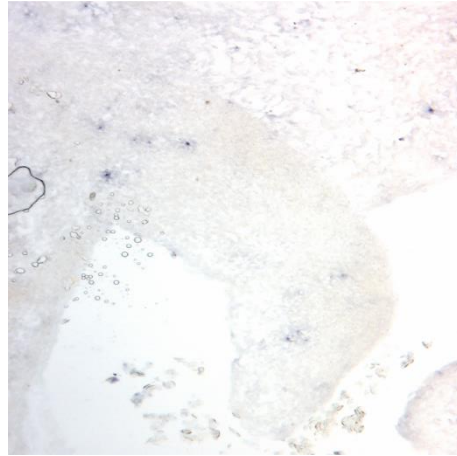
8 Appendices

Hybridization mix for riboprobes

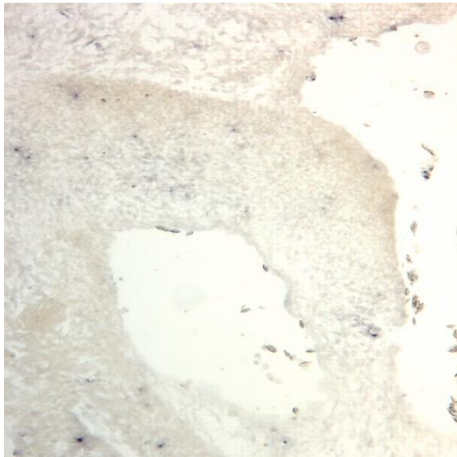
Stock concentration	Volume required	Final concentration
Deionized formamide	25ml	50%
20X SSC	12.5ml	5X
50X Denhardt's solution	1ml	1X
50% Dextran sulphate	10ml	10%
Tween 20	250µl	0.1%
Heparin (100mg/ml)	25µl	50µg/ml
tRNA (100mg/ml)	500µl	1mg/ml
DEPC-ddH ₂ O	Make up to 50ml	



KLF2 sense (NcoI/SP6)



TBX20 sense (NcoI/SP6)



Periostin sense (SpeI/T7)