



The University of
Nottingham

**Identification and analysis of the
synergistic targets of TBX5 and GATA-4**

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**Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy**

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DECLARATION

I declare that this thesis has not, whether in the same or different form, been presented to this or any other university in support of an application for any degree other than that for which I am now a candidate.

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LIST OF ABBREVIATIONS

ANF	Atrial natriuretic factor
ASD	Atrial septal defect
AV	Atrioventricular
AVSD	Atrioventricular septal defect
BAF	Brahma-associated factor
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic local alignment search tool
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
CAD	Coronary artery disease
cDNA	Complementary cDNA
CHD	Congenital heart defect
CNC	Cardiac neural crest
DCM	Dilated cardiomyopathy
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's minimum essential media
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DORV	Double outlet right ventricle
E.coli	Eschericia coli
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECR	Evolutionarily conserved region
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
EST	Expressed sequence tag
Exol	Exonuclease I

FAM	Fluorescein amidite
FDR	False discovery rate
HBSS	Hank's buffered salt solution
HCM	Hypertrophic cardiomyopathy
HH	Hamburger and Hamilton
HOS	Holt-Oram syndrome
HRP	Horseradish peroxidase
IDC	Idiopathic dilated cardiomyopathy
KD	Knockdown
LB	Luria-Bertani
MMD	Moyamoya disease
NBT	Nitroblue tetrazolium
NMD	Nonsense-mediated decay
OFT	Outflow tract
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Triton X-100
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PTA	Persistent truncus arteriosus
PTC	Premature termination codon
QPCR	Quantitative polymerase chain reaction
RAE	Retinoic acid embryopathy
RIN	RNA integrity number
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SGBS	Simpson-Golabi-Behmel syndrome
SMC	Smooth muscle cell
SOC	Super optimal broth with catabolite repression
SRE	Serum response element
SSC	Saline sodium citrate
TAAD	Thoracic aortic aneurysms and dissections
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% Tween20
TGA	Transposition of the great arteries
TOF	Tetralogy of Fallot
VSD	Ventricular septal defect
VSMC	Vascular smooth muscle cell
WISH	Whole mount in situ hybridisation
WT	Wild type

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ABSTRACT

Cardiac development is a complex multi-step process involving a diverse network of genes. Defects in cardiac genes can lead to congenital heart defects (CHDs), and understanding the processes involved in normal cardiogenesis is crucial in elucidating the pathogenesis of disease. Mutations in a number of cardiac transcription factors have been associated with CHDs, including TBX5 and GATA-4. These transcription factors are high in the regulatory hierarchy and form a complex that is thought to direct and synergistically regulate common cardiac pathways. However, little is currently known about their joint targets. This study aimed to identify and analyse genes important in cardiogenesis, with focus on the combined targets of TBX5 and GATA-4. Microarray expression analysis of TBX5 / GATA-4 double overexpression P19 cell lines led to the identification of a large number of genes, of which seven were selected for study; *PA2.26*, *PETA-3*, *FUCA1*, *FN*, *TPM1*, *DES*, and *RBMS1*. Expression of these was confirmed in the embryonic chick heart at Hamburger and Hamilton (HH) stages 12 – 26. The cell cycle regulator and transcription factor RBMS1 was selected for investigation of its role in cardiac development. Morpholino knockdown of RBMS1 expression in the developing chick resulted in defects in cardiac looping and atrial septation. Whilst further work is required to strengthen this data, this is a novel finding and opens up possibilities for future research into cardiac transcriptional networks. Microarray expression analysis using an *in ovo* model of TBX5 and GATA-4 double knockdown led to the identification of a number of interesting putative targets, including *TFAP2B*, *GPC3*, and *CRABP1*, which have known roles in cardiac development. *TFAP2B* is of particular interest as mutations in this gene are associated with the heart-hand disorder Char syndrome. *LOC420770*, a novel gene of unknown function, was also identified and displayed expression indicative of a heart-limb profile. Further studies to attempt elucidation of the function of this gene may provide a novel candidate gene for CHD. Investigation of these genes will form the basis of future research, contributing to our current knowledge of the vast network of genes involved in development of the heart.

CHAPTER 1

INTRODUCTION

1.1 CARDIAC DEVELOPMENT

The heart is the first organ to form in embryogenesis, developing by a complex and precisely co-ordinated process involving heart specification, formation of the linear heart tube, looping, and maturation to ultimately form a four-chambered structure [Martinsen, 2005]. Processes such as septation, valve development and coronary vasculature formation occur in parallel and are crucial. Cardiac development is regulated by a vast network of genes encoding growth factors, transcription factors, co-factors and regulators, signalling proteins, and structural molecules, some of which will be described here. Perturbations in the developmental process can lead to heart malformations, and understanding the molecular processes involved in normal cardiac development is important in elucidating the pathogenesis of congenital heart defects. The anatomical development of the heart has been widely studied in a number of organisms including human (Carnegie Stages / CS), mouse (Embryonic days / E), and chick (Hamburger and Hamilton stages / HH), and is detailed below. Processes that differ between mammals and the chick (namely atrial septation) are discussed.

1.1.1 Pre-gastrulation and gastrulation

At the pre-primitive streak stage, cardiac precursor cells are identifiable in the epiblast cell layer [Yutzey and Kirby, 2002]. The primitive streak is a triangular region found in the midline of the long axis of the oval disc in the area pellucida, darker than its surroundings due to cell ingression to this region before outward cell migration begins. At the onset of primitive streak formation, cardiac progenitor cells exist in the postero-lateral epiblast [Hatada and Stern, 1994; Yatskievych et al., 1997]. Interaction between the postero-lateral epiblast and hypoblast is required for anterior lateral plate mesoderm formation, and this region gives rise to the heart [Ehrman and Yutzey,

1999]. The intermediate primitive streak extends from the posterior into the centre of the area pellucida and contains precardiac cells in the anterior [Rawles, 1943] (Figure 1.1A). The definitive primitive streak extends over most of the area pellucida.

Gastrulation, where the three embryonic germ layers are established, is one of the first morphogenetic events to occur in development. Migration of cells from the upper layer of the primitive streak gives rise to the ectoderm, the earliest layer to form and the outermost. The endoderm is formed by inward migration of cells along the archenteron from the inner layer of the gastrula. The mesoderm, an additional layer between the ectoderm and endoderm, is formed from some of the cells migrating to form the endoderm. Each germ layer gives rise to specific body tissues.

1.1.2 Formation of the primary heart field

Precardiac cells are found in the anterior mesoderm and either side of the node [Linask and Lash, 1986]. The anterior intestinal portal, head process and head fold develop, and the cells that will form the heart move into two bilateral regions in the anterolateral region of the embryo called the primary heart field, first identified by Rawles in 1943 [Rawles, 1943] (Figure 1.1B). This region gives rise to the myocardium and endocardium. Cardiac precursor cells (not yet fully committed) segregate into the splanchnic mesoderm layer during migration into the primary heart fields. This layer is adjacent to the endoderm, which is thought to induce differentiation of myocardial cells [Abu-Issa et al., 2004]. Definition of the avian embryonic heart forming region (HFR) has been attempted through the use of early cardiac markers such as Nkx2.5 and GATA-4, and by cell lineage tracing studies [Redkar et al., 2001]. The two methods yielded differing results, as with many previous studies, and the extent of the heart forming region remains unclear. As cardiac precursor cells reach a higher level of commitment, expression of cardiac genes is initiated [van den Hoff et al., 2004]. The prospective heart forming regions migrate to form the cardiac crescent (Figure 1.1C) (week 2 of human gestation / E7.5

in mouse), whilst the neural plate forms from the ectoderm, and folds to become the neural tube.

1.1.3 Formation of the linear heart tube

The cardiac crescent moves towards the embryonic neck and the cardiac plate folds into the primary heart tube at the embryonic ventral midline (Figure 1.1D) [Moorman et al., 2003], connected to the body by a posterior inflow (venous pole) and anterior outflow (arterial pole). The linear heart tube comprises two concentric layers of cells separated by an extensive matrix of cardiac jelly; the outer layer is made up of myocardial cells, and the inner layer consists of endocardial cells [Brutsaert, 2003]. This structure, together with the remainder of the heart forming regions, takes the shape of an inverted Y. At this pre-looping phase, the heart is centrally positioned and undergoes rapid elongation along the craniocaudal axis of the embryo to form a long nearly symmetrical tube [Moorman et al., 2003]. The primitive tubular heart begins to beat spontaneously soon after it is formed, at CS 10 (22 – 23 days) in humans, E8 in mouse, and HH10 in the chick [Franco et al., 1998].

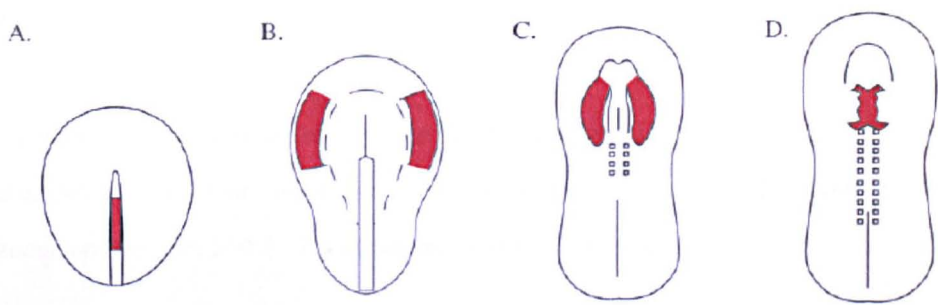


Figure 1.1 Embryonic origin of the heart. Heart forming regions are displayed in red. A) Heart progenitor cells are located in the upper region of the primitive streak, under Hensen's node. B) Bilateral heart primordia are present in the anterior lateral plate mesoderm. C) Formation of the cardiac crescent following fusion of the anterior end of the two heart fields. D) Fusion of heart primordia to form a beating heart tube at the embryonic midline. *Adapted from Yutzey et al., 2002*

1.1.4 Looping of the heart

The looping process can be subdivided into four phases following pre-looping (as described in 1.1.3); dextral-looping to form the c-shaped loop, transformation from the c-shaped loop to the s-shaped loop (early and late phases), and the positional changes which occur mainly in the primitive outflow tract (OFT) during cardiac septation [Manner, 2000]. These are represented in Figure 1.2.

During dextral looping, the linear heart tube loops to the right to become c-shaped and asymmetric (Figure 1.2, 1). This process is essential in ventricle orientation and connection to the appropriate vessels, and is dependent on asymmetric signalling cascades [Kathiriya and Srivastava, 2000]. The right lateral furrow flattens, the left deepens, and the diameter of the heart tube increases particularly at the caudal end (an early manifestation of the thinner outflow portion (primitive conus) and thicker primitive ventricular region) [Manner, 2000]. The primitive ventricular region, composed of the primordia of the apical trabeculated regions of the left and right ventricles, bends to the right resulting in the ventral and dorsal sides of the heart tube becoming the convex right and concave left sides of the bend [Manner, 2000]. Myocardial precursor cells from the posterior region of the primary heart field contribute to the atria forming region, and cells from a second population of heart precursor cells in the pharyngeal mesoderm termed the secondary heart field, migrate into the cranial (arterial) pole of the heart, supporting growth of the outflow tract and right ventricle forming regions [Abu-Issa et al., 2004; Kelly et al., 2001; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Moorman et al., 2007; Waldo et al., 2001]. Cardiac neural crest (CNC) cells undergo epithelial-to-mesenchymal transition (EMT) and migrate away from the neural tube into the heart via the outflow tract and pharyngeal arch arteries [Boot et al., 2003]. CNC cells are essential for correct outflow tract septation, cardiac innervation, aortic arch repatterning, and myocardial function [Kirby, 2002].

Cardiac looping continues transforming the heart from a c-shape to an s-shaped structure via an early and late phase (Figure 1.2, 2-3). In the early phase, the caudal wall of the primitive conus, and cranial wall of the primitive atria move closer together, and the primitive ventricular bend shifts from a cranial to caudal position from the atria [Manner, 2009; Martinsen, 2005]. This transforms the heart to an immature s-shaped structure. In the late phase of s-looping, the proximal part of the outflow tract shifts to the left and ballooning of the chambers also occurs (described in 1.1.5) [Manner, 2009]. The epicardium, myocardium, endocardium and cardiac jelly heart layers have also developed upon completion of this process.

The last phase of looping is often referred to as the cardiac septation phase, since this is the main anatomical event occurring at this stage. A number of morphological changes occur resulting in positional changes of cardiac regions (Figure 1.2, 4). The proximal two thirds of the outlet section of the outflow tract shifts from a position lateral to the atria, to a position ventral to the right atrium, and the outflow tract and inner curvature of the ventricular bend are remodelled [Manner, 2009].

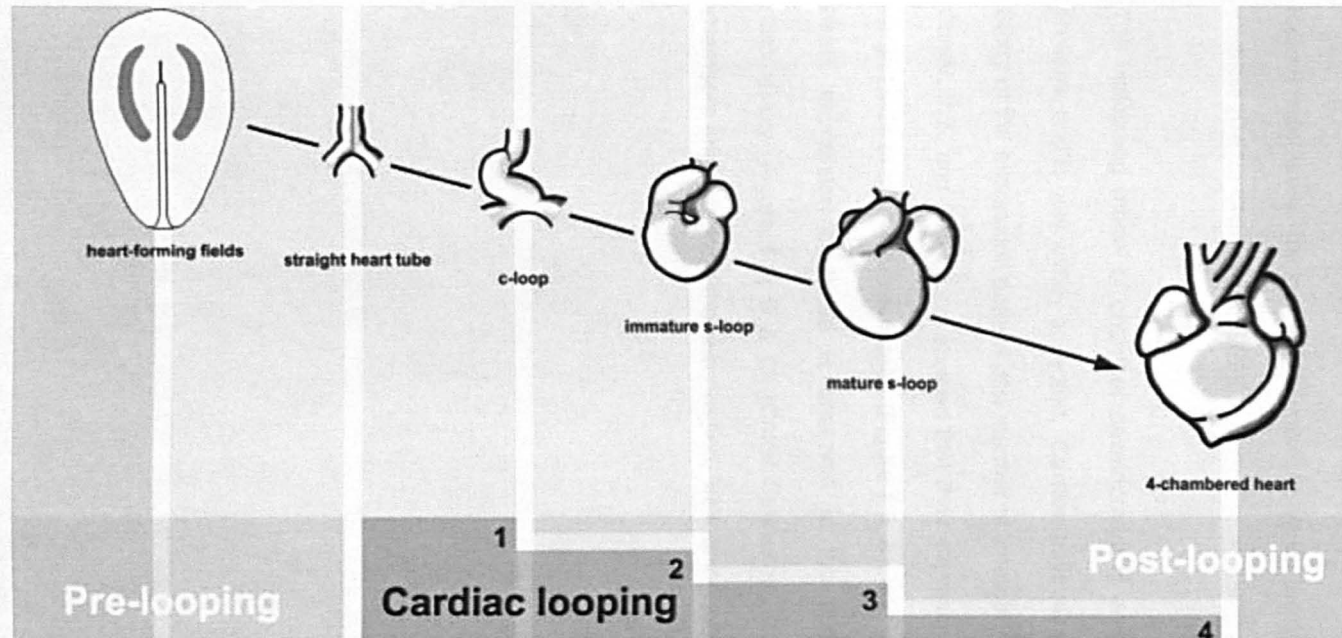


Figure 1.2 Cardiac looping

Looping of the heart is represented in four phases. 1) Dextral-looping, where the linear heart tube loops to the right to become c-shaped and asymmetric. 2) Early phase s-looping, where the heart is transformed to an immature s-shaped structure. 3) Late phase s-looping, where the proximal part of the outflow tract shifts to the left and the heart is transformed to a mature s-shaped structure. 4) Cardiac septation, where a number of positional changes of cardiac regions occur, mainly in the primitive outflow tract. *Adapted from Manner, 2009*

1.1.5 Chamber formation

There have been two main theories about how the cardiac chambers form – the segmental theory, and the ballooning theory. According to the segmental model, the cardiac chambers form from segmental primordia along the anteroposterior axis of the heart tube, and this has been supported by apparent segmental expression of various cardiac genes [Franco et al., 1998]. The heart tube contains caudal atrial and cranial ventricular regions which will give rise to the cardiac chambers (separated by the atrioventricular canal), and a cranial outflow tract. In this model, the outer curve of the looped heart gives rise to the right ventricle, whilst the inner bend gives rise to the left ventricle [De La Cruz et al., 1989].

The ballooning model of chamber development is an alternative theory behind this process, and is now widely accepted. Cardiac ballooning is represented in Figure 1.3. Study of the expression pattern of a number of 'chamber specific' cardiac genes (ANF, Chisel, *Irx5*, and SERCA2a) showed that their expression is spatially restricted to the ventral side of the linear heart tube (Figure 1.3, A), and in the looped heart and mature heart, expression of these is observed in the outer curvature and chambers respectively (Figure 1.3, B & C) [Christoffels et al., 2000]. This demonstrates the chambers originate from the outer region, 'ballooning' out from the heart tube.

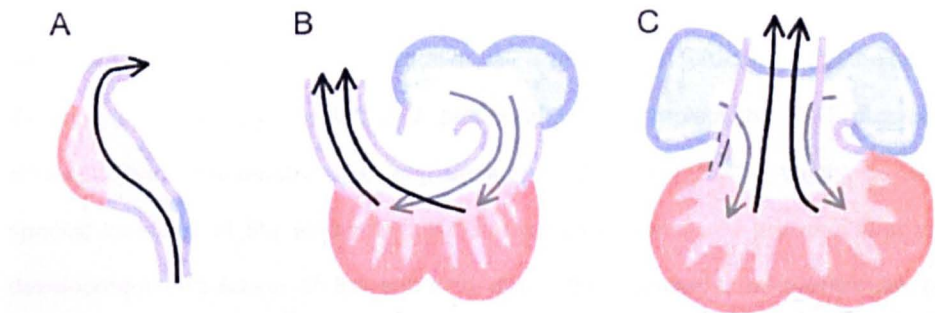


Figure 1.3 Representation of ballooning of the heart

Prospective ventricular and atrial myocardium regions are shown in pink and blue respectively. Arrows denote the direction of blood-flow; grey arrows indicate blood-flow into the ventricles, and black arrows indicate blood-flow through the outflow tract. Three phases of development are represented, A) the linear heart tube, B) the mature s-looped heart, and C) the four chambered heart. The chambers originate from the outer region of the heart tube, 'ballooning' out. *Adapted from Christoffels et al., 2000*

1.1.6 Endocardial cushion formation and function

Endocardial cushions are cell masses which initially appear as swellings in the cardiac jelly that form by EMT of the endothelial cell layer. In this process, endothelial cells delaminate, invade the cardiac jelly, proliferate, and complete the EMT process to differentiate into mesenchymal cells [Runyan and Markwald, 1983]. Cushions form in specific locations of the atrioventricular and conus regions, and are crucial in valve development and fusion of the septa to divide the chambers, atrioventricular (AV) canal, and outflow tract for formation of the aortic and pulmonary channels [Markwald et al., 1977].

1.1.7 Septation

Septation divides the heart into four distinct chambers, and separates the outflow tract region into the pulmonary and aortic channels. This compartmentalisation is critical in establishing and maintaining separate pulmonary and systemic circulations. Defects in septation, when left uncorrected, can lead to pulmonary vascular disease, atrial enlargement with a resulting predisposition to atrial arrhythmias, ventricular dilation, and a shortened life expectancy [Garg, 2006].

1.1.7.1 Atrial septation

Development of the interatrial septum, which separates the left and right atria, begins with downward growth of the septum primum from the dorsal cranial wall of the common atrium into the atrial lumen, towards the endocardial cushions in the AV canal [Anderson et al., 2003] (Figure 1.4A). The interatrial septum is identifiable from CS 13. The primary atrial opening (or foramen) between the septum primum and endocardial cushions is termed the ostium primum. The septum primum harbours a mesenchymal cap which becomes fused with the superior and inferior endocardial cushions in the AV canal (which also become fused with one another), closing the ostium primum [Wessels et al., 2000]. In parallel, perforations form in the upper part of the septum as a result of apoptosis, and these coalesce to form a secondary atrial foramen termed the ostium secundum [Abdulla et al., 2004] (Figure 1.4B).

Development of an additional septum, the septum secundum, begins from the roof of the atrium wall and alongside the remnants of the septum primum [Lamers and Moorman, 2002] (Figure 1.4C). This leaves an opening called the foramen ovale. Following breakdown of the upper portion of the septum primum, the remaining part acts as a valve in this region and is termed the valve of foramen ovale [Anderson et al., 2003]. Once lung circulation begins postnatally, increased pressure in the left atrium results in fusion of the foramen ovale valve with the septum secundum to close the foramen ovale and complete atrial septation [Anderson et al., 2003] (Figure 1.4D).

As mentioned, the process of atrial septation in the chick differs to that of mammals, and formation of the septum secundum does not occur. In the chick, the septum primum begins downward growth from the inner atrial wall at HH14 [Anderson et al., 2003]. It becomes fused with dorsal and ventral endocardial cushions in the AV canal at HH 24 (4 days), and small perforations (foramina secunda) form in the mid-dorsal portion of the septum primum in parallel, maintaining communication between the atria [Hendrix and Morse, 1977]. These increase in number and size from days 5 to 8, creating cords of endocardium covered tissue [Morse and Hendrix, 1980]. This cord tissue thickens during days 8 to 15, reducing the size of the foramina secunda. By day 2 post hatching, the interatrial perforations are closed, and atrial septation is complete [Hendrix and Morse, 1977]. This variation between species means that particular atrial septal defects which arise from disturbances in septum secundum formation cannot be modelled in the chick (discussed in section 1.2). Cardiac development in the chick is discussed further in Chapter 4.

1.1.7.2 Atrioventricular septation

The atrioventricular septum separates the atria and ventricles. Atrioventricular cushions form at the superior and inferior borders of the AV canal, and lateral cushions form on the left and right borders in parallel. The superior and inferior cushions continue to grow and extend into the lumen, becoming fused with one another and developing into the mature AV septum [Sadler, 2006].

1.1.7.3 Outflow tract septation

The aorticopulmonary septum divides the common outflow tract (truncus arteriosus) and conus region into aortic and pulmonary channels. Migration of cardiac neural crest cells (CNCs) into the outflow tract region via the pharyngeal arches contributes to the formation of cushions in this region [Kirby, 1987; Waldo et al., 1998]. A pair of opposing ridges termed the left and right superior truncus swellings form in the truncus, and distal growth of these towards one another results in fusion to form the aorticopulmonary septum [Sadler, 2006]. In parallel, cushions also form on the right dorsal and left ventral walls of the conus region. These grow distally, and towards one another to join with the truncus septum, dividing the conus into anterolateral and posteromedial portions [Sadler, 2006]. The vessels rotate to connect with the appropriate chamber, with the aorta joining the left ventricle, and the pulmonary artery connecting to the right ventricle, generating separate pulmonary and systemic circulations. Alignment and fusion of the aorticopulmonary septum, ventricular septum, AV septum, and base of the atrial septum occurs to produce a four-chambered heart.

1.1.7.4 Ventricular septation

The muscular interventricular septum, which separates the left and right ventricles, forms as a result of growth between the inlet and outlet regions of the ventricular section [Lamers et al., 1992] (Figure 1.4C-D). The ventricular medial walls become apposed and merge, giving rise to the muscular portion of the interventricular septum which is identifiable from week 5 (CS 15) [Lamers and Moorman, 2002]. At this stage, communication between the two ventricles via the interventricular foramen can still occur. Proliferation of the conus cushions and completion of conus septation (described in section 1.1.7.3) results in shrinkage of the interventricular foramen. Outgrowth from the inferior endocardial cushion to the muscular interventricular septum occurs, closing the interventricular foramen. The right and left conus cushion and inferior endocardial cushion proliferations form the membranous portions of the

interventricular septum. Ventricular septation is complete by CS 23 [Lamers et al., 1992].

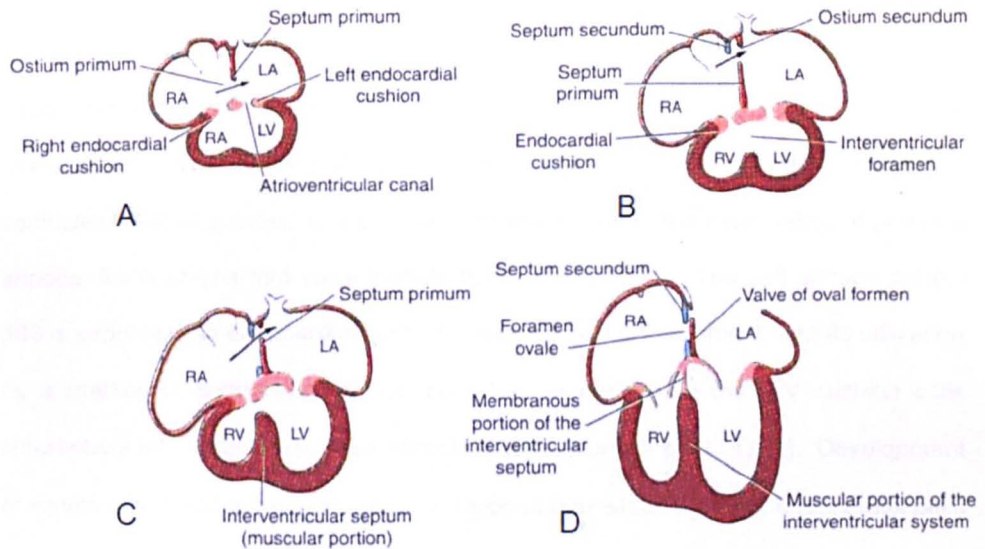


Figure 1.4 Human atrial and ventricular septation

A) 30 day embryo. The septum primum grows from the roof of the atrial chamber and extends towards the AV canal. The space between the septum primum and endocardial cushions is termed the ostium primum. B) 33 day embryo. The septum primum fuses with the endocardial cushions in the AV canal, resulting in closure of the ostium primum. The ostium secundum forms as a result of perforations in the septum primum. Development of the septum secundum, also from the roof of the atrial chamber, begins. This does not occur in the chick. C) 37 day embryo. Growth of the septum secundum towards the valve of foramen ovale occurs. The ventricular medial walls become apposed and merge, giving rise to the muscular portion of the interventricular septum. D) Newborn. The septum secundum and valve of foramen ovale fuse to divide the atria. Outgrowth from the inferior endocardial cushion to the muscular interventricular septum occurs, closing the interventricular foramen. The conus cushion and inferior endocardial cushion proliferations form the membranous portions of the interventricular septum. *Adapted from Langman's Medical Embryology, 10th Edition*

1.1.8 Valve development

Cardiac valves are thin fibrous structures which open and close in a precise manner, allowing unidirectional blood flow through the heart. Two sets of valves form in the heart; the semilunar valves (aortic and pulmonary) form in the outflow tract, and atrioventricular (AV) valves (tricuspid and mitral) in the AV canal between the atria and ventricles. Valvulogenesis is a complex process involving the remodelling of primitive endocardial cushions into valve leaflets [Chin et al., 1992]. The cell surface protein JB3 is expressed in endocardial cushion tissue early in development, and its utilisation as a marker of endothelial derived cells has demonstrated that AV cushion cells differentiate into connective tissue fibroblast cells [Wunsch et al., 1994]. Development of valves is initiated by signals from the myocardium which promote endocardial cells to undergo EMT forming endocardial cushions composed of proliferative valve progenitor cells in an extracellular matrix [Armstrong and Bischoff, 2004]. Endocardial cushions become cellularised by proliferation of mesenchymal cells, they elongate and are remodelled to form functional valves composed of three main layers – the atrialis (elastin-rich), spongiosa (proteoglycan-rich), and fibrosa (collagen-rich) [Hinton et al., 2006]. This is represented in Figure 1.5. Valve remodelling is characterised by this increase in ECM organisation and complexity, and also by a decrease in endocardial cushion cell proliferation [Hinton et al., 2006; Lincoln et al., 2004]. Altered expression of specific genes also marks this process; expression of remodelling enzymes (such as matrix metalloproteinases) decreases, and there is an increase in expression of constituents of valvular ECM such as the proteoglycans aggrecan and versican [Shelton and Yutzey, 2007]. The molecular mechanisms regulating valve morphogenesis and remodelling are not well understood. Correct positioning of valves is critical, and BMP2 and TBX2 play roles in regulating this process [Harrelson et al., 2004; Ma et al., 2005]. TBX20 expression antagonises the transition from endocardial cushions to remodelled valves [Shelton and Yutzey, 2007]. Notch signalling is important in development of the endocardial cushions that give rise to cardiac valves [Timmerman et al., 2004], and mutations in the human *NOTCH1* gene can result in abnormal aortic / mitral valve development [Garg et al., 2005].

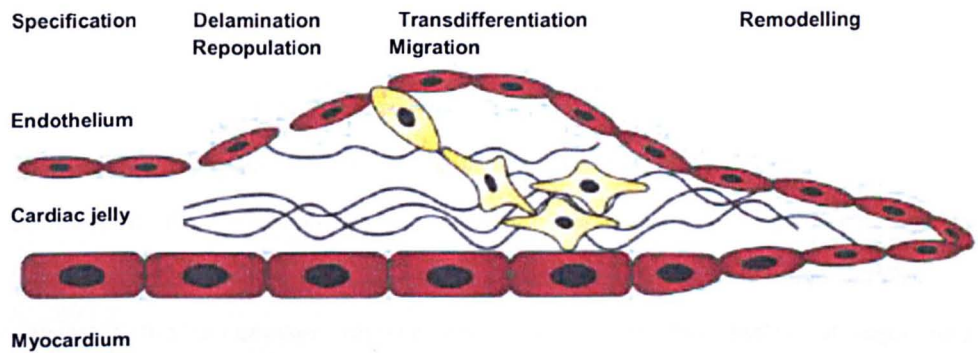


Figure 1.5 Cardiac valve formation

The heart tube is composed of an outer myocardial cell layer, and an inner endothelial cell layer, with a layer of extensive extracellular matrix (ECM) in between, termed the cardiac jelly. Specified endothelial cells at the future valve site undergo epithelial-to-mesenchymal transformation (EMT) (they delaminate, differentiate, and migrate into the cardiac jelly layer). Cardiac cushions undergo remodelling to form cardiac valves.

Adapted from Armstrong and Bischoff et al., 2004

1.1.9 The cardiac conduction system

The co-ordinated and rhythmic contraction of the heart occurs as a result of precisely timed action potentials generated via the cardiac conduction system (CCS) [Moorman et al., 1997]. The CCS comprises the sino-atrial node (SA node) also known as the pacemaker, the atrioventricular node (AV-node), and the His-Purkinje system [Moorman et al., 1997]. The SA node is found in the wall of the right atrium, and generates the pacemaker impulse which determines the rhythm of myocardial contraction [Moorman et al., 1997]. The AV node is located at the base of the interatrial septum, and serves to slow transmission of the impulse to the ventricles, causing a delay which allows blood to exit the atria prior to ventricular contraction [Moorman et al., 1997]. Signals from the AV node are transmitted via the AV bundle and bundle branches, activating contraction of the ventricles via the Purkinje fibre network system [Moorman et al., 1997]. As the atrial septum is important in this pathway, defects in atrial septation can lead to altered atrial conduction routes [James, 1970].

1.2 CONGENITAL HEART DEFECTS

Congenital heart defects (CHD) are a leading cause of infant mortality, with an incidence of around 7.5 cases per 1000 live births [Samson and Kumar, 2004]. Development of the heart is a complex process and defects can occur at a number of stages leading to a variety of heart malformations, some of which are represented in Figure 1.6. Selected CHDs are described below.

1.2.1 Cardiac septation defects

Defects in cardiac septation account for approximately 50% of CHD cases [Hoffman and Kaplan, 2002]. Atrial septal defects (ASDs), where the atria are not fully septated, can be divided into two categories. Ostium secundum defects are the most common form of ASD, and this type of defect occurs due to excessive degeneration of the septum primum, or inadequate development of the septum secundum [Blom et al., 2005]. This condition is associated with mitral valve prolapse in 10 – 20% of sufferers [Leachman et al., 1976]. Ostium primum defects occur less frequently than ostium secundum defects, usually in association with Trisomy21 [Kaur et al., 2008]. The ostium primum is usually sealed by fusion of superior and inferior endocardial cushions, and ostium primum defects usually occur as a result of failure of ostium primum closure due to defects in the AV endocardial cushions. As these endocardial cushions are also required for mitral and tricuspid valve formation, ostium primum defects are usually accompanied with valve abnormalities [Balchum et al., 1956].

Atrioventricular septal defects (AVSDs), where both the atria and ventricles are not fully septated, arise due to malformation of the atrioventricular canal cushions, which lead to inadequate fusion with the atrial or ventricular septum [Sadler, 2006]. Two types of ventricular septal defects (VSDs) may occur. Muscular ventricular septal defects arise as a result of incomplete proliferation of cardiomyocytes in the muscular portion of the interventricular septum [Soto et al., 1980]. Perimembranous ventricular septal defects arise due to defects in the membranous portion of the septum, occurring as a result of endocardial cushion defects [Soto et al., 1980].

1.2.2 Conotruncal and aortic arch artery defects

Cardiac outflow tract and aortic arch defects account for 20 – 30% of CHD cases [Hoffman and Kaplan, 2002]. Since cardiac neural crest cells contribute to this region, many conotruncal defects arise due to defects in their differentiation or migration. Double outlet right ventricle (DORV) is where both great arteries align with the right ventricle, and occurs as a result defects in neural crest cell migration, or incomplete rotation of the outflow tract [Obler et al., 2008]. Persistent truncus arteriosus (PTA) occurs due to a failure of conus ridge fusion and truncus septation, resulting in the pulmonary artery arising from the common truncus [Sadler, 2006]. This is always accompanied by a ventricular septal defect as the conus ridges also play a part in interventricular septation. This condition can arise through defects in neural crest cell migration [Kirby, 1987]. Transposition of the great arteries (TGA) occurs when the conotruncal septum does not follow its normal course, leading to connection of the aorta to the right ventricle, and the pulmonary artery to the left ventricle [Sadler, 2006]. This can be due to defects in looping, or in the migration of neural crest cells, which are important in forming the truncal cushions [Kirby et al., 1983]. Anterior displacement of the conotruncal septum and unequal division of the conus region leads to tetralogy of Fallot (TOF), a heart disorder comprising four anatomical defects; narrow right ventricular outflow region, a ventricular septal defect, overriding aorta, and right ventricular hypertrophy [Sadler, 2006]. The ductus arteriosus is the arterial connection between the pulmonary artery and aorta, and serves to shunt blood from the right ventricle away from the lungs, allowing them to be bypassed during foetal development. The ductus normally constricts and closes shortly after birth, and failure to do so results in patent ductus arteriosus (PDA) [Sadler, 2006]. There is evidence that this disorder may arise as a result of defects in cardiac neural crest cell differentiation or migration [Char, 1978], or altered smooth muscle cell contractility in the aorta [Guo et al., 2007; Zhu et al., 2006].

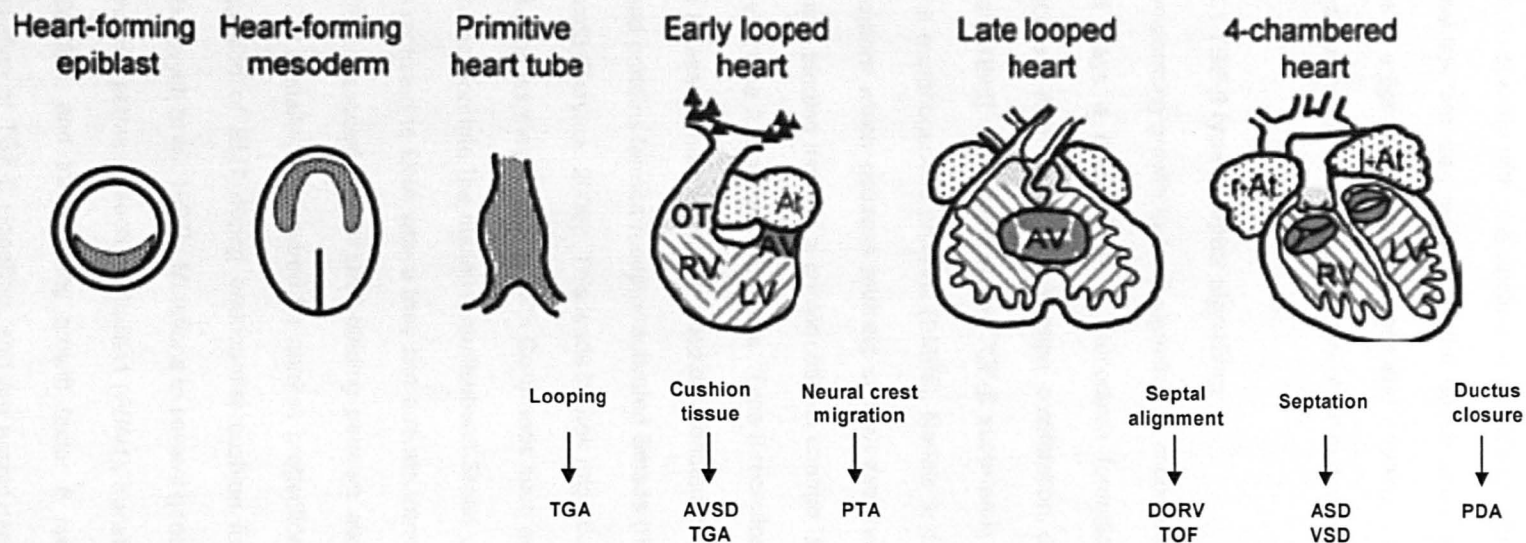


Figure 1.6 Stages of heart development and associated defects

Development of the heart is a complex process and defects can occur at a number of stages leading to a variety of heart malformations, some of which are represented. AVSD – atrioventricular septal defect, TGA - transposition of the great arteries, PTA – persistent truncus arteriosus, DORV – double outlet right ventricle, TOF – tetralogy of Fallot, ASD – atrial septal defect, VSD – ventricular septal defect, PDA – patent ductus arteriosus. OT – outflow tract, RV – right ventricle, LV – left ventricle, AV- atrioventricular canal, At – atrium. *Adapted from Sakabe et al., 2005*

1.3 GROWTH FACTOR SIGNALLING IN CARDIAC DEVELOPMENT

Patterning of the developing embryo is governed by a group of signalling pathways that co-operate with one another to provide positional information and allow complex cross-talk between tissues for the development of specific organs and structures. This is a tightly regulated process and involves a vast array of signalling molecules. Some of the signalling pathways involved in development of the heart are discussed.

1.3.1 TGF- β type receptor signalling

Transforming growth factor- β signalling is important in early developmental pathways, and plays a crucial role in mesoderm formation and cardiogenesis, mediating processes such as cell specification, proliferation, differentiation, and apoptosis [Ladd et al., 1998]. Members of the TGF- β superfamily of signalling molecules (such as bone morphogenetic proteins (BMPs), Nodals and Activins) bind to type I or type II receptors which possess intrinsic serine/threonine kinase activity [Derynck, 2008]. Ligand binding induces a conformational change leading to cell surface assembly of type I / type II receptor complexes. Type II receptors then phosphorylate and activate the kinase domain of type I receptors, leading to phosphorylation of a subgroup of Smad proteins termed receptor-activated Smads (R-Smads), for example Smad2 and Smad3 [Derynck, 2008]. This leads to their migration towards the nucleus, and during this process they associate with Co-Smads such as Smad4 and Smad4 β . Following translocation into the nucleus, multisubunit Smad complexes (Smad4/Smad2/Smad3) are recruited to DNA where they can activate transcription of target genes [Derynck, 2008]. Association with DNA-binding partners allows selective activation of genes. TGF- β signalling is required for cardiac progenitor differentiation, and has a role in regulation of EMT during endocardial cushion formation [Yamagishi et al., 2009; Yatskievych et al., 1997]. Mutations in several genes which are involved in the TGF- β signalling pathway, such as fibrillin1 (*FBN1*), transforming growth factor β receptor 1 (*TGF β R1*), and transforming growth factor β receptor 2 (*TGF β R2*), lead to up-regulation of TGF- β signalling, and are associated with aortic aneurysms [ten Dijke and Arthur, 2007]. Deletion of the type I receptor Alk3 (BMPRIa) in mouse embryonic

hearts results in impaired EMT in the AV canal with drastically reduced numbers of mesenchymal cells here, demonstrating that BMP signalling via this receptor is essential for endocardial cushion morphogenesis in this region [Song et al., 2007]. Deletion of Smad4 in embryonic cardiomyocytes during mouse development results in severe heart defects, and expression of the cardiac transcription factors Nkx2.5, GATA-4 and MEF2C is disrupted [Qi et al., 2007]. Targeted disruption of Smad6 (an inhibitory Smad) results in cardiac valve hyperplasia and defects in outflow tract septation, indicating a role for Smad6 in endocardial cushion development [Galvin et al., 2000].

1.3.1.1 BMPs

BMPs are multifunctional signalling proteins belonging to the TGF β family. During development, BMPs such as BMP2 and BMP4 secreted by the endoderm cause commitment of mesodermal cells lateral to the Hensen's node to the cardiac cell lineage following gastrulation [Barron et al., 2000; Lough and Sugi, 2000; Yamada et al., 2000]. BMP2 is involved in correct positioning of heart valves [Harrelson et al., 2004; Ma et al., 2005]. BMP2 induces expression of the cardiac transcription factors Nkx2.5 and GATA-4 in the developing chick [Andree et al., 1998], and also acts upstream of the cardiac transcription factor MEF2A, activating its expression in neonatal cardiomyocytes [Wang et al., 2007]. BMP2 null mice do not express Nkx2.5 and display severe defects in heart formation including a failure to undergo looping [Zhang and Bradley, 1996]. BMP4 is expressed asymmetrically in the heart tube, and is important in cardiac looping [Breckenridge et al., 2001]. BMP4 null mice display a reduction or total lack of mesodermal differentiation, and Brachyury (a mesodermal marker) is not expressed [Winnier et al., 1995]. GATA-4 is a downstream transcriptional mediator of BMP4 signalling in the lateral mesoderm in mice [Rojas et al., 2005]. BMP4 activates Smad1, 5 and 8 when added to the outflow tract of the developing chick (E4.5), inducing expression of Sox9 and aggrecan in the semilunar valves, a transcription factor and proteoglycan characteristic of cartilage cell types [Zhao et al., 2007].

1.3.2 Receptor tyrosine kinase signalling

Receptor tyrosine kinases (RTKs) mediate growth factor signalling and are important in processes such as embryogenesis, cell proliferation, and apoptosis [Schlessinger, 2000]. Ligand binding causes receptor dimerisation, activation of tyrosine kinase activity and autophosphorylation of the intracellular domain. Phosphorylated tyrosines in this region serve as binding sites for the Src homology 2 / phosphotyrosine binding domains of signalling proteins, which are phosphorylated upon binding to become activated [Schlessinger, 2000]. This can activate several pathways including the Ras / MAPK pathway, which leads to phosphorylation and activation of transcription factors in the nucleus, regulating expression of target genes. Some examples of growth factors that signal via receptor tyrosine kinases during heart development are discussed below.

1.3.2.1 Fibroblast growth factors

Fibroblast growth factors (FGFs) are a large family of polypeptide growth factors involved in a range of cellular processes including apoptosis, cell-cell adhesion, cell migration, differentiation, and proliferation [Bottcher and Niehrs, 2005]. FGF signalling during early embryonic development is important in induction, gastrulation, and patterning of the embryo [Bottcher and Niehrs, 2005], and is required for cardiomyocyte differentiation [Zhu et al., 1996]. FGFs signal via binding to fibroblast growth factor receptors (FGFRs), a subfamily of receptor tyrosine kinases. This can lead to activation of three possible FGF signal transduction pathways – the Ras / MAPK pathway (which leads to phosphorylation and activation of target transcription factors), the PLC γ / Ca²⁺ pathway (activating protein kinase C and also stimulating intracellular Ca²⁺ release), and the PI3 kinase / Akt pathway (involved in mesoderm induction) [Bottcher and Niehrs, 2005]. FGF2 signalling plays a role in commitment of mesodermal cells to a cardiac lineage during development [Sugi et al., 1993]. FGF2 is expressed early in cardiac development in the trabeculated region of the ventricular myocardium, and is involved in the regulation of myocyte proliferation and differentiation [Consigli and Joseph-Silverstein, 1991]. FGF4 increases MAPK

phosphorylation and signalling, resulting in increased expression of genes that characterise tendon cell types in avian semilunar valves [Zhao et al., 2007]. Inhibition of the FGF signalling pathway in *Xenopus* prevents mesoderm induction. The *Xenopus* genes *sprouty2* and *spred1* have been identified as targets and mediators of FGF signalling, with roles in mesodermal specification [Sivak et al., 2005]. FGF8 is expressed in the mesoderm and participates in directing mesodermal cells to the cardiogenic cell lineage, co-operating with BMP2 in this role [Alsan and Schultheiss, 2002; Lopez-Sanchez et al., 2002; Lough et al., 1996; Zhu et al., 1996]. Cardiogenic induction is thought to be via activation of expression of cardiac transcription factors such as members of the Tbox, GATA and NK families. Both FGF8 and BMP2 are required for expression of Nkx2.5 and MEF2C [Alsan and Schultheiss, 2002]. FGF8 can also direct limb development and induces expression of Wnt2b and Wnt8c (at the forelimb and hindlimb levels respectively) in the intermediate mesoderm of the chicken embryo [Crossley et al., 1996; Kawakami et al., 2001]. FGF10 plays a crucial role in limb development, with TBX5 as an upstream regulator, and there is reciprocal regulation of TBX5 expression by FGF signalling [Ohuchi et al., 1997]. In zebrafish, FGF24 has been shown to act downstream of TBX5 to regulate FGF10 expression [Fischer et al., 2003].

1.3.2.2 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) is important in vascular development and heart morphogenesis [Tomanek et al., 2006], and plays an important role in heart valve formation [Lee et al., 2006]. VEGF signalling occurs via VEGFR1 / Flt1 and VEGFR2 / Flk1 receptors. The downstream targets of VEGF signalling are not fully characterised. The gene Down syndrome candidate region 1 (DSCR1) has been identified as one downstream mediator of VEGF signalling, with a role in endothelial cell migration and angiogenesis [Iizuka et al., 2004]. VEGF has also been shown to up-regulate Nkx2.5 in differentiated embryonic stem cells [Chen et al., 2006]. Single allelic loss of VEGF in mice results in defects in endocardial cushion formation, septation, and chamber formation [Stalmans et al., 2003]. Overexpression of VEGF

results in altered expression of eHand (an important cardiac transcription factor) and consequent defects in heart looping [Nagao et al., 2007].

1.3.2.3 Epidermal Growth Factors

Epidermal Growth Factors (EGFs) signal via the four tyrosine kinase receptors of the ErbB family, ErbB1 – ErbB4. EGF signalling is important in embryonic development, in processes such as cell proliferation and differentiation. In the heart, EGF signalling via ErbB2 and ErbB4 is important in trabeculae development [Gassmann et al., 1995; Lee et al., 1995], whilst signalling via ErbB3 is important in endocardial cushion and cardiac valve formation [Erickson et al., 1997]. Mouse knockout of heparin-binding EGF-like growth factor (HB-EGF) results in enlarged heart valves and postnatal lethality in around 50% of cases, demonstrating a role for this type of signalling in correct valve development and function [Nanba et al., 2006].

1.3.2.4 Cytokine signalling

Janus kinases (JAKs) are a family of cytosolic tyrosine kinases associated with membrane receptors that do not possess intrinsic kinase activity. Cytokine binding to these transmembrane receptors leads to receptor-kinase complex dimerisation, kinase activation and receptor phosphorylation [Heinrich et al., 1998]. This allows binding of the SH2 domain of signal transducer and activators of transcription (STATs), a family of transcription factors that become phosphorylated upon binding and translocate into the nucleus to activate transcription of target genes [Heinrich et al., 1998]. The cytokine family members Interleukin-6 (IL-6) and Leukaemia Inhibitory Factor (LIF) are important in the heart, and function through activation of the JAK / STAT pathway which leads to phosphorylation of STAT3 [Heinrich et al., 1998]. STAT3 phosphorylation (mediated by JAK1 or JAK2) results in dimerisation, nuclear translocation, DNA binding, and transcriptional activation [Heinrich et al., 1998]. MAPK also phosphorylates STAT3 at a separate site to influence its transcriptional activity [Jain et al., 1998]. STAT3 directly regulates expression of the cardiac transcription factors TBX5, GATA-4 and Nkx2.5, and is required for differentiation of

P19 cells into cardiomyocytes [Snyder et al.]. In addition, the JAK/STAT pathway is important in ischemic preconditioning [Xuan et al., 2001], with STAT3 mediating cardioprotection against ischemia/reperfusion injury [Oshima et al., 2005]. LIF promotes cardiac cell commitment to the endothelial cell lineage for neovascularisation during tissue remodelling [Mohri et al., 2006]. In the heart, IL-6 is important in cardioprotection, formation of blood vessels, and cell-cell adhesion [Fujio et al., 2004; Oshima et al., 2005; Osugi et al., 2002].

1.3.3 Wnt signalling

Wnt genes, defined by their homology to the *Drosophila wingless* gene, encode secreted lipid-modified glycoproteins that are involved in the activation of different intracellular signalling pathways via interaction with seven-transmembrane frizzled receptors [Brade et al., 2006]. There are three known Wnt signalling pathways - the canonical Wnt/ β -catenin pathway, and non-canonical Wnt/PCP and Wnt/ Ca^{2+} pathways [Brade et al., 2006]. The best characterised is the Wnt/ β -catenin pathway, in which ligand binding stabilises cytoplasmic β -catenin, allowing it to enter the nucleus for regulation of gene expression through interaction with HMG-box transcription factors [Logan and Nusse, 2004]. This pathway plays a dual role in myocardial specification, promoting cardiac differentiation in early development, and inhibiting this process at later stages [Ueno et al., 2007]. Non-canonical Wnt signalling pathways, which are also important during cardiac development, act via G-protein mediated intracellular calcium influx for activation of calcium sensitive enzymes such as protein kinase C (PKC) or calcineurin, rho family GTPases, and jun N-terminal kinase (JNK) [Veeman et al., 2003]. Wnt11, via non-canonical signalling, promotes differentiation of mesodermal cells to a cardiac lineage in early development [Eisenberg and Eisenberg, 1999]. Loss of this gene is associated with abnormal heart tube formation [Pandur et al., 2002]. In addition, this type of signalling also regulates morphogenetic movements during development through regulation of cadherin-mediated cell adhesion and polarity [Torres et al., 1996; Toyofuku et al., 2000; Ulrich et al., 2005]. A large number of Wnt ligands (Wnt2, Wnt2b, Wnt3a, Wnt5a, Wnt5b,

Wnt7a, Wnt8c, Wnt9, Wnt11) and frizzled receptors (Fz2, Fz4, Fz9) have been identified during murine and/or avian cardiac development, suggestive of distinct but potentially overlapping roles [Dealy et al., 1993; DeRossi et al., 2000; Eisenberg and Eisenberg, 1999; Hume and Dodd, 1993; Jaspard et al., 2000; Liu et al., 1999; Takada et al., 1994; van Gijn et al., 2001; Wang et al., 1999].

1.4 TRANSCRIPTIONAL REGULATION OF CARIOGENESIS AND GENE REGULATORY NETWORKS

Cardiac transcription factors play a key role in regulating expression of the many regulatory and structural genes involved in growth and patterning of the heart, and mutations in a number of important cardiac transcription factors are associated with CHDs. Members of the NK2, Tbox, GATA, MEF2, and HAND gene families form a core cardiac regulatory network which transcriptionally regulates downstream cardiac genes, and additionally, these transcription factors can also regulate expression of one another. Key members of these gene families are described in this section. The target genes of these transcription factors are largely unknown and this area is being widely researched. Figure 1.7 represents this core network and known upstream activators. Nkx2.5, GATA-4, Isl1 and Foxh1 are targets of inductive signals. Nkx2.5 and GATA-4 are upstream activators in the primary heart field, and Isl1 and Foxh1 are upstream activators in the secondary heart field [Cai et al., 2003; Dodou et al., 2004; Lin et al., 1997; von Both et al., 2004]. Cardiac transcription factors function within complex gene regulatory networks, as previously described with respect to GATA-4, -5 and -6, GATA-4 and Nkx2.5 [Peterkin et al., 2005], and overall during various developmental stages of mesendoderm formation in *Xenopus* [Loose and Patient, 2004].

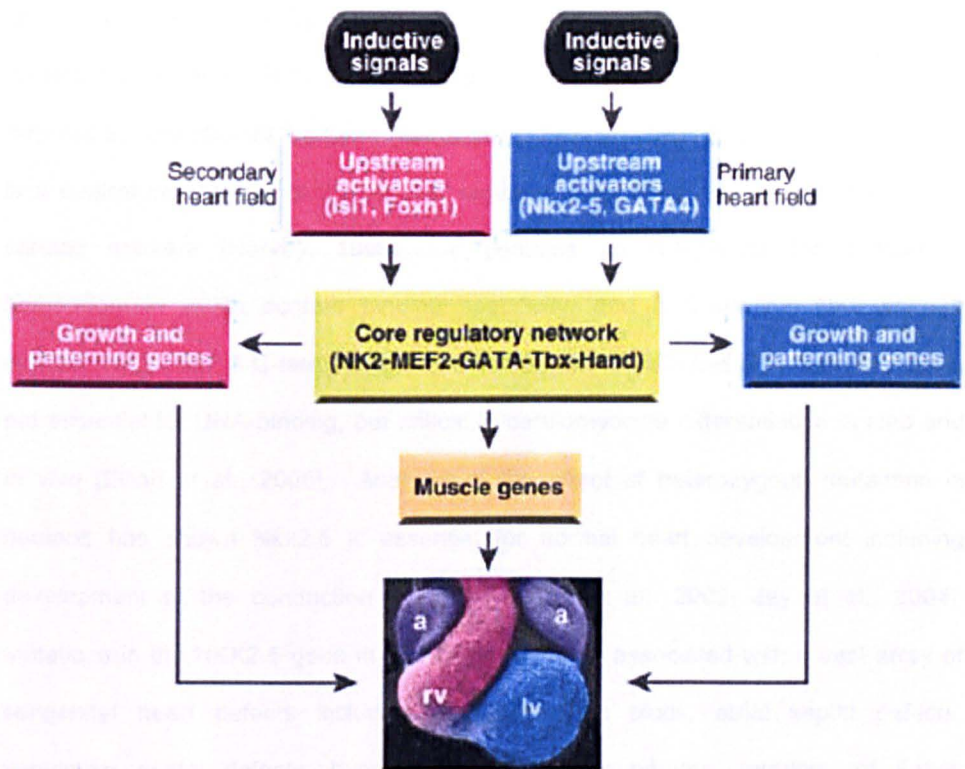


Figure 1.7 Transcriptional networks involved in heart development

The core cardiac regulatory network comprising members of the NK2, MEF2, GATA, Tbx and Hand gene families is represented with upstream activators, which are activated by inductive signals. Members of these gene families transcriptionally regulate important downstream genes (structural and regulatory), and can also regulate expression of one another. Nkx2.5 and GATA-4 are upstream activators of primary heart field formation, and Isl1 and Foxh1 are upstream activators of the secondary heart field. The scanning electromicrograph image is of a mouse heart at E14.5, and regions arising from the primary and secondary heart fields are displayed in blue and pink respectively. Regions arising from both i.e. the atria, are shown in purple. a - atrium, rv – right ventricle, lv – left ventricle. *Adapted from Olson, 2006*

1.4.1 Nkx2.5

Nkx2.5 is an evolutionarily conserved member of the NK2 class of homeodomain transcription factors. It is a homologue of the *Drosophila tinman* gene, which is required for formation of the heart [Azpiazu and Frasch, 1993; Bodmer, 1993]. Nkx2.5 is a central component of the cardiac regulatory hierarchy and one of the earliest cardiac markers [Harvey, 1996]. It contains an N-terminal TN domain, a homeodomain which confers binding specificity, and a C-terminal NK2 specific domain (NK2-SD). A C-terminal tyrosine rich domain (YRD) has also been identified, not essential for DNA-binding, but critical in cardiomyocyte differentiation *in vitro* and *in vivo* [Elliott et al., 2006]. Analysis of the effect of heterozygous mutations in humans has shown Nkx2.5 is essential for normal heart development including development of the conduction system [Harvey et al., 2002; Jay et al., 2004]. Mutations in the NKX2.5 gene in humans have been associated with a vast array of congenital heart defects including AV conduction block, atrial septal defects, ventricular septal defects, hypoplastic left heart syndrome, tetralogy of Fallot, persistent truncus arteriosus, double outlet right ventricle, and transposition of the great arteries [Benson et al., 1999; Elliott et al., 2003; McElhinney et al., 2003; Pashmforoush et al., 2004; Schott et al., 1998].

Nkx2.5 is expressed in heart precursor cells in the primary and secondary heart fields during mouse development [Komuro and Izumo, 1993], and is highly expressed in the adult heart, with the protein detectable in myocytes of the atria and ventricles [Kasahara et al., 1998]. Mouse knockout of Nkx2.5 results in an arrest in cardiac looping, leading to embryonic lethality at E9/10 [Lyons et al., 1995; Tanaka et al., 1999]. Ventricular restricted knockout has demonstrated a crucial role for Nkx2.5 in development and function of the cardiac conduction system [Pashmforoush et al., 2004], and in line with this, a transient increase in Nkx2.5 expression is seen in developing myocardial conduction cells during development of the conduction system [Thomas et al., 2001].

Nkx2.5 transcriptionally regulates expression of an array of cardiac genes including myosin light chain 2v (MLC2v), α -cardiac actin, connexin 40, myocardin, ANF, MEF2C, and eHAND [Biben and Harvey, 1997; Bruneau et al., 2001; Chen and Schwartz, 1996; Lyons et al., 1995; Tanaka et al., 1999; Ueyama et al., 2003]. Nkx2.5 interacts with several important cardiac transcription factors including some members of the GATA and T-box families, SRF, and others (section 1.5).

1.4.2 The T-box family of transcription factors

The T-box gene family encodes a group of transcription factors important in the patterning of the vertebrate embryo, and is characterised by a conserved 180 amino acid DNA-binding motif, the T-box. They regulate critical developmental processes such as cell type specification and morphogenesis, and mutations in several T-box genes lead to developmental defects.

TBX1 – *TBX5*, *TBX18* and *TBX20* are all expressed in the developing heart [Bussen et al., 2004; Chapman et al., 1996; de Lange et al., 2004; Stennard et al., 2003]. Mouse knockout studies have been carried out on each of these genes, and all result in perinatal or embryonic lethality [Bussen et al., 2004; Davenport et al., 2003; Harrelson et al., 2004; Jerome and Papaioannou, 2001; Lindsay, 2001; Lindsay et al., 2001; Stennard et al., 2005; Takeuchi et al., 2003]. *TBX1* heterozygotes display abnormal development of the aorta, pulmonary artery, and fourth pharyngeal artery [Jerome and Papaioannou, 2001; Lindsay et al., 2001], whilst heterozygotes of the other T-box genes are viable and fertile / apparently normal [Bussen et al., 2004; Davenport et al., 2003; Harrelson et al., 2004; Naiche and Papaioannou, 2003; Stennard et al., 2005]. *TBX1* is important in proliferation of cells forming the secondary heart field (which contributes to OFT formation) [Xu et al., 2004; Zhang et al., 2006]. In humans, *TBX1* haploinsufficiency results in DiGeorge syndrome [Merscher et al., 2001], a disorder whose symptoms include outflow tract defects. Mutations in *TBX3* have been associated with Ulnar Mammary syndrome [Bamshad et al., 1997], a rare disorder characterised by upper limb malformation and abnormal

development of the mammary glands, nipples, teeth, genitalia, and apocrine glands [Schinzel, 1987]. Ventricular septal defects have also been observed, therefore this gene is sometimes considered a 'heart-hand' gene [Linden et al., 2009]. *TBX4*, together with *wnt8c* and *fgf10*, is involved in development of the lower limbs [Takeuchi et al., 2003], and *TBX4* mutations are associated with small patella syndrome [Bongers et al., 2004]. There is evidence of a role for *TBX20* in promotion of cell proliferation in valve precursor cells in endocardial cushions during development [Shelton and Yutzey, 2007]. *TBX20* heterozygotes display mild dilated left ventricular cardiomyopathy [Stennard et al., 2005], and mutations in humans have been associated with cardiomyopathy and defects in septation and valve development [Kirk et al., 2007]. *TBX22* mutations are associated with cleft palate [Packham and Brook, 2003].

1.4.2.1 *TBX5*

TBX5 is a member of the T-box family of transcription factors, and plays an important role in heart development and specifying forelimb identity [Isaac et al., 1998]. Mutations in *TBX5* are associated with Holt-Oram syndrome (HOS) [Basson et al., 1997; Fan et al., 2003; Li et al., 1997], a congenital disorder characterised by upper limb and heart abnormalities. These are usually atrial septal defects (ostium primum or ostium secundum) or ventricular muscular septal defects, but other abnormalities may also occur including mitral valve defects, electrophysiological defects such as AV conduction block, tetralogy of Fallot, and left-sided abnormalities including hypoplastic left heart syndrome, defective trabeculation, and endocardial cushion defects [Basson et al., 1994; Bruneau et al., 1999; Newbury-Ecob et al., 1996; Sletten and Pierpont, 1996]. The majority of HOS associated *TBX5* mutations are found in the T-box [Mori and Bruneau, 2004].

TBX5 displays expression in the heart and upper limbs during embryogenesis [Chapman et al., 1996]. Expression is initiated early in development, where it is observed throughout the cardiac crescent and in the forelimb field [Begemann and

Ingham, 2000]. Analysis of the cardiac expression pattern of TBX5 in the developing mouse and chick has correlated its expression with the sites of defects observed in HOS; TBX5 is expressed throughout the early heart tube, later becoming restricted to the posterior regions which give rise to the sinus venosus, atria, and left ventricle [Bruneau et al., 1999]. TBX5 is subsequently expressed in the left ventricle, left side of the ventricular septum, trabeculae, and also in the atrial walls, atrial septa, and atrial side of the AV valves, consistent with the ASDs, VSDs, and left-sided malformations seen in HOS [Bruneau et al., 1999].

Studies involving TBX5 knockout / overexpression have allowed elucidation of its function in cardiogenesis. Overexpression of wild type TBX5 transcripts both *in vitro* and *in vivo* (in the embryonic chick) results in an inhibitory effect on cardiomyocyte proliferation, with a resulting decrease in heart size [Hatcher et al., 2001]. Conversely, TBX5 heterozygous null mice, in addition to ventricular and atrial septal defects, display an overall enlargement of the heart, with dilation of the right atrium and ventricle [Bruneau et al., 2001]. Complete knockout of mouse TBX5 results in embryonic lethality by E10.5; heart fails to undergo looping, and hypoplasia (underdevelopment) of the sinuatria and left ventricle occurs [Bruneau et al., 2001]. These findings indicate a regulatory role for TBX5 in chamber morphogenesis. TBX5 can interact with other transcription factors including Nkx2.5 and GATA-4, for synergistic regulation of cardiac genes (section 1.5).

1.4.3 The GATA family of transcription factors

GATA transcription factors contain two conserved zinc fingers (Cys-X₂-Cys-X₁₇-Cys-X₂-Cys) that bind to the consensus DNA sequence (A/T) GATA (A/G), a regulatory sequence first identified during studies into the tissue specific expression of erythroid-specific nuclear protein factors [Plumb et al., 1989]. Six members of the GATA family of transcription factors have been identified to date, GATA 1 – 6. Each is expressed in a lineage restricted pattern, regulating expression of genes within specific tissues and playing a major role during development. The GATA factors have been

subdivided into two groups based on their protein structure and expression pattern; GATA-1, -2 and -3 which are important in haematopoiesis and ectodermal patterning [Leonard et al., 1993], and GATA-4, -5 and -6, which are expressed in mesoderm and endoderm derived organs and are important in cardiac development [Peterkin et al., 2005]. Conservation of particular factors between species is higher than conservation of different GATA factors within the same species [Jiang and Evans, 1996] i.e. individuality of the GATA genes has been conserved through evolution implying a unique function for each.

1.4.3.1 GATA-4

Mutations in the human GATA-4 gene have been associated with atrial and ventricular septal defects [Garg et al., 2003], and tetralogy of Fallot [Nemer et al., 2006]. GATA-4 contains two zinc finger domains, a C-terminal nuclear localisation sequence (for DNA-binding and protein-protein interactions), and two N-terminal transcriptional activation domains (TAD), that are conserved within the GATA-4/5/6 subfamily [Morrissey et al., 1997b]. Expression of GATA-4 is seen in the cardiac ventral mesoderm at the time of linear heart tube formation, and subsequently in the developing atria and ventricles, and in the endocardium but not myocardium [Kelley et al., 1993].

A number of knockdown / knockout studies have been performed to assess the specific functions of GATA-4 in cardiogenesis. The P19 mouse embryonal carcinoma cell line can be induced to differentiate into beating cardiac muscle cells and is thus a widely used *in vitro* model system of cardiac cell differentiation. The effect of a reduction in GATA-4 protein has been investigated in this cell line through stable transfection with GATA-4 antisense expression vectors [Grepin et al., 1995]. Presence of antisense transcripts results in a marked reduction in active GATA-4 protein, but has no effect on the morphology of the cells. However, antisense clones displayed very poor differentiation in comparison to the wild type and control clones, indicating GATA-4 is required for differentiation of cardiac cells. In addition, clones

also failed to express important cardiac muscle markers such as BNP, TpC, α -MHC, β -MHC, and MLC-1A, consistent with the theory that GATA-4 is essential for activation of cardiac cell differentiation.

GATA-4 null mouse embryos do not survive past early embryonic development, arresting at E7.0 – E9.5 due to defects in ventral morphogenesis and linear heart tube formation [Molkentin and Olson, 1997]. This means they cannot be used to study GATA-4 function later in development or in the adult heart. This has been overcome through the use of Cre/LoxP technology to conditionally inactivate GATA-4 expression in the mouse myocardium at selected time points in order to study its role in cardiac development [Zeisberg et al., 2005]. *GATA-4-loxP*-targeted mice were crossed with mice containing either an *Nkx2-5* locus or an α -MHC promoter that drives Cre recombinase expression early or late in development respectively. Early myocyte restricted deletion of GATA-4 by *Nkx2-5^{Cre}* showed no effect on overall development at E9.5. Whole mount examination of embryos at E9.5 showed cardiac malformations, most commonly a single predominant ventricular chamber. Histological sections showed myocardial hypoplasia, and small atrioventricular and OFT endocardial cushions, suggesting a defect in the EMT of endocardial cells. This was confirmed by selective inactivation of GATA-4 in endothelial derived cells which resulted in failure of endothelium to undergo EMT resulting in hypocellular cushions i.e. GATA-4 is required to form AV cushion mesenchyme which is essential in valve development [Rivera-Feliciano et al., 2006]. GATA-4 is also required subsequently in the remodelling of AV endocardial cushions for ventricular septation. At E10.0, embryos displayed a mild delay in development and pericardial effusions which signify heart failure. Embryonic lethality occurred by E11.5. *GATA-4-loxP*-targeted mice (*Gata4^{fl/fl}*) have also been crossed with mice containing an α -MHC or β -MHC promoter driven Cre transgene to study the function of GATA-4 in the adult heart [Oka et al., 2006]. The resulting mice survived into adulthood despite a 70–95% reduction in GATA-4 protein. Cardiac function was compromised as a result of GATA-4 deletion from the heart; the cardiac hypertrophic response was attenuated, cardiac cell death

increased, and basal gene expression significantly altered. It was concluded that GATA-4 transcriptionally regulates gene expression, hypertrophy, stress compensation, and myocyte viability in the heart.

1.4.3.2 GATA-5

GATA-5 is expressed in the precardiac mesoderm from E7.0 / E8.0, in the atrial and ventricular chambers of the heart at E9.5, and is restricted to the atrial endocardium by E12.5 [Morrisey et al., 1997a]. It is also expressed in the developing lung, urogenital ridge, bladder and gut. The expression pattern of GATA-5 shows marked differences to that of GATA-4 and GATA-6, and unlike GATA-4 and GATA-6 null mice which do not survive into adulthood, GATA-5 null mice are viable and fertile [Molkentin et al., 2000]. Females display defects in the genitourinary system (males are unaffected) suggesting a unique role for GATA-5 in development of this region. One explanation for the mild phenotype is that GATA-4/6 may compensate for the loss of GATA-5.

1.4.3.3 GATA-6

Like *GATA4*^{-/-} mice, *GATA6*^{-/-} mice also fail to develop beyond gastrulation due to its necessity in the visceral endoderm [Koutsourakis et al., 1999; Morrissey et al., 1998]. This has similarly been overcome through the use of Cre / LoxP technology to create a conditional loss of function allele [Sodhi et al., 2006]. Conditional inactivation of GATA-6 *in vivo* in VSMCs and in the cardiac neural crest results in cardiovascular abnormalities including aortic arch and outflow tract defects, indicating a role for GATA-6 in patterning of these regions [Lepore et al., 2006].

As mice heterozygous for GATA-4 or GATA-6 null mutations appear to be normal, and GATA-4 and GATA-6 have a similar protein structure and expression pattern, it was hypothesised that they may act co-operatively during cardiac development. This was tested by crossing *GATA-4*^{+/-} and *GATA-6*^{+/-} heterozygotes to produce compound heterozygotes [Xin et al., 2006]. Lethality with complete penetrance occurred at

E13.5. E10.0 embryos displayed enlargement and disorganisation of cranial and intersomitic vasculature suggesting defects in vessel development. Furthermore, mutants displayed failure of septation of the cardiac outflow tract, thinning of the myocardium, and vascular defects. Expression of some cardiac genes was altered, for example β -MHC and MEF2C were down-regulated. This demonstrates that a certain level of GATA-4 and GATA-6 expression is required for cardiovascular development.

1.4.4 The MADS family of transcription factors

The MADS family of transcription factors contain a 57 amino acid 'MADS box' which is important in homodimerisation and DNA-binding [Shore and Sharrocks, 1995]. Members of the MADS family can recruit other transcription factors to form regulatory complexes, which are important in regulation of target genes [Shore and Sharrocks, 1995; Sprague, 1990]. Relevant members of this family are described below.

1.4.4.1 Serum Response Factor (SRF)

Serum response factor (SRF) is a member of the MADS family that was initially discovered as an activator of growth factor and cell cycle genes, now known to be highly expressed in differentiating cardiac, skeletal and smooth muscle cells [Belaguli et al., 1997]. SRF is a key regulator of the expression of number of contractile genes, acting through binding to the CArG box (CC(A/T)₆GG) [Belaguli et al., 1997]. The role of SRF in cardiovascular development has been studied by cardiac and smooth muscle cell-restricted inactivation in Cre recombinase mice [Miano et al., 2004]. Inactivation of the transgene at E8.5 in mice leads to progressively decreasing levels of SRF expression in cardiomyocytes and aortic smooth muscle cells, and poorly developed trabeculations and sarcomeric disorganisation at E10.5. Studies of SRF null mice also show that it is required for cardiac mesoderm formation [Arsenian et al., 1998]. Myocardin is a member of the SAP (SAF-A/B, Acinus, PIAS) domain family of nuclear proteins which have broad regulatory functions relating to transcription and chromatin organisation [Aravind and Koonin, 2000; Kipp et al., 2000]. Myocardin

associates with SRF to act as a highly potent transcriptional co-activator, and has an important function in cardiomyocyte differentiation [Wang et al., 2001]. Myocardin is expressed in cardiac and smooth muscle cells throughout the heart both during development (beginning at the same time as Nkx2.5) and postnatally [Wang et al., 2001], contributing to the muscle specificity of SRF [Wang et al., 2003b]. Mouse knockout of myocardin leads to embryonic lethality by E10.5, with mice displaying developmental delay and severe vascular defects [Li et al., 2003]. Interestingly, abnormalities are smooth muscle cell specific and cardiac morphogenesis is normal, possibly due to functional redundancy with additional myogenic regulators in cardiac muscle cells.

1.4.4.2 The myocyte enhancer factor (MEF) family of transcription factors

Myocyte enhancer factors 2 (MEF2s) belong to the MADS family of transcription factors and contain a conserved N-terminal MADS box and MEF2 domain, and C-terminal transactivation domain. Vertebrates possess four MEF2 genes, MEF2A – MEF2D, thought to have arisen from a common ancestral gene. They act as homo- or heterodimers and bind to the sequence CAT(A/T)₄TAG/A [Black and Olson, 1998; McKinsey et al., 2002], which has been identified in the regulatory region of a number of muscle genes for transcriptional control of muscle development. Targets include α -MHC [Molkentin and Markham, 1993], MLC1/3 [McGrew et al., 1996; Rao et al., 1996], MLC2v [Lee et al., 1994], α -skeletal actin [Muscat et al., 1992], tropomyosin I [Lin et al., 1996], cardiac troponin T [Wang et al., 1994], cardiac troponin C [Gahlmann and Kedes, 1993; Parmacek et al., 1994], cardiac troponin I [Nakayama et al., 1996], desmin [Kuisik et al., 1996; Li and Capetanaki, 1994], and dystrophin [Klamut et al., 1997; Nishio et al., 1994].

1.4.4.2.1 MEF2C

MEF2C is expressed early in cardiac development, detectable in the cardiac mesoderm (from which the heart tube originates) at E7.5, and in the myocardium at E8.5 [Edmondson et al., 1994]. MEF2C knockout mice die by E9.5 as a result of

cardiac and vascular defects [Lin et al., 1997]. Conditional $mef2c^{loxP/loxP}$ knockout in mice results in failure of hearts to undergo looping, absence of right ventricle formation, and lack of expression of a subset of cardiac muscle genes, highlighting its importance in early heart development [Lin et al., 1997]. *Nkx2.5* and *MEF2C* have an up-regulatory effect on the expression of one another and induce cardiomyocyte differentiation on P19 cells [Skerjanc et al., 1998]. *MEF2C* is involved in a physical interaction with *TBX5* which is essential in cardiogenesis [Ghosh et al., 2009].

1.4.4.2.2 MEF2A

MEF2A is expressed in the myocardium during mouse embryogenesis from E8.5 [Edmondson et al., 1994]. It is involved in cardiac development, and mouse knockout of this gene results in right ventricular dilation and an increased likelihood of sudden perinatal death due to conduction defects, thought to result from a mitochondrial deficiency in cardiac muscle [Naya et al., 2002]. A deletion in exon 11 of the human *MEF2A* gene was found to be associated with coronary artery disease (CAD) in a large family of sufferers [Wang et al., 2003a], and a number of missense mutations in this gene were subsequently identified in sporadic CAD patients [Bhagavatula et al., 2004; Gonzalez et al., 2006; Weng et al., 2005].

1.4.5 The HAND family of transcription factors

The *HAND* gene family are a group of basic helix-loop-helix cardiac transcription factors. *dHand* and *eHand* are co-expressed in the precardiac mesoderm at E7.75, and later display regionally restricted expression in the ventricle forming regions of the heart tube; *dHand* is expressed in the right ventricle forming regions, and *eHand* in the left ventricle forming regions [Srivastava et al., 1997].

Gene deletion of *dHand* results in embryonic lethality by E10.5, and a wide spectrum of abnormalities are observed in cardiac looping, septation, and ventricular formation [Srivastava et al., 1997]. Transgenic mice expressing *dHand* under control of the β -MHC promoter show it is essential for interventricular septum formation and

trabeculation during heart development [Togi et al., 2006]. dHand upregulates ANF, BNP and connexin 40 expression in H9c2 cells and also in transgenic embryos overexpressing dHand. Targeted deletion of dHand in CNC derived cells causes misalignment of the aortic arch arteries and outflow tract, resulting in DORV and VSDs [Holler et al.].

Mice homozygous for an eHand allele die at E8.5 – E9.5 with defects in heart looping, hypoplasia of the left ventricle, and outflow tract abnormalities, demonstrating the importance of this gene in cardiac development [Firulli et al., 1998]. In humans, mutations in the eHand gene are associated with septal defects, outflow tract abnormalities, and hypoplasia [Reamon-Buettner et al., 2008; Reamon-Buettner et al., 2009]. This left-sidedness in expression of eHand is abolished in mouse embryos lacking Nkx2.5, showing Nkx2.5 plays a role in control of this asymmetric expression [Biben and Harvey, 1997].

1.5 CO-OPERATIVE ACTIVITY OF CARDIAC TRANSCRIPTION FACTORS

A number of important cardiac transcription factors physically interact to direct and synergistically regulate important pathways and key genes.

TBX5 and GATA-4 form a complex, and the *TBX5* mutations G80R and R237W, which are associated with cardiac septal defects in HOS patients [Basson et al., 1999], disrupt this interaction [Garg et al., 2003]. Functional analysis of a number of other *TBX5* mutations found in HOS has shown diminished binding of TBX5 to both GATA-4 and Nkx2.5, and a resulting decrease in transcriptional activation of ANF and FGF10 [Boogerd et al.]. The *GATA-4* mutation G296S has been linked to ASDs in humans, and also disrupts the TBX5 / GATA-4 interaction [Garg et al., 2003]. These findings together account for the fact that similar heart defects are seen as a result of both GATA-4 and TBX5 mutations, and indicate that these transcription factors direct and synergistically regulate common pathways important in septation. *Gata4*^{+/-} *Tbx5*^{+/-} compound heterozygous mice were generated during the time of this research (2009) and displayed embryonic lethality with almost full penetrance [Maitra et al., 2009]. Mice displayed defects in AV septation and myocardial thinning. *MYH6*, encoding α -myosin heavy chain, is a joint target of TBX5 and GATA-4 [Ching et al., 2005; Huang et al., 1995; Molkenstein et al., 1994], and is down-regulated in *Gata4*^{+/-} *Tbx5*^{+/-} compound heterozygote mice [Maitra et al., 2009]. Brahma-associated factor (BAF) complexes belong to the SWI/SNF family of multi-subunit chromatin remodelling complexes which are important developmentally and can act both as activators and repressors of transcription [Ho and Crabtree]. BAF complexes can interact with a variety of transcription factors in different cell or tissue types, and show context-specific functions depending on their interaction partners [Ho and Crabtree]. Baf60c, a cardiac-specific subunit of BAF chromatin-remodelling complexes, acts by physically linking transcription factors to these complexes during cardiogenesis and is necessary for their function here [Lickert et al., 2004]. TBX5 and GATA-4, in combination with Baf60c (which is essential), can induce a full cardiac transcriptional programme and direct ectopic differentiation of the non-cardiogenic posterior mesoderm and

extraembryonic mesoderm of the amnion (of mouse) into beating cardiomyocytes [Takeuchi and Bruneau, 2009]. Interestingly, TBX5, GATA-4 and MEF2C in combination are able to reprogramme mouse postnatal fibroblasts into functional cardiomyocytes [Ieda et al.].

Tbx5 and Nkx2.5 specifically associate with one another via the N-terminal domain and N-terminal portion of the T-box of TBX5, and the homeodomain of Nkx2.5 [Hiroi et al., 2001]. This interaction is crucial for cardiomyocyte differentiation. TBX5 and Nkx2.5 directly bind to the ANF promoter for synergistic activation [Bruneau et al., 2001; Ghosh et al., 2001; Hiroi et al., 2001]. The *TBX5* mutations G80R and R237Q are both associated with HOS, and both disrupt the interaction with GATA-4 [Garg et al., 2003]. The former mutation results in a severe cardiac phenotype, whilst the latter results primarily in upper limb abnormalities with little effect on the heart [Basson et al., 1999]. The G80R mutation has an inhibitory effect on cardiomyocyte differentiation, and results in significantly diminished activation of the ANF promoter (with Nkx2.5), whilst the R237Q mutation has little or no effect on these, in line with the vast difference in severity of cardiac malformations these mutations underlie [Hiroi et al., 2001]. In a separate study, both mutations have been reported to eliminate target site binding [Ghosh et al., 2001].

TBX5 and MEF2C associate via their DNA binding domains to synergistically activate the MYH6 promoter [Ghosh et al., 2009]. TBX5 and Nkx2.5 act co-operatively to activate expression of connexin 40 [Bruneau et al., 2001], a gap junction protein crucial in conduction in the heart [Kirchhoff et al., 1998; Simon and Goodenough, 1998], suggesting one possible explanation for the conduction abnormalities seen in HOS. Purkinje fibres of the cardiac conduction system express high levels of Nkx2.5 and GATA-4 [Bruneau, 2002; Thomas et al., 2001]. Nkx2.5, GATA-4, and TBX5 act together with the nuclear factor Sp1 to regulate expression of connexin 40 via elements in its minimal promoter region [Linhares et al., 2004]. Nkx2.5 and GATA-4 are able to synergistically activate the promoter, whilst TBX5 counteracts this effect.

TBX20 directly associates with Nkx2.5 and GATA-4 [Stennard et al., 2003] and is also able to interact with the connexin 40 promoter. It represses activation by Nkx2.5 and GATA-4, whilst acting as an activator when the same reporter is stimulated with Nkx2.5 and GATA-5. This demonstrates that Tbx20 can function as a transcriptional activator or repressor depending on its binding partner. TBX20 also associates with TBX5, activating or repressing ANF promoter activity in a concentration dependent manner [Brown et al., 2005].

Mutations in both GATA-4 and Nkx2.5 are associated with familial ASD [Hirayama-Yamada et al., 2005]. Nkx2.5 and GATA-4 associate via helix III of the homeodomain of Nkx2.5 and the C-terminal zinc finger of GATA-4 [Lee et al., 1998]. These transcription factors can activate ANF individually, and in combination for synergistic activation in a manner which requires full transcriptional activity of both (including co-factor activity). The ANF promoter contains binding sites for TBX5, Nkx2.5, GATA (proximal and distal), and SRF [Small and Krieg, 2003]. Mutation of the TBX5, SRF or distal GATA site results in a strong reduction in ANF promoter activity. Mutation of the Nkx2.5 and proximal GATA site singly and together results in persistent expression in the ventricles and outflow tract. This suggests a repressor complex may bind to these sites for atrial specific ANF expression.

Nkx2.5 and SRF together stimulate promoter activity of α -cardiac actin, and the presence of serum response elements (SREs) is crucial [Chen et al., 1996]. The C-terminal and MADS box of SRF are required for activation with Nkx2.5, and Nkx2.5 alone has no effect. Nkx2.5 and SRF, with GATA-4, also synergistically activates the α -cardiac actin promoter [Sepulveda et al., 2002]. SRF and GATA-4, in combination with mutant Nkx2.5 which lacks binding capability, are able to activate this promoter. However, Nkx2.5, GATA-4 and mutant SRF (which lacks binding capability) are unable to activate the promoter, and sequential deletion of the four SRE sites in this region results in corresponding decreases in promoter activity. These findings

together demonstrate that SRF binding is necessary for recruitment of Nkx2.5 and GATA-4 to the α -cardiac actin promoter via a physical interaction. In addition, DNA recruitment assays have demonstrated that Nkx2.5 and GATA-4 together strongly enhance the formation of SRF dependent promoter binding complexes, possibly through conformational changes that increase SRF DNA binding efficiency [Sepulveda et al., 2002]. Combinatorial expression of these three transcription factors also synergistically activates the ANF gene in a co-operative manner [Nishida et al., 2002].

1.6 THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) consists of cells such as fibroblasts, collagens, glycoproteins, proteoglycans, growth factors, and proteases that form a dynamic macromolecular complex which plays an important role in cardiac development and growth by providing structural support for cells and facilitating intercellular communication. Throughout formation of the heart, the processes of cell differentiation, adhesion, migration, and apoptosis are crucial for tissue remodelling and occur in a precisely timed and spatially regulated manner, mediated extensively through signalling that occurs via the ECM. Additionally, ECM composition has an impact on the process of EMT, which is important in cardiac remodelling at various developmental stages including gastrulation, endocardial cushion formation, valvulogenesis, and neural crest cell differentiation (and also in chronic disease states such as hypertension and heart failure). Defects in the remodelling process can lead to loss of organisation and abnormalities such as valve dysfunction and abnormal septation. Components of the ECM and their specific functions are discussed below.

Non-myocyte cells make up approximately 70% of the heart cell population [Nag, 1980; Zak, 1974] and include cardiac fibroblasts, the proportion of which varies depending on species, developmental age and physiological condition [Banerjee et al., 2007]. Fibroblasts are connective tissue cells that synthesise and deposit various ECM components including collagens and fibronectin [Fries et al., 1994] in addition to secreting cytokines, growth factors, and matrix metalloproteinases [Powell et al., 1999]. Cardiac fibroblasts are interconnected to form a network of cells within a collagen matrix, which serves to provide structural integrity through cell-cell and cell-ECM interactions [Baxter et al., 2008].

Collagens are a family of fibrous proteins that are important in maintaining tissue structure. Collagens I, III, IV, V, and VI have been identified in the heart where they each have specific functions [Schaper and Speiser, 1992; Speiser et al., 1991; Speiser et al., 1992]. Collagens I and III account for over 90% of total collagens and

are important in maintaining tissue and blood vessel structure, whilst collagens IV and VI form part of the basal lamina, and allow interaction with receptors such as integrins [Weber, 1989].

Integrins are large transmembrane ECM receptors consisting of α and β subunits, a large extracellular domain, and a small cytoplasmic signalling domain [Hynes, 1992]. They are essential in interaction with cardiac fibroblasts and myocytes, and mediate cell-cell and cell-ECM communication bidirectionally to regulate cell migration and survival during development and in pathological hypertrophy [Bowers et al.]. Integrins act as receptors for a broad range of ligands which include collagen, laminin and fibronectin [Humphries et al., 2006].

Laminins are glycoproteins that are synthesised by cardiac myocytes, vascular smooth muscle cells (VSMCs), and endothelial cells, and are located in the basement membrane of cardiomyocytes [Corda et al., 2000; Jane-Lise et al., 2000]. Laminins contain binding domains for ECM components, and mouse knockout of laminin subunits results in cardiac abnormalities [Jane-Lise et al., 2000].

Fibronectin is an adhesive extracellular matrix glycoprotein involved in cytoskeletal organisation, cell adhesion, cell migration, and cell spreading [Smith et al., 1990]. Fibronectin associates with multiple ECM components and is crucial during development, with loss of this gene in mice resulting in severe developmental defects to the entire body including the heart [George et al., 1993].

Periostin is an ECM protein most often secreted by fibroblast or fibroblast-like cells, which can bind to several integrin receptors and also interact with ECM proteins such as collagens and fibronectin. During development, the expression pattern of periostin follows that of endocardial cushion and cardiac valve formation [Lindsley et al., 2005], and loss of this gene in mice results in valve defects [Norris et al., 2008]. Periostin is also involved in cardiac hypertrophy and remodelling [Borg and Caulfield, 1980].

Matrix metalloproteinases (MMPs) are a family of secreted zinc-dependent enzymes which are capable of ECM degradation and are therefore important developmentally and during pathologic remodelling in cardiovascular disease [Nagase and Woessner, 1999]. MMPs can be classified into four groups according to their substrate specificity; collagenases (MMP-1 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3), and membrane-type MMPs (MT1-MMP) [Fedak et al., 2005]. MMPs also have regulatory functions during normal ECM protein processing [Mann and Spinale, 1998].

1.7 THE CONTRACTILE APPARATUS

Contraction of the heart is dependent upon expression of a set of sarcomeric and Z-disc proteins that form the contractile apparatus, represented in Figure 1.8. The sarcomeric thick filament is composed of myosin heavy chains, myosin light chains, myosin binding protein C, and titin. Actins and troponins make up the thin filament. In addition to sarcomeric proteins having an important role in the structure and contractility of the heart, they are now also known to be important in developmental processes such as cardiac septation. Mutations in sarcomeric proteins have been linked to various cardiomyopathies and congenital cardiovascular malformations such as ASDs, VSDs, PDA, and Ebstein anomaly. The mechanism by which mutations in sarcomeric genes can lead to CHDs such as atrial septal defects is unknown. Genes that are mutated in human cardiovascular conditions are represented in Figure 1.8, and relevant muscle genes are described in this section.

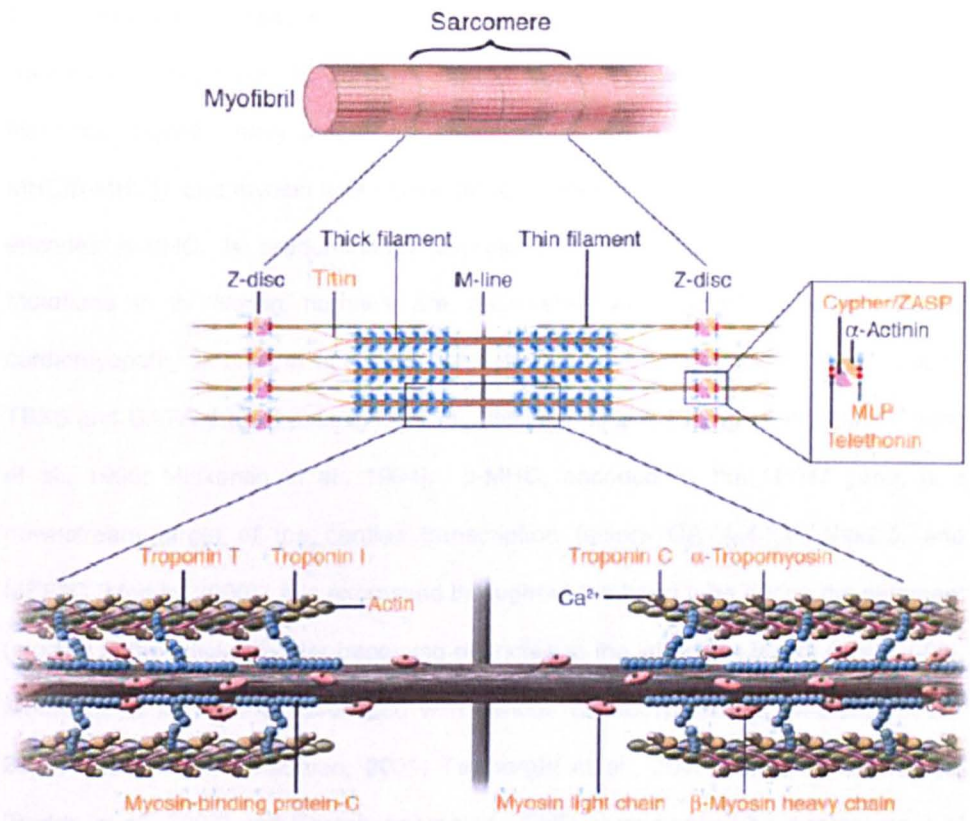


Figure 1.8 Sarcomeric and Z-disc proteins

The sarcomeric thick filament is composed of myosin heavy chains, myosin light chains, myosin binding protein C, and titin. Actins and troponins make up the sarcomeric thin filament. Genes that are mutated in human cardiovascular conditions are shown in orange. *Adapted from Morita, 2005*

1.7.1 Myosin heavy chains

Sarcomeric complexes in cardiac muscle contain myosin thick and actin thin filaments. Myosin heavy chains (comprising alpha- and beta-myosin heavy chain (α -MHC/ β -MHC)), and myosin light chains (MLC) form the thick filaments. *MYH6*, which encodes α -MHC, is predominantly expressed in the atria [Somi et al., 2006]. Mutations in *MYH6* in humans are associated with hypertrophic and dilated cardiomyopathy [Morita et al., 2005], and atrial septal defects [Ching et al., 2005]. TBX5 and GATA-4 are transcriptional regulators of *MYH6* [Ching et al., 2005; Huang et al., 1995; Molkentin et al., 1994]. β -MHC, encoded by the *MYH7* gene, is a downstream target of the cardiac transcription factors GATA-4/5/6, Nkx2.5, and MEF2C [Morkin, 2000]. It is expressed throughout the heart tube during development (studied in the chicken), later becoming restricted to the ventricles [Somi et al., 2006]. Mutations in *MYH7* are associated with various cardiomyopathies [Meredith et al., 2004; Seidman and Seidman, 2001; Tajsharghi et al., 2003], atrial septal defects [Budde et al., 2007] and Ebstein anomaly (a CHD characterised by displacement of the tricuspid valve leaflets) [Kaneda et al., 2008].

1.7.2 Myosin light chains

Myosin light chains are a component of the sarcomeric thick filament and can be classified as either essential or regulatory [Franco et al., 1998]. Regulatory myosin light chains are subject to phosphorylation which influences their sensitivity to calcium during muscle contraction, thereby modulating this process [Szczesna et al., 2002]. These categories can be subdivided into fast and slow isoforms, and multiple isoforms also exist. Three essential isoforms are expressed in the heart; MLC3f (a fast isoform), MLC1V (expressed in the ventricular myocardium), and MLC1a (expressed in the atrial myocardium) [Hailstones and Gunning, 1990; Kelly et al., 1995; Lyons et al., 1990; McGrew et al., 1996]. Three regulatory isoforms are expressed in the heart during cardiac development; MLC2a (expressed in the atrial myocardium), MLC2v (expressed in the ventricular myocardium), and MLC2f (a fast isoform that is transiently expressed in the heart during embryonic development) [Faerman and

Shani, 1993]. Mutations in essential myosin light chain (*MYL3*) and regulatory myosin light chain (*MYL2*) result in hypertrophic cardiomyopathy (HCM) [Bos et al., 2007; Burkett and Hershberger, 2005; Morita et al., 2005].

1.7.3 Actins

Actins are a major component of the sarcomeric thin filament. There are four isoforms of actin occurring in muscle; the striated α -skeletal and α -cardiac actins which are the primary isoforms in skeletal and cardiac muscle respectively, and α - and γ - smooth muscle actins, primarily expressed in smooth muscle cells. Alpha-smooth muscle (α -SM) actin is the earliest expressed actin isoform in the developing heart [Ruzicka and Schwartz, 1988]. Mouse knockout of the α -SM actin gene *ACTA2* results in impaired vascular contractility and blood flow [Schildmeyer et al., 2000; Tomasek et al., 2006]. Mutations in the human *ACTA2* gene are associated with a variety of vascular diseases such as coronary artery disease (CAD), ischemic strokes, Moyamoya disease (MMD), and thoracic aortic aneurysms and dissections (TAAD) [Guo et al., 2009]. PDA has also been reported in patients with *ACTA2* mutation [Guo et al., 2009]. The striated α -skeletal and α -cardiac actins become the predominantly expressed actin isoforms in the heart following dextral looping [Ruzicka and Schwartz, 1988]. Missense mutations affecting conserved amino acids in the cardiac actin gene *ACTC* have been identified in two separate families with idiopathic dilated cardiomyopathy (IDC) [Olson et al., 1998], and mutations in this gene are also associated with atrial and ventricular septal defects [Matsson et al., 2008; Monserrat et al., 2007].

1.7.4 Tropomyosin

Tropomyosin (TM) is a dimeric α -helical coiled actin-binding protein, important as a contractile component of the sarcomeric thin filament and necessary for myocardial function [Perry, 2001]. More than twenty isoforms of tropomyosin have been identified in mammalian species, encoded by four genes, *TPM1* – *TPM4* in humans, which give rise to α -, β -, γ -, and δ -tropomyosin respectively [Pittenger et al., 1994]. These are

expressed predominantly in skeletal, smooth, and cardiac muscle, and at lower levels in non-muscle cytoskeletal and actin associated cells [Pittenger et al., 1994]. Familial hypertrophic cardiomyopathy has been linked to missense mutations in TPM1 which encodes alpha-tropomyosin [Thierfelder et al., 1994]. E40K and E54K mutations in α -tropomyosin cause dilated cardiomyopathy due to decreased sensitivity to Ca^{2+} [Mirza et al., 2007]. Alpha-tropomyosin null mice do not survive past E11.5, whilst heterozygous mice are viable and able to reproduce but display a 50% reduction in cardiac muscle α -TM mRNA, with no differences in myocardial and myofilament function between these and control mice [Rethinasamy et al., 1998].

1.7.5 Troponins

Cardiac troponin is located along the actin thin filament, and the troponin complex comprises three troponin subunits, cardiac troponin C (cTnC), cardiac troponin T (cTnT), and cardiac troponin I (cTnI) which are involved in striated muscle contraction via interaction with Ca^{2+} [Farah and Reinach, 1995]. Each of these subunits has a distinct role; cTnC acts as a Ca^{2+} sensor, cTnT binds to tropomyosin and transmits the Ca^{2+} binding signal from troponin to tropomyosin, and cTnI is inhibitory and prevents actin and myosin association in the absence of Ca^{2+} [Farah and Reinach, 1995]. Mutations in cTnI and cTnT are associated with restrictive cardiomyopathy [Knollmann et al., 2003].

1.8 CARDIAC MODEL SYSTEMS RELEVANT TO THIS THESIS

1.8.1 The P19 cell line as a model of cardiomyocyte differentiation

Cardiac cell lines have been used extensively to model cardiomyocyte differentiation and gene regulation. The P19 mouse embryonal carcinoma cell line is pluripotent and can be induced to differentiate by the addition of non-toxic drugs. Retinoic acid induces differentiation to neuroectodermal derived cell types, and dimethyl sulfoxide (DMSO) induces differentiation to endodermal and mesodermal derived cell types such as skeletal and cardiac muscle cells [McBurney, 1993]. Addition of DMSO at 0.5 – 1% (v/v) induces the formation of beating cardiomyocytes at days 6 – 7, thus the P19 cell line provides an excellent *in vitro* model of early development and cardiomyocyte differentiation and is widely used. It has previously been used in a number of functional studies looking at the effect of a reduction or increase in expression of cardiac genes such as TBX5 and GATA-4 [Fijnvandraat et al., 2003; Grepin et al., 1995; Hiroi et al., 2001].

1.8.2 The developing chick as a model of cardiogenesis

The chicken embryo is one of the most widely used non-mammalian vertebrate models of development. Chickens have a short gestational period of 21 days, and development occurs *in ovo* rather than *in utero*, which means ease of manipulation with particular advantages for temporal studies. Complete sequencing of the chicken genome [2004] and the availability of expressed sequence tag (EST) libraries mean sequences are readily available and can be utilised for technologies such as morpholino antisense knockdown in order to study gene function. Additionally, avian cardiac development is very similar to human cardiac development. The primary difference occurs during atrial septation, where a septum secundum forms in the human but not the chicken. This means that ostium primum defects that arise from defects in the septum secundum cannot be modelled. Since this study will be focusing on defects of the septum primum, this is not a major concern.

1.9 BACKGROUND TO THIS THESIS

Holt-Oram syndrome (HOS) is a human genetic disorder associated with mutations in the *TBX5* gene [Li et al., 1997], characterised by upper limb deformities and heart defects, usually in atrial or ventricular septation [Lehner et al., 2003]. The *TBX5* mutations G80R and R237W, which are associated with cardiac septal defects in HOS patients [Basson et al., 1999], disrupt the physical interaction between *TBX5* and *GATA-4* [Garg et al., 2003]. The *GATA-4* mutation G296S has been linked to ASDs in humans, and also disrupts the *GATA-4* / *TBX5* interaction [Garg et al., 2003]. These findings together account for the fact that similar heart defects are seen as a result of both *GATA-4* and *TBX5* mutations, and highlight the potential importance of this complex in synergistic regulation of genes involved in septation of the heart. Identification of these downstream targets is crucial in gaining an understanding of the mechanism via which mutations in these transcription factors lead to heart defects. *TBX5* and *GATA-4*, and their combined downstream targets are the focus of this thesis.

This study is based on the work of Jonathan Ronksley (a former PhD student in Prof J David Brook's group) who aimed to identify genes co-ordinately regulated by *TBX5* and *GATA-4* using the P19CL6 mouse embryonal carcinoma cell line as a model of cardiac development [Ronksley, 2007]. The full length human *GATA-4* gene was cloned into a pcDNA3.1/Zeo vector and used to create cell lines overexpressing *GATA-4*. The full length human *TBX5* gene was then additionally cloned in to create double overexpression cell lines. Overexpression of *TBX5* and *GATA-4* was confirmed by western blot analysis and ANF luciferase reporter assays. Two P19 *TBX5* / *GATA-4* overexpression cell lines were selected for further analysis, named G10 and G22. Microarray expression analyses were then performed using the Ocumum Biosolutions Mouse 1400 pmol 30K V2 Oligo set (A/B/C), which contains 50-base pair oligonucleotide probes (1 per gene) for detection of 27 530 targets. A dye swap method (Cy3 and Cy5) was employed in order to eliminate dye bias.

For identification of downstream targets of TBX5 and GATA-4, microarray expression analysis of TBX5 / GATA-4 overexpression cell line RNA in comparison to wild type RNA (at day 0) was carried out. This revealed 80 up-regulated, and 154 down-regulated genes (by >2 fold on 3 out of 4 replicate arrays). Surprisingly, ANF and connexin 40 (which are known targets of TBX5 and GATA-4) could not be detected in any of the wild type or overexpression arrays. However, cardiac troponin I, which is synergistically regulated by TBX5 and GATA-4 (Dr Tushar Ghosh, unpublished data), and alpha cardiac actin (a predicted target of TBX5) [Ghosh et al., 2001] were up-regulated post-differentiation in response to overexpression of TBX5 and GATA-4.

For additional expression profiling of wild type P19 cell differentiation, cells were induced to differentiate by addition of DMSO, and RNA extracted at days 0, 2, 5, 7 and 10. This provided a means to study expression of genes of interest during normal differentiation into cardiomyocytes. QPCR analysis of TBX5 and GATA-4 expression was also carried out during wild type P19 cell differentiation, and revealed significant up-regulation of GATA-4 on days 2, 5 and 7. TBX5 expression was not detectable at day 2, and displayed significant up-regulation on days 5 and 7.

A subset of genes was chosen for further study based on expression changes and current literature. Six down-regulated genes are shown in Table 1.1, and sixteen up-regulated genes in Table 1.2, with average fold changes in expression.

Table 1.1 Genes down-regulated in microarray expression analysis of TBX5 / GATA-4 overexpression cell lines

Gene	NCBI Accession ID	Fold change
Annexin A2	AK012563	2.91
VEGF-b	BC046303	2.57
RBBP6	NM_011247	2.22
Annexin A4	AK032703	3.01
Galanin R2	AF042784	2.41
A2-macroglobulin	NM_175628	9.87

Average expression of double overexpression cell lines G10 and G22 at day 0, measured against wild type P19 cells at day 0.

Table 1.2 Genes up-regulated in microarray expression analysis of TBX5 / GATA-4 overexpression cell lines

Gene	NCBI Accession ID	Fold change
Gamma Filamin	BC060276	2.24
Tropomyosin	M22479	2.23
Glutaredoxin	AB013137	2.97
S100A10	NM_009112	3.84
Fibronectin	X93167	2.45
Calreticulin	AK075605	2.77
ERp57	AK088721	2.18
Mest / Peg1	AF482999	2.34
HNRPDL	NM_016690	2.50
RBMS1	BC057866	2.51
PMM2	XM_354942	2.65
PA2.26	AJ250246	3.36
PETA-3	AF033620	2.65
Neurofilament 3	AK051696	2.19
AP4e1	BC042530	3.17
Alpha-l-fucosidase	AK002230	2.22

Average expression of double overexpression cell lines G10 and G22 at day 0, measured against wild type P19 cells at day 0.

1.10 AIMS AND OBJECTIVES

This P19 overexpression study was the first study of its kind to attempt identification of the combined targets of TBX5 and GATA-4. Microarray expression profiling resulted in identification of a large number of potential targets. This thesis aimed to build on this research as follows:

- 1) Use of the P19 overexpression study as a starting point for identification of a subset of genes likely to be important in cardiogenesis, taking into account expression, bioinformatic, and functional information
- 2) Development of a novel model system of combined knockdown of TBX5 and GATA-4 allowing phenotypic characterisation, and independent assessment of the responsiveness of candidate genes to these transcription factors
- 3) Investigation of the role of one or more selected genes in cardiogenesis, with the aim to validate and characterise function in a developmental system for evidenced presentation of new cardiac genes
- 4) Use of the model system for identification of new synergistic targets of TBX5 and GATA-4

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Molecular methods

50x TAE buffer (1 litre)	242 g Tris, 100 ml 0.5M EDTA pH 8.0, 57.1 ml glacial acetic acid
DNA size markers	Hyperladder IV (Bioline) and 1 Kb ⁺ DNA ladder (Invitrogen)
<i>PfuUltra</i> II Fusion HS DNA polymerase	2.5 U / μ l (Stratagene)
HotStar Taq	5 U / μ l (Qiagen)
DNase I	Lyophilised DNase I (1500 kunitz units) dissolved in 550 μ l RNase free water (Qiagen)
RNaseOUT	40 U / μ l (Invitrogen)
SuperScript III	200 U / μ l (Invitrogen)

2.1.2 Microbial techniques

LB media (1 litre)	10 g tryptone, 5 g yeast extract, 10 g NaCl
SOC media (1 litre)	20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl, 20 mM glucose, 5 ml 2 M MgCl ₂ , 20 ml 1 M glucose. pH adjusted to 7.0 with NaOH
MacConkey agar plates	52 g MacConkey agar per litre, ampicillin added to a final concentration of 50 µg / ml
Ampicillin stock	50 mg / ml in H ₂ O
T4 DNA ligase	3 U / µl (Promega)
<i>Taq</i> DNA polymerase	5 U / µl (NEB)

2.1.3 DNA purification techniques

Shrimp Alkaline Phosphatase	1 U / µl (Promega)
Exonuclease I	20 U / µl (NEB)

2.1.4 Whole mount *in situ* hybridisation (WISH)

Chick saline solution (1 litre)	71.9 g NaCl
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PBST	PBS (Sigma) containing 0.1% Triton X-100
4% paraformaldehyde (1 litre)	40 g PFA, 100 ml 10x PBS, pH adjusted to 9.1-9.3 using NaOH
Proteinase K	10 mg / ml in DEPC treated H ₂ O
Pre-hybridisation solution	50% deionized formamide, 5x SSC, 2% blocking powder, 0.1% Triton X-100, 0.1% CHAPS, 50 µg / ml yeast RNA, 5 mM EDTA, 50 µg / ml heparin
DEPC treated H ₂ O	0.5 ml DEPC per litre H ₂ O
20x SSC (1 litre)	175 g NaCl, 88 g sodium citrate, pH adjusted to 7.0 with HCl
2.0x SSC with 0.1% Tween20 (1 litre)	100 ml 20x SSC, 10 ml 10% Tween20
0.2x SSC with 0.1% Tween20 (1 litre)	10 ml 20x SSC, 10 ml 10% Tween20
KTBT	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM KCl, 1% Triton X-100
NTMT	100 mM NaCl, 100 mM Tris (pH 9.5), 50 mM MgCl ₂ , 0.1% Triton X-100
Levamisole	50 mg / ml in DEPC treated H ₂ O

Colour reagent (1 ml)	3 μ l 75 mg / ml NBT in 70% DMF, 2.3 μ l 50 mg / ml in DMF BCIP, 1 ml NTMT
SacII	20 U / μ l (NEB)
SpeI	10 U / μ l (NEB)
T7 RNA polymerase	20 U / μ l (Roche)
SP6 RNA polymerase	20 U / μ l (Roche)

2.1.5 Western blotting

RIPA buffer (100 ml)	50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 μ l 0.1 M PMSF, one complete mini inhibitor cocktail tablet (Roche)
PMSF (0.1 M)	0.17 g PMSF, made up to 10 ml in isopropanol
5x sample loading buffer (50 ml)	1.25 g SDS, 24.02 g urea, 5 ml β -mercaptoethanol, 10 ml glycerol, 20 ml 0.5 M Tris-HCl, 0.05 g bromophenol blue
Electrophoresis buffer (1 litre)	3 g Tris, 14 g glycine, 1 g SDS
Transfer buffer (1 litre)	9 g Tris, 42 g glycine, 600 ml methanol

TBS (1 litre)	1.2 g Tris, 8.75 g NaCl, pH adjusted to 7.5 with HCl
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TBST	1x TBS containing 0.1% Tween20
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2.1.6 Morpholino knockdown

F127 pluronic gel (100 ml)	15 g F127 pluronic gel made up to 100 ml with HBSS (Sigma)
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Table 2.1 Primers used for PCR amplification of mouse genomic regions of RBMS1

Primer name	Primer sequence (5' – 3')
RBMS1 mouse intron 1 F1	CACCTCTCTCTACCTGAAAAGATGG
RBMS1 mouse intron 1 F2	ATGGCTAATTATGTTAGGGCCTTGT
RBMS1 mouse intron 1 R1	AAAGCTTTGCATCACAGCATCTTAT
RBMS1 mouse intron 1 R2	GAGATCATGAAGTCACTCACGGTAA
RBMS1 mouse 3' UTR F1	TAAGACAAAAGATGACGAAGCACTG
RBMS1 mouse 3' UTR F2	TGTTCCCCTGAACAGGTTTATTTTA
RBMS1 mouse 3' UTR R1	AAAAACCGACAGGAAGAAGAAAGTT
RBMS1 mouse 3' UTR R2	AATGTCGCTGACATCTATCATCAAA
RBMS1 mouse 5' UTR F1	CTCCTCCAAAGACACTTTCCAAATA
RBMS1 mouse 5' UTR F2	CCCAAGTTTGTAGCTCCTACTGAAA
RBMS1 mouse 5' UTR F3	GTGGGACAATTTGAGCATCTACAAA
RBMS1 mouse 5' UTR R1	TGCAGATACTGGGGGTAGTAATAGG
RBMS1 mouse 5' UTR R2	GGGTAGTAATAGGTGGCGTACTGAG
RBMS1 mouse 5' UTR R3	CTATAGTTAGCAACCCCAGCACTTG

Table 2.2 Primers used for introduction of SacI restriction sites

Primer name	Primer sequence (5' – 3')
RBMS1 Int1 SacI primer F	GATC <u>GAGCTC</u> CACCTCTCTCTACCTGAAAAGATGG
RBMS1 Int1 SacI primer R	GATC <u>GAGCTC</u> AAAGCTTTGCATCACAGCATCTTAT

Table 2.3 QPCR assays designed to candidate genes in the chick

Gene	Accession ID	Primer name	Primer sequence (5' – 3')	Position	Probe *
PA2.26	XM_001231272.1	PA2.26 F1	CAGAGTTTGAGGAATTACCGACA	368 – 390	44
		PA2.26 R1	CTTCCAAACTGCAAGAGATTTGT	418 - 440	
PA2.26	XM_001231272.1	PA2.26 F2	TTGGAATCATAGTAGCTGTTGGAA	602 - 625	151
		PA2.26 R2	CGAGTACCTGCCTGACATCTT	658 - 678	
PETA-3	NM_001006472.1	PETA-3 F	CGTCGGAATTTGCTAAGAGTGT	510 - 531	2
		PETA-3 R	GCCAGGATTCCAGCAATG	569 - 586	
FUCA1	XM_417835.2	FUCA1 F	AACTGGAATCTTTGGACACG	409 - 429	33
		FUCA1 R	CAGTCCGTAGCGTATGTTGTTT	476 - 498	
TPM1	NM_205401.1	TPM1 F	GTGGCTCGTAAGCTCGTGAT	668 – 687	1
		TPM1 R	TAGTTCAGCACGCTCCTCAG	711 - 730	
Fibronectin	XM_421868.2	Fibronectin F	GAGGGTGATGAGCCACAGTAT	2287 – 2307	76
		Fibronectin R	CAGGAAGCAAGTCAGGGATG	2340 - 2359	
Desmin	XM_427965.2	Desmin F	CTGCTTTCAGAGCTGACGTG	684 - 703	80
		Desmin R	TCTCCTCCTGCAGGGACTC	746 - 764	
RBMS1	NM_205024.1	RBMS1 F	CTGTGAGATGCCCCGGTGT	1228 - 1245	80
		RBMS1 R	ACACCGGGCATCTCACAG	1266 - 1287	
GAPDH	NM_204305.1	GAPDH F	GGTCTTATGACCACTGTCCA	568 - 587	76
		GAPDH R	TCCACAGCTTCCAGAGG	622 - 640	
TBP	NM_205103.1	TBP F	GATGTCTCCGGATGTTTCA	98 - 118	151
		TBP R	CGAAAAGGTTTTGACCCTCT	153 - 173	
RPLPO	NM_204987.1	RPLPO F	ATGGGGAAGAACACGATGAT	186 - 205	80
		RPLPO R	GCAGCAGCTTCTCCAAGG	244 - 261	
EEF1A1	NM_204157.2	EEF1A1 F	GTTGGAACGGGGACAACAT	584 - 602	37
		EEF1A1 R	CTTCCGGGTAACCTTCCAT	639 - 658	
α -MHC	NM_001001302.1	α -MHC F	GACCTGGAGAATGACAAGCAG	190 - 210	65
		α -MHC R	CGAGCATTGAGTGTGTGAGTT	245 - 266	

*Probe identifiers refer to pre-designed Universal ProbeLibrary probes (Roche). Each probe binds to a specific nucleotide sequence. Probe sequence information can be found online at <https://www.roche-applied-science.com>).

Table 2.4 Primers designed to the chicken RBMS1 gene

Primer name	Primer sequence (5' – 3')
RBMS1 exon1F	CCAGTATCTGCAGGCGAAC
RBMS1 exon2F	ACCCAATGGCTCCTCCTAGT
RBMS1 exon5F	TTGCCACTTTCAATGGATGA
RBMS1 exon8F	CCAACCACAGCTGCTTTACA
RBMS1 exon3R	TGCACTTGTTTGTTGTCTTATCC
RBMS1 exon5R	TCTTGTTGCTTTGCCATTTG
RBMS1 exon9R	TATGTGGAAACCGGAGAAGC
RBMS1 exon10R	GCTGCATCCAAGAAGGACTC
RBMS1 intron2F	GCACTCAGGTTAGGGCAGAG
RBMS1 intron2R	CCTCACCAGAAACGGACAAT

Table 2.5 QPCR assays designed to the α -actin genes in the chick

Gene	Ensembl ID	Primer name	Primer sequence (5' – 3')	Position	Probe *
α -sma	ENSGALT00000039927	α -sma F	GCACCTGAGGACATTGACATAG	3 – 24	56
		α -sma R	TTACAGAGCCCTGAGCCATT	67 – 86	
α -ske a	ENSGALT00000018064	α -ske a F	GCTCTGGACTTTGAGAACGAG	439 - 459	65
		α -ske a R	CCCATCAGGCAGCTCATAG	498 - 516	
α -ca	ENSGALT00000016005	α -ca F	TGAGAATGAAATGGCCACAG	625 - 644	80
		α -ca R	ACCATCAGGCAATTCGTAGC	672 – 691	

*Probe identifiers refer to pre-designed Universal ProbeLibrary probes (Roche). Each probe binds to a specific nucleotide sequence. Probe sequence information can be found online at <https://www.roche-applied-science.com>).

Table 2.6 Primers used in RT-PCR expression analysis of genes identified in microarray analysis of TBX5 / GATA-4 double knockdown embryos

Gene	Accession ID	Primer name	Primer sequence
TFAP2B	NM_204895.1	TFAP2B F	GGCCGTTTGTCTTTGCTTAG
		TFAP2B R	CCACCAGGGAGGTGAGTAAA
PTPRZ1	XM_001231942.1	PTPRZ1 F	CCTTCTGGTTACGTGGGAAA
		PTPRZ1 R	CGTGCAGACCATTAGAGCAA
FIGF	NM_204568.1	FIGF F	ACCAGATTTGCTGCTGCTTT
		FIGF R	TGCAGCTCAGACTCTCCTCA
NR2E1	NM_205170.1	NR2E1 F	CCAGGCTTTACAGGAGGTTG
		NR2E1 R	GATTGGTACATTCCCGATGG
LOC420770	XM_429784.2	LOC420770 F	TGCTTGAGGAAACGAGTGTG
		LOC420770 R	TTTGCTGCAGGCACTATTTG
SUZ12	XM_415658.2	SUZ12 F	ATGAAGCACGGGTTTATTGC
		SUZ12 R	GGGAGGTCGATTCTCCTTTC
CRABP1	NM_001030539.1	CRABP1 F	AGGACGGGGACCAGTTCTAC
		CRABP1 R	CCTCATCCCAGGTGACAAGT
GOLGA4	XM_418856.2	GOLGA4 F	GAAAGAGGCACTGCAGGAAC
		GOLGA4 R	TGCATTTGTCGCTTTGTCTC
GPC3	XM_001232891.1	GPC3 F	GGAGAGGTACAGTCCCCACA
		GPC3 R	AGCCTCTGCCACAGTCATCT

Table 2.7 Morpholinos used in the TBX5 / GATA-4 knockdown study

Morpholino	Sequence (5' – 3')	3' tag
Standard control	CCTCTTACCTCAGTTACAATTATA	Fluorescein
TBX5 AUG	GGCTCCTTCCTCCCGGTGCAAAGC	Fluorescein
TBX5 AUG 5-mismatch	GG <u>ATC</u> ATTCC <u>TGCC</u> GGT <u>ACAAA</u> AC	Fluorescein
TBX5 AUG 7-mismatch*	G <u>CCT</u> GCTT <u>GCT</u> G <u>CCC</u> G <u>TG</u> (G)CAA <u>ACC</u>	Fluorescein
GATA-4 AUG	CATGGCTAAGCTCTGGTACATCTCG	Lissamine
GATA-4 AUG 5-mismatch	CAT <u>GCT</u> AA <u>CTCT</u> <u>CGTA</u> GATGTCG	Lissamine
GATA-4 AUG 7-mismatch	CAT <u>CCCT</u> AA <u>CTGT</u> <u>CGTA</u> GATGTCG	Lissamine

Bases altered in mismatch control morpholinos are underlined and shown in bold. All morpholinos were synthesised by Gene-Tools USA.

*An error was made by Gene-Tools during the design of this morpholino and an extra base (guanine, shown in brackets) inserted at position 19

Table 2.8 Morpholinos used in the RBMS1 knockdown study

Morpholino	Sequence (5' – 3')	3' tag
RBMS1 E2I2	AAGTATCGTACTTACGGTTGGCATA	Fluorescein
RBMS1 E2I2 5-mismatch	AA <u>CTAT</u> <u>CC</u> TA <u>GTTAC</u> <u>CGTTG</u> <u>C</u> CATA	Fluorescein
RBMS1 E8I8	GTGTACCAGCACTTAACATACCCAT	Lissamine

Bases altered in mismatch control morpholinos are underlined. All morpholinos were synthesised by Gene-Tools (USA).

2.2 METHODS

2.2.1 Molecular methods

2.2.1.1 Genomic DNA extraction

Genomic DNA extractions were performed using the Wizard SV Genomic DNA Purification system (Promega), following manufacturer's instructions. A 275 µl volume of digestion solution mastermix was added to the tissue sample (maximum 20 mg). Samples were incubated overnight at 55 °C in a heat block, and centrifuged at 2000 x *g*. Supernatant was transferred to a new microcentrifuge tube and 250 µl lysis buffer added followed by vortexing. This was transferred to a minicolumn assembly and centrifuged at 13000 x *g* for 3 minutes. Following removal of eluate, 650 µl wash solution was added to the column followed by centrifugation at 13000 x *g* for 1 minute. This wash step was repeated 3 times followed by an additional centrifugation step without addition of wash solution in order to remove any traces of ethanol. The column was placed in a new microcentrifuge tube and 250 µl H₂O pipetted directly onto the membrane followed by incubation for 2 minutes at room temperature. DNA was eluted by centrifugation at 13000 x *g* for 1 minute. This elution step was repeated to obtain a final elution volume of 500 µl, and DNA concentration assessed by spectrophotometry (section 2.2.1.3).

2.2.1.2 RNA extraction

RNA extractions were performed using the Qiagen RNeasy minikit or microkit as appropriate, following manufacturer's instructions. RNA/*later* stabilised tissue was placed in 600 µl buffer RLT containing 1% β-mercaptoethanol and homogenised using a sterile needle (BD Microlance 25G 5/8) and syringe. The resulting lysate was centrifuged at maximum speed for 3 minutes, and supernatant transferred to a new microcentrifuge tube for addition of 1 volume of 70% ethanol. This was transferred to an RNeasy spin column placed in a 2 ml collection tube, centrifuged for 15 seconds at maximum speed, and the flow-through discarded. On-column DNase digestion was

carried out in all cases using the RNase-free DNase set (Qiagen). Buffer RW1 (350 µl) was added to the column and centrifugation carried out at maximum speed for 15 seconds. A 10 µl volume of DNase I stock solution was added to 70 µl buffer RDD and pipetted into the membrane for a 15 minute incubation at room temperature. A 350 µl volume of buffer RW1 was added to the column and centrifugation carried out at maximum speed for 15 seconds. A 500 µl volume of wash buffer RPE was added to the column followed by centrifugation for 15 seconds at maximum speed to wash the column-bound RNA. The flow-through was discarded, and a further 500 µl of buffer RPE added to the column for centrifugation for 2 minutes at maximum speed. The column was placed in a new collection tube and centrifuged at full speed for 1 minute to remove any traces of ethanol. The column was placed in a new microcentrifuge tube, and 30 µl RNase-free H₂O pipetted directly onto the centre of the membrane followed by centrifugation at full speed for 1 minute for RNA elution. RNA purity and concentration was assessed by spectrophotometry (section 2.2.1.3), and RNA stored at -80 °C.

2.2.1.3 Nucleic acid quantification

Quantifications were performed using the NanoDrop® ND-1000 spectrophotometer. A 1 µl volume of sample was pipetted onto the lower measurement pedestal, followed by lowering of the sampling arm, drawing the sample between the two measurement pedestals for spectral analysis.

2.2.1.4 Reverse Transcription

Reverse transcription reactions were carried out in 20 µl reaction volumes. Total RNA (1 µg) was combined with 2 µl 50 ng / µl random hexamers and 1 µl 10 mM dNTPs, and made up to 12 µl with H₂O. Samples were heated at 65 °C for 5 minutes then placed on ice before addition of 4 µl 5x first strand buffer, 2 µl 0.1 M DTT and 1 µl 40 U / µl RNaseOUT (Invitrogen). Samples were incubated at 37 °C for 2 minutes prior to addition of 1 µl SuperScript III (Invitrogen), excluding -RT controls. Samples were

incubated at 25 °C for 10 minutes, 37 °C for 50 minutes, 70 °C for 10 minutes before storage at -80 °C.

2.2.1.5 Primer design

Primers were most frequently designed using online Primer3 software (<http://frodo.wi.mit.edu/primer3/>), or manually when a very specific primer location was required (such as in cases of high homology with other genes or transcripts). Primers were generally 18 – 25 bp in length, with melting temperatures of 55 – 65 °C. Blast searches were carried out for all primers in *Ensembl* (<http://www.ensembl.org/Multi/blastview>).

2.2.1.6 Polymerase chain reaction (PCR)

2.2.1.6.1 HotStar Taq

PCR reactions were carried out in 25 µl reaction volumes using 2 µl 10 µM forward and reverse primers, 2.5 µl 10x buffer (Qiagen), 1 µl 10 mM dNTPs, 0.25 µl 5U / µl HotStar Taq (Qiagen), 16.25 µl H₂O, and 1 µl DNA template. Thermal cycling conditions were based on the following: 95 °C for 15 mins, 35 cycles of 94 °C for 30 secs, 60 °C for 30 secs and 72 °C for 30 secs, and 72 °C for 10 mins.

2.2.1.6.2 PfuUltra 11 Fusion HS DNA Polymerase

PCR reactions were carried out in 50 µl reaction volumes using 1.0 µl 10 µM forward and reverse primers, 5.0 µl 10x *PfuUltra* II reaction buffer (Stratagene), 5.0 µl 10 mM dNTPs, 1.0 µl *PfuUltra* II Fusion HS DNA polymerase (Stratagene), 36.0 µl H₂O, and 1 µl 100 ng / µl genomic or plasmid DNA template. Thermal cycling conditions were based on the following: 95 °C for 5 mins, 35 cycles of 94 °C for 30 secs, 60 °C for 30 secs and 72 °C for 30 secs, and 72 °C for 5 mins.

2.2.1.7 Agarose Gel Electrophoresis

Agarose gels were made up and electrophoresed in TAE buffer. Ethidium bromide was added to a final concentration of 0.1 µg / ml. DNA samples were combined with

5x loading buffer (BioLine) and loaded alongside 10 µl 1 Kb⁺ DNA ladder (Invitrogen) or 5 µl Hyperladder IV (BioLine). Gels were run at 100 - 150 V.

2.2.1.8 DNA sequencing

Sequencing reactions were carried out using 0.5 µl BigDye Terminator v3.1 (Applied Biosystems), 2 µl 5x sequencing buffer (Applied Biosystems), 0.4 µl 10 µM primer, and 3 – 20 ng PCR product / 150 – 300 ng plasmid DNA, made up to a total volume of 10 µl with water. Reactions were performed under the following conditions: 96 °C for 16 min, followed by 28 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 minutes. Samples were ethanol precipitated (section 2.2.3.4) and run on an ABI prism™ 3130xl Genetic Analyser (Applied Biosystems) at the Biopolymer Synthesis and Analysis Unit (University of Nottingham, UK). Sequences were analysed using Chromas software (Technelysium Pty Ltd).

2.2.1.9 Quantitative PCR (QPCR) for validation of mouse targets

2.2.1.9.1 Methodology

There are two main methodologies for QPCR analysis of gene expression, the Relative Standard Curve method and the Comparative C_T ($\Delta\Delta C_T$) method, described here. The Relative Standard Curve method involves the construction of standard curves from known quantities of DNA for both the target and endogenous control on each reaction plate, with sample DNA amplified simultaneously under identical conditions. This allows interpolation of sample target and endogenous reference quantities from the standard curves, and assay efficiency data is generated in parallel, producing highly accurate data. Due to the requirement of standard curves and all samples on each reaction plate, this method uses a large quantity of reagents and is suitable when sample numbers are low. The Comparative C_T method uses arithmetic formulae for relative quantification calculations. It requires the construction of standard curves for all target and control genes for assessment of amplification efficiencies prior to quantification experiments, and these values are incorporated into

relative quantification calculations. The use of standard curves on each plate is not required, which means this method has economical advantages over the Relative Standard Curve method. It is ideal for high throughput studies where there are a large number of targets and / or samples, such as in the validation of microarray data, and will be used for this purpose here.

2.2.1.9.2 Assay design and experimental conditions

Assays were designed by Jonathan Ronksley using the online Roche Universal ProbeLibrary assay design centre (<https://www.roche-applied-science.com>). QPCR analyses of gene expression in the mouse P19 cell line were performed using the Comparative C_T method [Livak and Schmittgen, 2001]. Quantitative PCR was performed in a total volume of 25 μ l using 12.5 μ l SensiMix (Quantace), 0.25 μ l 10 μ M probe (5' FAM labelled and 3' labelled with a dark quencher dye, Universal ProbeLibrary, Roche), 1.0 μ l 10 μ M forward primer, 1.0 μ l 10 μ M reverse primer, 1.0 μ l 50mM Mg^{2+} , 4.0 μ l cDNA and 5.25 μ l dH₂O. Reactions were performed using the Applied Biosystems 7500 Fast Real-time PCR system under the following thermal cycling conditions: 95 °C for 15 mins, and 40 cycles of 94 °C for 15 secs and 60 °C for 1 min. Negative RT and water controls were included. Primer sequences and probe numbers are shown in Table 2.1. Real-time PCR efficiencies for each assay were calculated from the exponential phase of the slope in the SDS v1.3.1 software, using the equation $E = 10^{-1/\text{slope}}$. Reactions were performed in duplicate at five serial dilutions of HH19 wild type cDNA (1, 0.1, 0.01, 0.001, and 0.0001). Quantitative PCR reactions were performed in triplicate on cDNA reverse transcribed from RNA used in the initial microarray analysis. Expression quantifications were corrected for PCR efficiency, with the equation $R = 2^{-\Delta\Delta C_t}$ modified to the equation $R = (E_{\text{target}})^{\Delta C_t} / (E_{\text{ref}})^{\Delta C_t}$ target (control – sample) / (Eref) ΔC_t ref (control – sample).

2.2.1.10 QPCR for expression analysis in the chick

2.2.1.10.1 Assay design

QPCR assays were designed using the online Roche Universal ProbeLibrary assay design centre (<https://www.roche-applied-science.com>). For genes with high similarity to other sequences, regions of lowest homology were input and only specific assays selected. Blast searches were carried out for all primers to ensure specificity.

2.2.1.10.2 Amplification conditions

Quantitative PCR was performed in a total volume of 28 µl using 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 µl 10 µM probe (5' FAM labelled, Universal ProbeLibrary, Roche), 0.75 µl 10 µM forward primer, 0.75 µl 10 µM reverse primer, 5.0 µl cDNA, and 5.5 µl dH₂O. Reactions were performed using the Applied Biosystems 7500 Fast Real-time PCR system under the following thermal cycling conditions: 95 °C for 15 mins, and 40 cycles of 94 °C for 15 secs and 60 °C for 1 min. Negative RT and water controls were included in each plate. Primer sequences and probe numbers are shown in Tables 2.4 and 2.5. Data was analysed using Applied Biosystems 7500 v2.0.1 software.

2.2.1.10.3 Comparative C_T method vs Relative Standard Curve method

QPCR analyses of gene expression in the chick were carried out using the Relative Standard Curve method. This method is ideal for the detection of small changes in expression as would be expected in an *in ovo* system, and also when sample numbers are low in order that both standard curves and all samples can be run simultaneously in one reaction plate. Reliable quantification depends on accurately constructed standard curves; therefore data generated in this study has only been used when both standard curves meet a stringent set of parameters. These are discussed further in the next section.

2.2.1.10.4 Specification of experimental parameters

For generation of accurate and reproducible data, a strict set of experimental parameters was set out. Data was used only if both standard curve slopes were in the range -3.2 to -3.6 (a linearity of -3.33 corresponds to an assay efficiency of 100%). Standard curve linearity was a minimum of 0.99 for both genes on the reaction plate.

2.2.1.10.5 Preparation of standard curves

The preparation of a standard curve requires a cDNA standard which expresses the target and endogenous control genes. This can be obtained from a source independent to the study samples, which has advantages when working with material in low supply. It also allows cDNA standard production on a large scale for comparison of data across all experiments. HH24 whole embryo cDNA was the chosen standard for this study. Dilutions were carried out immediately after cDNA synthesis for storage in single use aliquots to prevent repeated freeze / thaw cycles, and for consistency across reaction plates. RNA preparations often contain inhibitors (such as proteins) which can affect subsequent QPCR reactions. To minimise this, all cDNA was diluted prior to use. cDNA for standards was used at a starting dilution of 1 in 16, and subsequent dilutions were made from this. Sample cDNA dilutions were carried out such that following amplification they would fall within the mid-range of the standard curve. The fold-dilution of standards was selected according to the expected magnitude of expression change. A 4-fold dilution series was chosen as relatively small expression changes were expected due to the fact this experiment was performed *in ovo*.

2.2.1.11 Microarray expression analysis

Microarray technology allows gene expression profiling of different sample groups on a genome-wide scale. It involves the use of individual spotted probes immobilised on a support in a known arrangement for detection of hybridisation. Sample RNA is reverse transcribed to produce cDNA which is then labelled with fluorescent dyes, most commonly Cy3 / Cy5. Samples are then hybridised onto arrays and scanned

using a confocal laser to produce images from which the relative fluorescent intensity of spots can be assessed for calculation of relative gene expression. Microarray studies were carried out using the Affymetrix service provided by the Nottingham Arabidopsis Stock Centre (NASC), using the Affymetrix GeneChip® Chicken Genome Array (900590). Microarray expression data was analysed using GeneSpring version 11 software (Agilent).

2.2.2 Microbial techniques

2.2.2.1 A-tailing procedure for cloning into the pGEM-T Vector

PCR products amplified using the *PfuUltra* 11 Fusion HS DNA polymerase are blunt ended and need to be modified using an A-tailing procedure for cloning into the pGEM-T Vector. Reactions were set up as follows: 1 - 7 µl gel purified PCR product, 1 µl 10x *Taq* standard PCR buffer containing MgCl₂ (NEB), 0.4 µl 5 mM dATP, 1 µl 5U / µl *Taq* DNA polymerase (NEB), and dH₂O to a final volume of 10 µl. Samples were incubated at 70 °C for 30 minutes, and 1 - 2 µl used per ligation reaction.

2.2.2.2 Ligation of vector – insert

Ligations were carried out using the TOPO cloning kit (Promega). Reactions were set up with 5 µl 2x rapid ligation buffer, 1 µl vector (50 ng / µl), 1 µl T4 DNA ligase, and insert at an approximate insert:vector molar ratio of 3:1, made up to a total volume of 10 µl with dH₂O. Samples were incubated at room temperature for 1 hour, or at 4 °C overnight. Positive controls (containing vector and control insert) and background controls (containing vector and no insert) were included in each set of ligations / transformations.

2.2.2.3 Transformation

The *Escherichia coli* (*E.coli*) DH5α strain was used in all bacterial work. DH5α cells were thawed on ice (30 µl aliquots) and 2 µl ligation reaction added, mixed by flicking, then placed on ice for 25 – 30 minutes. Cells were subjected to heat-shocking at 42 °C for 45 seconds, placed on ice for 2 minutes, and 950 µl SOC media added for incubation at 37 °C shaking for 1 hour. Cells were pelleted by centrifugation at 1000 g for 10 minutes, resuspended in 300 µl SOC media, and spread onto MacConkey agar plates containing 50 µg / ml ampicillin for overnight incubation at 37 °C. MacConkey agar is selective for gram negative bacteria (due to the fact it contains crystal violet and bile salts). It also differentiates between lactose fermenting bacteria (which do not contain an insert) and appear pink, and non lactose fermenting bacteria

(containing an insert) which appear colourless. Colour screening can therefore be used to accurately detect transformed colonies.

2.2.2.4 Colony PCR

Colony PCR reactions were performed for detection of the presence / absence of insert. PCR reactions were carried out in 25 µl reaction volumes using 2 µl 10 µM forward and reverse primers, 2.5 µl 10x buffer (Qiagen), 1 µl 10 mM dNTPs, 0.25 µl 5U / µl HotStar Taq (Qiagen), and 16.25 µl H₂O. Reactions were inoculated using individual colonies picked from agar plates. Colonies picked from the positive control plate were used as negative insert controls, in addition to a standard negative PCR control. Thermal cycling conditions were based on the following: 95 °C for 15 mins, 35 cycles of 94 °C for 30 secs, 60 °C for 30 secs and 72 °C for 30 secs, and 72 °C for 10 mins.

2.2.2.5 Overnight cultures

Single colonies were used to inoculate 5 ml LB media containing ampicillin (50 µg / ml), and a negative control was included with each set of overnight cultures to verify sterility. Cultures were incubated at 37 °C shaking overnight.

2.2.2.6 Preparation of glycerol stocks

A 200 µl volume of glycerol was added to 800 µl fresh overnight culture (final concentration 20%). Samples were mixed and stored at -80 °C.

2.2.2.7 Plasmid DNA isolation (Miniprep)

For plasmid minipreps, DNA was extracted from 3 ml of bacterial culture. This was divided into two 1.5 ml aliquots which were sequentially centrifuged at maximum speed for 1 minute to pellet the cells. DNA was extracted using the GenElute Plasmid Miniprep Kit (Sigma) according to manufacturer's instructions. The bacterial pellet was resuspended by addition of 200 µl resuspension solution and vortexing. Cells were lysed by addition of 200 µl lysis solution and gentle inversion. Cell debris was

precipitated by addition of 350 µl of neutralisation/binding solution and gentle mixing, followed by centrifugation at maximum speed for 10 minutes. Following column preparation, the cleared lysate was transferred to the column and centrifuged at maximum speed for 1 minute. A 750 µl volume of wash solution was added to the column, a 1 minute centrifugation performed, and the flow-through discarded prior to a second centrifugation for 2 minutes to remove any traces of ethanol. The column was transferred to a clean microcentrifuge tube and 100 µl H₂O pipetted directly onto the membrane followed by centrifugation for 1 minute at maximum speed to elute DNA. DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (section 2.2.1.3).

2.2.2.8 Plasmid DNA isolation (Maxiprep)

A single colony was picked from a plate and used to inoculate a 3 ml starter culture in LB media which was incubated for 8 hours at 37 °C shaking. A volume of 200 µl starting culture was made up to 100 ml (1 / 500 dilution) with LB media and grown at 37 °C shaking overnight. DNA was extracted from 100 ml bacterial culture using the Plasmid Maxi kit (Qiagen). Cells were harvested by centrifugation at 6000 x g for 15 minutes at 4 °C. The bacterial pellet was resuspended in 4 ml buffer P1, followed by addition of 4 ml of lysis buffer P2 and incubation at room temperature for 5 minutes. Chilled buffer P3 was then added at a volume of 4 ml, mixed, and incubated on ice for 15 minutes. This was followed by centrifugation at maximum speed at 4 °C for 30 minutes. The supernatant was removed and re-centrifuged under identical conditions for 15 minutes. The supernatant was then applied to an equilibrated QIAGEN-tip and allowed to enter the resin. The tip was then washed twice with 10 ml buffer QC, and DNA eluted in 5 ml buffer QF. DNA was precipitated by addition of 3.5 ml isopropanol, mixing, and centrifugation at maximum speed for 30 minutes at 4 °C. The supernatant was carefully removed and the DNA pellet washed with 2 ml 70% ethanol followed by centrifugation at maximum speed for 10 minutes. The supernatant was carefully decanted and the DNA pellet allowed to air-dry prior to

being re-dissolved in an appropriate volume of H₂O. DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (section 2.2.1.3).

2.2.2.9 Plasmid linearization by restriction digestion

Digests were performed using restriction enzymes, buffers, and BSA (if required) from NEB. Reactions were set up as follows: 1 - 2 µg plasmid DNA, 1 µl restriction enzyme, 2 µl 10x buffer, 1 µl 20x BSA if necessary, made up to 20 µl with H₂O. Samples were incubated at 37 °C for 3 - 4 hours, or overnight. Agarose gel electrophoresis was performed using 100 ng to confirm digestion was complete.

2.2.2.10 Introduction of restriction sites by PCR amplification for subcloning into the pGL3-Basic and pGL3-Promoter vectors

Primers incorporating restriction sites (in this case SacI) were designed and used for PCR amplification of insert (as described in section 2.1.1.5.2) using plasmid DNA as template, for subcloning into a second vector. Products were subjected to agarose gel electrophoresis, and purified by gel extraction (section 2.1.3.3).

2.2.2.11 Restriction digestion and vector dephosphorylation

For subcloning into a second vector, the destination vector and modified insert were cut using the same restriction enzyme to produce complementary ends. Linearised vector DNA was dephosphorylated using Shrimp Alkaline Phosphatase (SAP) to prevent recircularisation by removal of 5' phosphate groups from both termini.

2.2.3 DNA purification techniques

2.2.3.1 Shrimp Alkaline Phosphatase / Exonuclease I treatment

This is a simple enzymatic procedure for preparing PCR products for sequencing. SAP catalyses the dephosphorylation of 5' phosphates from DNA, removing excess dNTPs. Exonuclease I (ExoI) digests single stranded DNA in the 3' to 5' direction, removing unincorporated primers. PCR products were purified for sequencing in 7 µl reaction volumes using 1.5 µl 1 U / µl SAP (Promega) and 0.5 µl 20 U / µl ExoI (NEB) with 5 µl PCR product. Samples were incubated at 37 °C for 1 hour, followed by an enzyme inactivation step of 80 °C for 20 mins.

2.2.3.2 Column purification of PCR products

PCR products > 100 bp were purified using the Sigma GenElute PCR clean up kit according to manufacturer's instructions. Briefly, following column preparation, 5 volumes of binding solution was combined with PCR product. This was applied to the column for centrifugation at maximum speed for 1 minute, and the eluate discarded. Column-bound DNA was then washed by addition of 0.5 ml wash solution and centrifugation at maximum speed for 1 minute. Following eluate disposal, the column was centrifuged for a further 2 minutes at maximum speed to remove any traces of ethanol. The column was transferred to a fresh collection tube and 50 µl water pipetted directly onto the centre of the membrane followed by incubation at room temperature for 1 minute. DNA was eluted by centrifugation at maximum speed for 1 minute, and stored at -20 °C.

2.2.3.3 DNA extraction from agarose gels

DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. Briefly, the DNA fragment was visualised under UV light and excised from the agarose gel using a scalpel blade. The gel slice was then weighed and 3 volumes of buffer QG added followed by incubation at 50 °C for 10 minutes with vortexing every 2 - 3 minutes, allowing the gel to dissolve. Isopropanol

was added to the sample at 1 gel volume, mixed, and applied to the column for centrifugation for 1 minute at maximum speed. The eluate was discarded and column-bound DNA washed by addition of 0.75 ml buffer PE and centrifugation for 1 minute at maximum speed. The column was re-centrifuged for an additional minute to remove all traces of ethanol. The column was placed in a clean microcentrifuge tube and 50 µl water pipetted directly onto the centre of the membrane for DNA elution by centrifugation at maximum speed for 1 minute. DNA was stored at -20 °C.

2.2.3.4 Ethanol precipitation of DNA

Ethanol was added at 2.5 volumes, and 1 / 10 volume of 3 M NaOAc pH 5.5 added to each sample, mixed by inversion, and incubated at room temperature for 15 minutes. Samples were centrifuged at 13000 rpm for 30 minutes and the supernatant carefully removed. The DNA pellet was washed with 70% ethanol, and centrifuged at 13000 rpm for 10 minutes. The supernatant was carefully removed and the pellet air-dried before resuspension in an appropriate volume of H₂O.

2.2.4 Chick embryo techniques

2.2.4.1 General care and conditions

Fertile white Leghorn eggs (Henry Stewart & Co Ltd) were stored at 12 °C prior to use. Eggs were incubated at 37 °C gently rotating in a humidified incubator for the appropriate time.

2.2.4.2 Embryo staging

A small opening was created at the top of each egg and 4 ml albumen removed. Individual embryos were staged visually whilst in the egg according to Hamburger & Hamilton (HH) chicken developmental stages [Hamburger and Hamilton, 1992]. Chick developmental stages are further described in Appendix A.

2.2.5 Chick expression studies

2.2.5.1 Collection of chick embryonic tissue for RT-PCR, QPCR, and microarray expression analysis

Embryos were excised and placed in sterile ice cold PBS (Sigma). Membranes were removed and the relevant tissue was isolated at the required stage of development and immediately placed in RNAlater (Ambion) for storage at -20 °C. In the case of morpholino treated embryos (section 2.1.4.5), embryos were excised from the egg and placed in sterile ice cold PBS. The outer and inner membranes were quickly removed, and the embryo placed in RNAlater for assessment of morpholino uptake under fluorescent light using a Zeiss Stemi SV11 microscope. Embryos were photographed where necessary and morpholino positive hearts isolated and stored in RNAlater at -20 °C prior to extraction of RNA.

2.2.5.2 Whole mount in situ hybridisation (WISH)

2.2.5.2.1 Embryo collection and preparation

Embryos were placed in ice-cold sterile chick saline solution for membrane removal followed by overnight fixation in 4% paraformaldehyde (PFA). Embryos were subjected to 3x 5 minute washes in PBS with 0.5% Tween (PBST), bleached for 1 hour in 3% H₂O₂ (Sigma) in PBS, followed by 5 minute washes in a graded methanol series of PBST, 25% methanol (in PBST), 50% methanol (in PBS), 75% methanol (in DEPC treated water), and 100% methanol, then stored in 10 ml of methanol for a minimum overnight at -20 °C (maximum 1 week). Embryos were subjected to 5 minute washes in the methanol series ending in PBST, then digested with proteinase K in PBS (HH16: 10 µg / ml, HH19 and HH24: 20 µg / ml) for 20 minutes. Samples were washed for 10 minutes in PBST, fixed for 20 minutes in 4% PFA with 0.2% glutaraldehyde, and subjected to two 10 minute washes in PSBT. PBST was removed and samples incubated in pre-hybridisation solution at 65 °C for 2 hours.

The pre-hybridisation solution was replaced with fresh pre-hybridisation solution and embryos stored at -20 °C for up to 10 days prior to use.

2.2.5.2.2 Probe synthesis and digoxigenin (DIG) labelling

Single stranded DIG-labelled RNA probes were synthesised using the DIG RNA Labelling Mix (Roche) by *in vitro* transcription with SP6 or T7 RNA polymerases, which incorporate DIG-11-UTP at approximately every 20 – 25 bases. Reactions were set up on ice as follows: 500 ng - 1 µg linearised plasmid DNA, 2 µl 10x DIG RNA labelling mix, 2 µl 10x transcription buffer, 2 µl 20 U / µl SP6 or T7 RNA polymerase, sterile RNase free water to a final volume of 20 µl. Reactions were incubated at 37 °C for 3 hours, then template DNA was removed by addition of 2 µl DNase I (RNase free) and further incubation at 37 °C for 30 minutes. Reactions were stopped by addition of 1 µl 0.5 M EDTA pH 8.0, and 9 µl DEPC treated H₂O added.

2.2.5.2.3 Probe purification and quantification

Probes were purified using Microspin G-50 columns (Illustra) according to manufacturer's instructions. Briefly, column preparation involved resuspension of the resin by vortexing, the cap was then loosened by one quarter of a turn, and the bottom closure snapped off prior to placement in the collection tube. The column was pre-spun for 1 minute at low speed (735 x *g*). The column was placed in a new microcentrifuge tube. DIG labelling reactions were made up to a volume of 50 µl with Probe Elution and Storage Buffer, carefully applied to the top centre of the resin, and centrifuged at 735 x *g* for 2 minutes for elution. Probes were quantified using the NanoDrop® ND-1000 spectrophotometer and stored at -80 °C.

2.2.5.2.4 *In situ* hybridisation

Prepared embryos were warmed in a hybridisation oven (Biometra OV3) at 65 °C for 2 hours. Pre-hybridisation solution was replaced with fresh pre-warmed pre-hybridisation solution containing probe at 150 ng / µl, and samples incubated at 65 °C for 22 hours. Embryos were subjected to 10 minute wash steps at 65 °C in each of

the following: 100% pre-hybridisation solution, 75% pre-hybridisation solution / 25% 2x SSC, 50% pre-hybridisation solution / 50% 2x SSC, 25% pre-hybridisation solution / 75% 2x SSC, 100% 2x SSC, then 4x 15 minute washes at 65 °C in 0.2 x SSC. Embryos were then subjected to 5 minute washes at room temperature in the following: 75% 0.2x SSC / 25% KTBT, 50% 0.2x SSC / 50% KTBT, 25% 0.2x SSC / 75% KTBT, 100% KTBT. Embryos were blocked in KTBT containing 10% sheep serum (Invitrogen) and 2 mg / ml BSA (Fisher Scientific) for 3 hours at room temperature (sheep serum was inactivated by incubation at 65 °C for 30 minutes prior to use). This was followed by incubation with anti-DIG-AP Fab fragment (Roche) at 1 / 5000 in blocking solution at 4 °C overnight. Embryos were subjected to 5x 1 hour washes in KTBT gently rotating at room temperature, and then 2x 15 minute washes in fresh NTMT (containing levamisole at 25 µg / ml). Colour reagent was made immediately before use and 2 ml added to each sample for initial incubation in the dark at room temperature for 20 minutes, then longer as necessary. The colour reaction was stopped by 2x 15 minute rinses in KTBT and one 15 minute wash in PBS. Embryos were taken through in a graded methanol series ending in 100% methanol, where they were incubated until sufficient background was removed. Embryos were then rehydrated through the methanol series ending in PBS, and transferred to 70% glycerol for photography. Embryos were washed in PBS and stored in pH 5.5 PBS containing 1 mM EDTA at 4 °C in the dark.

2.2.5.3 Western blotting

2.2.5.3.1 Collection of tissue

HH24 wild type embryonic tissue was isolated as sample material for initial technique development. Embryos were excised from the egg and placed in sterile ice cold PBS. The outer and inner membranes were quickly removed, and if necessary, assessment of morpholino uptake carried out under fluorescent light using a Zeiss Stemi SV11

microscope. Embryos were photographed where necessary and morpholino positive hearts isolated and snap frozen in liquid nitrogen prior to extraction of protein.

2.2.5.3.2 Preparation of tissue lysates

Embryonic tissue was isolated and placed in approximately 5x volume of chilled RIPA buffer (containing 1 mM PMSF and protease inhibitor cocktail (Roche)). Samples were immediately homogenised using a sterile needle (BD Microlance 25G 5/8) and syringe, and placed on ice for 30 minutes. Samples were centrifuged at maximum speed for 10 minutes and the supernatant removed and stored at -80 °C prior to use.

2.2.5.3.3 HeLa cell growth and isolation

Cells were grown and isolated by Sukrat Arya. Passage 8 HeLa cells were grown in 20 ml complete media (Dulbecco's minimal essential media (DMEM) with 10% v/v foetal bovine serum and peninstrep). Media was discarded and dead cells removed by washing with PBS. A 2 ml volume of trypsin was spread gently around the flask and incubated at 37 °C for 4 minutes to detach and round the cells. A volume of 18 ml complete media was added to the cells, mixed, and centrifuged at 1500 rpm for 3 minutes. The supernatant was discarded and cells were resuspended in 10 ml PBS prior to centrifugation at 1500 rpm for 3 minutes. The supernatant was discarded and the pellet stored on ice prior to immediate extraction of protein.

2.2.5.3.4 Preparation of HeLa cell lysates

HeLa cell pellets were dissolved in 300 µl chilled RIPA buffer and placed on ice for 30 minutes. Samples were mixed by flicking then subjected to two 5 – 10 minute freeze / thaw cycles at -80 °C. Samples were centrifuged at 12000 rpm for 15 minutes, and the supernatant removed and stored at -80 °C.

2.2.5.3.5 Protein quantification

Protein content was measured by Bradford assay using the Biorad protein assay kit, using a 50 µg / ml – 1 mg / ml BSA standard curve. Absorbances were measured at 750 nm on a Biorad SmartSpec™ 3000 spectrophotometer.

2.2.5.3.6 SDS PAGE and western blotting

Samples were combined with 5x sample buffer and boiled for 10 minutes at 100 °C prior to loading onto 5 - 20% SDS PAGE gradient gels (20 - 30 µg total protein loaded per lane). Samples were electrophoresed using Atto apparatus alongside the Precision Plus Protein Standard (BioRad) at 100 V for approximately 90 minutes in electrophoresis buffer. Protein was transferred onto a Hybond C Extra nitrocellulose membrane (Amersham Biosciences) by a semi-dry blotting technique, using a transfer sandwich comprising gel and membrane, sandwiched between 2 layers of absorbent paper (Atto) soaked in transfer buffer on either side. Electroblothing was performed with Atto apparatus at 20 V for 2 hours. Following transfer, membranes were washed in deionised water and blocked in the appropriate agent overnight at 4 °C with gentle shaking. Membranes were subjected to three 10 minute washes in TBST, then incubated with primary antibody at the appropriate dilution for 1 hour at room temperature with gentle shaking. Membranes were again subjected to three 10 minute washes in TBST, and then incubated with secondary antibody for 1 hour at room temperature with gentle shaking. Membranes were again subjected to three washes in TBST. ECL reactions were then performed using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer) for protein detection.

2.2.5.3.7 Conditions tested for antibody optimisation

For GAPDH detection, membranes were blocked with 5% milk powder in TBS and incubated with a mouse monoclonal antibody (ab8245, Abcam) at a dilution of 1 / 1000. Rabbit anti-mouse HRP conjugated secondary antibody (P0260, Dako) was used at 1 / 1500, and a 36 kDa band successfully detected.

For TBX5 detection, a rabbit polyclonal antibody to TBX5 (ab49308, Abcam) was tested in 5% milk powder / PBS. Swine anti-rabbit HRP conjugated secondary antibody (P0217, Dako) was used at 1 / 5000. A band of unexpected size (~ 75 kDa, expected size 50 – 53 kDa) was detected.

For detection of GATA-4, a rabbit polyclonal antibody to GATA-4 (ab25992, Abcam) was tested in two blocking agents - 5% milk powder / TBS, and 5% BSA / TBS. Swine anti-rabbit HRP conjugated secondary antibody (P0217, Dako) was tested at 1 / 1500. A very high level of background resulted in both cases and it was not possible to accurately distinguish any bands.

For RBMS1 detection, membranes were blocked with 5% milk powder in TBS and incubated with a rabbit polyclonal antibody (ARP40428_T100, Aviva Systems Biology) at a dilution of 1 / 100. Swine anti-rabbit HRP conjugated secondary antibody (P0217, Dako) was used at 1 / 1500. Antibodies were tested on HeLa cell lysate and chick tissue lysate. A band of unexpected size (~ 120 kDa, expected size 46 kDa) was detected.

2.2.6 Morpholino knockdown

2.2.6.1 Morpholino knockdown technology

Morpholino knockdown is an antisense loss-of-function technology used widely in developmental studies. A morpholino oligomer is a synthetic DNA analogue usually consisting of around 25 subunits, with a morpholine moiety replacing the riboside moiety and phosphorodiamidate intersubunit linkages instead of phosphodiester linkages [Summerton and Weller, 1997]. These modifications confer stability to the oligomer, and cellular enzymatic degradation does not occur [Hudziak et al., 1996]. Morpholinos have high affinity for RNA and do not elicit an immune response. They can be end-labelled with 3' carboxyfluorescein or lissamine (sulforhodamine B) for confirmation of uptake.

Morpholinos function by two major gene targeting strategies. They can be designed to bind to the initiating AUG sequence (or in close proximity) in order to sterically blocking ribosomal binding and inhibit translation [Summerton and Weller, 1997]. Translational blocking morpholinos are most effective when designed within a 100 bp region between the 75 bp upstream and 25 bp downstream region of the initiating AUG. Morpholinos can also be designed to target nuclear processing and modify pre-mRNA splicing through complementary binding to splice sites [Morcos, 2007].

Morpholinos have been utilised to study the effect of gene knockdown in cultured cells, and in the developing embryos of a number of organisms such as *Xenopus* [Falk et al., 2007], zebrafish [Draper et al., 2001], and chick [Voiculescu et al., 2008]. They are particularly useful for temporal gene knockdown studies, where the role of a particular gene at specific stages of development can be elucidated.

2.2.6.2 Identification of target regions for morpholino design

Human homologues of genes of interest were identified on the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and their genomic location noted. Protein sequences

were blasted in the BBSRC ChickEST database (<http://www.chick.manchester.ac.uk/>). ESTs were then blasted in Ensembl (<http://www.ensembl.org>) against the chick genome in order to locate the corresponding chick gene, and viewed in a chromosomal contig view. Synteny of the chick genomic location against the expected human genomic region was confirmed. Translation software (<http://expasy.org/tools/>) was used to aid identification of the initiating ATG. ESTs around this region were aligned with the theoretical mRNA sequence for sequence confirmation using sequence alignment software (<http://align.genome.jp/>). The region 75 base pairs upstream and 25 base pairs downstream of the initiating ATG was identified for morpholino design. Where it was not possible to design a morpholino to this region, morpholinos were designed to modify pre-mRNA splicing by targeting exon / intron boundaries (splice sites). These were predicted to result either in deletion of an exon and cause a frameshift and premature termination, or retention of an intron causing insertion of incorrect amino acids followed by a premature stop.

2.2.6.3 Morpholino design and preparation

Morpholino antisense oligonucleotides were designed to a 25 base pair region using the following guidelines: 40% - 60% GC content, maximum 36% G, maximum run of 3 Gs, avoidance of G or A on 3' end where possible (<http://www.genetools.com>). Morpholinos had either 3' carboxyfluorescein or 3' lissamine end modifications for confirmation of uptake. Morpholinos were resuspended at 2 μ M in HBSS and stored at -20 °C.

2.2.6.4 Morpholino delivery

Morpholinos are most commonly introduced into embryos by microinjection, and in the case of the chick, by electroporation [Kos et al., 2003]. However, this method is not always effective for knockdown outside the central nervous system, particularly mesenchymal tissues [Kos et al., 2003]. In recent years the use of F127 pluronic gel has been employed as an alternative method of morpholino delivery into the chick embryo [Ching et al., 2005; Rutland et al., 2009].

F127 pluronic gel is a non-ionic co-polymer of polyethylene oxide and polypropylene oxide with thermosensitive properties. Upon reaching physiological temperatures it undergoes a liquid to gel phase transition, and in terms of *in vivo* application, this means it is injectable and will polymerise locally *in situ* i.e. it has potential uses as a vehicle for drug delivery. It has been tested in a number of biomedical applications such as the local delivery of an anti-tumour agent into mice [Yang et al., 2009], ocular delivery of ophthalmic agents [El-Kamel, 2002; Miyazaki et al., 2001], and in vaccine delivery systems [Coeshott et al., 2004]. It has also been used for local application of antisense phosphorothioate oligodeoxynucleotides into major blood vessels *in vivo* for loss of function studies [Abe et al., 1994; Castier et al., 1998; Herbert et al., 1997]. For gene knockdown in the developing chick, pluronic gel can be combined with morpholino antisense oligonucleotides and applied directly onto the embryo where it undergoes gelation, facilitating morpholino entry into the embryo. This is the method of choice for this study.

2.2.6.5 Application of morpholino via F127 pluronic gel to embryos in ovo

Eggs were opened as described previously and embryo ages assessed. Prior to use, morpholinos were heated to 65 °C for 5 minutes, incubated on ice, and combined with HBSS and F127 pluronic gel (BASF) at a final concentration of 15%, for a final morpholino concentration of 250 µM or 500 µM. In the case of double knockdown, morpholinos were combined at this stage, each at a final concentration of 250 µM. A 7 µl volume of morpholino / F127 pluronic gel mix was pipetted directly onto the chick (usually at HH12 – HH16). Eggs were sealed using tape and incubated at 37 °C until the required stage of development was reached.

2.2.7 Phenotypic analysis of embryos

2.2.7.1 Embryo isolation and processing

Chick embryos were excised from the egg, placed in PBS, and the outer and inner membranes carefully removed to avoid damage to the embryo. Morpholino uptake was assessed under fluorescent light, and embryos photographed where appropriate at 1.6x and 4.0x zoom using a Zeiss Stemi SV11 microscope. Embryos were incubated in 4% PFA at room temperature for 90 mins, and stored at 4 °C in PBS for up to 1 week. Embryos were dehydrated by 2x 15 minute incubations in each of the following; dH₂O, 75% ethanol, 95% ethanol, 100% ethanol, and xylene (room temperature). A final 50% xylene / 50% wax incubation was carried out at 65 °C before transfer into wax at 65 °C overnight.

2.2.7.2 Embedding and sectioning

Embryos were correctly positioned and left to set at room temperature overnight. Embryos were sectioned in a coronal orientation at 10 microns using a Leica DSC1 microtome. Sections were floated in a heated water bath (40 °C) and mounted onto glass slides. Slides were air-dried overnight at room temperature.

2.2.7.3 Haemulum staining and mounting of slides

Sections were de-waxed and rehydrated by 5 minute incubations in the following: 100% xylene x 2, 100% ethanol x 2, 95% ethanol, 75% ethanol, 50% ethanol, dH₂O. Excess water was blotted away before incubation in Mayer's Haemulum (Raymond A Lamb) for 3 minutes. Slides were washed then dehydrated by 5 minute incubations in the following: dH₂O, H₂O, dH₂O, 50% ethanol, 75% ethanol, 95% ethanol, 100% ethanol, 100% xylene. Slides were removed from xylene, mounted with DPX (Bios Europe) and allowed to dry overnight before photography.

2.2.7.4 Photography and analysis

Sections were photographed at 2.5x and 5.0x magnification under a Zeiss Axioscope 40 microscope connected to a Macintosh computer and OpenLab software (PerkinElmer, UK).

CHAPTER 3

IDENTIFICATION OF DOWNSTREAM TARGETS OF TBX5 and GATA-4 IN THE MOUSE P19 CELL LINE

3.1 AIMS

This chapter aims to use the P19 study as a basis for further research into the combined targets of TBX5 and GATA-4 as follows:

- a) Validation of previous microarray data by QPCR
- b) Selection of genes for further study based on expression data and review of current literature
- c) Bioinformatic analysis of candidate gene promoter regions for identification of evolutionarily conserved TBX5 and GATA-4 binding sites
- d) Luciferase reporter assays to test promoter activation of one or more genes by TBX5 and GATA-4

3.2 RESULTS

3.2.1 QPCR validation of microarray data

Genome-wide microarray expression analyses allow identification of a large number of targets which display differential regulation. These results are often variable, and validation by an independent method with greater sensitivity is necessary. QPCR is one of the most precise methods of expression quantification available, and was used to confirm and more accurately quantify previous microarray gene expression data. QPCR validation experiments were performed in collaboration with Jonathan Ronksley.

3.2.1.1 Assay design

QPCR assays were designed by Jonathan Ronksley using the online Roche Universal ProbeLibrary assay design centre (<https://www.roche-applied-science.com>). Assays spanned a 60 – 100 bp region including an exon-exon boundary where possible, and were used with an annealing temperature of 60 °C. Primer sequences and assay information are given in Appendix B. These primers were used for RT-PCR amplification to confirm products sizes and single product amplification prior to QPCR, using cDNA from wild type (day 0 and 7) and overexpression cell lines (day 0) (not shown).

3.2.1.2 Selection of endogenous controls

Selection of an appropriate endogenous control gene for normalisation is crucial and allows correction of any skewing of data arising from small differences in starting quantity of total nucleic acid. The endogenous control must be expressed at the same time as the target gene, and display uniform expression across all samples i.e. expression should not be affected by sample treatments. A total of six genes were selected for assessment of their suitability as controls, based on stable expression in the original microarray study - β -tubulin, non-POU-domain containing / nono,

ribosomal protein L13a / rpl13a, bolA-like 2 / bola2, dynactin, and transmembrane protein 131 / tmem131.

3.2.1.2.1 Analysis of endogenous control variation across study samples

In order to determine suitability of the above genes as endogenous controls, their expression across all study samples was measured for assessment of variability. Real time reactions were performed in triplicate, and relative expression was measured against undifferentiated P19 wild type cells. Baseline and threshold values were set manually. Fold change in expression was calculated using the following equation:

$$\begin{aligned} R &= 2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ control})} \\ &= 2^{-\Delta \Delta Ct} \end{aligned}$$

R = relative expression, ΔCt : Ct difference between samples, control = wild type day 0 sample, sample = overexpression cell line day 0 or wild type sample at day 2, 5 or 7.

Expression results are shown in Table 3.1. Variation between samples was assessed by calculation of the range of variation (maximum value – minimum value), and the coefficient of variation was also calculated. Tmem131 displayed the smallest range of variation between samples (0.36), and also the smallest coefficient of variation across experimental samples (0.18).

Table 3.1 Relative expression of six candidate endogenous control genes in study samples

	Relative expression					
	β -tubulin	nono	rpl13a	bola2	dynactin	tmem131
G10	0.79	1.01	1.05	0.75	0.82	0.71
G22	0.95	1.19	1.03	1.21	1.30	1.07
WT Day 2	0.98	1.32	0.96	0.94	1.09	0.86
WT Day 5	0.67	0.83	0.84	0.64	0.81	0.74
WT Day 7	0.59	0.84	0.64	0.61	0.76	0.77
Range of variation	0.39	0.49	0.41	0.6	0.54	0.36
Coefficient of variation	0.21	0.22	0.19	0.30	0.24	0.18

Expression of six candidate endogenous control genes was measured in G10 and G22 overexpression cell lines, and wild type differentiating P19 cells at days 2, 5 and 7, measured against undifferentiated P19 cells (day 0).

3.2.1.2.2 Test normalisation using short-listed endogenous control genes

Tmem131 and dynactin were used for test normalisation of α -l-fucosidase, which was up-regulated in microarray analysis of the two overexpression cell lines. Relative expression values are shown in Table 3.2. The two controls gave almost identical results and confirmed up-regulation of alpha-l-fucosidase. Tmem131 was selected for use as the endogenous control as this gene previously displayed the lowest range of variation and coefficient of variation across experimental samples (Table 3.1).

Table 3.2 Relative expression of FUCA1 when normalised against candidate endogenous control genes tmem131 and dynactin

Sample	Relative expression of FUCA1	
	Normalisation with tmem131	Normalisation with dynactin
G10 day 0	1.627	1.625
G22 day 0	1.131	1.094

Expression of alpha-l-fucosidase (up-regulated in the microarray study) was measured in G10 and G22 overexpression cell lines against undifferentiated P19 cells (day 0), normalised to both tmem131 and dynactin. The two controls gave highly similar results.

3.2.1.3 Analysis of target and control gene amplification efficiency

The maximum PCR efficiency of a reaction (E) is 2, if every single template is replicated in each cycle [Pfaffl, 2001]. Target and control genes should ideally show similar amplification efficiency, but data can be corrected for differences in assay efficiency by prior calculation of these values. Real-time PCR efficiencies for each gene were calculated from the exponential phase of the slope in the SDS v1.3.1 software, using the equation $E = 10^{-1/\text{slope}}$. Reactions were performed in duplicate

using a 5-point 10-fold dilution series of P19 wild type day 0 cDNA (1, 0.1, 0.01, 0.001, and 0.0001). Assay properties are shown in Table 3.3. A value of -3.33 corresponds to an assay efficiency of 100%, and generally -3.2 to -3.6 is considered acceptable. However, as expression quantifications will be corrected for PCR efficiency (see section 3.2.1.4), assays with efficiency outside this range are also usable. Linearity values ≥ 0.99 are desirable, and assays to all genes with the exception of RBMS1 met this.

Table 3.3 Target and endogenous control real time assay linearity and efficiency

Gene	Linearity (R^2)	Slope	Efficiency (E)
PETA-3	0.995	- 4.01	1.78
RBMS1	0.986	- 3.03	1.89
VegfB	0.992	- 3.62	1.89
Annexin A4	0.994	- 3.37	1.98
Tropomyosin	0.998	- 3.29	2.01
Gamma-filamin	0.997	- 3.62	1.89
mRbp46	0.998	- 3.53	1.92
PMM2	0.997	- 3.42	1.96
Neurofilament 3	0.998	- 5	1.58
Glutaredoxin	0.997	- 3.27	2.02
HNRPDL	0.991	- 3.27	2.02
Erp57	0.994	- 3.42	1.97
PA2.26	0.998	- 3.42	1.90
α -l-fucosidase	0.995	- 4.22	1.72
Fibronectin	0.997	- 3.51	1.93
α -2-macroglobulin	0.997	- 3.78	1.84
Calreticulin	0.998	- 3.72	1.85
Ap4e1	0.996	- 3.34	2
S100A10	0.996	- 3.74	1.85
Tmem131	0.992	- 3.83	1.82

Standard curves were constructed for all target and control genes using cDNA from P19 wild type undifferentiated cells. Reactions were performed in duplicate at five serial dilutions of P19 wild type day 0 cDNA (1, 0.1, 0.01, 0.001, and 0.0001).

3.2.1.4 Expression analysis

Quantitative PCR reactions were performed in triplicate on cDNA reverse transcribed from RNA used in the initial microarray analysis. For correction of expression quantifications to take into account PCR efficiency (E), the equation $R = 2^{-\Delta\Delta Ct}$ was modified to the equation below:

$$R = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control - sample)}}}{(E_{\text{reference}})^{\Delta Ct \text{ ref (control - sample)}}$$

R = relative expression, E = efficiency of QPCR assay, ΔCt : Ct difference between samples, target = gene of interest, reference = endogenous control gene, control = wild type day 0 sample, sample = overexpression cell line at day 0 / wild type sample at day 2, 5 or 7.

Expression data is represented in Table 3.4 and Figures 3.1 and 3.2. Figure 3.1 shows relative expression of 18 genes of interest in the two TBX5 / GATA-4 overexpression cell lines at day 0 of differentiation, displayed as \log_2 fold change. A number of genes displayed average up-regulation of 2-fold or greater including S100A10, glutaredoxin, γ -filamin, RBMS1, PETA-3, PA2.26, neurofilament 3, and α -tropomyosin.

Figure 3.2 displays expression profiles of the 18 genes of interest in wild type differentiating P19 cells, represented as \log_2 fold change. As mentioned previously, wild type TBX5 and GATA-4 expression in differentiating P19 cells is switched on at day 2 and day 0 of differentiation respectively, and shows a massive increase over days 5 and 7. A number of genes followed the same pattern of expression including glutaredoxin, γ -filamin, RBMS1, PETA-3, α -tropomyosin, calreticulin, erp57, and annexin A4.

Table 3.4 Relative expression of candidate genes in TBX5 / GATA4 overexpression cell lines and wild type differentiating wild type cells, measured by QPCR

Gene	Fold change					
	Overexpression cell lines			Wild type		
	G10 day 0	G22 day 0	G10/G22 average	WT day 2	WT day 5	WT day 7
S100A10	3.74	3.18	3.46	4.40	1.87	2.75
Glutaredoxin	2.45	2.75	2.60	1.75	5.94	11.6
Gamma filamin	3.85	4.06	3.96	1.50	6.11	9.89
RBMS1	3.22	2.71	2.97	9.29	9.66	13.2
PETA-3	3.76	3.23	3.50	0.94	1.84	2.25
PA2.26	3.57	2.34	2.96	0.68	0.43	0.58
Neurofilament	2.48	3.37	2.93	5.19	0.17	2.60
Tropomyosin 1	3.32	1.40	2.36	1.37	3.11	10.5
AP4e1	1.65	1.47	1.56	1.65	1.12	1.57
Fibronectin	1.64	1.61	1.63	34.7	18.9	34.2
α -L-fucosidase	1.40	1.39	1.40	1.97	1.92	3.64
Mrbpa46	1.84	1.61	1.73	1.39	1.60	1.49
Calreticulin	1.26	1.47	1.37	1.08	1.59	1.99
Erp57	1.01	1.33	1.17	0.85	0.92	1.42
Pmm2	1.06	1.23	1.15	1.20	0.85	1.44
HnrpdI	0.72	0.83	0.78	0.63	0.54	1.13
Vegfb	0.43	0.55	0.49	0.53	0.50	0.42
Annexin A4	0.85	0.55	0.70	0.51	0.74	1.23

QPCR analysis of candidate genes was carried out in TBX5 / GATA-4 overexpression cell lines G10 and G22, and P19 wild type cells at days 2, 5 and 7, measured against undifferentiated wild type P19 cells. Data was normalised against *tnfrsf11b* and corrected for real time assay efficiencies.

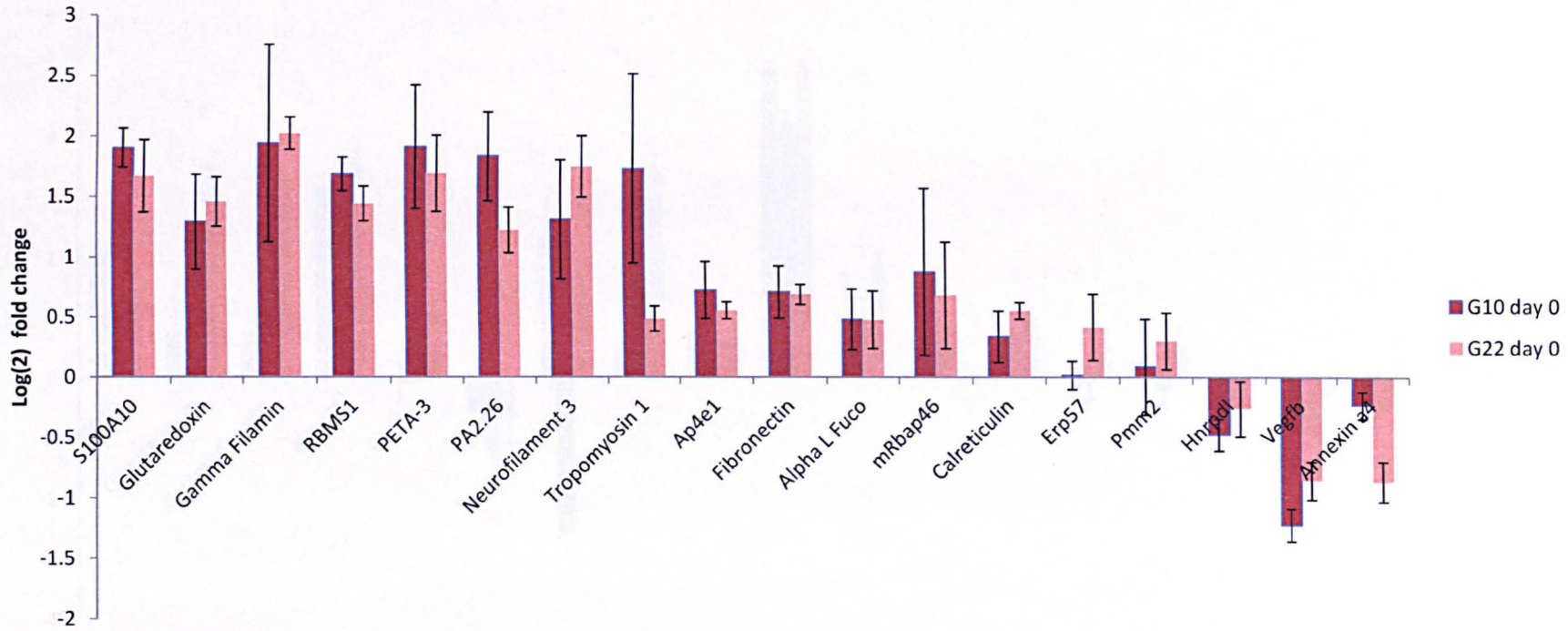


Figure 3.1 Relative expression of candidate genes in TBX5 / GATA4 overexpression cell lines G10 and G20

QPCR analysis of candidate genes was carried out in overexpression cell line G10 and G22 at day 0, measured against wild type P19 cells on day 0. Expression is represented as log₂ fold change, and error bars represent standard deviation. Data was corrected for real time assay efficiencies and normalised against tmem131.

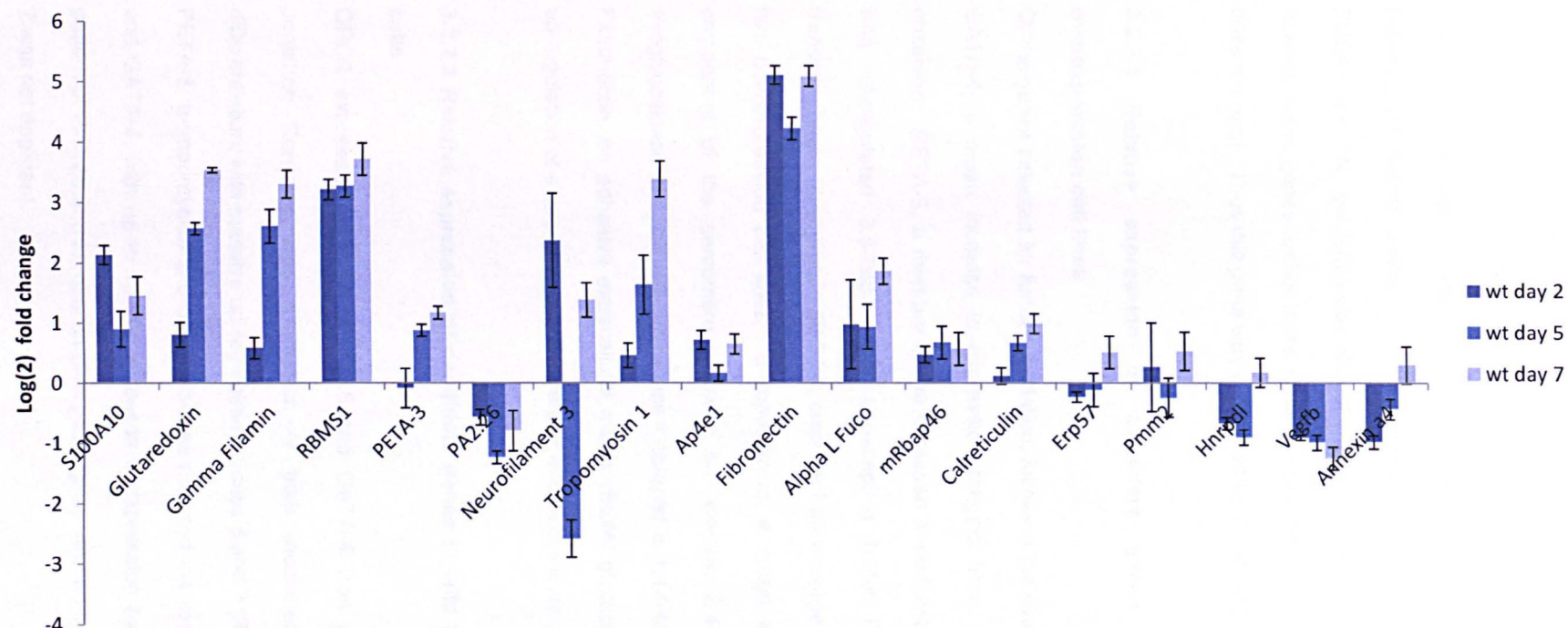


Figure 3.2 Relative expression of candidate genes in wild type differentiating wild type cells

QPCR analysis of candidate genes was carried out in P19 wild type cells at days 2, 5 and 7, measured against wild type P19 cells on day 0. Expression is represented as log₂ fold change, and error bars represent standard deviation. Data was corrected for real time assay efficiencies and normalised against tmem131.

3.2.2 Selection of genes for further study

Genes displaying the highest fold increases in expression in the overexpression cell lines, and following the same pattern of expression as TBX5 and GATA-4, were selected for further analysis. These included TPM1, PETA-3, PA2.26, RBMS1, FUCA1, and FN. γ -Filamin was also of interest, but a future aim of this research was to study these genes further in the chick embryo, and a chick homologue of this gene does not exist. Thus this gene was eliminated from further study.

3.2.2.1 Relative expression of candidate genes in TBX5 / GATA-4 overexpression cell lines

Of the genes selected for further validation, following the overexpression of TBX5 and GATA-4, a mean increase in expression ranging from 1.4-fold to 3.5 fold was observed. PETA-3, a member of the tetraspan superfamily of membrane proteins, was up-regulated 3.5-fold. The transcription factor RBMS1, and the small transmembrane glycoprotein PA2.26, displayed an average increase of 3-fold in the two overexpression cell lines. α -tropomyosin, a coiled actin-binding protein and component of the sarcomere, showed an average 2.4-fold increase, and the exoglycosidase enzyme α -L-fucosidase displayed a 1.4-fold increase in expression. Fibronectin, an adhesive extracellular matrix (ECM) glycoprotein, displayed 1.6-fold up-regulation of expression in both overexpression cell lines.

3.2.2.2 Relative expression of candidate genes in wild type differentiating P19 cells

QPCR expression analysis of TBX5 and GATA-4 was performed previously by Jonathan Ronksley, and expression of both increased during cardiomyocyte differentiation, with massive up-regulation on days 5 and 7 [Ronksley, 2007]. RBMS1, PETA-3, α -tropomyosin and α -L-fucosidase mirrored the expression pattern of TBX5 and GATA-4, with up to 13-fold increases in expression by day 7. The expression pattern of PA2.26 and fibronectin diverged slightly, and an increase from day 2 to day 7 was not apparent.

3.2.3 Candidate genes

The candidate genes *TPM1*, *PETA-3*, *PA2.26*, *RBMS1*, *FUCA1*, and *FN* were chosen for further study of their function during cardiac development. An additional gene (*DES*), encoding the sarcomeric filament protein desmin, was added to the study. This was based on a similar microarray study carried out by Dr Lynn Amy (a former Post-Doctoral researcher in the lab), where *TBX5* and *GATA-4* were transiently overexpressed in the P19 cell line and desmin was identified as a potential target.

3.2.3.1 TPM1

TPM1 belongs to the tropomyosin gene family and encodes α -tropomyosin, a dimeric α -helical coiled actin-binding protein. It is a contractile component of the sarcomeric thin filament, crucial for myocardial function [Perry, 2001]. The tropomyosins are expressed predominantly in skeletal, smooth, and cardiac muscle, and at lower levels in non-muscle cytoskeletal and actin associated cells [Pittenger et al., 1994]. Familial dilated and hypertrophic cardiomyopathy have been linked to mutations in *TPM1* [Lakdawala et al., ; Thierfelder et al., 1994]. E40K and E54K mutations in *TPM1* cause dilated cardiomyopathy due to decreased sensitivity to Ca^{2+} [Mirza et al., 2007]. *TPM1* null mice do not survive past E11.5, whilst heterozygous mice are viable and fertile [Rethinasamy et al., 1998].

3.2.3.2 Fibronectin

Fibronectin (discussed in section 1.6.5) is an ECM glycoprotein expressed in the myocardium, endocardium, and foregut [Waterman and Balian, 1980], and also in the cardiac jelly, with expression highest in regions adjacent to the myocardium where endocardial cushion cell migration occurs [Ffrench-Constant and Hynes, 1988]. There is evidence of a role for fibronectin in endocardial cell migration [Icardo et al., 1992]. Fibronectin null homozygous embryos display abnormalities such as a shortened anterior-posterior axes, and defects in the neural tube, heart, embryonic vessels and mesoderm derived tissues become apparent at E7.5 - E8.0, worsening as development proceeds and eventually leading to embryonic lethality [George et al.,

1993]. Heart development defects occur, with heart primordia remaining unfused. Less severely affected embryos have fused primordia with abnormal morphology. Myocardial tissue thickening, a deficiency in cardiac jelly, and abnormal endocardium morphology has also been observed.

3.2.3.3 Desmin

Desmin is a sarcomeric type III intermediate filament protein which associates with Z-discs and connects myofibrils to one another and to the plasma membrane. It has an important contractile role in cardiac, skeletal and smooth muscle. Mouse knockout of desmin results in cardiomyopathy, and heart tissue from these animals displays abnormally shaped and distributed mitochondria [Thornell et al., 1997]. Disturbances in cardiac conduction in desmin null mice have also been observed [Schrickel et al.]. A range of mutations in the desmin gene have been associated with familial cardiac and skeletal myopathy [Bergman et al., 2007; Fidzianska et al., 2005; Goldfarb et al., 1998; Goudeau et al., 2001; Olive et al., 2007; Sung et al., ; Taylor et al., 2007; Vernengo et al., ; Vrabie et al., 2005]. Features of this disease include atrial dilation, cardiac arrhythmia, conduction block, and sudden death due to conduction defects [Vernengo et al.].

3.2.3.4 PETA-3

Platelet endothelial tetraspan antigen-3 or PETA-3 is a member of the transmembrane 4 or tetraspan superfamily of membrane proteins, which strongly associate with laminin binding integrins such as $\alpha 3\beta 1$, and other tetraspans (e.g. CD9 or CD63). PETA-3 is thought to have a role in cell-cell adhesion, cell migration, platelet adhesion, and angiogenesis [Wright et al., 2004]. PETA-3 expression is observed in the endothelium and epithelium, Schwann and dendritic cells, megakaryocytes, platelets, and in skeletal, smooth and cardiac muscle [Sincock et al., 1997]. In cardiac muscle, PETA-3 expression is seen (at high levels) in the sarcolemma of muscle cells and capillary endothelium [Sincock et al., 1997]. The phenotype of PETA-3 null mice has been characterised in three separate studies. The first study described PETA-3

null mice as viable, healthy and fertile, but displaying defects in haemostasis with extended bleeding times and greater blood loss [Wright et al., 2004]. A separate study examining the role of PETA-3 in angiogenesis additionally revealed PETA-3 null mice do not display defects in vasculature during normal development [Takeda et al., 2007]. However, alternative *in vivo* and *in vitro* assays did show an effect as a result of mouse gene knockout, with changes in adhesion, spreading and invasion, all of which are important processes in angiogenesis [Takeda et al., 2007]. Nonsense mutations in the human PETA-3 gene have been associated with kidney failure, and a third study of PETA-3 knockout mice revealed that with age, these mice developed a similar pathological state to that seen in human patients; knockout mice displayed proteinuria due to focal glomerulosclerosis, glomerular basement membrane disorganisation, and tubular cystic dilation [Sachs et al., 2006].

3.2.3.5 PA2.26

PA2.26 is a small 172 amino acid transmembrane glycoprotein originally identified as a cell surface antigen, that is expressed in skeletal muscle, heart, lung and placental tissue [Martin-Villar et al., 2005]. Its function has been studied by transfection of PA2.26 into immortalised keratinocytes that do not express the protein [Scholl et al., 1999]. These cells displayed differences in cell-cell adhesion, and actin cytoskeletal reorganisation, altering the cell morphology from epithelial to fibroblastoid, indicating PA2.26 plays a role in cell migration. A specific role for PA2.26 in the heart has not yet been demonstrated. As a protein involved in the extracellular matrix, it may be involved in cardiac remodelling.

3.2.3.6 FUCA1

Alpha-L-fucosidases are exoglycosidases that are involved in fucosylated glycoconjugate processing (they catalyse terminal L-fucose residue removal), and also possess transglycosylation properties [Intra et al., 2006]. These enzymes are involved in inflammation, cancer development, cystic fibrosis, and lysosomal storage disease [Intra et al., 2006].

3.2.3.7 RBMS1

RBMS1 is a multifunctional gene involved in transcriptional regulation, activation of DNA replication, cell cycle regulation, and apoptosis. The human RBMS1 gene gives rise to multiple splice variants encoding proteins of approximately 400 amino acids [Takai et al., 1994]. RBMS1 contains two ribonucleoprotein consensus sequences, each containing an RNA-binding protein consensus motif (RNP1-A and RNP1-B), which are essential to its function [Dreyfuss et al., 1988]. RBMS1, via its RNP domains, interacts with the N-terminal region of a catalytic subunit of DNA polymerase α , stimulating its polymerase activity [Niki et al., 2000a]. RBMS1, via these domains, also binds to the c-myc protein which has important functions in cell cycle regulation, cell transformation, and apoptosis [Niki et al., 2000b]. RBMS1 transcriptionally regulates a number of genes including α -smooth muscle actin [Kimura et al., 1998] (discussed in Chapter 5), MHC Class I and II genes [Balducci-Silano et al., 1998], and the thyrotropin receptor [Shimura et al., 1995]. RBMS1 expression has been studied by northern blot analysis in the adult mouse, where expression was detected in all tissues examined except testis [Fujimoto et al., 2000]. A more detailed spatial analysis of expression has not been performed. Expression of RBMS1 is induced by mitogens such as serum [Fujimoto et al., 2001]. *Rbms1*^{-/-} mice once born appear healthy but display an atypical Mendelian ratio i.e. disruption of this gene results in embryonic lethality in some homozygous mice [Fujimoto et al., 2001]. RBMS1 knockout embryos display growth and developmental abnormalities at E2.5 when cultured *in vitro* for 5 days, and female adult mice show a reduced progesterone concentration. These findings together account for the embryonic lethality observed. It is not clear whether cardiac defects occur in these mice. RBMS1 suppresses transcription of α -smooth muscle actin, which is important in myofibrillogenesis, via *cis*-elements in its promoter region [Kimura et al., 1998]. This could indicate a potential regulatory role for RBMS1 in the heart.

3.2.4 Bioinformatic identification of conserved TBX5 and GATA-4 binding sites within putative targets

In order to identify evolutionarily conserved transcription factor binding sites in genes of interest, multi-species sequence alignments were performed using online Mulan software (<http://mulan.dcode.org/>). This software allows detection of evolutionarily conserved regions (ECRs) and specific consensus sequences within them [Ovcharenko et al., 2005]. Full genomic sequences of candidate genes plus 10 kb upstream from the first exon and 10 kb downstream from the last exon, were manually exported from the *Ensembl* database (www.ensembl.org) and input into Mulan. TBX5 binds to the consensus sequence [A/G/T]GGTG[T/C][C/T/G][A/G] [Ghosh et al., 2001] and GATA-4 to the consensus sequence [A/T]GATA[A/G] [Plumb et al., 1989]. The search input sequences DGGTGYBR and WGATAR were used in accordance with IUPAC-IUB codes for nucleic acid bases (Appendix C).

Jonathan Ronksley previously performed similar analyses between human and mouse and found multiple binding sites for both TBX5 and GATA-4 in all of the genes examined, with *RBMS1* displaying the highest number of conserved consensus binding sites for TBX5 and GATA-4 [Ronksley, 2007]. In the next chapter of this thesis, candidate genes will be studied in the developing chick embryo, including assessment of their responsiveness to TBX5 and GATA4. In light of this, alignments have been performed between chicken and human sequences in addition to mouse and human sequences, for identification of regions conserved between all three species.

Figure 3.3i-vii displays Mulan analysis results for the seven genes of interest. No multi-species conserved consensus binding sites for either TBX5 or GATA-4 were identified upon analysis of the *PA2.26*, *PETA-3* and *FUCA1* genes (Figures 3.3i, 3.3ii and 3.3iii). Analysis of the *TPM1* gene revealed two conserved binding sites for TBX5, and one for GATA4, all within the coding region (Figure 3.3iv). Figure 3.3v shows Mulan analysis of the *DES* gene. Two conserved TBX5 consensus binding

sites were identified the coding region, and no GATA-4 sites were found. Figure 3.4vi shows analysis of the *FN* gene. Three conserved sites for both GATA-4 and TBX5 were identified, all within the coding region. RBMS1 analysis resulted in identification of three putative binding sites for TBX5 (all within the coding region), and four GATA-4 sites were identified, two in the coding region, and two in the 3' UTR (Figure 3.3vii). Of the genes studied, *RBMS1* displayed the highest number of multi-species conserved putative binding sites for both TBX5 and GATA-4.

Figure 3.3i Mulan analysis of the PA2.26 gene in human, mouse and chicken

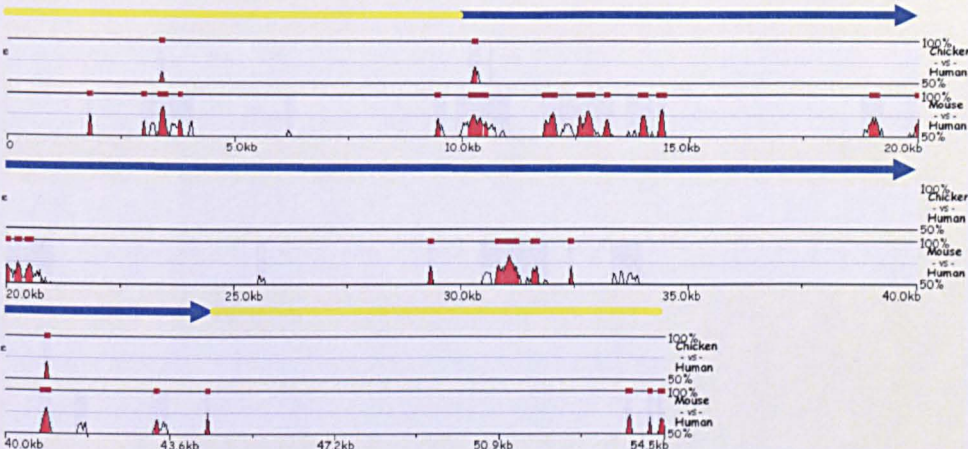


Figure 3.3ii Mulan analysis of the PETA-3 gene in human, mouse and chicken

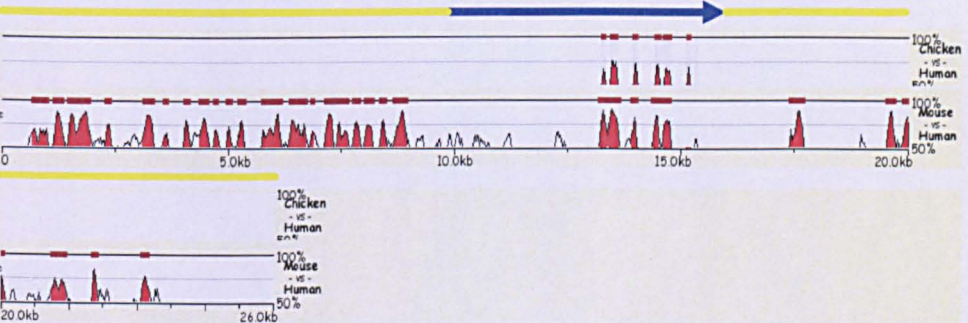


Figure 3.3iii Mulan analysis of the FUCA-1 gene in human, mouse and chicken

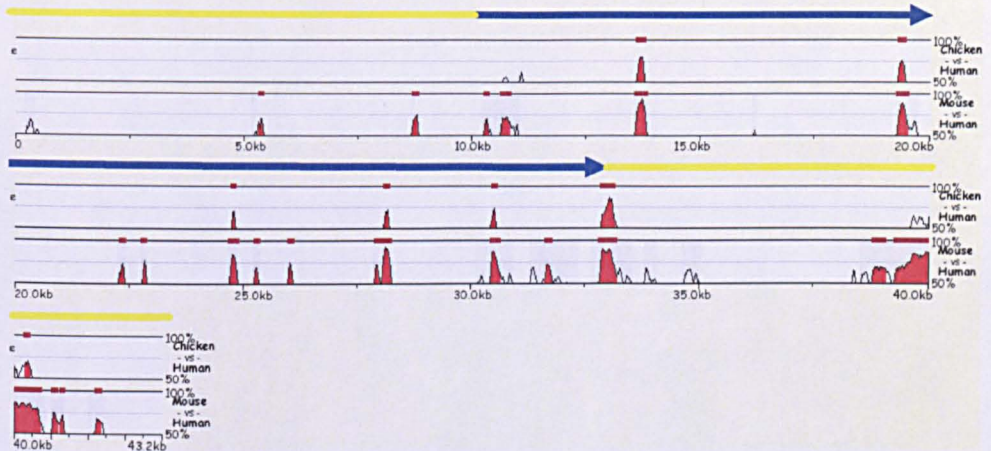


Figure 3.3iv Mulan analysis of the TPM1 gene in human, mouse and chicken

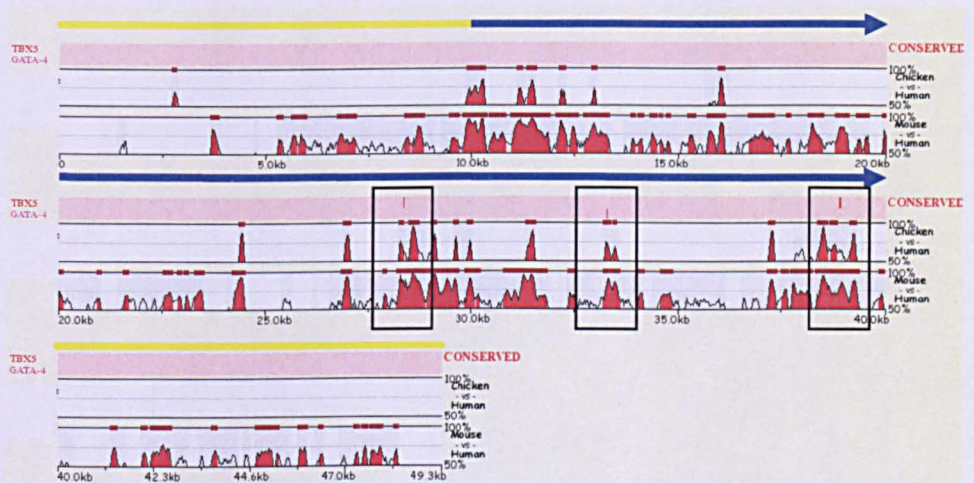


Figure 3.3v Mulan analysis of the desmin gene in human, mouse and chicken

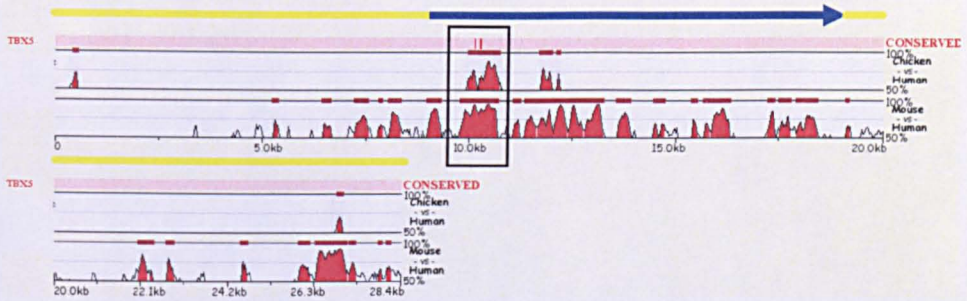


Figure 3.3vi Mulan analysis of the fibronectin gene in human, mouse and chicken

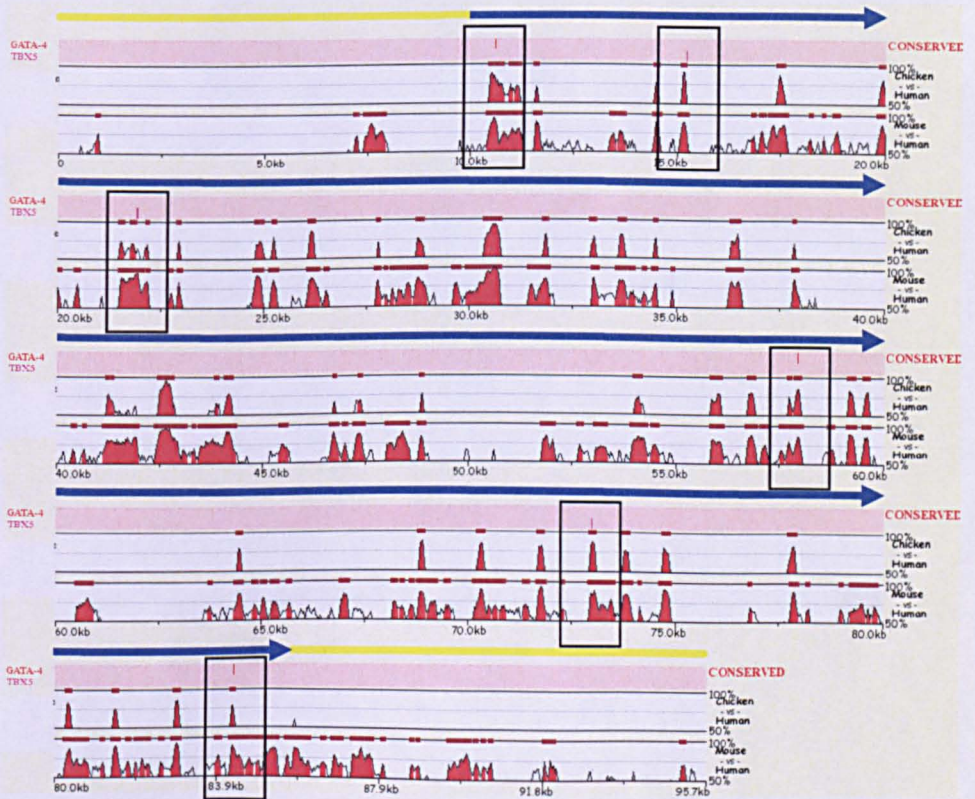
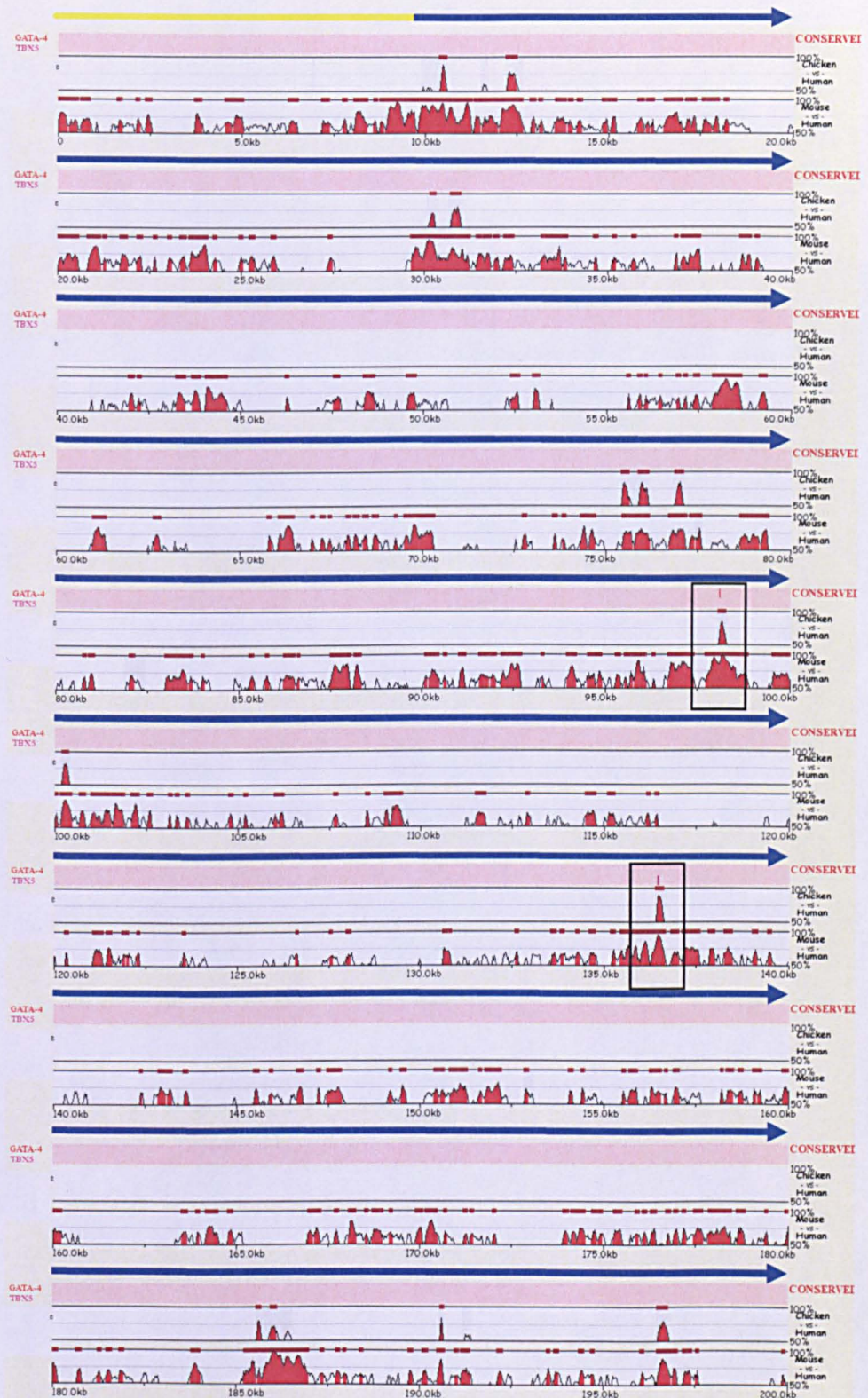


Figure 3.3vii Mulan analysis of the RBMS1 gene in human, mouse and chicken

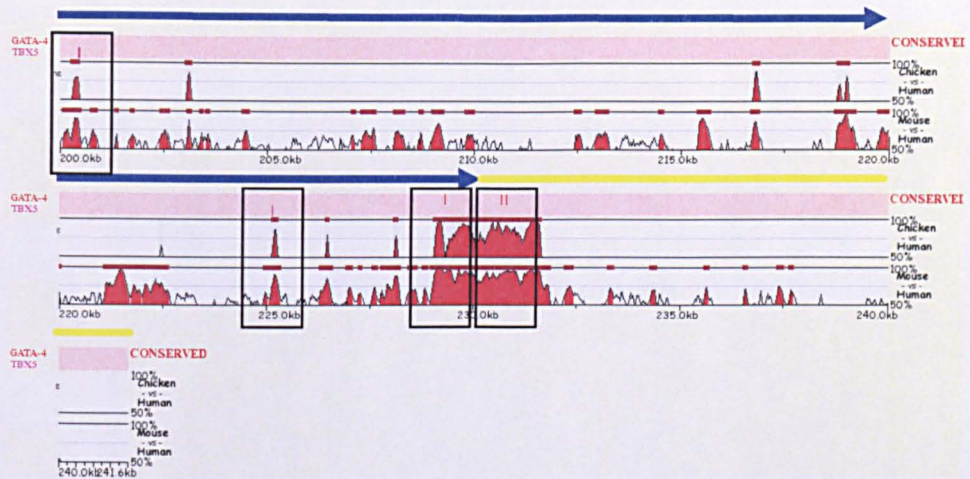


Figure 3.3i-vii Mulan analysis of candidate genes in human, mouse and chicken

Alignment of full length genes (marked by blue arrows) plus 10 kb upstream and downstream (marked by yellow lines) in human, mouse and chicken was performed for identification of consensus TBX5 and GATA-4 binding sites using the transcription factor binding site identification tool in Mulan software (<http://mulan.dcode.org/>). Evolutionarily conserved regions with $\geq 50\%$ homology between species are plotted, and regions meeting the input criteria (100 nucleotide run, minimum 70% identity) are indicated by the red rectangles on top of each plot. The lower scales represent mouse / human sequence alignment, and the upper scales represent chicken / human sequence alignment. Consensus sites conserved between all three species are represented by vertical red or purple dashes for TBX5 or GATA-4 respectively, and have been boxed. Genes analysed are PA2.26 (i), PETA-3 (ii), FUCA1 (iii), TPM1 (iv), DES (v), FN (vi), and RBMS1 (vii). No conserved consensus binding sites were identified for either TBX5 or GATA-4 in PA2.26, PETA-3 or FUCA1. Two conserved binding sites for TBX5 and one for GATA-4 were identified within the coding region of TPM1. Two conserved TBX5 consensus binding sites were identified the coding region, and no GATA-4 sites were found in the DES gene. In the FN gene, three conserved sites for both GATA4 and TBX5 were identified, all within the coding region. RBMS1 analysis resulted in identification of three putative binding sites for TBX5 (all within the coding region), and four GATA-4 sites were identified, two in the coding region, and two in the 3' UTR.

3.2.5 Cloning of RBMS1 fragments for functional analysis of evolutionarily conserved regions (ECRs) in RBMS1

Whilst bioinformatic analysis is a useful tool for identifying conserved consensus transcription factor binding sites, the functional significance of these regions needs to be confirmed experimentally. Luciferase reporter studies are useful for measuring promoter response in cells. RBMS1 was selected for further study based on its high number of conserved putative binding sites and the fact that this is a novel gene in terms of a cardiac function.

3.2.5.1 Identification of regions of the RBMS1 gene for further study

In order to assess transcriptional activation of RBMS1 by TBX5 and GATA-4, luciferase reporter studies were carried out on regions of interest in the mouse promoter. For this purpose Mulan alignment between human and mouse sequences only was performed and is displayed in Figure 3.4. Conserved regions containing putative binding sites for both TBX5 and GATA-4 were identified across the gene. Three regions of particular interest are boxed. The first region of interest is the main promoter region of the 5' UTR, containing single conserved TBX5 and GATA-4 sites 4 kb – 5kb upstream from the ATG translational start site. The transcriptional start site of this gene has not been determined (this was attempted in the next section, see 3.3.5.2). Another conserved region of approximately 1 kb was identified within intron 1, found to contain single putative sites for both TBX5 and GATA-4 within 100 bp of one another. Both sites are conserved between the human and mouse, and the TBX5 site is also conserved with the chick. The high degree of conservation of this non-coding region indicates this may serve as an enhancer or repressor element. A third 3 kb region was identified within the 3' UTR, containing one putative binding site for TBX5 and multiple conserved GATA-4 sites, three of which are also present in the chicken sequence.

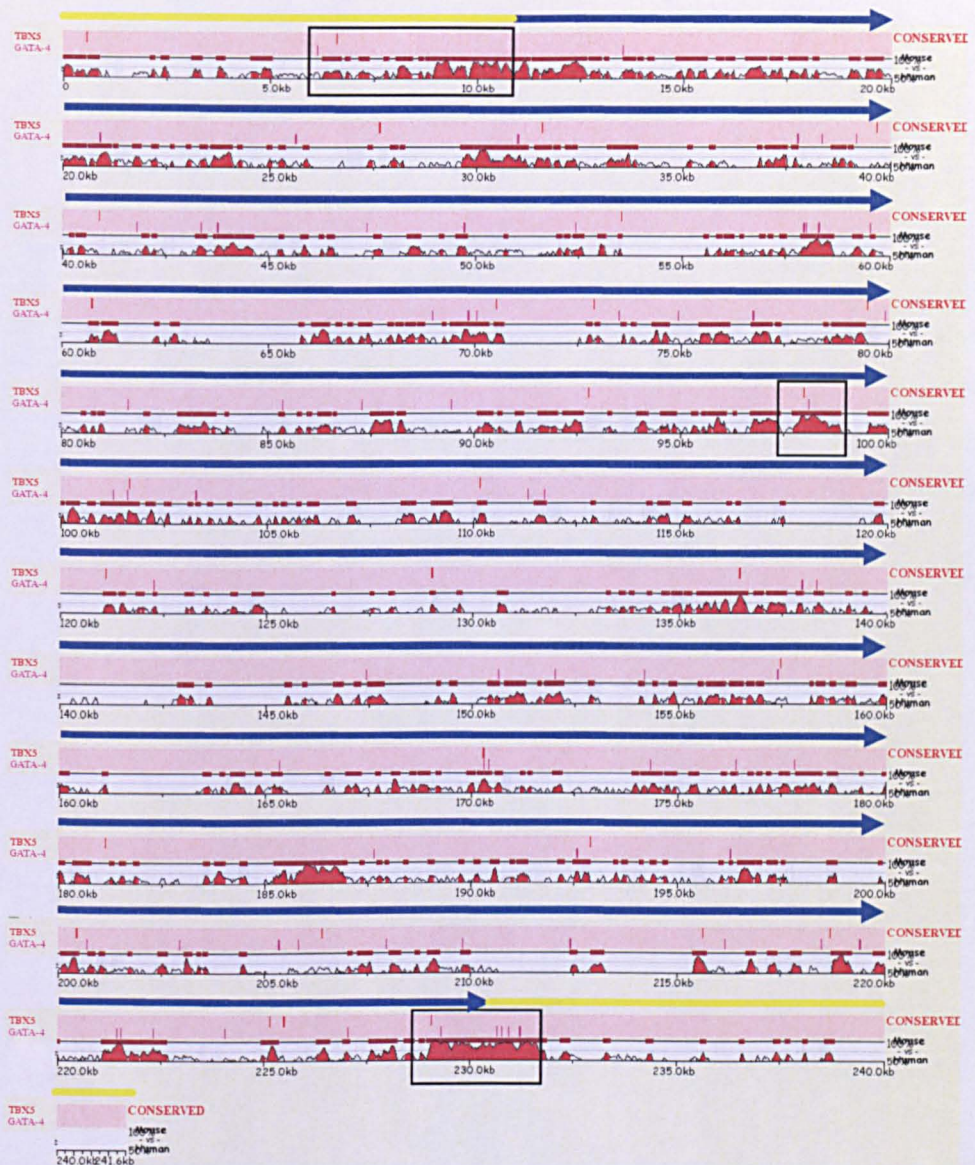


Figure 3.4 Mulan analysis of the RBMS1 gene in human and mouse

Alignment of the full RBMS1 gene plus 10 kb upstream and downstream in human and mouse was performed for identification of consensus TBX5 and GATA-4 binding sites using the online Mulan transcription factor binding site identification tool (<http://mulan.dcode.org/>). Evolutionarily conserved regions with $\geq 50\%$ homology between species are plotted. Conserved consensus sites are represented by vertical red or purple dashes for TBX5 or GATA-4 respectively. Multiple conserved GATA-4 and TBX5 sites were identified throughout the gene. Regions of interest in the 5' UTR, intron 1, and in the 3' UTR are boxed.

3.2.5.2 Primer design and PCR amplification

In order to assess the regulatory activity of regions of interest, they needed to be PCR amplified and cloned into suitable reporter vectors. PCR reactions were carried out using *PfuUltra II Fusion HS* DNA polymerase, a proof-reading enzyme that requires dramatically shortened extension times and is therefore ideal for accurate amplification of long targets. Initially, two primer pairs were designed to each region (sequences in Table 2.2), and PCR amplification attempted with all four primer combinations. The intron 1 region was successfully amplified with all primer combinations, and the fragment selected for cloning is displayed in Figure 3.5. PCR of the 5' and 3' UTR regions resulted either in no amplification, or amplification of multiple bands.

The large size of the 5' UTR region (4.6 kb from the GATA-4 site to the initiating ATG) may have contributed to the difficulties in its amplification, and could also pose problems in subsequent cloning. For study of this region as a promoter the transcriptional start site (but not the translational start site) needs to be included. Two strategies were employed in order to minimise the size of this region; mouse EST searches were carried out to identify the transcribed region, and determination of the transcriptional start site was attempted using a number of online prediction tools. One matching EST was found extending 690 bp 5' of the ATG translational start site (GenBank Accession ID: BP765823). Transcription start site identification tools all gave differing results, so this data was not taken into account. An additional two primer pairs were designed to the shortened region (4 kb) and multiple combinations tested for PCR amplification under various conditions. This again resulted either in no amplification, or amplification of multiple bands.

The 3' UTR region contains long runs of adenine nucleotides which can cause DNA polymerase slippage, resulting in difficulties in PCR amplification. An additional two primer pairs were designed and multiple combinations tested for PCR amplification, again without success.

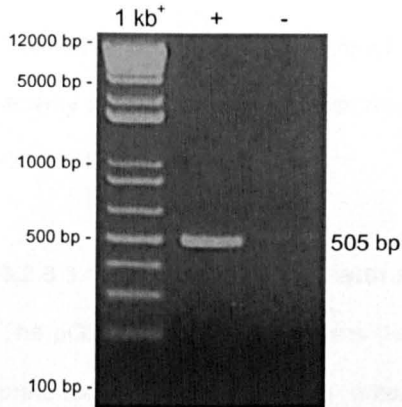


Figure 3.5 PCR amplification of the conserved region within intron 1 of RBMS1

PCR reactions were electrophoresed on 2% agarose gels using 1 kb⁺ DNA ladder (Invitrogen) as a size marker. Negative PCR controls (where water was added in place of template) were included.

3.2.5.3 Destination vectors

The intron 1 fragment was cloned into two destination vectors for assessment of its activity as a regulatory element and the individual and combined effects of TBX5 and GATA-4.

3.2.5.3.1 pGL3-Basic vector with ANF minimal promoter

The pGL3-Basic vector contains the luciferase gene but lacks upstream promoter or enhancer sequences, which means that luciferase expression depends on the insertion of a functional promoter. The human ANF minimal promoter (70 bp) was previously cloned into the KpnI site of this vector by Dr Tushar Ghosh, who kindly provided this for use as a destination vector in this study. The ANF promoter displays basal promoter activity which means this is a sensitive system and allows detection of discrete changes in activation. The intron 1 fragment was cloned into the SacI site of the multiple cloning region of this vector, and is represented in Figure 3.6.

3.2.5.3.2 pGL3-Promoter vector

The pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene, and regions of DNA containing putative enhancer elements can be inserted upstream or downstream of this unit. The SV40 promoter displays strong activity and has advantages when quantifying large changes in activation. The intronic fragment was cloned into the SacI site of the multiple cloning region of this vector, and is displayed in Figure 3.7.

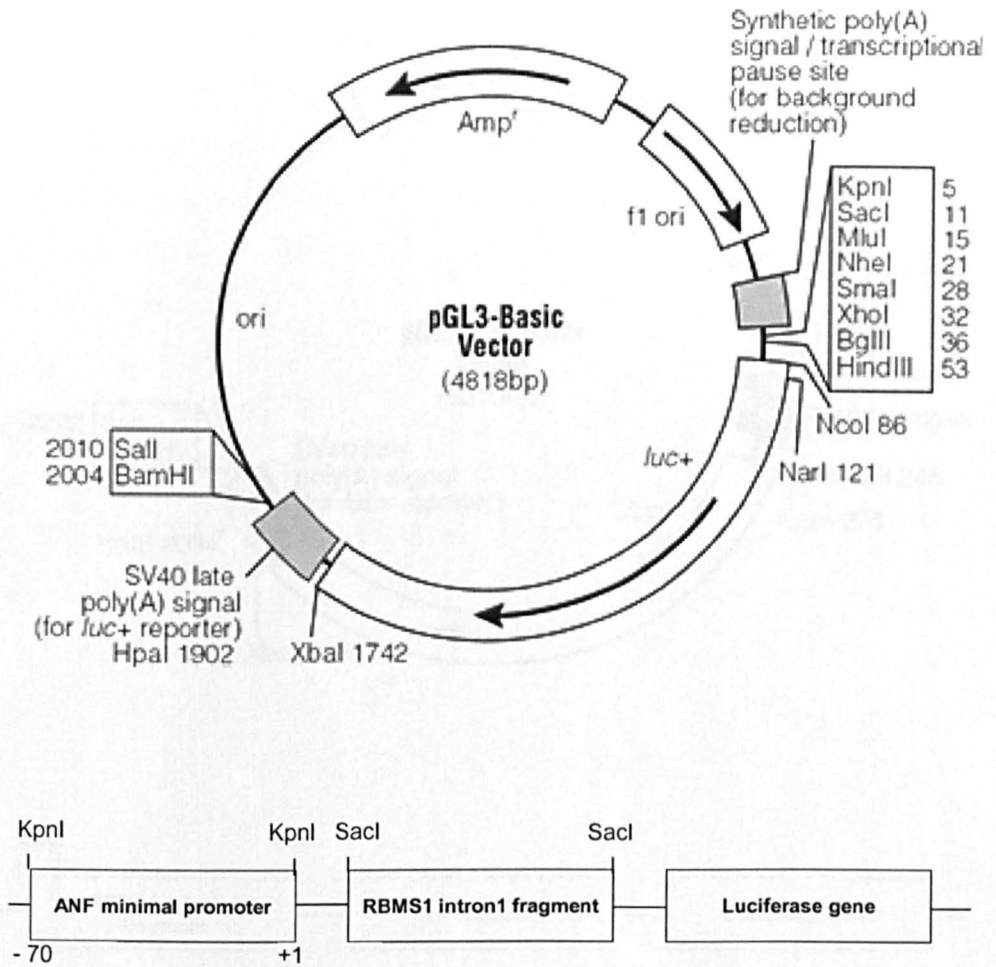


Figure 3.6 pGL3-Basic Vector circle map and schematic displaying position of inserts

The pGL3-Basic vector contains the luciferase gene but lacks upstream promoter or enhancer sequences, which means that luciferase expression depends on the insertion of a functional promoter. The human ANF minimal promoter (70 bp) was previously cloned into the KpnI site of this vector by Dr Tushar Ghosh, and this was kindly provided for use as a destination vector in this study. The intron 1 fragment was cloned into the SacI site of the multiple cloning region of this vector.

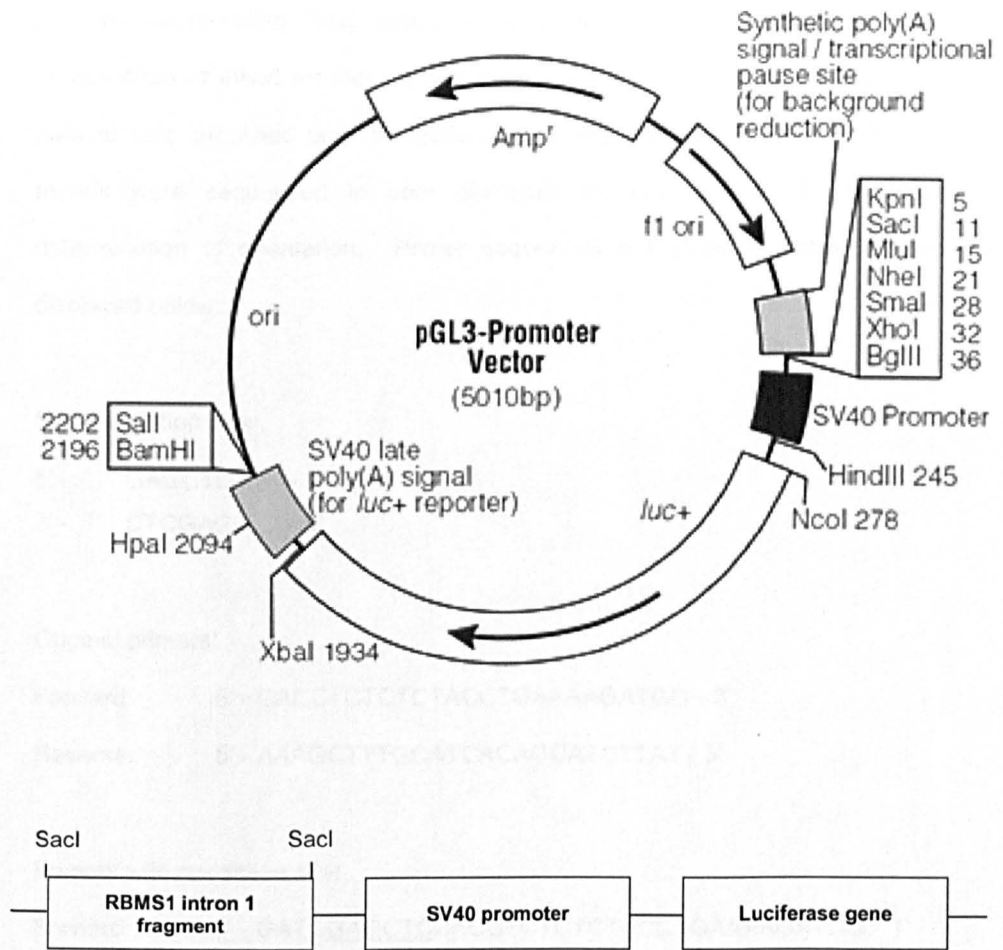


Figure 3.7 pGL3-Promoter Vector circle map and schematic displaying position of insert

The pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene, and regions of DNA containing putative enhancer elements can be inserted upstream or downstream of this unit. The intronic fragment was cloned into the SacI site of the multiple cloning region of this vector.

3.2.5.4 Cloning of the RBMS1 intron 1 fragment

Primers incorporating SacI restriction sites were designed and used for PCR amplification of insert for cloning into destination vectors. PCR products were gel-purified and quantified prior to ligation and initial cloning into the pGEM-T vector. Inserts were sequenced in both directions for confirmation of sequence and determination of orientation. Primer sequences are given in Table 2.3 and are displayed below:

SacI restriction site:

```

5' -   GAGCTC   - 3'
      ▼
3' -   CTCGAG   - 5'
      ▲

```

Original primers:

Forward: 5' - CACCTCTCTCTACCTGAAAAGATGG - 3'

Reverse: 5' - AAAGCTTTGCATCACAGCATCTTAT - 3'

Primers with restriction site:

Forward: 5' - GATCGAGCTCCACCTCTCTCTACCTGAAAAGATGG - 3'

Reverse: 5' - GATCGAGCTCAAAGCTTTGCATCACAGCATCTTAT - 3'

Luciferase reporter assays were performed by Dr Tushar Ghosh, and results are displayed in Appendix D. GATA-4 had a repressive effect on promoter activity via this region, whilst TBX5 had no effect.

CHAPTER 4

STUDY OF CANDIDATE GENES IN THE EMBRYONIC CHICK

4.1 AIMS

This chapter aims to investigate the expression and responsiveness of candidate genes to TBX5 and GATA-4 in the developing chick as follows:

- a) Confirmation of expression of candidate genes in the developing chick heart including investigation of temporal expression
- b) Development of an *in ovo* model of TBX5 and GATA-4 knockdown with appropriate controls
- c) Model validation by phenotypic analysis of embryos subjected to single and double knockdown of TBX5 and GATA-4 during key stages of cardiac development
- d) Development of a robust QPCR method for cardiac expression analysis, and study of candidate gene expression in TBX5 / GATA-4 double knockdown embryonic hearts

4.2 RESULTS

4.2.1 Investigation of candidate gene temporal expression profiles by RT-PCR

Expression of the candidate genes (*PA2.26*, *PETA-3*, *FUCA1*, *FN*, *TPM1*, *DES*, and *RBMS1*) in the developing chick heart was examined at seven stages of development (HH12, HH14, HH16, HH19, HH22, HH24 and HH26). These developmental stages encompass the early stages of heart looping, chamber formation, atrial septation, and the start of ventricular septation. Wild type embryonic age was assessed visually according to Hamburger and Hamilton stages [Hamburger and Hamilton, 1992], and hearts removed under sterile conditions before immediate storage in RNA*later*. Hearts were pooled prior to RNA extraction and cDNA synthesis. PCR amplification was performed using HotStar Taq. Primers were designed using the online Roche Universal ProbeLibrary assay design centre (<https://www.roche-applied-science.com>) as these primers would subsequently be used for QPCR expression analysis (section 4.3.4). Consequently, all products are 60 – 90 bp. Assays spanning exons were selected wherever possible. In the case of α -tropomyosin, alignments with other members of this gene family were performed during assay design to ensure specificity, and the RT-PCR product generated was cloned into the pGEM-T Easy vector for sequencing to confirm amplification of the correct product. Primer sequences and positional information is given in Table 2.4. Expression of all candidate genes was detected in the developing heart at all stages examined (Figure 4.1). Negative RT controls were included and did not display amplification (Appendix E). In addition, amplicons were electrophoresed on the same gel in order to confirm relative sizes (Appendix F).

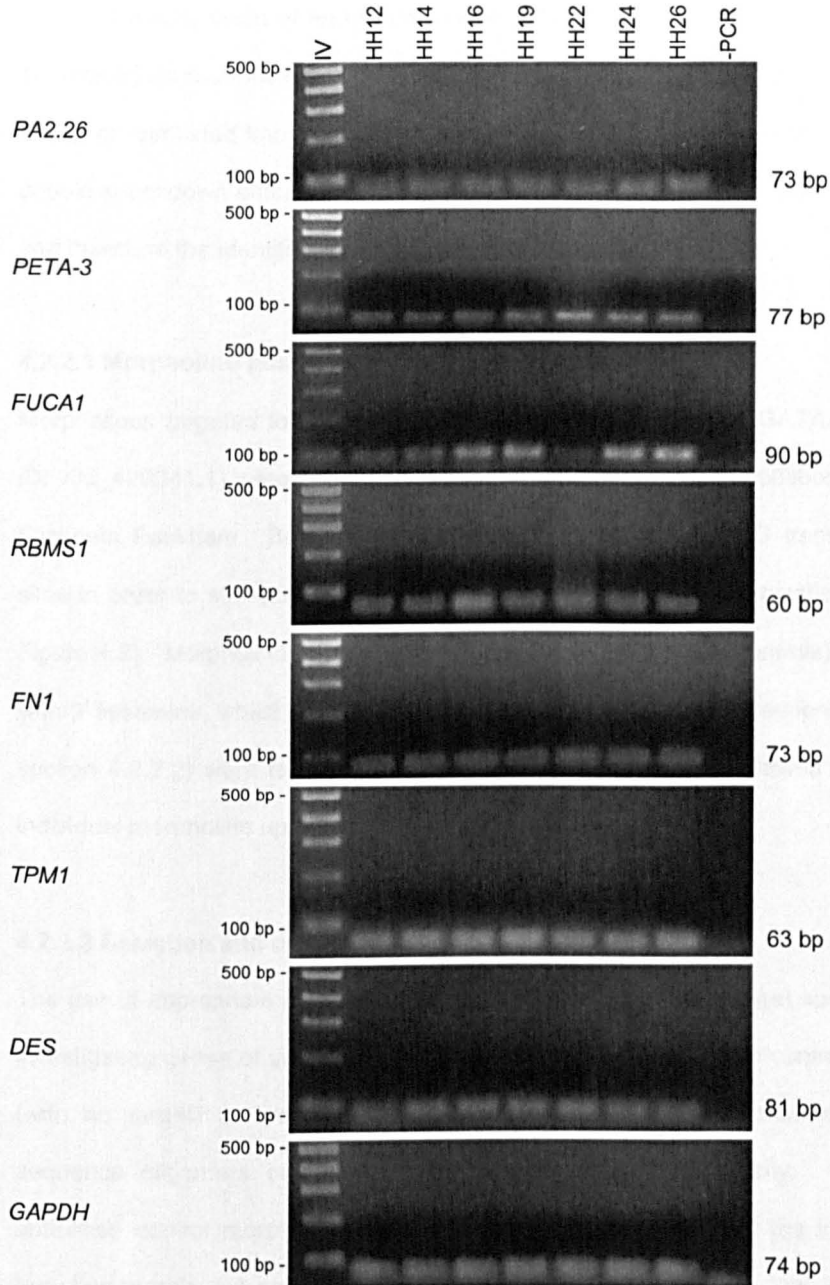


Figure 4.1 RT-PCR expression profiles of candidate genes in the chick heart

RT-PCR products were electrophoresed on 2% agarose gels using Hyperladder IV as a size marker (500 bp and 100 bp bands are indicated along with product sizes). Expression was studied at HH12, HH14, HH16, HH19, HH22, HH24 and HH26. GAPDH was included as a housekeeper gene. Negative PCR controls (where water was added in place of template) are shown (-PCR). Negative RT controls (where reverse transcriptase enzyme was not added) were also included and did not display amplification (Appendix E).

4.2.2 Development of an *in ovo* model of TBX5 and GATA-4 knockdown

To investigate the functional significance of the TBX5 / GATA-4 interaction, an *in ovo* model of combined knockdown was generated. This enabled phenotypic analysis of double knockdown embryos, and expression analysis of candidate downstream genes and therefore the identification of new genes for analysis.

4.2.2.1 Morpholino position and design

Morpholinos targeted to TBX5 (Accession ID: AF033671.1) and GATA-4 (Accession ID: XM_420041.1) were previously designed by Gene-Tools in collaboration with Dr Elizabeth Packham. Both morpholinos are designed to the AUG translational start sites in order to sterically block ribosomal binding and protein translation (Table 2.7, Figure 4.2). Morpholinos targeted to TBX5 (and corresponding controls) were tagged with 3' lissamine, whilst morpholinos targeted to GATA-4 (and corresponding controls, section 4.2.2.2) were tagged with 3' carboxyfluorescein. This allowed evaluation of individual morpholino uptake during combinatorial use.

4.2.2.2 Selection and design of specificity controls

The use of appropriate controls is essential for confirmation of target specificity when investigating genes of unknown function. A pre-designed standard control morpholino (with no target) is available from Gene-Tools (USA), and a number of custom sequence oligomers can also be used as controls of specificity. The inverse-antisense control morpholino is an oligomer whose sequence is the inverse of the targeting morpholino sequence. The advantage of this control is that its length and base composition are identical to that of the antisense morpholino. This is not however widely used as a control of specificity. The sense control morpholino is an oligomer with the reverse complement sequence of the targeting morpholino (identical to the target region). In some cases these have been reported to have an up-regulatory effect on target expression, and are not strongly recommended. The 5-base mismatch control incorporates five mismatched bases into the sequence of the targeting morpholino, most effective when three of out five of these are cytosine-

guanine substitutions or vice versa. At the time this research was carried out (2008), this was considered the most rigorous control of specificity, and was used in addition to the standard control morpholino. Sequences are given in Table 2.7, and Figures 4.2 and 4.3 additionally display morpholino target regions. Actual bases to be mis-paired were selected by Gene-Tools. 5-base mismatch control morpholinos were subsequently replaced with 7-base mismatch morpholinos (section 4.2.2.5), and sequences of these are also shown in Table 2.7 and Figures 4.2 and 4.3.

Chicken TBX5 AUG morpholino

Actual AUG '7-mismatch' MO*
Planned AUG 7-mismatch MO*
AUG 5-mismatch MO
AUG MO
mRNA
Protein

3'- CCAAACGCTGCCGTCGTTCGTCCG - 5'
3'- CGAAAGCTGCCGTCGTTCGTCCG - 5'
3'- CAAACATGGCCGTCCTTACTAGG - 5'
3'- CGAAACGTGGCCCTCCTTCCTCGG - 5'
5'- CUCUCCGCUUUGCACCGGGAGGAAGGAGCCGGAAUGCUCCGCGCCCGCGGACAGUGGGGGAUUCGGCGAAGGAAGCUCGUAACAUGGCGGACACCGAGG - 3'
M L R A R G P V G D S A K E A R N M A D T E

Figure 4.2 TBX5 targeting morpholino sequence and target region

Morpholino sequences are shown in the 3' – 5' direction, and target binding regions are boxed, with AUG start sites shaded in grey. Bases altered in mismatch control morpholinos are underlined. *An error was made during the design of this morpholino and an extra base (guanine) inserted at position 19 in the 5' – 3' direction.

Chicken GATA-4 AUG morpholino

AUG 7-mismatch MO

AUG 5-mismatch MO

AUG MO

mRNA

Protein

3' - GCTGTAGATGCTGTCCAATCCTAC - 5'3' - GCTGTAGATGCTCTCCAATCGTAC - 5'

3' - GCTCTACATGGTCTCGAATCGGTAC - 5'

5' - AUUGCGCUCGGAGACCAUCUGGGGUUGGGGGCCCCGCGAGAGUUACCAGAGCUUAGCCAUGGCGGCGAACCCCGGCCCGGUCCUACGAAGGG - 3'

M Y Q S L A M A A N P G P P S Y E G

Figure 4.3 GATA-4 targeting morpholino sequence and target region

Morpholino sequences are shown in the 3' – 5' direction, and target binding regions are boxed, with the AUG start site shaded in grey. Bases altered in mismatch control morpholinos are underlined.

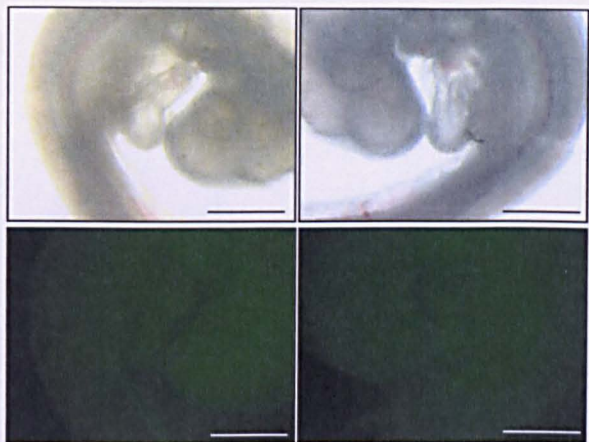
4.2.2.3 Selection of a developmental window for morpholino application

One of the practical considerations in selecting a timeframe for morpholino application is the ease with which embryonic age can be assessed. Based on initial experiments, it was possible to accurately assess the developmental stage of embryos *in ovo* from HH10 onwards, and this is also the stage at which the heart starts to beat. Prior to this a dye such as India ink (commonly used to view vasculature in chicken embryos [Latker et al., 1986]), would be required for visualisation, rendering the embryo unusable for a knockdown study. Also, at any selected time-point, there is a large degree of variation in embryonic age. This means it would be extremely laborious to attempt knockdowns at one focused stage of development, particularly on the scale intended in this study. In light of these considerations, morpholinos were applied at HH10 – HH16, and embryos harvested for analysis at HH19. These are key developmental stages which encompass looping of the heart (dextral looping occurs at HH10 – HH12, post dextral-looping at HH12 – HH17/18), and initiation of atrial septation (HH14). Since TBX5 and GATA-4 mutations have been associated with atrial septal defects, knockdown of these transcription factors in the early stages of atrial septation is ideal for elucidation of their individual and joint functions, and analysis of the effect on postulated downstream targets.

4.2.2.4 Morpholino application and assessment of uptake

Embryos were staged visually prior to morpholino application, as described in section 2.1.6.5. In cases of dual morpholino treatment, two morpholinos each at a concentration of 250 μ M were combined (resulting in a final concentration of 500 μ M). Standard control morpholino was applied singly at 500 μ M in order to match the total concentration of experimental morpholino. Wild type embryos were simultaneously opened and staged. Following incubation, extra-embryonic membranes were removed for assessment of morpholino uptake and photography. Fluorescence levels of two embryos classed as positive for morpholino uptake are shown in Figure 4.4 and indicate typical levels for all embryos used in the study.

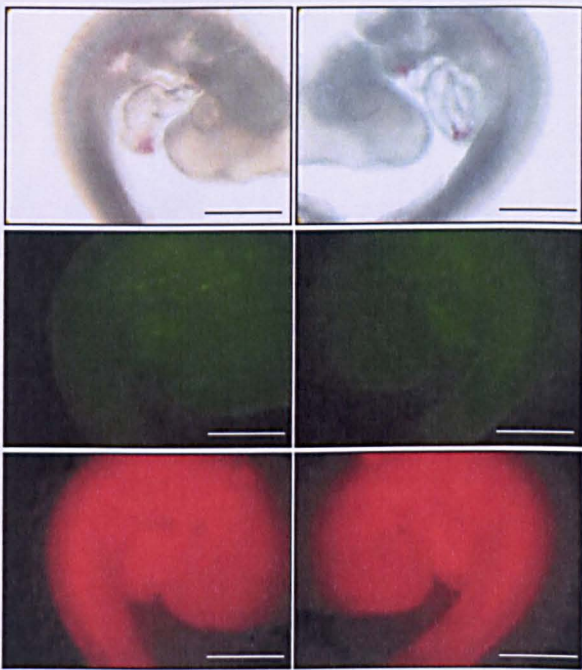
A



Single morpholino treatment:

Standard control 500 μ M
(fluorescein tag)

B



Dual morpholino treatment:

TBX5 5-base mismatch
250 μ M (fluorescein tag)

+

GATA-4 5-base mismatch
250 μ M (lissamine tag)

Figure 4.4 Assessment of morpholino uptake

Bright field and fluorescent views of embryos classed as positive for morpholino uptake are displayed and indicate typical fluorescence levels for all embryos used in the study. (A) Single embryo treated with a single fluorescein tagged standard control morpholino at 500 μ M (displayed from the left and right). (B) Single embryo treated with two morpholinos, TBX5 5-base mismatch at 250 μ M, and GATA-4 5-base mismatch at 250 μ M (displayed from the left and right). Scale bars represent 1000 μ m.

4.2.2.5 Preliminary testing of targeting and control morpholinos *in ovo*

Initial phenotypic analysis of embryos was carried out externally and is displayed in Figure 4.5. Outflow tract and ventricular regions are labelled. Figure 4.5 A1-A2 and B1-B2 depict wild type and standard control treated embryonic hearts, which display normal curvature and shape. C1 and C2 represent examples of TBX5 / GATA-4 double 5-base mismatch treated embryos, which respectively display a constricted outflow tract and dilated ventricle. Of the double mismatch control embryos, 3 / 10 externally harboured a cardiac defect. Since these were similar to, but less severe than, the abnormalities observed in knockdown embryos (discussed later in section 4.2.7), it was hypothesised that one or both of the control morpholinos was still able to bind to the target region, but with lower affinity than the targeting morpholinos. In order to overcome this problem, and upon recommendation by Gene-Tools, both 5-base mismatch morpholinos were replaced with 7-base mismatch controls. Where possible, different bases were altered to those originally modified (Table 2.7, Figures 4.2 and 4.3). The new controls were again tested prior to use to rule out low affinity binding or mis-targeting effects.

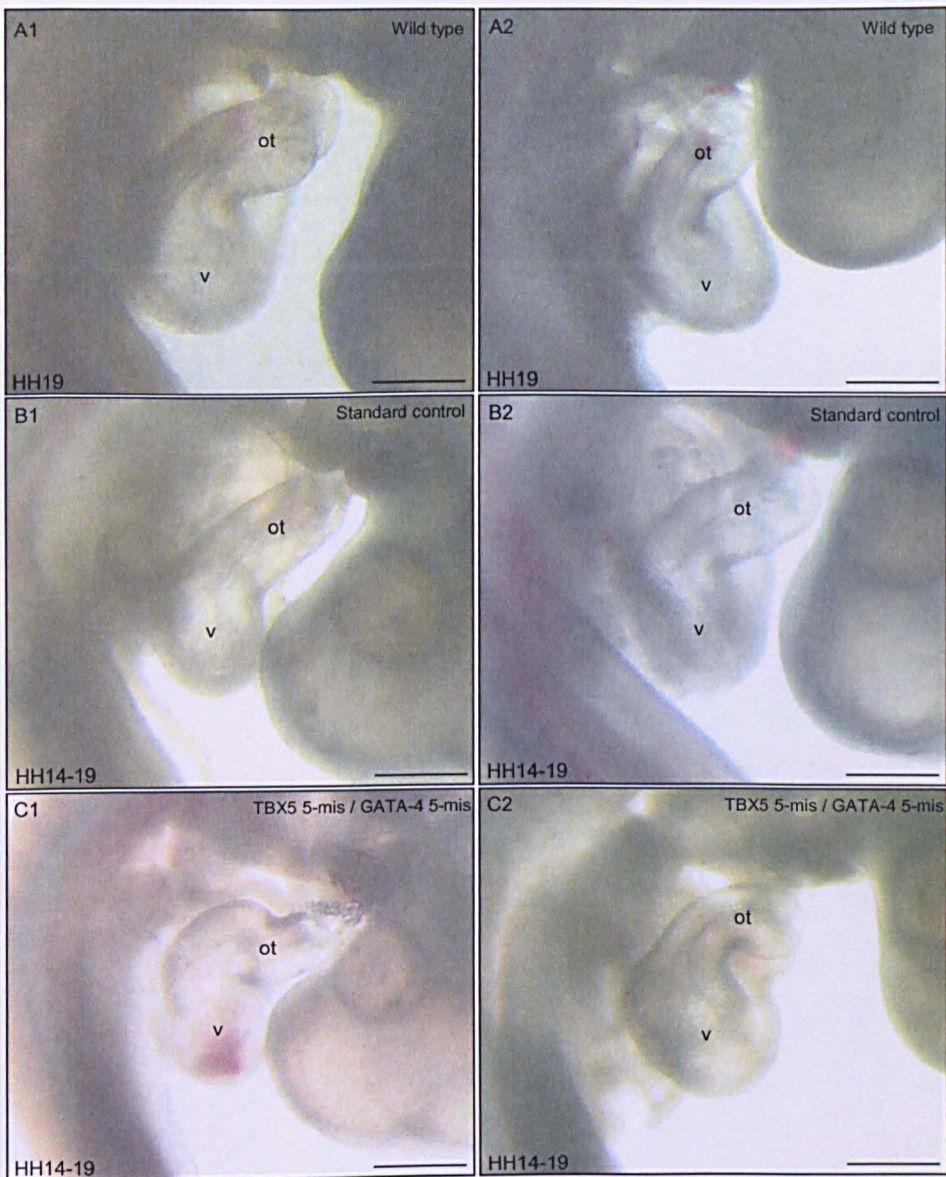


Figure 4.5 External phenotypic analysis of embryos treated with 5-mismatch morpholinos in comparison to standard control-treated and wild type embryos

Bright field lateral embryonic views are displayed with developmental stages of morpholino application and embryo harvest. Standard control morpholino was applied at a concentration of 500 μ M (B1 and B2), and 5-mismatch control morpholinos each applied at 250 μ M for a final morpholino concentration of 500 μ M (C1 and C2). Scale bars represent 500 μ m. ot – outflow tract, v - ventricle

4.2.2.6 External phenotypic analysis of single and double knockdown embryos

4.2.2.6.1 Whole body analysis

Figure 4.6 shows whole embryonic views of animals subjected to various morpholino treatments. Wild type, standard control and double mismatch control embryos display a normal body shape and 'c' shaped curvature (Figure 4.6 A1, B1 and C1 respectively). Embryos subjected to individual knockdown of TBX5 and GATA-4 (Figure 4.6 D1 and E1) diverted only slightly from this. In contrast, double knockdown embryos (Figure 4.6 F1 – F5) displayed a variety of defects including growth retardation (F3), body kinking and/or twisting (F1, F3, F4), and severe defects in heart looping (F2 and F5).

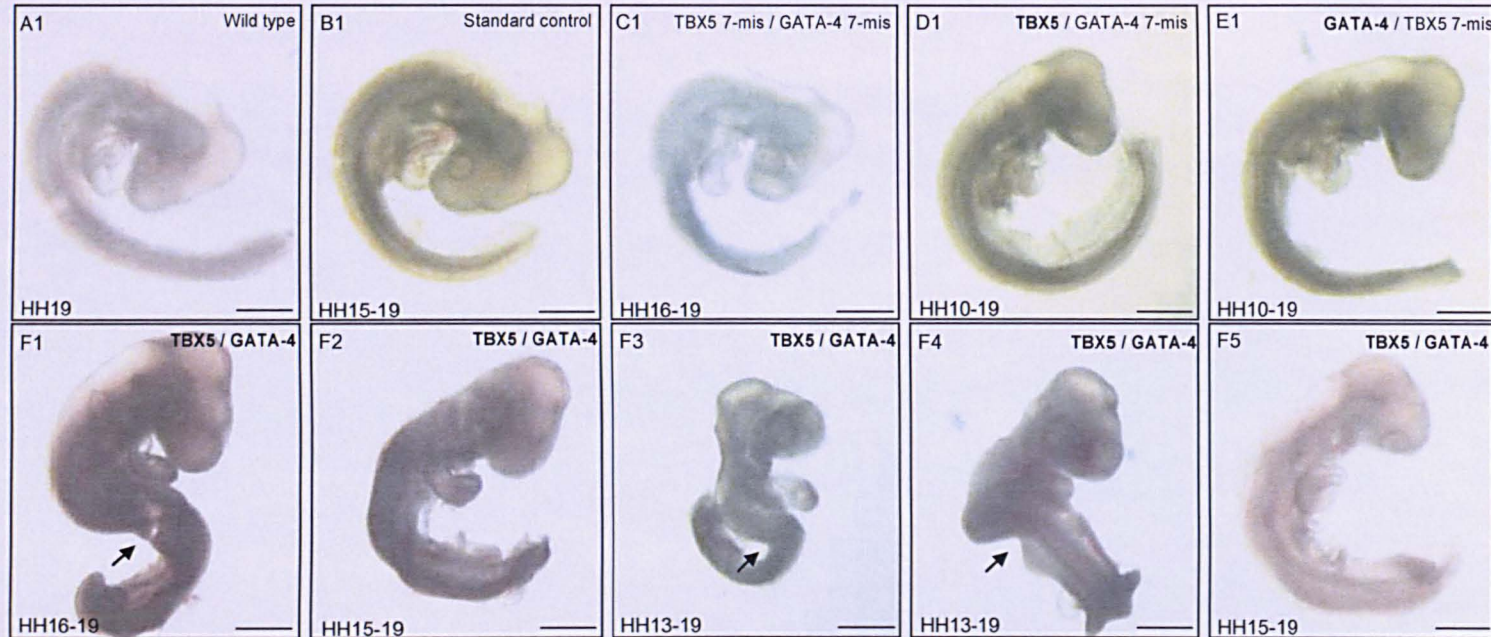


Figure 4.6 External whole body analysis of TBX5 / GATA-4 single and double knockdown embryos

Bright field lateral embryonic views are displayed with developmental stages of morpholino application and embryo harvest. Embryos were treated with a final morpholino concentration of 500 μM. A1, B1 and C1 show a wild type, standard control and double mismatch control embryo respectively. D1 and E1 are examples of TBX5 single knockdown and GATA-4 single knockdown embryos respectively. F1 – F5 are examples of double knockdown embryos. Arrows indicate abnormalities in body shape. Scale bars represent 1000 μm.

4.2.2.6.2 Cardiac analysis

External analysis focusing on embryonic hearts was also performed and is displayed in Figure 4.7. Outflow tract and ventricular regions are marked where identifiable. Embryos in the three control groups showed no outward abnormalities (Figure 4.7 A1 – C1). TBX5 knockdown resulted in heart malformations, primarily ventricular (Figure 4.7 D1) in 2 / 3 embryos. GATA-4 single knockdown embryos displayed a similar phenotype (Figure 4.7 E1), and 3 / 4 embryos were affected. A variety of heart defects were observed in double knockdown animals and five examples are shown (Figure 4.7 F1 – F5). These included severe heart-restricted growth retardation (F3), ventricular constriction (F4), and overall malformations with abnormal heart looping in all embryos shown (F1 – F5). Cardiac defects were present in 9 / 12 double knockdown embryos. External phenotypic analysis data is summarised in Table 4.2 (with additional internal analysis data, see next section). These findings are consistent with the phenotype of *Gata4*^{+/-} *Tbx5*^{+/-} mice [Maitra et al., 2009], published during the time of this study.

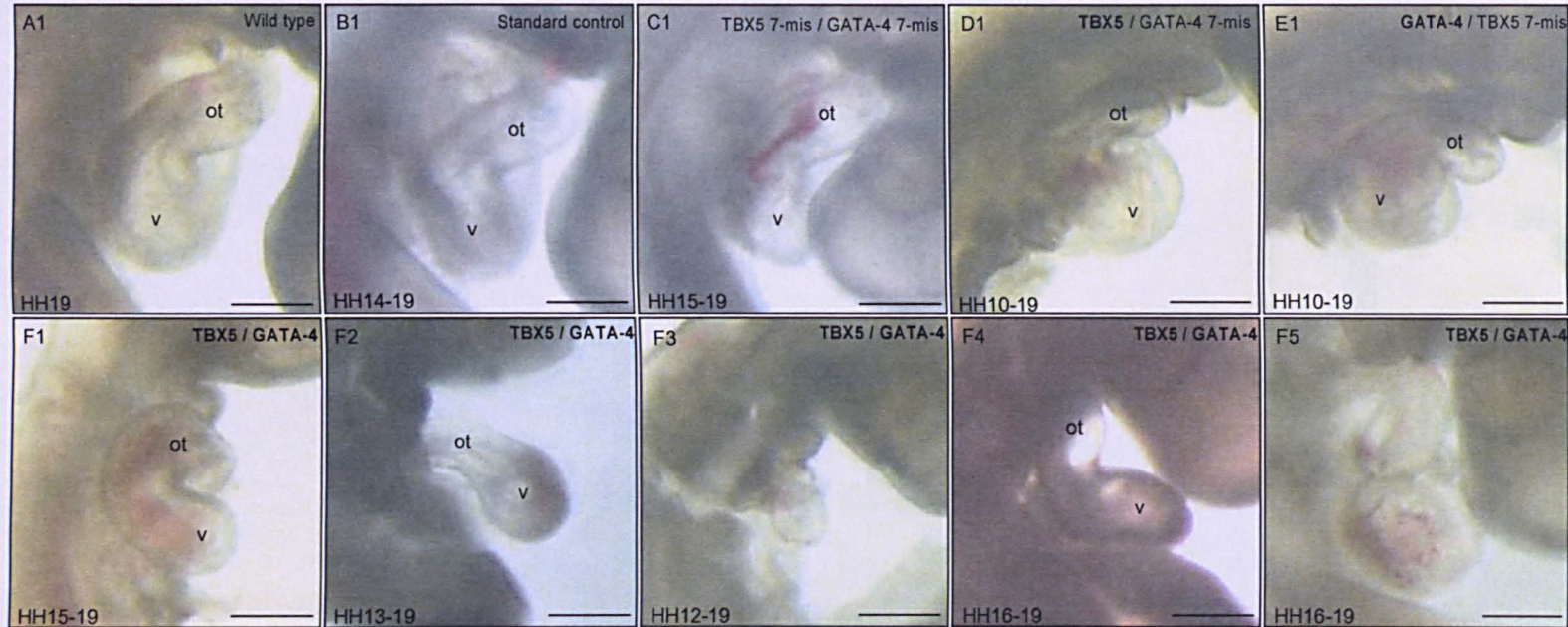


Figure 4.7 External cardiac analysis of TBX5 / GATA-4 single and double knockdown embryos

Bright field lateral embryonic views are displayed with developmental stages of morpholino application and embryo harvest. Embryos were treated with a final morpholino concentration of 500 μM. A1, B1 and C1 show a wild type, standard control and double mismatch control embryo respectively. D1 and E1 are examples of TBX5 single knockdown and GATA-4 single knockdown embryos respectively. F1 – F5 are examples of double knockdown embryos. Scale bars represent 500 μm. ot – outflow tract, v - ventricle

4.2.2.7 Histological analysis of embryos

Following photography, embryos were fixed in 4% PFA then dehydrated in a graded ethanol series prior to being embedded in wax. Embryos were sectioned coronally at 10 microns and sections were transferred onto slides for haemalum staining and mounting with DPX. Due to the high proportion of knockdown embryos with gross cardiac malformations (particularly in cardiac looping), consistent orientation of hearts for sectioning in the same plane was not possible in all cases, with resulting difficulties in comparative phenotypic analysis.

Figure 4.8 shows haemalum stained sections through embryos in the three control groups; (A) wild type, (B) standard control, and (C) TBX5 7-base mis / GATA-4 7-base mis, and two example embryos from each group are displayed. Regions of atrial septum formation are shown at their maximum point upon viewing serial sections throughout the embryo, and are boxed. All embryos displayed normal sized atrial septa.

Figure 4.9 shows haemalum stained sections through TBX5 and GATA-4 single and double knockdown embryos. Again, regions of atrial septum formation are shown at their maximum size. TBX5 knockdown embryos, in addition to the external cardiac defects seen, displayed a slight reduction in atrial septum size (Figure 4.9 A1 and A2). GATA-4 knockdown also resulted in a less pronounced atrial septum size in one embryo (Figure 4.9 B1). Unfortunately the orientation of other sectioned GATA-4 knockdown embryos did not allow analysis of septa size (e.g. B2).

Double knockdown of TBX5 and GATA-4 resulted in a number of internal cardiac defects (Figure 4.9 C1 and C2). C1 displayed an abnormal shape, and upon viewing sections throughout the heart, a complete lack of trabeculation was also observed. The beginnings of an atrial septum were visible. The second double knockdown embryo shown (C2) displayed a localised thickening of the cardiac jelly at the point of atrial septum formation, but no septum, despite the large overall size of the heart.

Phenotypic analysis data is summarised in Table 4.2. Approximately 75% of double knockdown embryos displayed a cardiac defect.

These findings are consistent with the phenotype of $Gata4^{+/-}$ $Tbx5^{+/-}$ compound heterozygote mice [Maitra et al., 2009], published at the time of this study (2008/9). Due to the phenotypic characterisation of compound heterozygote embryos performed in this publication, analysis of phenotype was not carried out on as large a scale as originally planned, and was used primarily for the purposes of technique validation and confirmation of knockdown.

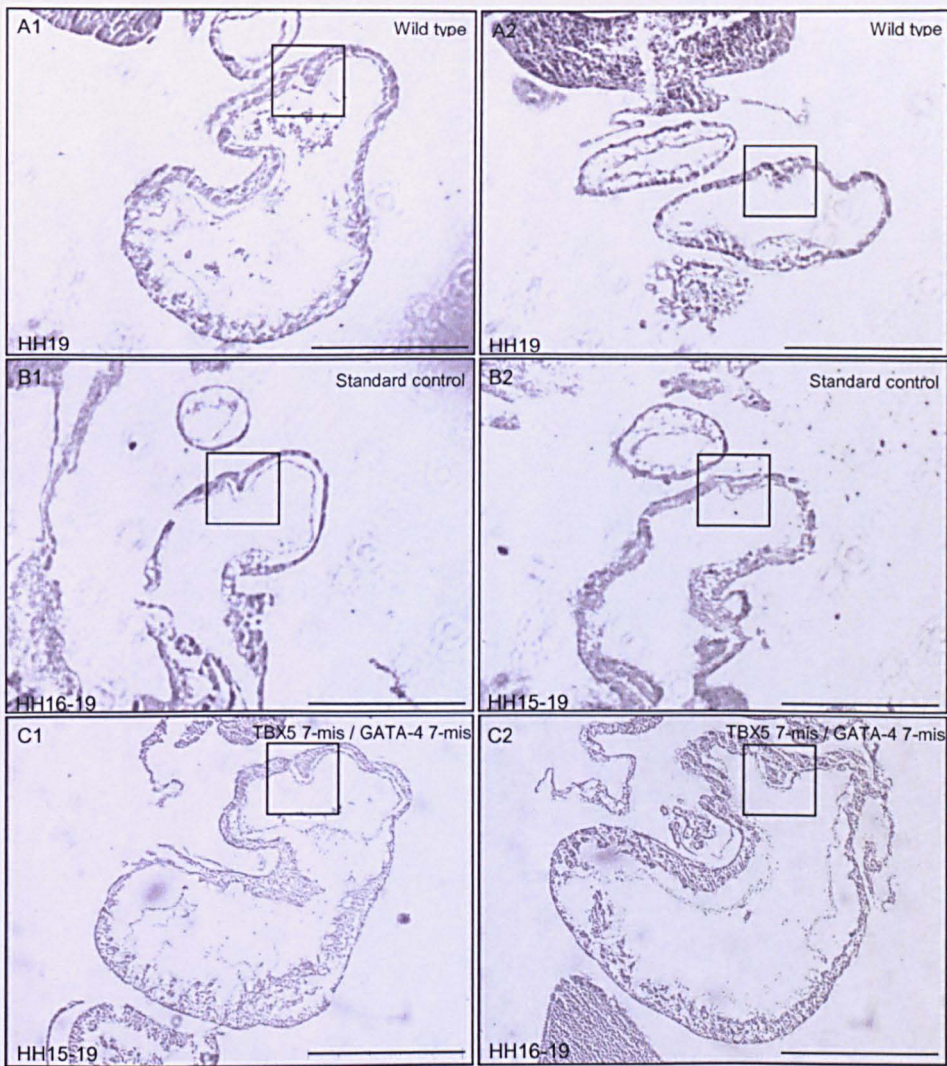


Figure 4.8 Histological analysis of the three control groups

Haemalum stained coronal embryonic sections through two embryos in each group are displayed. Three control groups were studied; (A) wild type, (B) standard control, and (C) TBX5 7-base mis / GATA-4 7-base mis. Developmental stages of morpholino application (where applicable) and embryo harvest are indicated. A final morpholino concentration of 500 μM was used in cases of morpholino treatment. Regions of atrial septa formation are boxed, shown at their maximum size upon viewing serial sections throughout the embryo. Scale bars represent 500 μm.

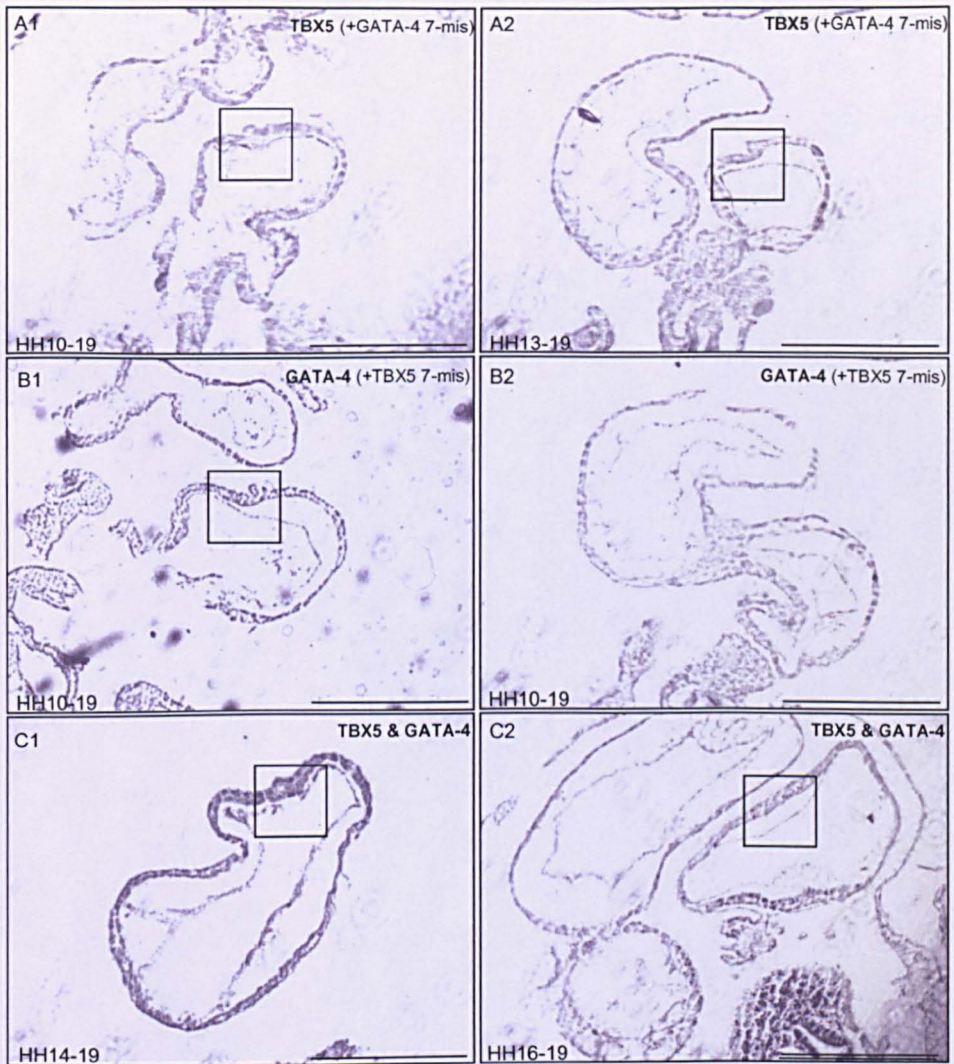


Figure 4.9 Histological analysis of TBX5 and GATA-4 knockdown embryos

Haemulium stained coronal embryonic sections through two embryos in each group are displayed. Three knockdown groups are represented; (A) TBX5 single knockdown (+ GATA-4 7-base mis), (B) GATA-4 single knockdown (+TBX5 7-base mis), and (C) TBX5 / GATA-4 double knockdown. Developmental stages of morpholino application (where applicable) and embryo harvest are indicated. Each morpholino was applied at a concentration of 250 μM , resulting in a final morpholino concentration of 500 μM . Regions of atrial septa formation are boxed, shown at their maximum size upon viewing serial sections throughout the embryo. Scale bars represent 500 μm .

Table 4.1 Summary of abnormalities seen in TBX5 / GATA-4 single and double knockdown embryos and controls

Nature/region of heart abnormality		Wild type	Standard control	Double mismatch control	TBX5 knockdown / GATA-4 mismatch	GATA-4 knockdown / TBX5 mismatch	TBX5 / GATA-4 double knockdown
External analysis	Body size / shape	0 / 3	0 / 3	0 / 3	0 / 3	1 / 4	7 / 12
	Heart size / shape	0 / 3	0 / 3	0 / 3	2 / 3	2 / 4	8 / 12
	Heart looping	0 / 3	0 / 3	0 / 3	2 / 3	1 / 4	8 / 12
	Other	0 / 3	0 / 3	0 / 3	1 / 3	1 / 4	2 / 12
	Total	0 / 3	0 / 3	0 / 3	2 / 3	3 / 4	9 / 12
Internal analysis	Atrial septum	0 / 3	0 / 3	0 / 3	2 / 2	1 / 1	1 / 3
	Trabeculae	0 / 3	0 / 3	0 / 3	0 / 3	-	2 / 3
	Cardiac jelly	0 / 3	0 / 3	0 / 3	1 / 3	0 / 3	2 / 3
	Total	0 / 3	0 / 3	0 / 3	2 / 3	1 / 3	3 / 3

Groups represented are wild type, standard control, TBX5 / GATA-4 double mismatch control, TBX5 single knockdown (with GATA-4 mismatch control), GATA-4 single knockdown (with TBX5 mismatch control), and TBX5 / GATA-4 double knockdown.

4.2.3 QPCR expression analysis of candidate genes in TBX5 / GATA-4 double knockdown embryos

For assessment of the responsiveness of candidate genes to TBX5 and GATA-4 in the developing chick heart, expression was analysed by QPCR in double knockdown embryos and controls.

4.2.3.1 Sample collection and preparation

Approximately 600 eggs were opened in total for this study. Morpholino was applied to around half following exclusion of embryos which were no longer viable or not within the desired range of development (HH12 – HH16). Morpholino uptake was confirmed in HH19 stage embryos prior to tissue isolation. Eight hearts were pooled per sample, and three biological replicates collected per group (3 groups: wild type (calibrator), double 7-base mismatch control, and double knockdown). Exactly 1 µg RNA was used per 20 µl reverse transcription reaction for identical loading in subsequent reactions. cDNA for all samples was prepared simultaneously, appropriately diluted (section 2.2.1.10.5), aliquoted, and stored at -80 °C prior to use. HH24 whole embryo cDNA was used for generation of standard curves.

4.2.3.2 QPCR assay design

QPCR assays were designed as set out in section 2.2.1.10.1, generally spanning a 60 – 100 bp region including an exon-exon boundary, and were suitable for use with an annealing temperature of 60 °C. Primer sequences and assay information are given in Table 2.4. These primers were also used for RT-PCR expression profiling (Figure 4.1) which additionally served to confirm products sizes and single product amplification prior to QPCR. Assays were designed to all seven candidate genes (*PA2.26*, *PETA-3*, *FUCA1*, *FN*, *TPM1*, *DES*, and *RBMS1*), and four potential endogenous control genes - *GAPDH*, *TBP*, *EEF1A1*, and *RPLPO*. The gene *MYH6*, a known target of TBX5 and GATA-4 [Ching et al., 2005; Huang et al., 1995; Molkenin et al., 1994], was also included as a positive control. During the time of this study,

MYH6 was additionally shown to be down-regulated in *Gata4*^{+/-} *Tbx5*^{+/-} compound heterozygote mice at E11.5 and E13.5 [Maitra et al., 2009].

4.2.3.3 Comparative C_T method ($\Delta\Delta C_T$) versus Relative Standard Curve method

In Chapter 3, the Comparative C_T method was used for QPCR expression analysis in the P19 cell line using transmembrane protein 131 (*tmem131*) as an endogenous control, generating a data set that was both accurate and reproducible. This method was attempted here using GAPDH as an endogenous control, but it was not possible to obtain reproducible results (not shown). The Comparative C_T method does not require the use of standard curves on each reaction plate, and whilst this has economical and time-savings advantages (particularly for high-throughput screening) it also means that it is not possible to assess the efficiency or reliability of each individual run. An alternative method, the Relative Standard Curve method, overcomes this limitation and was tested and optimised for quantification of gene expression in the chick. This method involves the construction of standard curves from known quantities of DNA for both the target and endogenous control, with sample DNA amplified simultaneously under identical conditions. Sample target and endogenous reference quantities are determined using this information, and assay efficiency data is generated in parallel, producing highly accurate data.

4.2.3.4 Selection of an endogenous control for relative quantification

Selection of an appropriate endogenous control gene is a crucial step in relative quantification experiments. Normalisation to an endogenous control allows correction of any skewing of data arising from small differences in starting quantity of template. The endogenous control must be expressed at the same time as the target gene, and display uniform expression across all samples i.e. expression should not be affected by sample treatments. Four genes were selected as potential candidates – *TBP* (TATA box binding protein), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *EEF1A1* (eukaryotic translation elongation factor 1 alpha 1) and *RPLPO* (large ribosomal protein). These are commonly used as endogenous controls in QPCR.

Primer sequences and assay information are given in Table 2.4. RT-PCR products generated using these primers were checked for size and specificity prior to QPCR (data not shown).

4.2.3.4.1 Preparation of standard curves

In order to assess QPCR assay efficiency and suitability of these four genes for use as endogenous controls, standard curves were constructed for each gene (using a 10 point dilution series of 4-fold). These are shown in Figures 4.10 – 4.13, and standard curve linearity and assay efficiency values are summarised in Table 4.2. As mentioned, a linearity of ≥ 0.99 and slope in the range -3.2 to -3.6 is desirable. All assays generated standard curves that met the linearity criteria. The slope (and therefore efficiency) of the GAPDH assay was outside the acceptable range so this gene was excluded. TBP, whilst meeting the criteria set out, generated a poor amplification plot (Figure 4.11a) with amplification starting at the high cycle number of 26 (EEF1A1 and RPLPO expression was observed from 18 and 16 cycles respectively). On this basis, TBP was also excluded, and EEF1A1 and RPLPO were short-listed for normalisation of data.

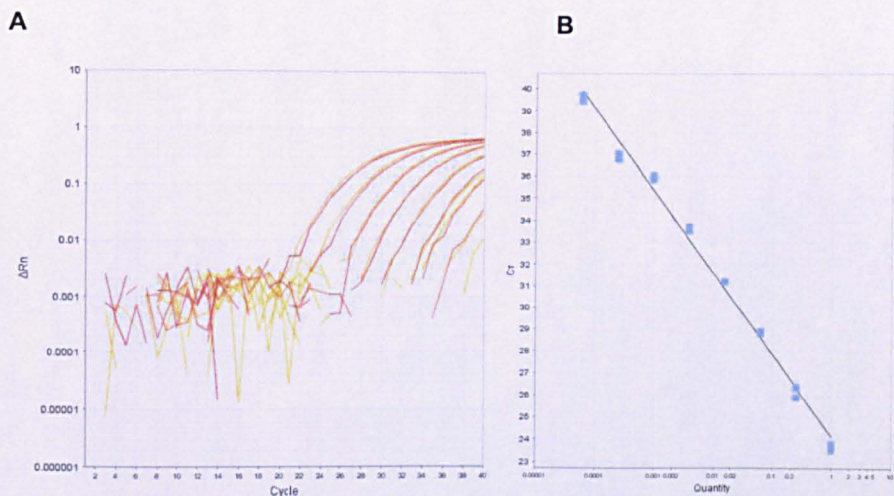


Figure 4.10 GAPDH amplification plot (A) and resulting standard curve (B)

Two replicate reactions were performed at ten concentrations of cDNA, based on a 4-fold dilution series. Amplification efficiency was calculated from the slope of the standard curve using the equation $E = 10^{[-1/\text{slope}]}$. Slope: -3.738, R^2 : 0.992, Efficiency (%): 85.132

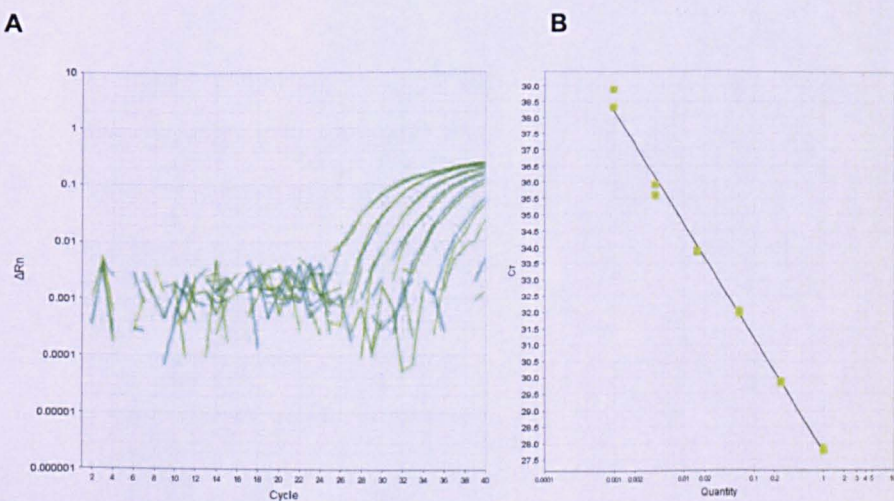


Figure 4.11 TBP amplification plot (A) and resulting standard curve (B)

Two replicate reactions were performed at ten concentrations of cDNA, based on a 4-fold dilution series. Amplification efficiency was calculated from the slope of the standard curve using the equation $E = 10^{[-1/\text{slope}]}$. Slope: -3.49, R^2 : 0.994, Efficiency (%): 93.427

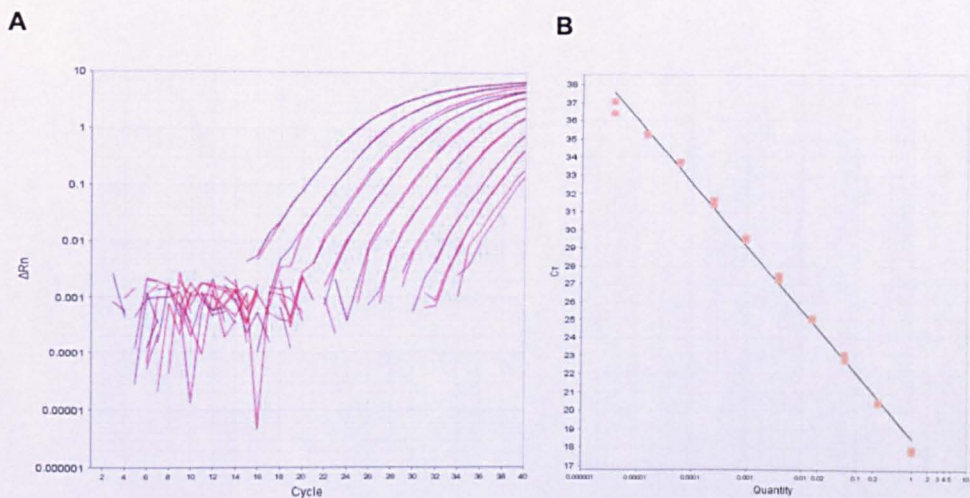


Figure 4.12 EEF1A1 amplification plot (A) and resulting standard curve (B)

Two replicate reactions were performed at ten concentrations of cDNA, based on a 4-fold dilution series. Amplification efficiency was calculated from the slope of the standard curve using the equation $E = 10^{-1/\text{slope}}$. Slope: -3.513, R^2 : 0.995, Efficiency (%): 92.611

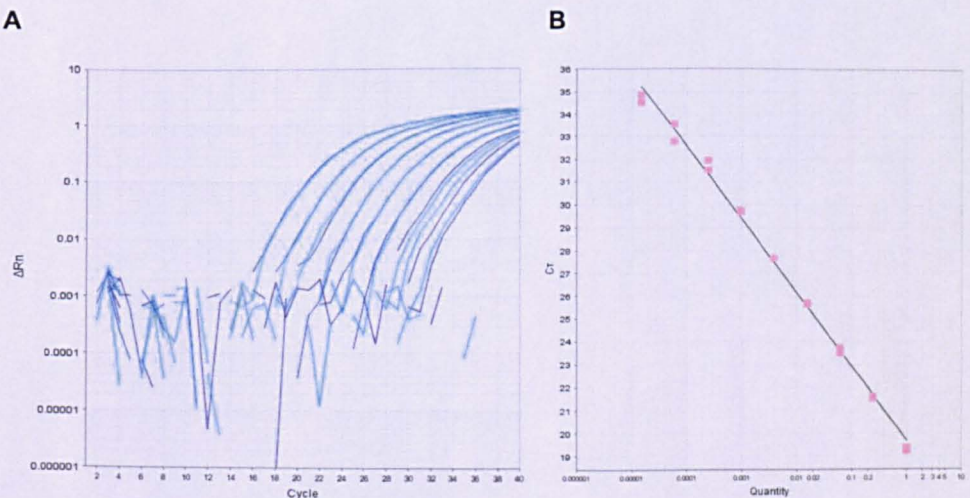


Figure 4.13 RPLPO amplification plot (A) and resulting standard curve (B)

Two replicate reactions were performed at ten concentrations of cDNA, based on a 4-fold dilution series. Amplification efficiency was calculated from the slope of the standard curve using the equation $E = 10^{-1/\text{slope}}$. Slope: -3.226, R^2 : 0.995, Efficiency (%): 104.142

Table 4.2 Summary of endogenous control gene assay properties

Gene	Linearity (R^2)	Slope	Efficiency (%)	Starting cycle
GAPDH	0.992	-3.738	85.1	22
TBP	0.994	-3.490	93.4	26
EEF1A1	0.995	-3.513	92.6	18
RPLPO	0.995	-3.226	104.1	16

Values are based on standard curves prepared from a 10-point 4-fold dilution series using HH24 whole embryo cDNA (Figures 4.10 – 4.13).

4.2.3.4.2 Analysis of variation of endogenous control expression levels in study samples

Expression of an endogenous control genes used for normalisation of data must not be affected by experimental treatments, so should display uniform expression levels across all sample groups (3 groups: wild type (calibrator), double 7-base mismatch control, and double knockdown). To investigate this, C_T values of EEF1A1 and RPLPO were measured in triplicate for each sample following their identical preparation and dilution. ΔC_T values were calculated for each group (Table 4.3) from mean C_T values of biological and experimental replicates using the equation:

$$\Delta C_{T(\text{sample})} = \text{Average } C_{T(\text{calibrator})} - \text{Average } C_{T(\text{sample})}$$

Samples with higher template concentrations than the calibrator result in lower C_T values and positive ΔC_T values, and vice-versa. A ΔC_T value of 1 equates to a two-fold difference in expression, and the ΔC_T value of an ideal endogenous control will vary only slightly from zero indicating stable expression. Both RPLPO and EEF1A1 displayed relatively constant expression across samples (maximum ΔC_T values of 0.36 and 0.44 respectively) and were suitable for use.

RPLPO was selected for use as the endogenous control since the range of ΔC_T values observed across samples was narrowest, and assay linearity (0.995) and efficiency (104%) were closest to ideal.

Table 4.3 Mean C_T and ΔC_T values of EEF1A1 and RPLPO in study samples

Sample	RPLPO		EEF1A1	
	Mean C_T	ΔC_T	Mean C_T	ΔC_T
Wild type (calibrator)	27.17389	0	26.54730	0
7-mismatch control	26.85162	0.322276	26.71622	-0.16892
Double knockdown	26.81002	0.363879	26.99526	-0.44797

Three biological samples were analysed per group, each assayed in triplicate. Standard curves for both genes were run in parallel on the same reaction plate for confirmation of assay quality.

4.2.3.5 QPCR expression analysis of candidate genes

The calibrator sample (in this case untreated or wild type) serves as a baseline for comparison of target and endogenous control levels, and also allows comparison across reaction plates. Baseline and threshold values were set manually, and absolute quantities of each gene generated from the standard curves in Applied Biosystems 7500 v2.0.1 software. Standard curves for target and endogenous control genes were included on each plate, with each point measured in duplicate. All samples were run simultaneously on the same plate, each assayed in triplicate. Reaction plates were run twice. Biological and experimental replicates were grouped for data normalisation and standard deviation calculations.

Results of QPCR expression analysis are displayed in Table 4.4 and Figure 4.14. Expression is displayed relative to expression of the endogenous control RPLPO. PA2.26 and FUCA1 displayed very low expression levels which were not accurately

quantifiable, and it was therefore necessary to exclude these from the QPCR study. Expression of the other five candidate genes (PETA-3, DES, TPM1, FN, and RBMS1) was accurately quantified but no significant differences were observed between the double knockdown and control groups (measured by the student's t test using $p < 0.05$ as the threshold value). MYH6 was used as a positive control in this study as it known to be regulated by TBX5 and GATA-4 [Ching et al., 2005; Huang et al., 1995; Molkentin et al., 1994]. This gene did not however display any change in expression.

Table 4.4 QPCR expression analysis of candidate genes in TBX5 / GATA-4 knockdown animals and controls

Gene	Relative expression		
	Double knockdown	Double mismatch control	Wild type (untreated control)
PETA-3	1.80 ± 0.64	1.69 ± 0.47	1.50 ± 0.31
Desmin	9.79 ± 2.66	10.1 ± 2.58	10.9 ± 2.26
α-tropomyosin	2.34 ± 0.75	2.45 ± 0.67	2.86 ± 0.44
Fibronectin	4.31 ± 1.38	3.43 ± 1.14	4.53 ± 0.81
RBMS1	2.10 ± 0.49	1.81 ± 0.49	1.84 ± 0.41
MYH6	31.0 ± 10.9	25.3 ± 7.1	26.9 ± 7.2

QPCR expression analysis was performed on cardiac tissues from TBX5 / GATA-4 double knockdown embryos, TBX5 7-mis / GATA-4 7-mis control embryos, and wild type embryos (3 biological replicates per group). Each reaction plate was run in duplicate. Data was normalised to RPLPO. No significant differences in expression were observed for any gene (measured between the knockdown group and both control groups by the student's t test using $p < 0.05$ as the threshold value).

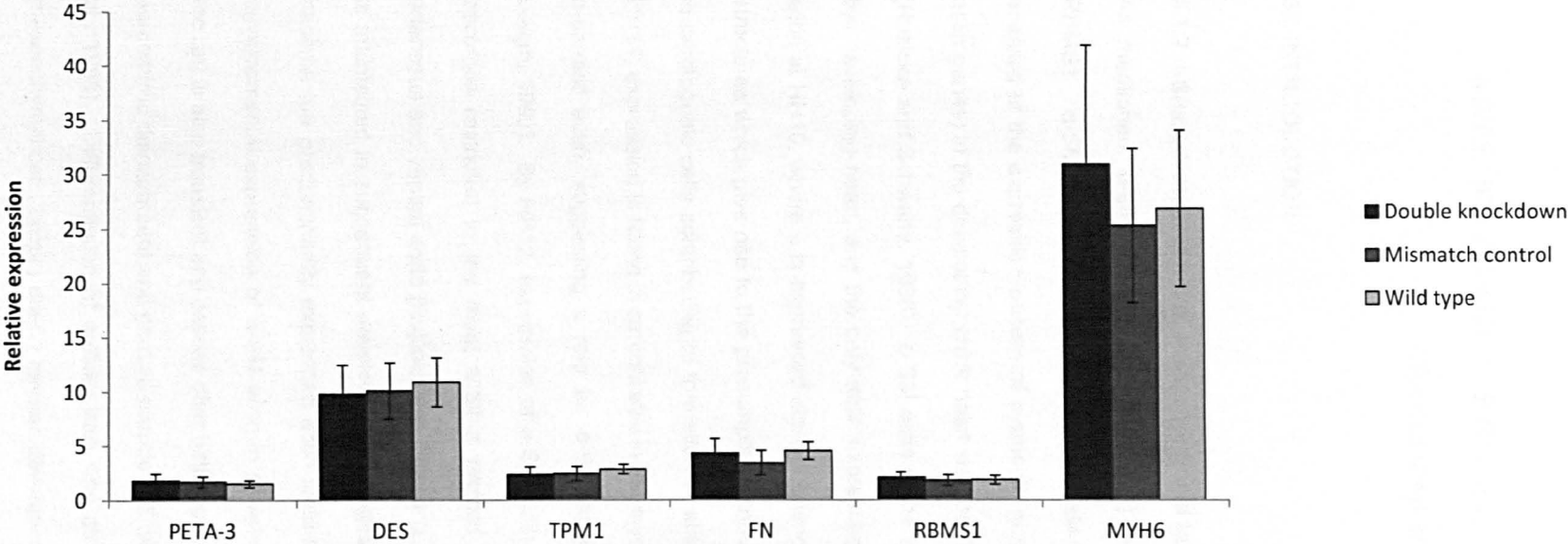


Figure 4.14 Relative expression of candidate genes in TBX5 / GATA-4 knockdown animals and controls

QPCR expression analysis was performed on TBX5 / GATA-4 double knockdown embryos, TBX5 7-mis / GATA-4 7-mis controls, and wild type embryos (3 biological replicates per group). Each reaction plate was run in duplicate. Data was normalised to RPLPO. Error bars represent standard deviation. No significant differences in expression were observed for any gene (measured between the knockdown group and both control groups by the student's t test, using $p < 0.05$ as the threshold value).

CHAPTER 5

INVESTIGATION OF THE ROLE OF RBMS1 IN CARDIAC DEVELOPMENT

5.1 INTRODUCTION

5.1.2 α -Smooth muscle actin, a transcriptional target of RBMS1

As mentioned, α -smooth muscle actin (α -SM) is a known downstream target of RBMS1. α -SM is the major actin isoform present in vascular tissue. A detailed analysis of the expression pattern of α -smooth muscle actin (and the two striated α -actin genes) in the developing chick heart was performed by Ruzicka and co-workers [Ruzicka and Schwartz, 1988]. α -SM actin is the earliest expressed actin isoform in the developing heart, and the only actin expressed at HH8/9. It is the predominant actin at HH10, where it is expressed along the length of the tubular heart, in anterior structures which give rise to the presumptive conus arteriosus and ventral aorta, and in cardiogenic cells contributing to formation of atrial and sinus venosus regions. At HH11, expression is found in sarcomeres in the myocardium, alongside α -skeletal and α -cardiac actin, suggesting a role for α -SM actin in myofibrillogenesis [Sugi and Lough, 1992]. By HH12, expression of α -SM actin is selectively down-regulated and becomes restricted to the most anterior regions of the heart, namely the conus arteriosus and ventral aorta [Ruzicka and Schwartz, 1988]. This pattern of expression is maintained in subsequent developmental stages. The two striated α -actin genes become the predominantly expressed actin isoforms from HH12 in the heart. The developmental expression of α -SM actin in skeletal and myocardial cells (studied in the rat) is also transient and ceases after birth, again indicating a role for α -SM actin in sarcomeric development and cardiac muscle cell differentiation [Woodcock-Mitchell et al., 1988]. Differentiation of outflow tract and atrioventricular endothelial cells into mesenchymal cells occurs during cardiac development, forming endocardial cushions

which are essential for heart valve and septa formation. α -SM actin plays an important role in this crucial process of endothelial-mesenchymal transformation [Nakajima et al., 1997], indicating an additional role in cardiogenesis.

Mouse knockout of the α -SM actin gene (*ACTA2*) results in impaired vascular contractility and blood flow, with no reported effects on development of the heart [Schildmeyer et al., 2000; Tomasek et al., 2006]. However, these findings were based upon gross examination of embryos and cross-sectional analysis of cardiac muscle. Internal heart structures were not examined for abnormalities. Mutations in *ACTA2* in humans are associated with a variety of vascular diseases such as coronary artery disease (CAD), ischemic strokes, Moyamoya disease (MMD), and thoracic aortic aneurysms and dissections (TAAD) [Guo et al., 2009]. In addition, *ACTA2* mutations are also associated with the CHD patent ductus arteriosus [Guo et al., 2007].

5.1.3 Transcriptional regulation of α -smooth muscle actin

Expression of α -SM actin is mediated by positive and negative cis-elements and their corresponding trans-acting factors. A number of positive regulators have been identified e.g. serum response factor (SRF) which acts via the CArg box [Hautmann et al., 1997; Shimizu et al., 1995; Simonson et al., 1995], and essential transcriptional enhancer factor-1 (TEF-1) which acts via the purine rich motif [Cogan et al., 1995]. Less is known about the negative regulation of this gene. Until recently, only two negative regulators of α -SM actin, vascular α -SM actin single-strand binding factors 1 and 2 (VACssBF1 and VACssBF2), were identified, both acting via the TEF-1 binding domain in single stranded DNA [Cogan et al., 1995]. A novel negative regulatory element has since been identified in the α -SM actin promoter region, ranging from -238 to -219. Mutation and deletion analyses revealed the sequence TATCTTA (-228 to -222) is essential for negative regulation [Kimura et al., 1998]. Gel shift assays (using smooth muscle cell nuclear extracts) were designed to identify protein factors specifically interacting with this sequence, and resulted in identification of the nuclear protein RBMS1. RBMS1 was found to interact with both single and double stranded

DNA containing this sequence. Furthermore, RBMS1 overexpression resulted in suppression of α -SM actin promoter activity. This provided evidence of a novel role for RBMS1 in the negative transcriptional regulation of α -SM actin.

5.1.4 α -cardiac and α -skeletal actin

The striated actins, α -skeletal and α -cardiac actin, are the major actin isoforms in skeletal and cardiac muscle respectively. Due to the common ancestry and high level of homology between these and α -smooth muscle actin, it is possible these genes may have retained some common regulatory elements, and the regulatory effect of RBMS1 on all three actins will be assessed in this chapter. Both α -cardiac and α -skeletal actin are expressed in the heart where they each have an important function. Expression of α -cardiac actin begins at HH9 (at lower levels than α -smooth muscle actin) in the ventricular myocardium, subsequently spreading in a spatial pattern corresponding with the progression of myofibrillogenesis [Ruzicka and Schwartz, 1988]. Mutations in alpha cardiac actin produce atrial and ventricular septal defects [Matsson et al., 2008]. Expression of α -skeletal actin is detectable from HH12, with the pattern of expression largely following that of α -cardiac actin, with greater expression in the conus region [Ruzicka and Schwartz, 1988]

5.2 AIMS

This chapter aims to study RBMS1 in the developing chick embryo as follows:

- a) Bioinformatic characterisation of the chicken RBMS1 gene and transcripts
- b) Determination of the expression pattern of RBMS1 in the developing chick
- c) Morpholino knockdown of RBMS1 for phenotypic analysis
- d) Characterisation of the effect of morpholinos on RBMS1 mRNA splicing and / or mRNA levels
- e) Bioinformatic characterisation of α -smooth muscle actin and related genes, and QPCR expression analysis of these in RBMS1 knockdown embryos versus controls

5.3 RESULTS

5.3.1 Bioinformatic Identification and characterisation of the chicken RBMS1 gene

The human RBMS1 gene was identified at chromosome 2: 161,101,000–161,378,000 using NCBI. The corresponding chicken gene was identified at chromosome 7: 23,214,270–23,352,271 (method described in section 2.2.6.1). Synteny of the chick genomic location with the known human genomic region of RBMS1 was confirmed, establishing identification of the true orthologue (Figure 5.1). Gene sequence alignments were performed in the online ECR browser (<http://ecrbrowser.dcode.org>) for ten species against the human (base) genome (Figure 5.2), showing this gene is conserved across a multitude of species. Two chicken RBMS1 protein transcripts were identified and multispecies alignment of RBMS1 protein sequences again showed this gene is highly conserved between the chicken, human, chimpanzee, mouse and rat (Figure 5.3). Sequence alignment of the two corresponding chicken mRNA transcripts is shown in Figure 5.4. ESTs matching the RBMS1 transcripts were identified and aligned with the theoretical mRNA sequences for sequence confirmation, represented in Figure 5.5. Transcript ENSGALT00000018146 contains a 9 bp insertion in exon 7, likely to have been introduced through an alternative splicing mechanism. Each transcript has been assigned a different AUG translational start site based on the postulated protein sequences. It is unclear which (if either) is the predominant transcript.

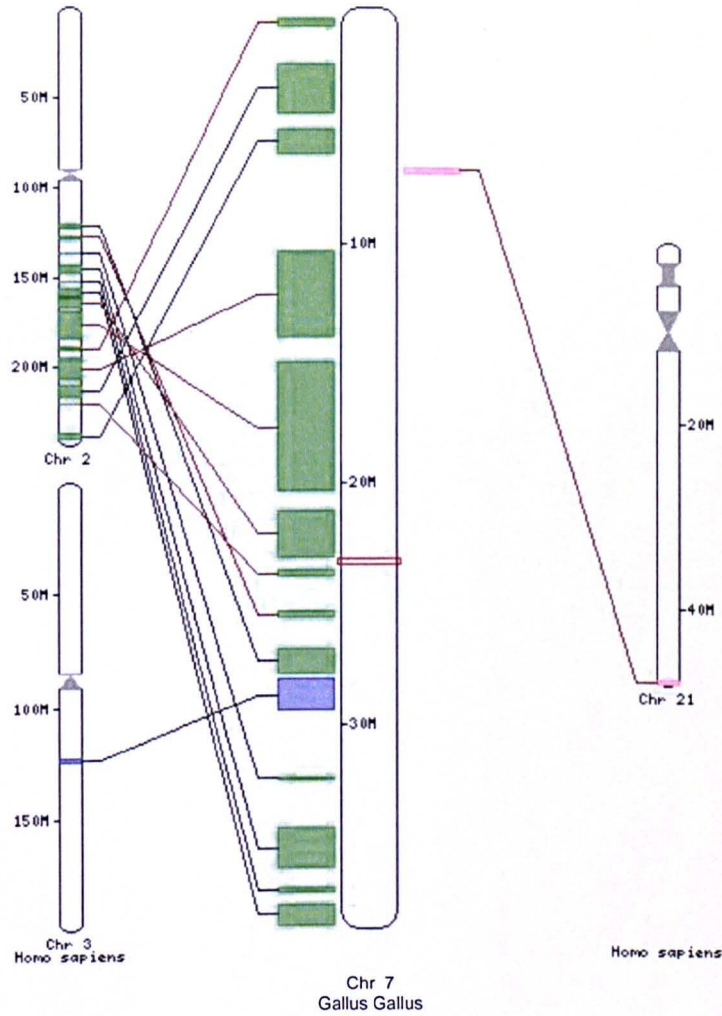


Figure 5.1 Synteny of the chicken and human RBMS1 genes

Synteny between the chromosomal location of the chicken RBMS1 gene and the known chromosomal location of the human RBMS1 gene was confirmed in *Ensembl* (<http://www.ensembl.org>). The centre block represents the species and region of interest (chicken, chromosome [7:23214240-23352391](#)), and the smaller chromosomes represent syntenic regions with the second species (human). Chromosome [2:161128662-161350305](#) is of interest. Blocks are coloured according to the chromosome of the second species. Black lines connect syntenic blocks with the same orientation, and brown lines connect syntenic blocks with opposite orientation. The RBMS1 gene is marked with the small red box.

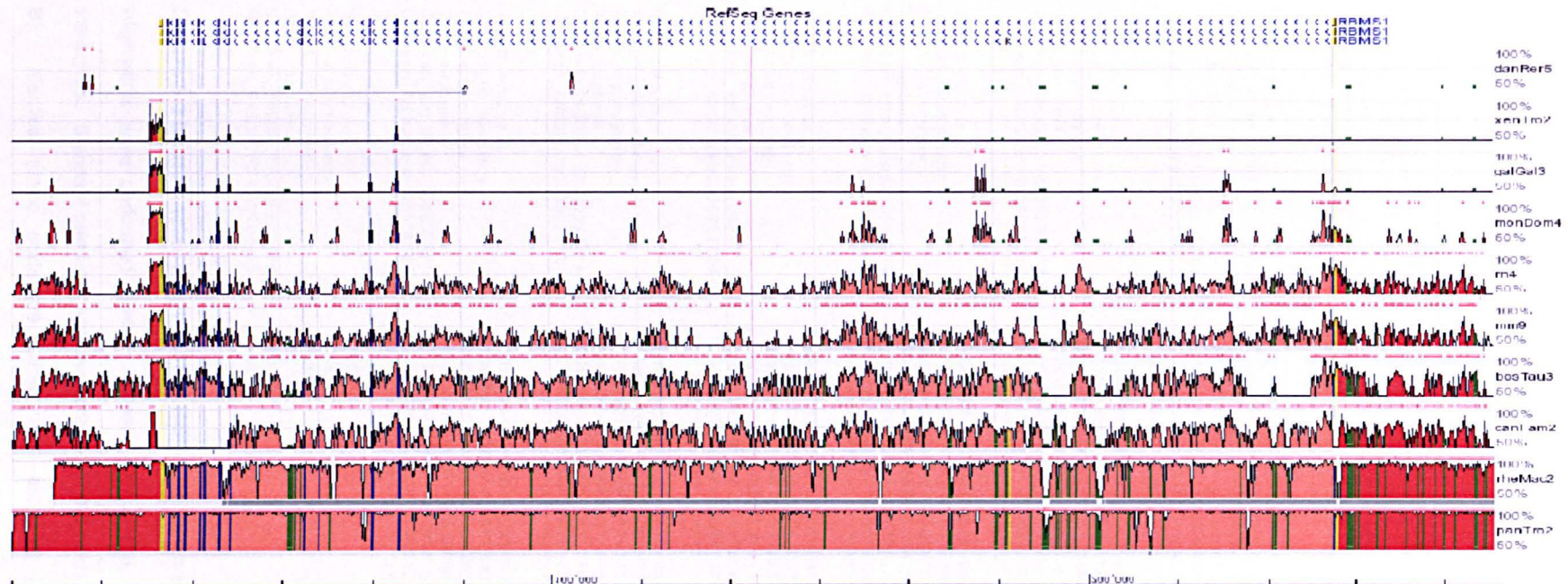


Figure 5.2 Multispecies conservation of the RBMS1 gene

Sequence alignments were performed in the online ECR browser (<http://ecrbrowser.dcode.org>) for ten species against the human (base) genome. The y-axis represents the percentage identity between the base and aligned genomes, and regions meeting the input criteria (100 nucleotide run, minimum 70% identity) are indicated by the pink rectangles on top of each plot. Blue lines indicate coding exons and salmon regions indicate intronic regions. Untranslated regions are shown in yellow, and red areas indicate intergenic regions. Green regions represent transposable elements and simple repeats.


```

Mus_musculus                                     MAPPSPTTSSNNNSSS
Rattus_norvegicus                               MGKVKWQQMYPQYATYYYPQYLQAKQSLVPAHPMAPSPSTTSSNNNSSS
Pan_troglodytes                                 -MIFPSSSGNPGGSSNCRTPYRKQQLVPAPHPMAPSPSTTSSNNNSSS
Homo_sapiens                                    MGKVKWQQMYPQYATYYYPQYLQAKQSLVPAHPMAPSPSTTSSNNNSSS
Chicken_ENSGALP00000032479                     -----MYPQYTYYYYPQYLLAKQSIVPAHPMAPSPSTTSSNNNSSS
Chicken_ENSGALP00000018124                     -----MAPPSPTTSSNNNSSS
*****

Mus_musculus                                     SSNSGWDQLSKTNLYIRGLPPNTTDQDLVKLCQPYGKIVSTKAILDKATN
Rattus_norvegicus                               SSNSGWDQLSKTNLYIRGLPPNTTDQDLVKLCQPYGKIVSTKAILDKATN
Pan_troglodytes                                 SSNSGWDQLSKTNLYIRGLPPHTTDQDLVKLCQPYGKIVSTKAILDKTTN
Homo_sapiens                                    SSNSGWDQLSKTNLYIRGLPPHTTDQDLVKLCQPYGKIVSTKAILDKTTN
Chicken_ENSGALP00000032479                     SSNSGWDQLSKTNLYIRALPPNTTDQDLVKLCQPYGKIVSTKAILDKTTN
Chicken_ENSGALP00000018124                     SSNSGWDQLSKTNLYIRALPPNTTDQDLVKLCQPYGKIVSTKAILDKTTN
*****

Mus_musculus                                     KCKGYGFVD FDS PAAQA KAVSALKANGVQAQMAKQQEQDPTNL YISNLPL
Rattus_norvegicus                               KCKGYGFVD FDS PAAQA KAVSALKASGVQAQMAKQQEQDPTNL YISNLPL
Pan_troglodytes                                 KCKGYGFVD FDS PAAQA KAVSALKASGVQAQMAKQQEQDPTNL YISNLPL
Homo_sapiens                                    KCKGYGFVD FDS PAAQA KAVSALKASGVQAQMAKQQEQDPTNL YISNLPL
Chicken_ENSGALP00000032479                     KCKGYGFVD FDS PAAQA KAVSALKASGVQAQMAKQQEQDPTNL YISNLPL
Chicken_ENSGALP00000018124                     KCKGYGFVD FDS PAAQA KAVSALKASGVQAQMAKQQEQDPTNL YISNLPL
*****

Mus_musculus                                     SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIGH
Rattus_norvegicus                               SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIGH
Pan_troglodytes                                 SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIGH
Homo_sapiens                                    SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIGH
Chicken_ENSGALP00000032479                     SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIAH
Chicken_ENSGALP00000018124                     SMDEQELENMLKPFQGVISTRLRDSSGTSRGVGFARMESTEKCEAVIAH
*****

Mus_musculus                                     FNGKFIKTPPGVSAPTEPLLCKFADGGQKKRQNPNKYIPNGRPWPRED--
Rattus_norvegicus                               FNGKFIKTPPGVSAPTEPLLCKFADGGQKKRQNPNKYIPNGRPWPREG--
Pan_troglodytes                                 FNGKFIKTPPGVSAPTEPLLCKFADGGQKKRQNPNKYIPNGRPWHREG--
Homo_sapiens                                    FNGKFIKTPPGVSAPTEPLLCKFADGGQKKRQNPNKYIPNGRPWHREGEV
Chicken_ENSGALP00000032479                     FNGKFIKTPAGVSVPAEPLLCKFADGGQKKRQNPNKYIQNGRAWHREG--
Chicken_ENSGALP00000018124                     FNGKFIKTPAGVSVPAEPLLCKFADGGQKKRQNPNKYIQNGRAWHREGEV
*****

Mus_musculus                                     -EAGMTLTYPDPTTAALHNGFYSPSPYSIATNRMITQTSLTPYIASPVSA YQ
Rattus_norvegicus                               -EAGMTLTYPDPTTAALHNGFYSPSPYSIATNRMITQTSLTPYIASPVSA YQ
Pan_troglodytes                                 -EAGMTLTYPDPTTAAIQNGFYSPSPYSIATNRMITQT SITPYIASPVSA YQ
Homo_sapiens                                    RLAGMTLTYPDPTTAAIQNGFYSPSPYSIATNRMITQT SITPYIASPVSA YQ
Chicken_ENSGALP00000032479                     -EAGMTLTYPDPTTAAIQNGFYSPSPYSITANRMITQT SITPYIASPVSTY Q
Chicken_ENSGALP00000018124                     RLAGMTLTYPDPTTAAIQNGFYSPSPYSITANRMITQT SITPYIASPVSTY Q
*****

Mus_musculus                                     VQSPSWMQPPYILQHPGA VLT PSMEHTMSLQ PASMISPLAQQM SHLSLG
Rattus_norvegicus                               VQSPSWMQPPYILQHPGA VLT PSMEHTMSLQ PASMISPLAQQM SHLSLG
Pan_troglodytes                                 VQSPSWMQPPYILQHPGA VLT PSMEHTMSLQ PASMISPLAQQM SHLSLG
Homo_sapiens                                    VQSPSWMQPPYILQHPGA VLT PSMEHTMSLQ PASMISPLAQQM SHLSLG
Chicken_ENSGALP00000032479                     VQSPSWMQPPYIMQHPGA VLT PSDHMTSLQ PASMISPLTQQM SHLSLG
Chicken_ENSGALP00000018124                     VQSPSWMQPPYIMQHPGA VLT PSDHMTSLQ PASMISPLTQQM SHLSLG
*****

Mus_musculus                                     STGTYPATSAMQGAYLPQYTHMQTA AVPVVEEASGQQQVAVETSNDHS PY
Rattus_norvegicus                               STGTYPATSAMQGAYLPQYTHMQTATVPVVEEASGQQQVTVETSDNHS PY
Pan_troglodytes                                 STGTYPATSAMQGAYLPQYAHMQTATVPVVEEASGQQQVAVETSNDHS PY
Homo_sapiens                                    STGTYPATSAMQGAYLPQYAHMQTATVPVVEEASGQQQVAVETSNDHS PY
Chicken_ENSGALP00000032479                     STGTYPATTAMQGAYIPQYTHVQTAAVPVVEEASGQQQVTVETSSDHS PY
Chicken_ENSGALP00000018124                     STGTYPATTAMQGAYIPQYTHVQTAAVPVVEEASGQQQVTVETSSDHS PY
*****

Mus_musculus                                     TFPQNK
Rattus_norvegicus                               TFPQNK
Pan_troglodytes                                 TFQPNK
Homo_sapiens                                    TFQPNK
Chicken_ENSGALP00000032479                     TYQQNK
Chicken_ENSGALP00000018124                     TYQQNK
** **

```

Figure 5.3 Multispecies alignment of RBMS1 protein sequences

Alignments were performed between human, chimpanzee, mouse, rat, and both chicken transcripts. Standard one letter abbreviations for amino acids have been used (see Appendix B). Identical amino acids are marked with asterisks, and RNP1 motifs are boxed.

Investigation of the role of RBMS1 in cardiac development

ENSGALT00000033119	CCCCAACCCCTGCACTGCAGGCTACATTTCAGCTTCNTGGGCATAGTGTGGAACAGCAG
ENSGALT00000018146	-----AGCTTCNTGGGCATAGTGTGGAACAGCAG *****
ENSGALT00000033119	ENIG TACCCCCAGTACACCACCTACTACTACCCCCAGTATCTGCTGGCGAAGCAGTCCATT
ENSGALT00000018146	ATGTACCCCCAGTACACCACCTACTACTACCCCCAGTATCTGCTGGCGAAGCAGTCCATT *****
ENSGALT00000033119	GTACCAGCCCACCAATGGCTCCTCCTAGTCCCAGCACCACCAGCAGTAATAACAACAGT
ENSGALT00000018146	GTACCAGCCCACCA ENIG GCTCCTCCTAGTCCCAGCACCACCAGCAGTAATAACAACAGT *****
ENSGALT00000033119	AGTAGCAGCAGCAACTCAGGATGGGATCAATTAAATAAAACAACCTTTACATCAGAGCA
ENSGALT00000018146	AGTAGCAGCAGCAACTCAGGATGGGATCAATTAAATAAAACAACCTTTACATCAGAGCA *****
ENSGALT00000033119	CTGCCTCCAAACACCACTGATCAGGACCTGGTAAAATTATGCCAACCGTATGGGAAAATT
ENSGALT00000018146	CTGCCTCCAAACACCACTGATCAGGACCTGGTAAAATTATGCCAACCGTATGGGAAAATT *****
ENSGALT00000033119	GTATCCACAAAGGCTATTTTGGATAAGACAACAACAAGTG CA AAAGGTTATGGTTTGTG
ENSGALT00000018146	GTATCCACAAAGGCTATTTTGGATAAGACAACAACAAGTG CA AAAGGTTATGGTTTGTG *****
ENSGALT00000033119	GACT TCGACAGCCAGCAGCTGCTCAGAAGGCAGTTTCTGCTCTAAGGCTAGTGGAGTC
ENSGALT00000018146	GACT TCGACAGCCAGCAGCTGCTCAGAAGGCAGTTTCTGCTCTAAGGCTAGTGGAGTC *****
ENSGALT00000033119	CAGGCACAAATGGCAAAGCAACAAGAACAGGATCCAACAAATTTATACATTTCCAATTTG
ENSGALT00000018146	CAGGCACAAATGGCAAAGCAACAAGAACAGGATCCAACAAATTTATACATTTCCAATTTG *****
ENSGALT00000033119	CCACTTTCAATGGATGAGCAGGAGCTTGAGAACATGCTTAAGCCTTTTGGGCAGGTTATC
ENSGALT00000018146	CCACTTTCAATGGATGAGCAGGAGCTTGAGAACATGCTTAAGCCTTTTGGGCAGGTTATC *****
ENSGALT00000033119	TCTACGAGGATATTGCGTGACTCGAGTGGAAACAG CGTGGTGTGGCTTTGCCAGGATG
ENSGALT00000018146	TCTACGAGGATATTGCGTGACTCGAGTGGAAACAG CGTGGTGTGGCTTTGCCAGGATG *****
ENSGALT00000033119	GAATCAACAGAAAAATGTGAAGCAGTGATTGCTCATTTTAACGGAAAAATCATAAAGACA
ENSGALT00000018146	GAATCAACAGAAAAATGTGAAGCAGTGATTGCTCATTTTAACGGAAAAATCATAAAGACA *****
ENSGALT00000033119	CCAGCAGGAGTTTCTGTTCCCTGCAGAACCTTTATTGTGCAAGTTTCTGATGGAGGACAG
ENSGALT00000018146	CCAGCAGGAGTTTCTGTTCCCTGCAGAACCTTTATTGTGCAAGTTTCTGATGGAGGACAG *****
ENSGALT00000033119	AAAAAGAGACAGAATCAGAATAAATACATACAGAATGGAAGAGCATGGCACAGAGAAGGC
ENSGALT00000018146	AAAAAGAGACAGAATCAGAATAAATACATACAGAATGGAAGAGCATGGCACAGAGAAGGC *****
ENSGALT00000033119	GAGG-----CTGGAATGACACTCACTTATGATCCAACCACAGCTGCTTTACAAAAT
ENSGALT00000018146	GAGGTGAGACTTGCTGGAATGACACTCACTTATGATCCAACCACAGCTGCTTTACAAAAT *****
ENSGALT00000033119	GGATTTTATCCATCACCTACAGTATTACAGCAAACAGAATGATCACTCAAACATCTATT
ENSGALT00000018146	GGATTTTATCCATCACCTACAGTATTACAGCAAACAGAATGATCACTCAAACATCTATT *****
ENSGALT00000033119	ACGCCATATATTGCTTCTCCGGTTTCCACATACCAGGTTTACAGTCTCTTCTGGATGCAG
ENSGALT00000018146	ACGCCATATATTGCTTCTCCGGTTTCCACATACCAGGTTTACAGTCTCTTCTGGATGCAG *****
ENSGALT00000033119	CCTCAACCATATATAATGCAGCACCCAGGTGCTGTACTGACTCCCTCCATGGACCACACC
ENSGALT00000018146	CCTCAACCATATATAATGCAGCACCCAGGTGCTGTACTGACTCCCTCCATGGACCACACC *****
ENSGALT00000033119	ATGTCACTACAGCTGCATCAATGATAAGCCCTTTGACGCAGCAGATGAGTCATCTTTCA
ENSGALT00000018146	ATGTCACTACAGCTGCATCAATGATAAGCCCTTTGACGCAGCAGATGAGTCATCTTTCA *****
ENSGALT00000033119	TTAGGCAGTACTGGAACATACATGCCAGCCACAACAGCTATGCAAGGAGCCTACATACCC
ENSGALT00000018146	TTAGGCAGTACTGGAACATACATGCCAGCCACAACAGCTATGCAAGGAGCCTACATACCC *****
ENSGALT00000033119	CAATACACACATGTCCAGACAGCAGCGGTTCTGTGTGAGGAAGCCAGTGGTCAACAGCAG
ENSGALT00000018146	CAATACACACATGTCCAGACAGCAGCGGTTCTGTGTGAGGAAGCCAGTGGTCAACAGCAG *****



Figure 5.4 ClustalW alignment of the two chicken RBMS1 mRNA transcripts

Identical bases are marked with asterisks. ATG translation start sites are shown in green, and stop codons in red. RNP1 motif encoding regions are boxed. Sequences are from *Ensembl* (www.ensembl.org), April 2010.

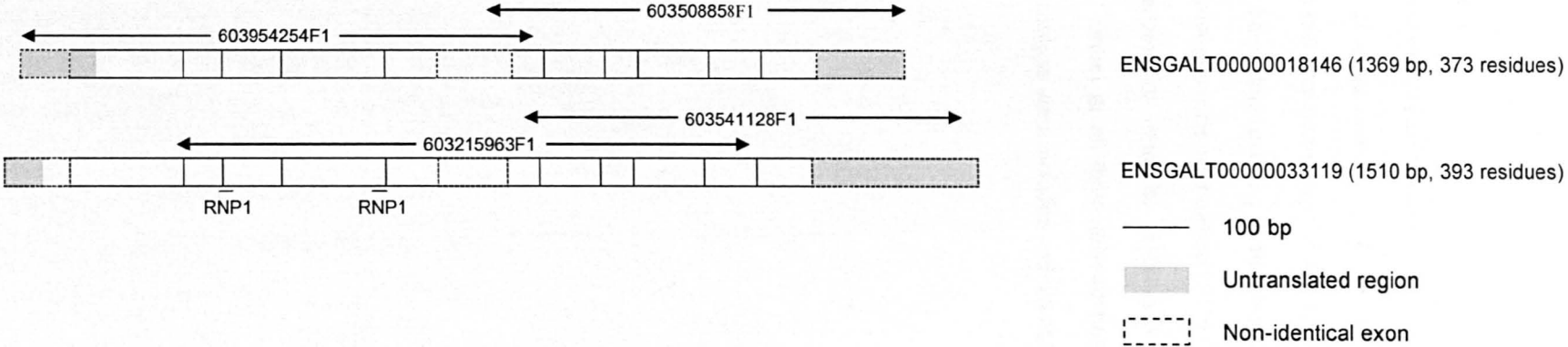


Figure 5.5 Representation of the two chicken RBMS1 mRNA transcripts and EST coverage

Diagrams are drawn to scale based on the transcripts ENSGALT00000018146 and ENSGALT00000033119 (*Ensembl*, April 2010). Exons that differ between transcripts are shown with dashed lines, and untranslated regions are shown in grey. Regions encoding the two RNP1 motifs are marked. Matching ESTs and regions of sequence identity are indicated.

5.3.2 Investigation of RBMS1 expression in the developing chick

5.3.2.1 RT-PCR

RBMS1 expression was initially examined by RT-PCR in chick embryonic segments at three stages of development (HH16, HH19 and HH24) as a preliminary means of temporal / spatial characterisation of expression. Primers were designed to bind to both RBMS1 transcripts resulting in amplification of a 488 bp fragment (sequences in Table 2.5, primer positions are represented in Figure 5.7, A). Figure 5.6 shows results of RT-PCR expression analysis. Expression was detected in all segments (head, heart, body / limbs) at all three developmental stages examined (Figure 5.6, A). Negative RT controls were included and did not display amplification (Figure 5.6, B).

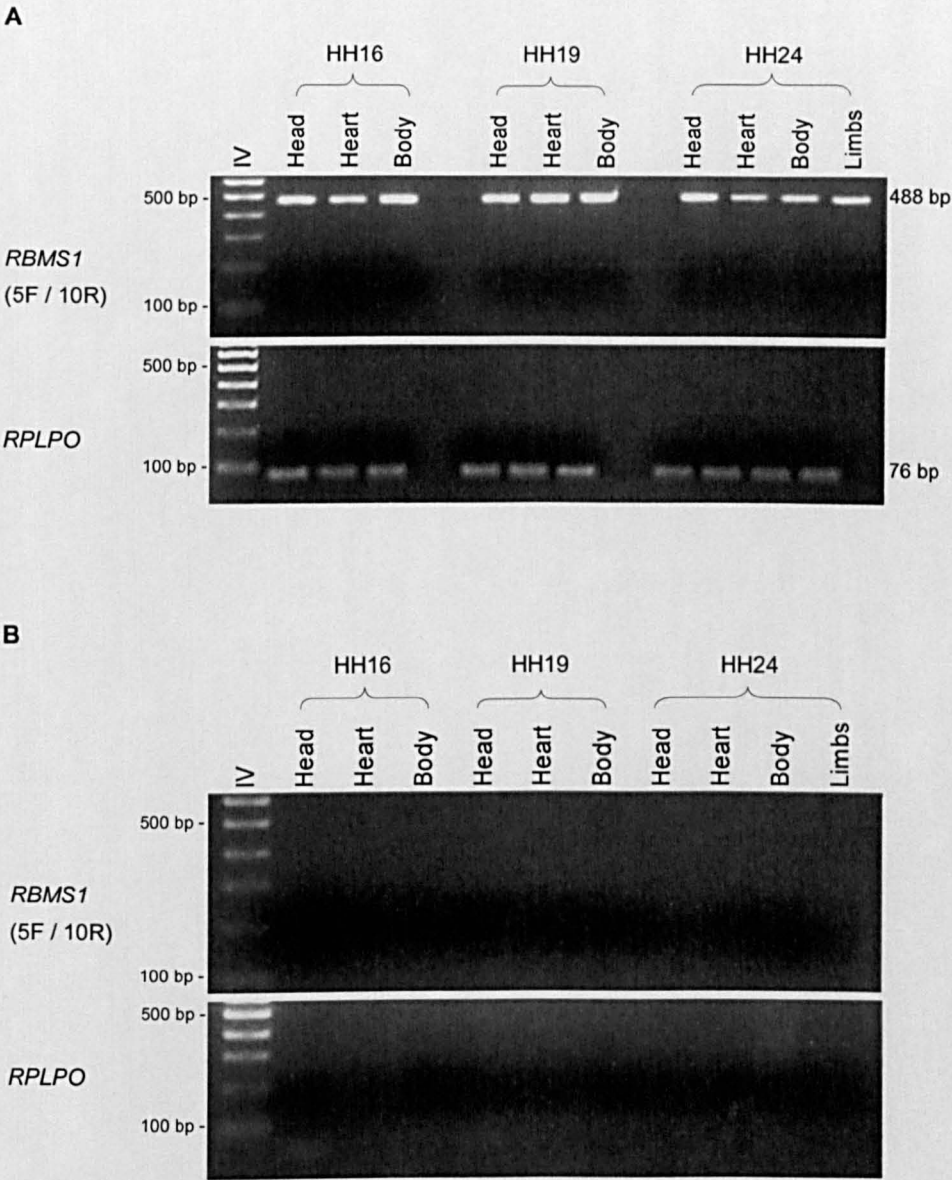


Figure 5.6 RT-PCR expression analysis of RBMS1

Products were electrophoresed on 2% agarose gels using Hyperladder IV (Bioline) as a size marker (500 bp and 100 bp bands are indicated along with product sizes). (A) Expression was examined in wild type chick embryonic body segments at HH16, HH19 and HH24. RPLPO was used as a housekeeping gene. (B) Negative RT controls where reverse transcriptase enzyme was not added were included for both genes and did not result in amplification.

5.3.2.2 Whole mount *in situ* hybridisation

RT-PCR expression analyses show that RBMS1 is expressed in the developing chick heart at HH12 – HH26 (Chapter 4), and throughout the embryo at HH16, HH19 and HH24 (Figure 5.6). These did not however allow identification of specific regions or structures of the heart giving rise to these signals. In order to obtain a more detailed picture of the localisation of RBMS1 transcripts, whole mount *in situ* hybridisation was performed. Two non-overlapping probes were generated against RBMS1 using the RT-PCR products shown in Figure 5.7, designed to bind to both RBMS1 transcripts. RT-PCR products were gel purified, cloned into the pGEM-T Easy vector, and sequenced in both directions for confirmation of sequence and determination of orientation. Clones selected contained insert in the forward orientation. Plasmid linearisation was carried out using an appropriate enzyme whose restriction site was not present within the insert, in this case *SpeI* for antisense probes, and *SacII* for sense probes (Appendix F). Single stranded DIG-labelled RNA probes were synthesised using a DIG RNA labelling mix by *in vitro* transcription with T7 RNA polymerase (antisense) or SP6 RNA polymerase (sense). Expression was examined at HH16, HH19 and HH24. For colour development, samples were incubated in fresh colour reagent at room temperature for 20 minutes in the dark, and longer as appropriate up to a maximum of 90 minutes due to the high strength of the signal. Background was reduced by washing in KTBT and a graded methanol series. Samples were stored in PBS and photographed in 70% glycerol.

RBMS1 expression was detected in the heart and throughout the embryo at all three stages of development, and represented in Figures 5.8 – 5.10. *In situ* hybridisation results for HH16 and HH19 early stage embryos (using the 5F/10R probe) are displayed in Figure 5.8. At HH16, staining to the heart was very weak, and other regions of the embryo including the somites displayed strong expression. The HH19 embryo displayed general expression, and staining to the heart was stronger at this stage. HH24 stage embryos are displayed in Figure 5.9 (probe 1F/5R) and Figure

5.10 (probe 5F/10R). RBMS1 displays expression in all areas of the heart, and is highly expressed in the developing forelimb and hind limb buds, somites, and brain.

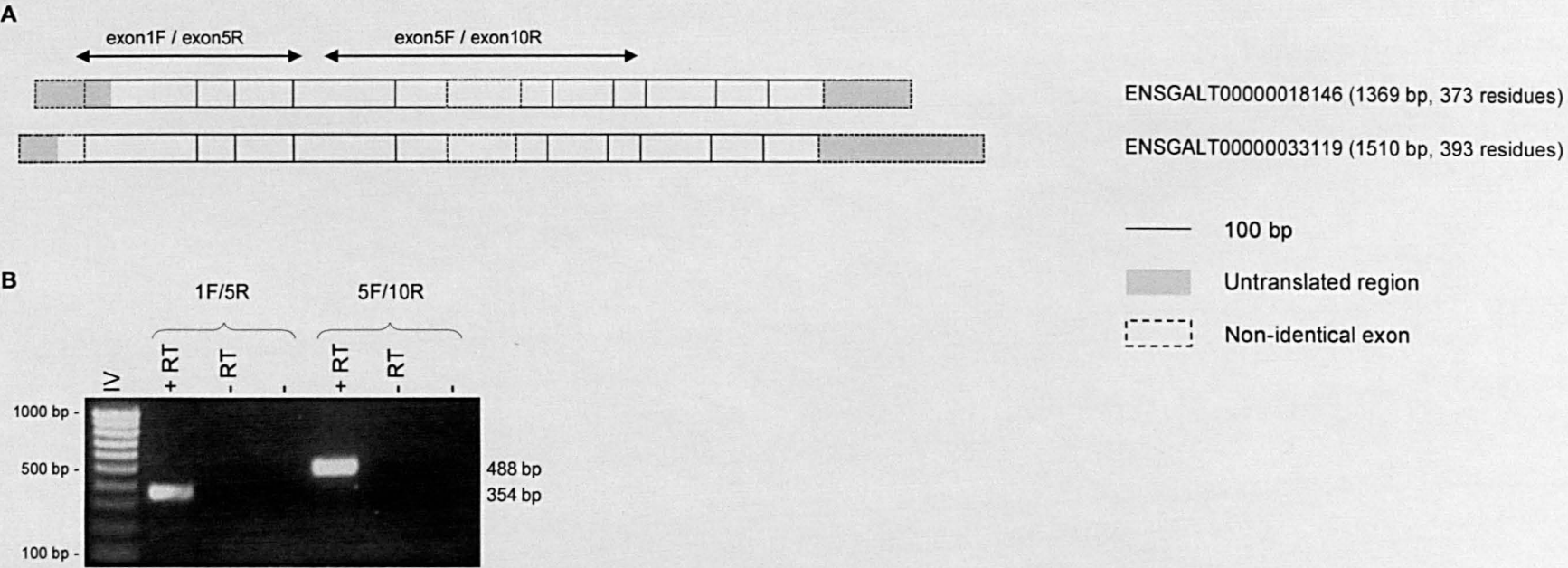


Figure 5.7 Amplicons used for RNA probe synthesis

(A) Representation of the two chicken RBMS1 mRNA transcripts, ENSGALT00000018146 and ENSGALT00000033119 are shown, and primer positions are indicated by the arrows. (B) RT-PCR products were amplified from HH19 chick heart cDNA and are displayed in the gel image, and product sizes are indicated

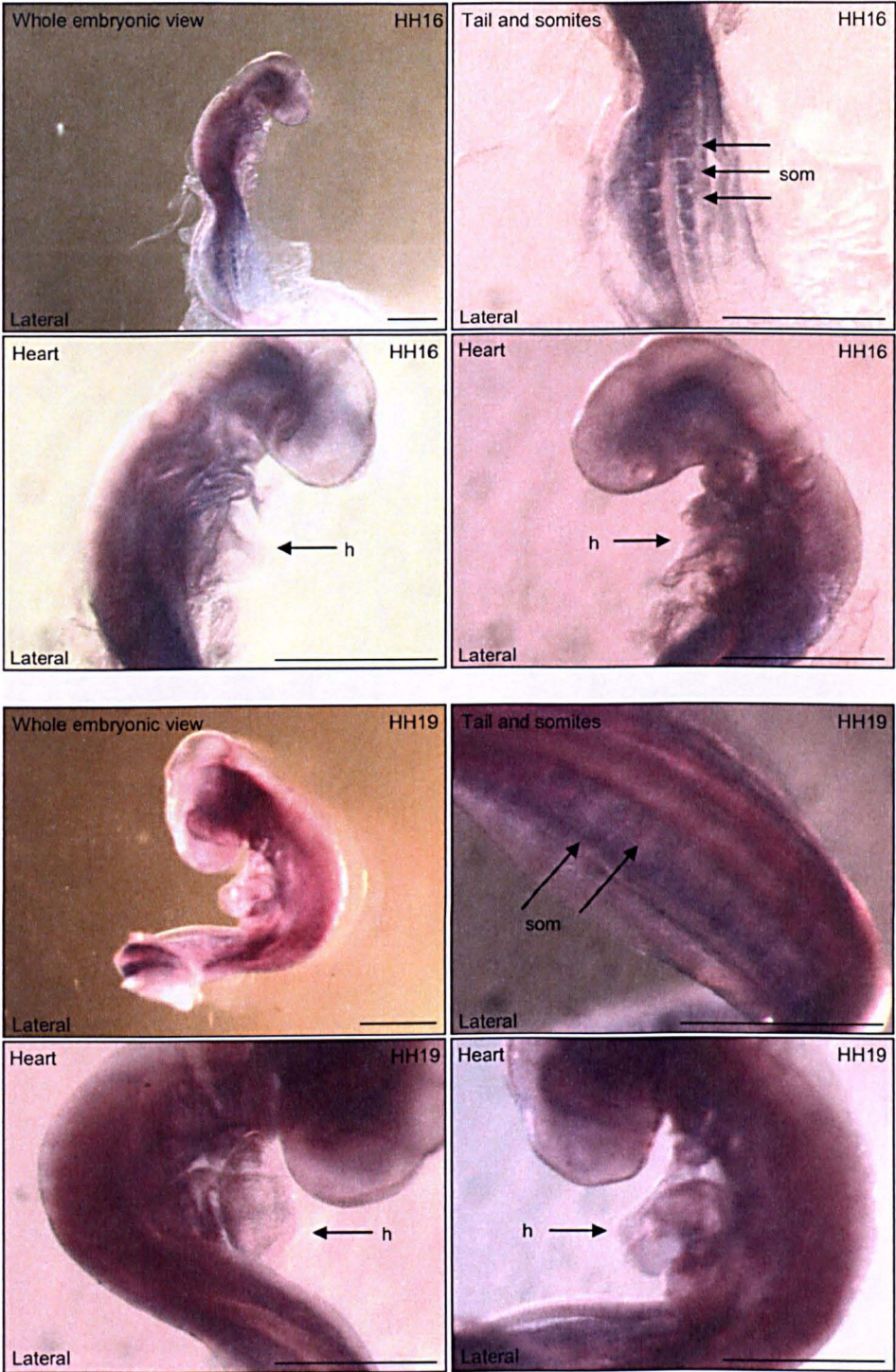


Figure 5.8 *In situ* hybridisation expression analysis of RBMS1 in the HH16 and HH19 chick embryo using the 5F/10R probe

Lateral embryonic views of embryos are displayed, and embryonic age is indicated. Expression was detected with the 1F/5R antisense probe, using the corresponding sense probe as a control (shown in Figure 5.11). Scales bars represent 500 μm . h – heart, som - somites

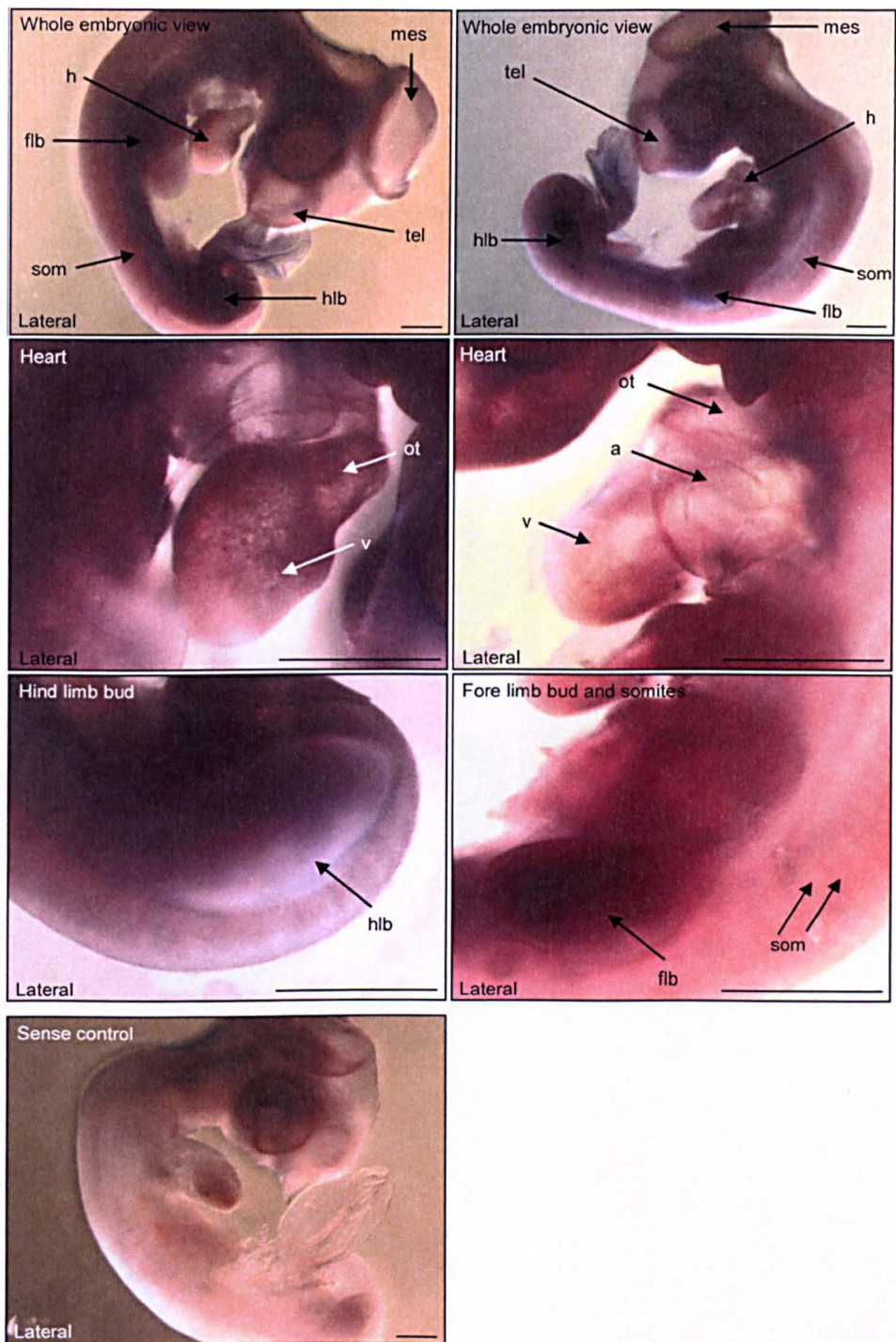


Figure 5.9 *In situ* hybridisation expression analysis of RBMS1 in the HH24 chick embryo using the 1F/5R probe

Lateral embryonic views of embryos are displayed. Expression was detected with the 1F/5R antisense probe, using the corresponding sense probe as a control. Scales bars represent 500 μ m. tel – telencephalon, mes – mesencephalon, h – heart, ot – outflow tract, a – atrium, v – ventricle, hlb – hind limb bud, flb – forelimb bud, som - somite

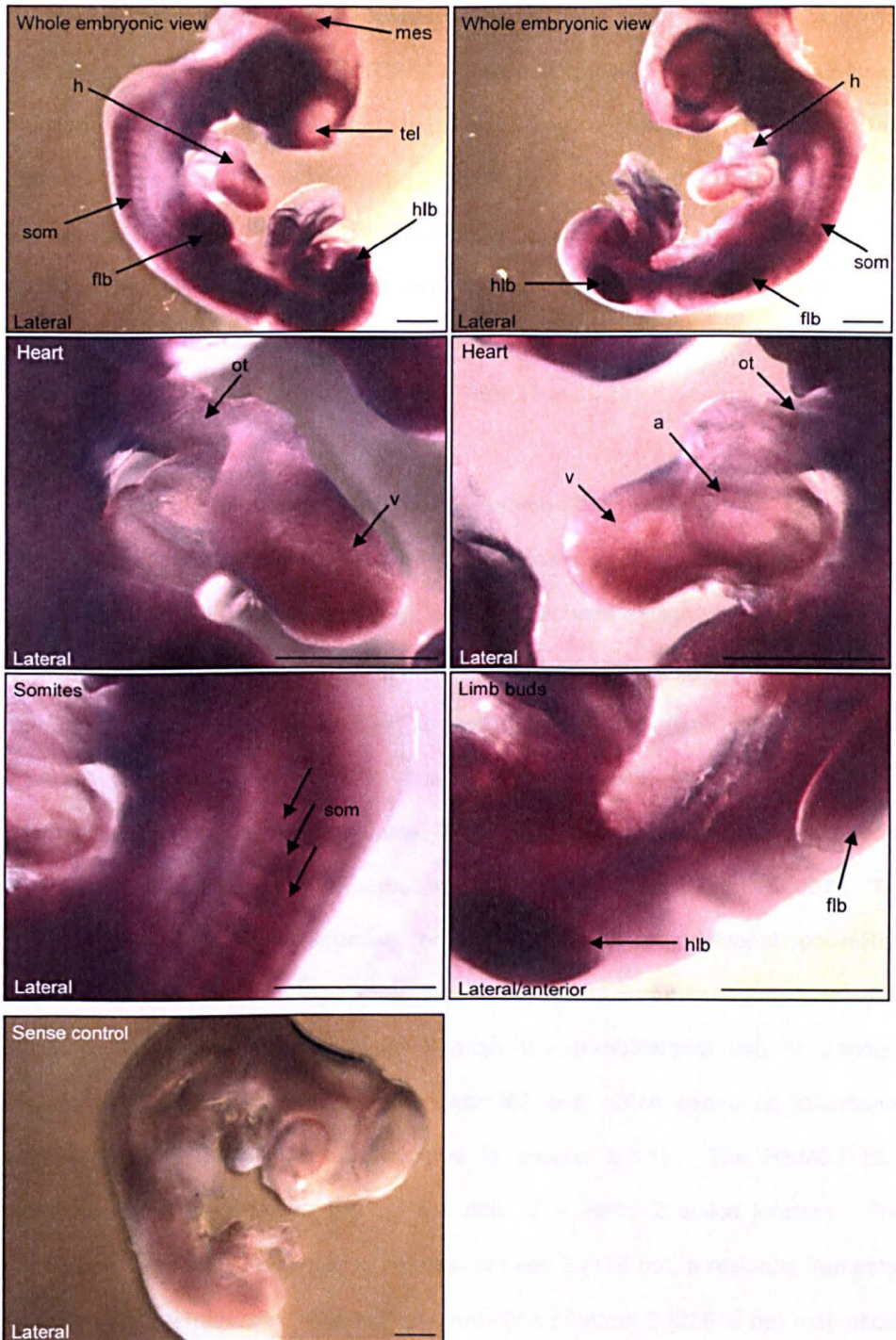


Figure 5.10 *In situ* hybridisation expression analysis of RBMS1 in the HH24 chick embryo using the 5F/10R probe

Lateral embryonic views of embryos are displayed. Expression was detected with the 5F/10R antisense probe, using the corresponding sense probe as a control. Scales bars represent 500 μ m. tel – telencephalon, mes – mesencephalon, h – heart, ot – outflow tract, a – atrium, v – ventricle, hlb – hind limb bud, flb – forelimb bud, som – somite

5.3.3 RBMS1 morpholino knockdown studies

In order to investigate the role of RBMS1 in cardiac development, morpholino loss of function studies were carried out. Morpholinos were applied at HH10 – HH16, and embryos harvested for analysis at HH19. At HH10 - HH12, dextral looping of the heart occurs, and looping continues until HH17/HH18. Endocardial cushion formation in the AV canal has begun by HH12, and development of the interatrial septum begins at HH14. These are crucial stages in cardiac development, and gene knockdown at this time will provide information into the role of RBMS1 in these processes.

5.3.3.1 Design of splice-junction targeting morpholinos and controls

Due to the presence of two RBMS1 mRNA transcripts, each potentially with a distinct ATG translational start site (Figure 5.4), morpholinos were designed to common splice junctions in order to modify gene splicing in both transcripts. Splice-blocking morpholinos can either be targeted to splice donor sites (exon-intron) or splice acceptor sites (intron-exon), and most commonly result in exon skipping, or alternatively in intron retention [Morcos, 2007]. Activation of cryptic splice sites may also occur, which can result in partial insertions or deletions [Morcos, 2007]. The design of splice-junction targeting morpholinos requires additional pre-mRNA sequence information, with well defined exon-intron and intron-exon junctions. This sequence information was obtained through the combinatorial use of genomic sequence information (extracted from *Ensembl*) and cDNA sequence information (verified with EST evidence, as outlined in section 5.3.1). The RBMS1 E2I2 morpholino was designed to bind to the exon 2 – intron 2 splice junction. This morpholino was predicted to cause deletion of exon 2 (176 bp), a resulting frameshift and premature termination. Alternatively, retention of intron 2 (25676 bp) may occur, also resulting in a premature termination. Morpholino sequence, target region and possible effects are displayed in Figure 5.11. A corresponding 5-base mismatch morpholino was also designed as a control of specificity and is displayed. Morpholino sequences are displayed in Table 2.8.

Chicken RBMS1 E2I2 morpholino

E2I2 5-mismatch morpholino

E2I2 morpholino

Pre-mRNA

mRNA

Protein

3'- ATACCGTTGCCATTGATGCTATCAA - 5'

3'- ATACGGTTGGCATTTCATGCTATGAA - 5'

5'- GCACUGCCUCCAAACACCACUGAUCAGGACCUUGUAAAAUAUGCCAACCGUAAGUACGAUACUUAAAUACGGAAUACGGAAUCGUACCUUAUAAGUUUG - 3'

5'- GCACUGCCUCCAAACACCACUGAUCAGGACCUUGUAAAAUAUGCCAACC - 3'

A L P P N T T D Q D L V K L C Q

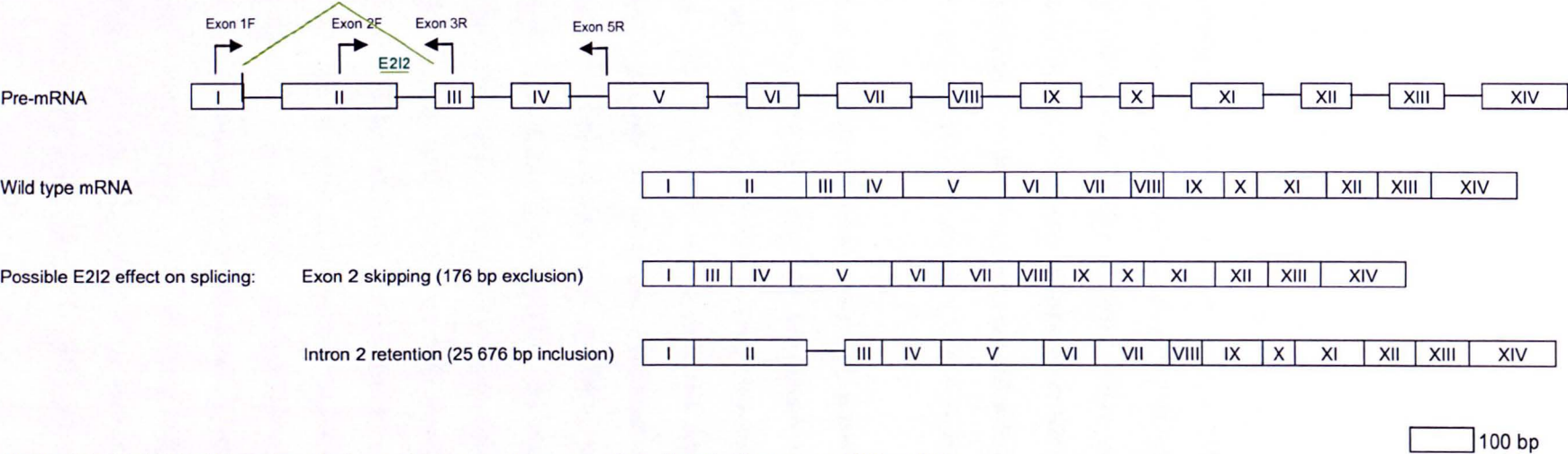


Figure 5.11 RBMS1 E2I2 and control morpholino sequences and predicted effects on mRNA splicing

The shorter of the two mRNA transcripts (ENSGALT00000018146) is represented for simplicity; all morpholinos, primers etc have been designed to bind to common regions of both transcripts. Exons are drawn to scale, and intron sizes have been standardised. Morpholino sequences are shown in the 3' – 5' direction, and target binding regions are boxed.

Bases altered in mismatch control morpholinos are underlined.

5.3.3.2 Preliminary testing of morpholinos *in ovo*

Initial phenotypic analysis of embryos was carried out externally and is displayed in Figure 5.12. The degree of abnormality was determined by gross examination of embryo size and shape, followed by visual assessment of heart shape and structure. Figure 5.12 A1 and B1 depict wild type and standard control treated embryonic hearts, which display normal size, curvature and shape. Figure 5.12 C1 and C2 are examples of embryos treated with the E2I2 5-mismatch morpholino at 250 μ M and 500 μ M respectively, both displaying an abnormal heart shape and constriction to the ventricle. In total, 2 / 8 embryos treated with the mismatch control at 250 μ M, and 5 / 18 embryos treated at 500 μ M harboured a cardiac defect. Since these were similar to the abnormalities observed in knockdown embryos (discussed later in section 5.3.3.4), it was thought that the 5-base mismatch morpholino was still able to bind to the target region (as in Chapter 4), and a new control of specificity was required.

5.3.3.3 Selection and design of an alternative control of specificity

At the time of this research, it emerged that the use of mismatch morpholinos was no longer considered the best control of specificity (personal communication with Dr Jon Moulton, Gene-Tools). This was based on the frequency with which mismatch morpholinos were able to generate an effect due to retained binding capability to target sites, or off-target effects, as demonstrated in this thesis. Instead of moving to a 7-base mismatch control (as previously in Chapter 4), the use of a second non-overlapping targeting morpholino to the same gene was recommended in order to look for morpholino specificity upon generation of a common phenotype, since it was deemed unlikely the same off-target effect would be created by separate morpholinos. Morpholino specificity and effect on target mRNA or protein could then be determined by other means. Consequently, a second splice site morpholino was designed; the RBMS1 E8I8 morpholino was designed to bind to the exon 8 – intron 8 splice junction, and predicted to cause deletion of exon 8 (50 bp) and a resulting frameshift, or retention of intron 8 (92 bp) which would result in a premature stop. This is represented in Figure 5.13, and morpholino sequences are given in Table 2.8.

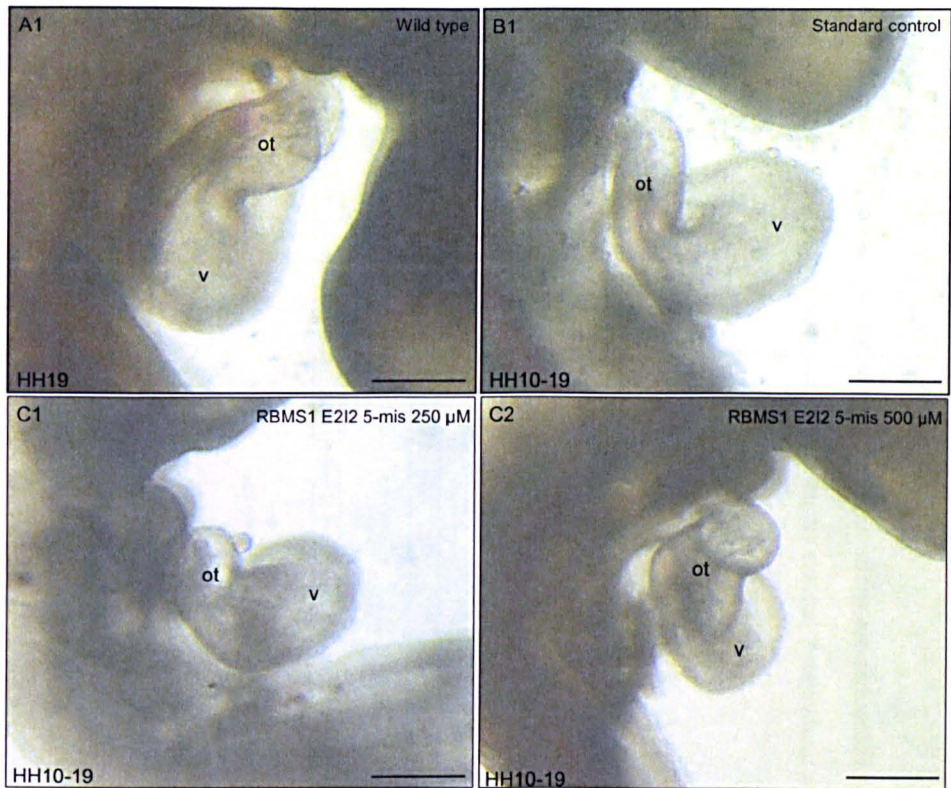


Figure 5.12 External phenotypic analysis of embryos treated with RBMS1 E2I2 5-base mismatch morpholinos in comparison to standard control-treated and wild type embryos

Bright field lateral embryonic views of wild type (A1), standard control (B1), and E2I2 5-mismatch control (C1-C2) are displayed with developmental stages of morpholino application and embryo harvest. Standard control morpholino was applied at a concentration of 500 μM , and mismatch control morpholinos applied at both 250 μM and 500 μM as indicated. Scale bars represent 500 μm . ot – outflow tract, v – ventricle

Chicken RBMS1 E8I8 morpholino

E8I8 morpholino
3' - TACCCATACAATTTCACGACCATGTG - 5'

Pre-mRNA
5' - GCUGGAAUGACACUCACUUAUGAUCCAACCACAGCUGCUUUACAAAUGGGUAUGUUAAGUGCUGGUACACUAAGAAAAAAUGUUUGCAUUAUGAAAGUU - 3'

mRNA
5' - GCUGGAAUGACACUCACUUAUGAUCCAACCACAGCUGCUUUACAAAUGG - 3'

Protein
A G M T L T Y D P T T A A L Q N

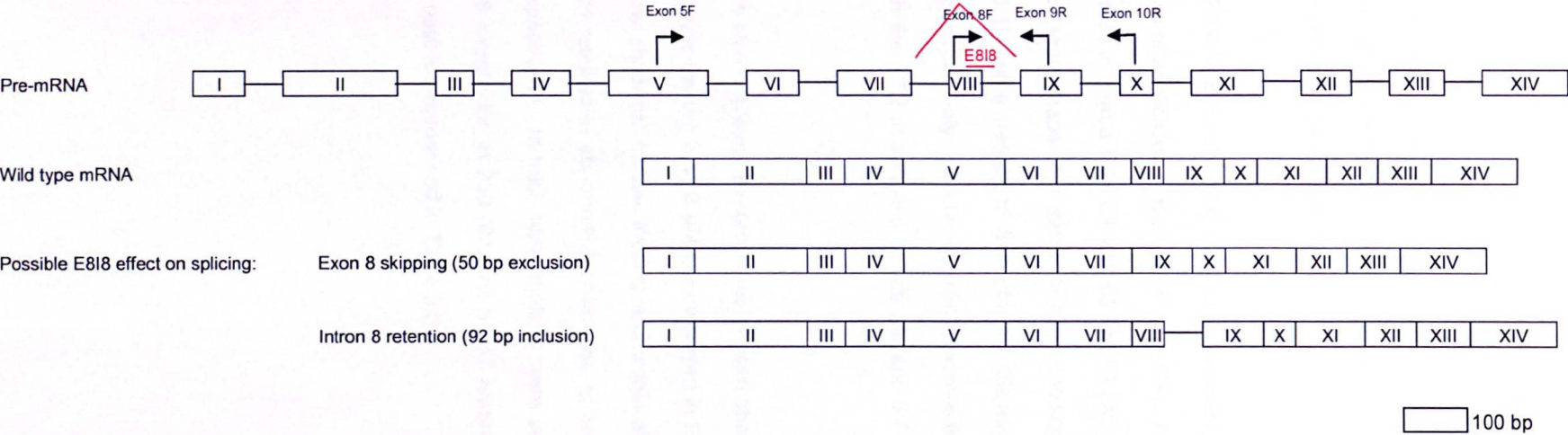


Figure 5.13 RBMS1 E8I8 morpholino sequence and predicted effect on splicing

The shorter of the two mRNA transcripts (ENSGALT00000018146) is represented for simplicity; all morpholinos, primers etc have been designed to bind to common regions of both transcripts. Exons are drawn to scale, and intron sizes have been standardised. Morpholino sequences are shown in the 3' – 5' direction, and target binding regions are boxed. Bases altered in mismatch control morpholinos are underlined.

5.3.3.4 External phenotypic analysis of RBMS1 knockdown embryos

External analysis focusing on embryonic hearts was performed on animals subjected to various morpholino treatments (Figure 5.14). Wild type and standard control treated embryos displayed no outward abnormalities (Figure 5.14 A1 and B1 respectively) whilst treatment with RBMS1 targeting morpholinos resulted in a range of cardiac abnormalities (Figure 5.14 C1-C4, D1-D4).

Treatment with the RBMS1 E2I2 targeting morpholino resulted in heart malformations at both concentrations of application (Figure 5.14 C1 – C4). Morpholino was applied to the embryos depicted in Figure 5.14 C1 and C2 at 250 μ M, and these respectively display an abnormal heart shape and defect in heart looping. Embryos shown in Figure 5.14 C3 and C4 were treated at 500 μ M and display a bulbous heart and irregular heart shape respectively. In total, cardiac abnormalities were seen in 2 / 11 embryos treated with the E2I2 morpholino at 250 μ M and 5 / 16 embryos treated at 500 μ M.

E8I8 treated embryos also displayed abnormalities in heart shape and looping (Figure 5.14 D1 – D4). Embryos treated at 250 μ M are displayed in Figure 5.14 D1 and D2, and respectively show abnormal cardiac looping and a thin almost tubular ventricle. Treatment at 500 μ M resulted in abnormal cardiac looping and heart shape (Figure 5.14 D3 and D4 respectively). In total, abnormalities were seen in 2 / 15 embryos treated with the E8I8 morpholino at 250 μ M, and 8 / 17 embryos treated at 500 μ M. Phenotypic analysis data is summarised in Table 5.2.

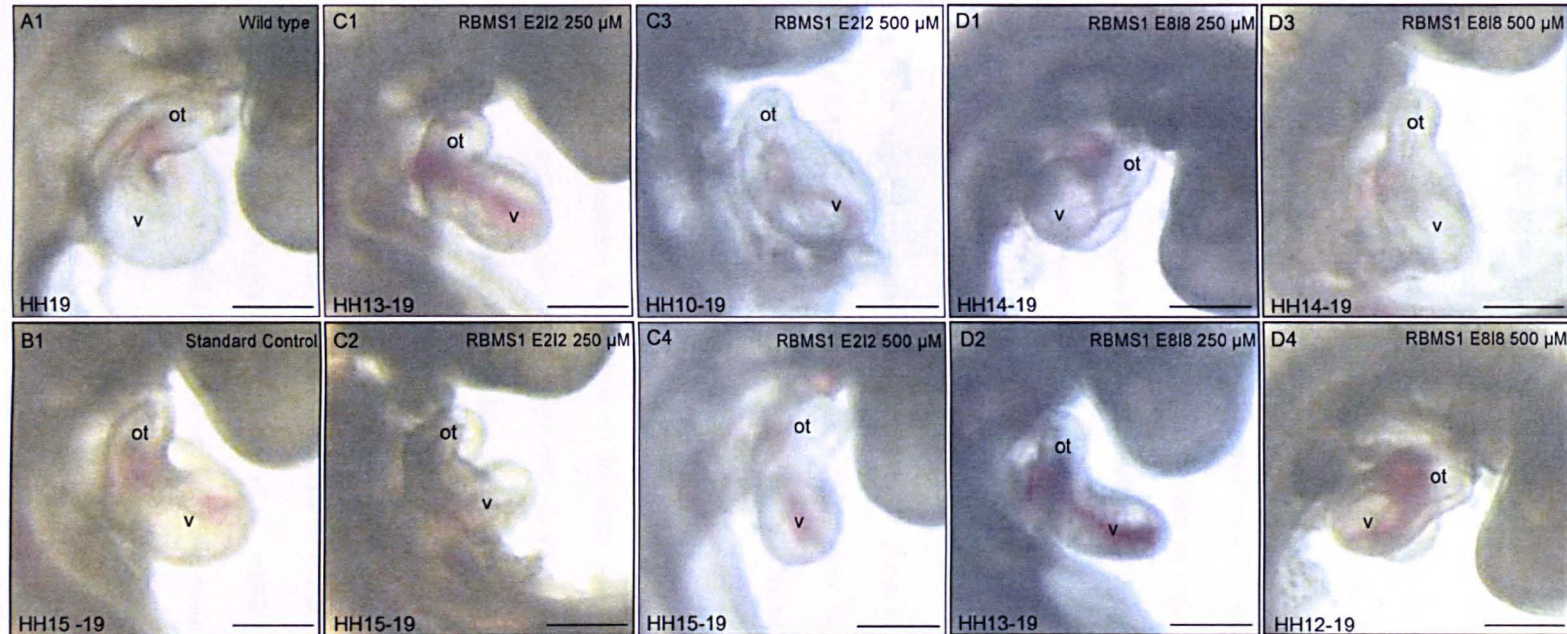


Figure 5.14 Altered cardiac morphology of RBMS1 knockdown embryos

Bright field lateral embryonic views of wild type (A1), standard control (B1), RBMS1 E2I2 applied at 250 μ M (C1-C2) and 500 μ M (C3 and C4), and RBMS1 E8I8 applied at 250 μ M (D1-D2) and 500 μ M (D3-D4) are displayed. Developmental stages of morpholino application and embryo harvest are indicated. Two examples of embryos subjected to each treatment are displayed. Scale bars represent 500 μ m. ot – outflow tract, v - ventricle

5.3.3.5 Histological analysis of RBMS1 knockdown embryos

Embryos were fixed in 4% PFA and dehydrated in a graded ethanol series prior to wax embedding. Embryos were sectioned coronally at a thickness of 10 microns, and sections transferred onto slides for haemalum staining and mounting with DPX. The high frequency of looping abnormalities did not always allow sectioning in the correct plane, resulting in difficulties in assessment of septum size in some cases. Figure 5.15 shows haemalum stained sections through control and knockdown embryos at points where the atrial septum is visible at its maximal size. Wild type and standard control embryos displayed a normal overall heart shape and structure including atrial septum size (Figure 5.15 A1 and B1 respectively). Treatment with the splice targeting morpholinos resulted in a reduction in the size of the atrial septum. Figure 5.15 C1 and C2 are two examples of embryos treated with the E2I2 morpholino at 250 μ M; C1 displayed a complete lack of septum, and C2 was normal. In total, 1 / 7 embryos in this treatment group displayed an internal cardiac defect. A larger proportion of embryos treated at 500 μ M displayed cardiac abnormalities (5 / 11), primarily in septal size, with secondary defects also present in some cases. Both examples shown (Figure 5.15 C3 and C4) are abnormal, with an atrial septum absent in C3. C4 shows septal growth but the heart is under-developed. Treatment with the E8I8 morpholino at 250 μ M (Figure 5.15 D1 and D2) did not have an effect on the internal structure of the heart (based on 8 embryos). However, treatment with this morpholino at 500 μ M resulted in a reduction in the size of the atrial septum in 6 / 13 cases. The two morpholinos produced overlapping but distinct abnormalities, which may be explained by the fact that each was targeted to a different region of the RBMS1 gene, resulting in different functional domains being affected in surviving proteins. This is observed with genes such as *TBX5* in HOS, where mutations in the 5' end of the T-box most significantly affect the heart, whilst mutations in the 3' end of the T-box have a more predominant effect on the limbs [Basson et al., 1999] (although there are exceptions [Boogerd et al.]). Overall, knockdown of RBMS1 resulted in refinement of the original phenotype observed in *TBX5* / *GATA-4* double knockdown embryos, with abnormalities restricted to the heart. This was surprising in view of the widespread

expression pattern of RBMS1, particularly when compared to the more restricted expression patterns of both TBX5 and GATA-4.

Figure 5.15 Histological analysis of altered cardiac morphology of RBMS1 knockdown embryos at HH19

Haemulium stained coronal embryonic sections through two embryos in each group are displayed. Groups represented are; (A) wild type, (B) standard control (500 μ M), (C) RBMS1 E212 (250 μ M and 500 μ M), and (D) RBMS1 E818 (250 μ M and 500 μ M). Developmental stages of morpholino application (where applicable) and embryo harvest are indicated. Regions of atrial septa formation (or expected septation) are boxed, shown at their maximum size upon viewing serial sections throughout embryo. Scale bars represent 500 μ m.

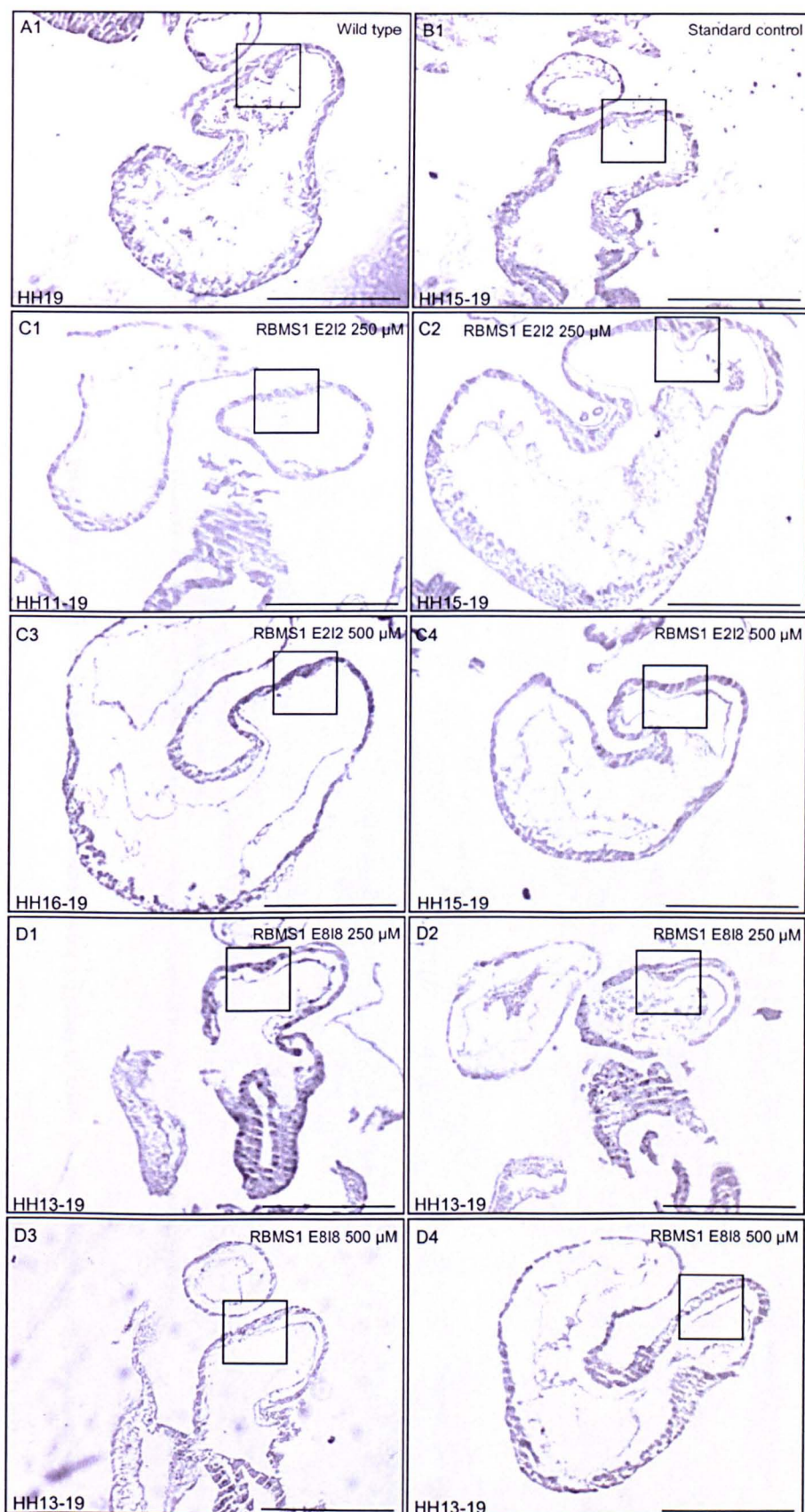


Table 5.1 Summary of heart abnormalities seen in RBMS1 knockdown embryos in comparison to controls

		Embryos with heart abnormality							
Nature/region of heart abnormality		Untreated (wild type)	Standard control 500 µM	RBMS1 E2I2 5-mis 250 µM	RBMS1 E2I2 5-mis 500 µM	RBMS1 E2I2 250 µM	RBMS1 E2I2 500 µM	RBMS1 E8I8 250 µM	RBMS1 E8I8 500 µM
External analysis	Heart size / shape	1 / 30	0 / 10	2 / 8	2 / 18	1 / 11	3 / 16	2 / 11	6 / 17
	Heart looping	1 / 30	0 / 10	2 / 8	3 / 18	1 / 11	2 / 16	0 / 11	2 / 17
	Ventricle formation	0 / 30	0 / 10	0 / 8	0 / 18	0 / 11	0 / 16	0 / 11	0 / 17
	Total	2 / 30	0 / 10	2 / 8	5 / 18	2 / 11	5 / 16	2 / 15	8 / 17
Internal analysis	Atrial septation	1 / 10	0 / 10	1 / 1	2 / 3	1 / 7	5 / 11	0 / 8	6 / 13
	Trabeculation	0 / 10	0 / 10	1 / 1	1 / 3	1 / 7	3 / 8	0 / 8	0 / 6
	Cardiac jelly	0 / 10	0 / 10	0 / 1	0 / 3	0 / 7	0 / 8	0 / 8	0 / 13
	Total	1 / 10	0 / 10	1 / 1	2 / 3	1 / 7	5 / 11	0 / 8	6 / 13

Groups represented are wild type, standard control (500 µM), RBMS1 E2I2 (250 µM and 500 µM), and RBMS1 E8I8 (250 µM and 500 µM).

5.3.4 Characterisation of morpholino effects on splicing

For characterisation of morpholino effects on splicing, RT-PCR was carried out using strategically placed primers for detection of alternatively spliced products.

5.3.4.1 RBMS1 E2I2 morpholino

5.3.4.1.1 Sample collection and preparation

RBMS1 E2I2 morpholino was applied to approximately 100 eggs following exclusion of embryos which were no longer viable or not within the desired range of development (HH12 – HH16). Morpholino-treated embryos were excised from the egg at HH19, placed in sterile chilled PBS, and membranes quickly removed. Morpholino uptake was assessed under fluorescent light and positive hearts isolated and stored in *RNAlater* at -20 °C. Eight hearts were pooled per sample, and three biological replicates collected per group (2 groups: knockdown and wild type). RNA was column-extracted and concentration assessed by spectrophotometry. Exactly 1 µg RNA was used per 20 µl reverse transcription reaction, and samples made up to 100 µl prior to use.

5.3.4.1.2 Primer placement for detection of alternatively spliced transcripts

As previously mentioned, the E2I2 morpholino would be expected to result either in exon 2 skipping, or intron 2 retention (Figure 5.12). Morpholino binding to splice junctions can also result in activation of cryptic splice sites, leading to partial insertions or deletions. Primers were therefore designed to flank the entire region which may be affected, in order to determine the nature of the morpholino effect through amplification of alternatively spliced transcripts and assessment of their size. Thus, the initial set of primers was placed in exon 1 and exon 5. An additional set of primers was also designed to exon 2 and exon 3; these could amplify any products with partial intronic retention (dependent on the size of the intronic region retained). Due to the large size of intron 2 (25676 bp), it would not be possible to amplify this whole region by PCR in the event of full intronic retention. Instead, a third set of primers was

designed within intron 2 in order to attempt PCR amplification of a small portion of this region (243 bp) for comparison between knockdown animals and untreated controls. Primer sequences are given in Table 2.5. RPLPO was used as an internal control and amplified in parallel.

5.3.4.1.3 RT-PCR amplification and sequencing of mRNA transcripts

PCR products generated using the above primers are shown in Figure 5.16. PCR using exon 1F and exon 5R primers resulted in sole amplification of the wild type transcript (354 bp) in all samples. Amplification using a second set of primers, exon 2F and exon 3R, also generated a single wild type product (213 bp) in all samples. Amplification using the above primer pairs was performed using extension times ranging from 30 seconds to 5 minutes, all yielding the same result. Products were also sequenced (one knockdown and one wild type from each group) to assess whether alternative splicing events (which may not have altered product sizes) had occurred, and all transcripts detected were normal. A third set of primers designed to detect retention of intron 2 produced an intronic band of the expected size (243 bp) in all samples. This was thought to be a pre-mRNA transcript, as amplification did not occur in negative RT controls (where no reverse transcriptase enzyme was added). RPLPO was also assayed as an internal control.

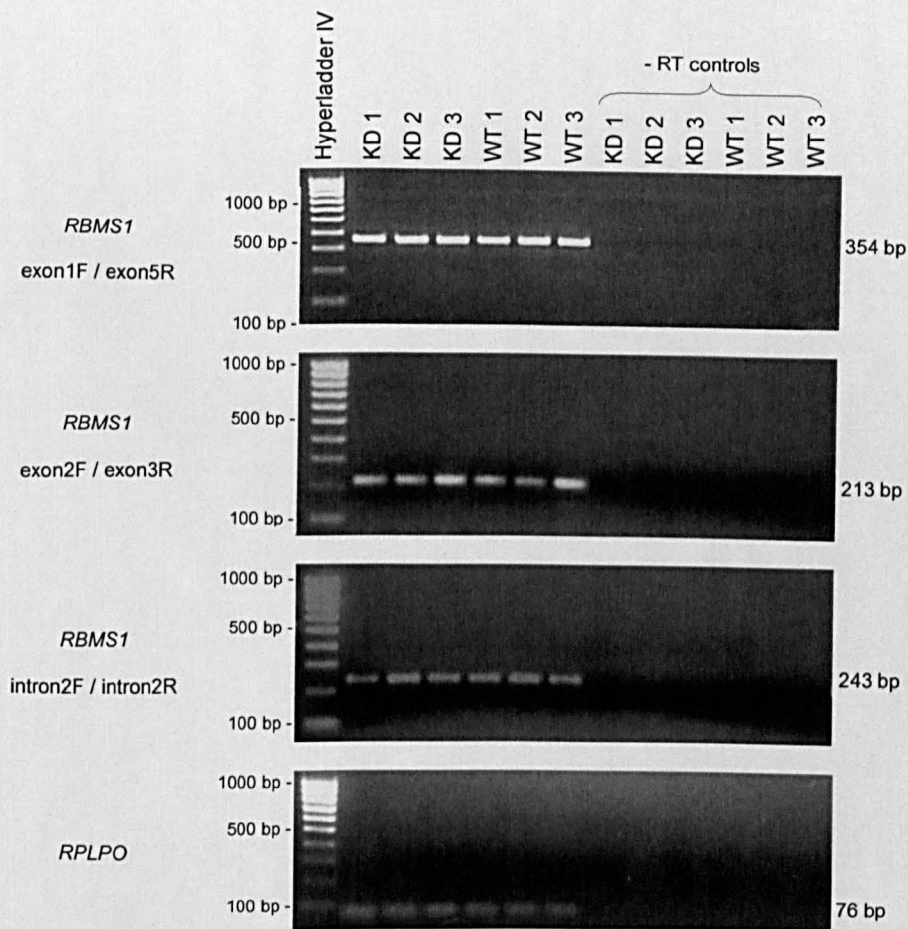


Figure 5.16 Characterisation of the effect of the RBMS1 E2I2 morpholino on mRNA splicing

PCR was performed at an annealing temperature of 60 °C with 35 cycles of amplification, and samples electrophoresed on 2% agarose gels. Three biological replicates were assayed for morpholino-treated and wild type groups. Three sets of primers were used for amplification of RBMS1 transcripts, and RPLPO was assayed in parallel as an endogenous control.

5.3.4.2 RBMS1 E8I8 morpholino

5.3.4.2.1 Sample collection and preparation

Embryos were treated with morpholino as previously (section 5.3.4.1.1). Three wild type and three morpholino positive whole embryos were harvested at HH19, and RNA column extracted and quantified for cDNA synthesis using 1 µg RNA in a 20 µl volume. Samples were made up to 100 µl prior to use.

5.3.4.2.2 Primer placement for detection of alternatively spliced transcripts

The E8I8 morpholino would be expected to result in exon 8 skipping (50 bp), or intron 8 retention (92 bp) (Figure 5.13). Flanking primers were designed to exon 5 and exon 10 for detection of alternatively spliced transcripts. An additional set of primers was also designed to exon 8 / exon 9, which would result in amplification of products harbouring partial/full intron retention. Primer sequences are given in Table 2.5. RPLPO was included as an endogenous control.

5.3.4.2.3 RT-PCR amplification and sequencing of mRNA transcripts

Figure 5.17 shows results of RT-PCR amplification using the above primers. Both sets of primers resulted in amplification of a single wild type transcript in all samples i.e. alternatively spliced products were not found. The product generated from the KD1 sample appeared to be fainter than the others in both RT-PCR assays, and QPCR was used to quantify any changes (see next section, 5.3.5). RT-PCR products from a single wild type and knockdown sample were also sequenced to assess any changes, but only wild type sequences were detected.

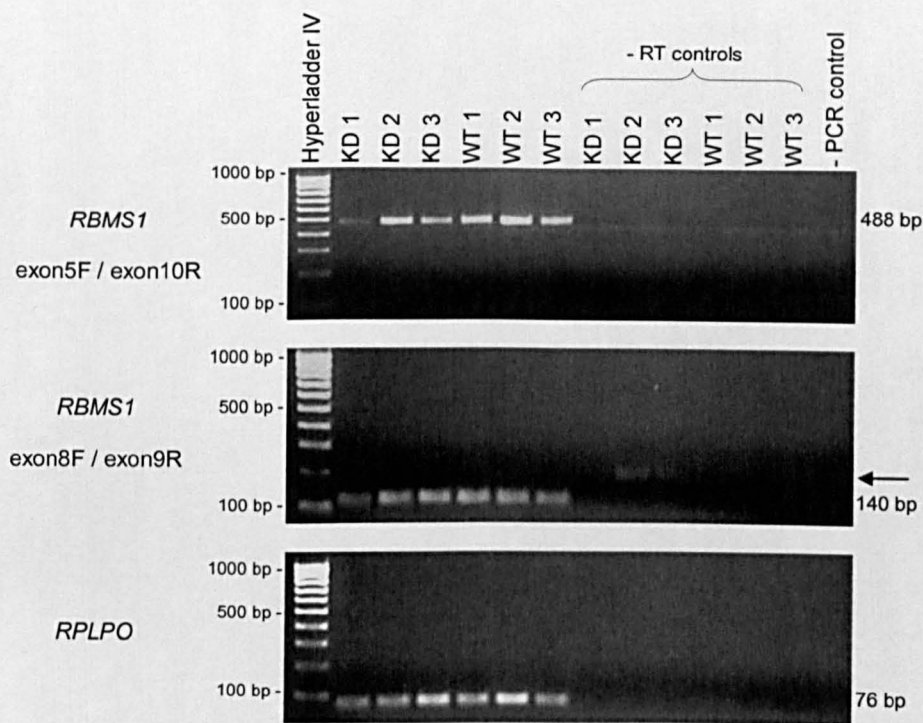


Figure 5.17 Characterisation of the effect of the RBMS1 E8I8 morpholino on mRNA splicing

PCR was performed at an annealing temperature of 60 °C with 35 cycles of amplification, and samples electrophoresed on 2% agarose gels. Three biological replicates were assayed per group. Two sets of primers were used for amplification of RBMS1 transcripts, and RPLPO was assayed in parallel as an endogenous control. The arrow indicates bands generated from low level genomic contamination in –RT samples KD2 and KD3 (152 bp product).

5.3.5 QPCR analysis of morpholino effects on mRNA levels

RT-PCR did not result in detection of alternatively spliced products as a result of treatment with either morpholino. Nonsense-mediated decay (NMD) is a cellular mechanism in which aberrant mRNAs are detected (through recognition of premature termination codons (PTCs)), and degraded [Hentze and Kulozik, 1999]. It was possible that NMD was eliminating alternatively spliced transcripts in morpholino-treated embryos, which would result in a corresponding decrease in wild type RBMS1 mRNA transcripts. QPCR expression analysis was performed in order to quantify any morpholino-induced change in RBMS1 mRNA levels. RNA isolated previously for splicing characterisation was used, and new reverse transcriptase reactions were performed. Dilutions of cDNA were carried out immediately upon synthesis, as described in 2.2.1.10.5.

5.3.5.1 Validation of RPLPO as an appropriate endogenous control

RPLPO was used in Chapter 4 as an endogenous control for normalisation of data, where its suitability was assessed by study of assay properties and analysis of variation of expression levels in study samples. In order to confirm that RPLPO expression was not affected by samples treatments here, C_T values of RPLPO were measured in triplicate for each sample following their identical preparation and dilution, and are displayed in Table 5.2. RPLPO displayed constant expression across samples for both experimental groups (ΔC_T values of -0.24 (E2I2 treatment) and 0.16 (E8I8 treatment)), and was used as an endogenous control gene.

Table 5.2 Mean C_T and ΔC_T values of RPLPO in study samples

Morpholino	Sample	Mean C_T	ΔC_T
RBMS1 E2I2	Wild type (calibrator)	25.42368	0
	Morpholino treated	25.66415	-0.24048
RBMS1 E8I8	Wild type (calibrator)	18.03443	0
	Morpholino treated	17.87218	0.162246

Three biological samples were analysed per group, each assayed in triplicate. A standard curve was run in parallel on the same reaction plate for confirmation of assay quality.

5.3.5.2 QPCR analysis of RBMS1 in morpholino treated embryos

Baseline and threshold values were set manually, and absolute quantities of each gene generated from the standard curves in Applied Biosystems 7500 v2.0.1 software. Standard curves for target and endogenous control genes were included on each plate, with each point measured in duplicate. All samples were run simultaneously on the same plate, each assayed in triplicate. Biological and experimental replicates were grouped for data normalisation and standard deviation calculations. Results of QPCR analysis are shown in Table 5.3 and Figures 5.18 and 5.19. No difference in mRNA levels of RBMS1 was observed between morpholino treated and wild type animals (data non-significant by student's t test). It is not clear why these approaches (both RT-PCR and QPCR) did not show any change at the mRNA level, given the identical and specific phenotype generated by the two morpholinos.

Table 5.3 QPCR expression analysis of RBMS1 in E2l2 and E8l8 morpholino treated animals and controls

	RBMS1 relative expression	
Morpholino	Morpholino treated	Wild type
RBMS1 E2l2	1.01 ± 0.44	1.04 ± 0.19
RBMS1 E8l8	0.39 ± 0.11	0.41 ± 0.12

QPCR expression analysis was performed on RBMS1 E2l2 morpholino treated embryonic hearts (8 per sample), and E8l8 treated whole embryos (1 per sample), each with corresponding wild type controls. 3 biological replicates were assayed per group. Expression is measured relative to the endogenous control RPLPO. Data was not significant (student's t test, p<0.05).

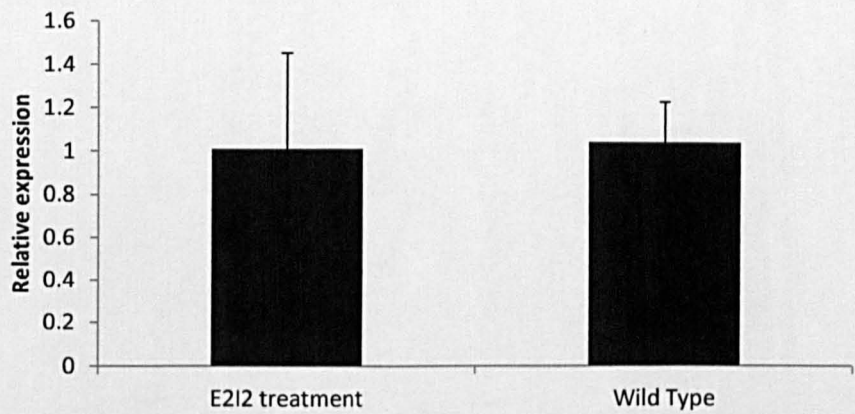


Figure 5.18 QPCR expression analysis of RBMS1 in E2I2 morpholino treated animals and controls

QPCR expression analysis was performed on RBMS1 E2I2 morpholino treated embryonic hearts (8 per sample), with corresponding wild type controls. 3 biological replicates were assayed per group. Expression is measured relative to RPLPO. Error bars represent standard deviation. Data was not significant by student's t test.

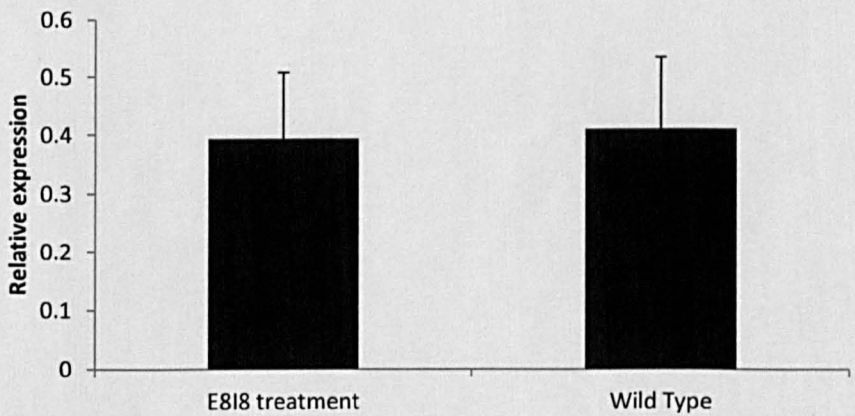


Figure 5.19 QPCR expression analysis of RBMS1 in E8I8 morpholino treated animals and controls

QPCR expression analysis was performed on E8I8 morpholino treated whole embryos (1 per sample), with corresponding wild type controls. 3 biological replicates were assayed per group. Expression is measured relative to RPLPO. Error bars represent standard deviation. Data was not significant by student's t test.

5.3.6 Bioinformatic promoter analysis of the α -actin genes

The α -actins are likely to have arisen from a common ancestral gene and show a high level of sequence similarity. Work by others has shown that RBMS1 negatively regulates expression of α -smooth muscle actin via a TATCTTA sequence found in the promoter region at position -228 to -222 [Kimura et al., 1998]. Due to the high degree of conservation between α -smooth muscle actin and other α -actins (α -skeletal and α -cardiac actin), it was postulated that RBMS1 may also play a role in the transcriptional regulation of these genes. The TATCTTA motif in α -smooth muscle actin displays only partial conservation (57%) with other species such as human, rat and mouse [Kimura et al., 1998]. This means that multi-species alignment for promoter analysis (as performed in Chapter 3) would not be an appropriate tool for identification of this motif. Promoter analysis was instead performed to examine the presence of the TATCTTA regulatory sequence solely in chicken striated α -actin genes, using the 'fuzznuc' search function (<http://emboss.bioinformatics.nl/>) which searches for patterns in nucleotide sequences. The TATCTTA motif was identified in both striated actin genes, located approximately 7 kb and 4.5 kb upstream of the first exon of α -cardiac and α -skeletal actin respectively. The position of these in relation to transcriptional start sites has not been determined. Additional motifs were identified within the coding regions and further 3'.

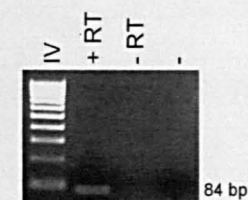
5.3.7 QPCR expression analysis of the actin genes in RBMS1 E2l2 morpholino treated animals and controls

RBMS1 negatively regulates transcription of α -smooth muscle actin, which means this gene can act as a positive control for knockdown. QPCR expression analysis was performed to assess whether α -smooth muscle actin and other α -actin genes were up-regulated in RBMS1 E2l2 morpholino treated embryonic hearts. The effect of the E8l8 morpholino on expression of these genes would also have been performed, but time restrictions did not allow this.

5.3.7.1 QPCR assay design

As previously, QPCR assays were designed using the online Roche Universal ProbeLibrary assay design centre (<https://www.roche-applied-science.com>) following the same general guidelines. Sequence alignments between the three α -actins were performed for design of assays to regions of maximal diversity for gene-specificity. The relevant regions including primer and probe binding sequences and RT-PCR products generated are shown in Figure 5.20. Amplicons were cloned into the pGEM-T Easy vector for sequencing to confirm assay specificity. Primer sequences and positional information are given in Table 2.6.

α-smooth muscle actin	<u>GCACCTGAGGACATTGACATAGAAGATGTGTGAGGAGGA</u> <u>GGACAGCA</u> CTGCCCTTGTTTGTGACAATGGCTCAGGGCTCTGTAA
α -skeletal muscle actin	-----
α -cardiac muscle actin	-----ATCAGCCAAGATGTGTGACGACGAGGAGACCACCGCGCTGGTGTGCGACAACGGCTCGGGGCTGGTCAA
α -smooth muscle actin	AGCATATTACTGTGAATGTATTCAGGAAAATACATTCTTAAATTTTCATTCCATAAATCTTTCATCGTAATGGCTGGTTAATTGGATACGGTGTA
α-skeletal muscle actin	<u>TGCCAACAC</u> ----- <u>ACTCAGGATGACA-ATCTTGTAGGTT</u> <u>CCAGGCTG</u> CTGAGGACC---TGCACCAGCCATGCAACTT-----TC
α -cardiac muscle actin	TTCCCACTCAG-----GATGACGACAGTATGCTTCTTGAGTCT--TCTGGCAGCCCTTC--CTGAACTCCTCCGTCATTGTACAGTTTGT
α -smooth muscle actin	TGAAAACGAGATGGCCACTGCTGCCTCTTCCTCCTCTCTGGAAAAAGCTATGAGCTTCCTGATGGC
α -skeletal muscle actin	TGAGAACGAGATGGCCACCGCTGCCTCCTCCTCCTCCCTGGAGAAGAGCTATGAGCTGCCTGATGGG
α-cardiac muscle actin	<u>TGAGAATGAAATGGCCACAGCTGCTTCGTCAT</u> <u>CCTCCCTGGAGA</u> AGAGCTACGAATTGCCTGATGGT



84 bp



73 bp



67 bp

Figure 5.20 QPCR assays used for expression analysis of the three actin genes

Primer binding sites are underlined, and probe binding sites are boxed. Sequences have been aligned with the corresponding region of the other two α -actin genes (shown in grey). 2% agarose gel images displaying the RT-PCR products generated are shown on the right. Specificity of each assay was confirmed by sequencing of RT-PCR products following cloning into the pGEM-T Easy vector.

5.3.7.2 Expression analysis

QPCR expression analysis was performed using the same samples as previously, on RBMS1 E2I2 morpholino treated embryonic hearts (8 per sample) and corresponding wild type controls. Three biological replicates were assayed per group. Data was normalised to RPLPO. Table 5.5 and Figure 5.21 show results obtained from QPCR analysis of the α -actins in E2I2 treated embryos and controls. Expression was measured relative to RPLPO. In wild type HH19 embryonic hearts, α -cardiac actin was the most highly expressed actin, followed by α -skeletal actin, with α -smooth muscle actin displaying the lowest level of expression. Expression of α -smooth muscle actin was found to be greater in the E2I2 treatment group than in wild type embryos (significant by the student t test, $p < 0.05$). No significant differences in expression of the striated actins were observed between the treated and untreated groups.

Table 5.5 QPCR expression analysis of actin genes in RBMS1 E2I2 morpholino treated animals and controls

Gene	Relative expression	
	RBMS1 E2I2 treatment	Wild Type
α -smooth muscle actin	$3.87 \pm 1.13^*$	2.25 ± 0.95
α -skeletal muscle actin	30.3 ± 8.28	23.4 ± 8.48
α -cardiac muscle actin	29.3 ± 11.6	33.1 ± 4.31

QPCR expression analysis was performed on RBMS1 E2I2 treated animals and wild type controls (3 biological replicates per group). Expression is measured relative to the endogenous control RPLPO. *Significant difference between E2I2 treated sample and wild type control ($p < 0.05$, student's t test).

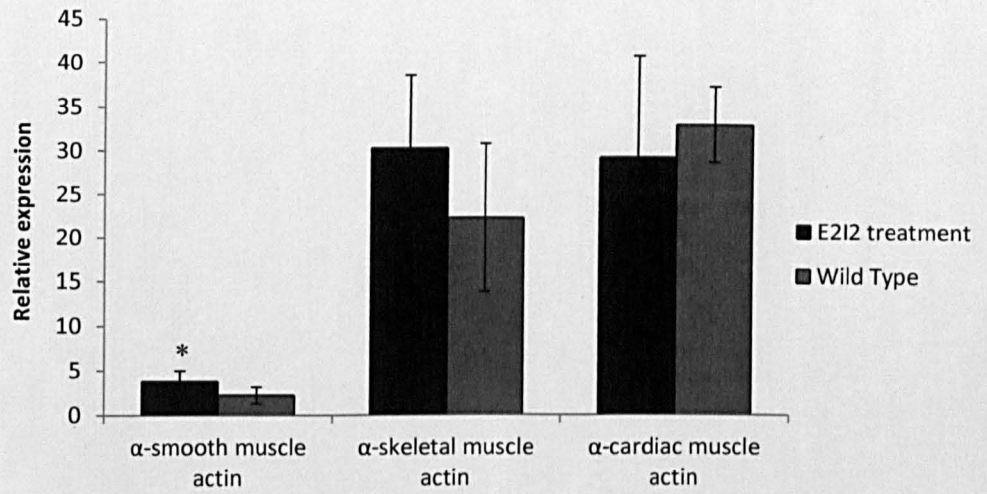


Figure 5.21 QPCR expression analysis of the three actin genes in RBMS1 E2I2 morpholino treated animals and controls

QPCR expression analysis was performed on RBMS1 E2I2 treated animals and wild type controls (3 biological replicates per group). Expression is measured relative to the endogenous control RPLPO. Error bars represent standard deviation. *Significant difference between E2I2 treated sample and wild type control ($p < 0.05$, student's t test).

CHAPTER 6

MICROARRAY EXPRESSION ANALYSIS OF TBX5 / GATA-4 DOUBLE KNOCKDOWN EMBRYOS

6.1 AIMS

This chapter aims to identify new downstream targets of TBX5 and GATA-4 in the developing chick heart by performing microarray analysis of double knockdown embryos as follows:

- a) Morpholino knockdown and collection of tissue. Extraction of RNA and assessment of concentration and purity
- b) Microarray expression analysis of TBX5 / GATA-4 double and single knockdown embryos and controls
- c) Review of literature and selection of candidate genes for further study
- d) Preliminary expression analyses in the chick embryo

6.2 RESULTS

6.2.1 Morpholino knockdown and tissue collection

Morpholinos were applied as described previously (Chapter 4) to create five sample groups:

- 1) TBX5 and GATA-4 double knockdown
- 2) TBX5 single knockdown (with GATA-4 7-mismatch control)
- 3) GATA-4 single knockdown (with TBX5 7-mismatch control)
- 4) TBX5 7-mismatch control and GATA-4 7-mismatch control
- 5) Wild type

Approximately 300 eggs were opened in total for this study. Morpholino was applied to around half following exclusion of embryos which were no longer viable or not at the required stage of development. Morpholinos were applied at HH15 for harvest at HH19 at a concentration of 250 μ M each, and uptake was assessed by fluorescent microscopy. Morpholino positive embryonic hearts were isolated and stored in RNA_{later} prior to appropriate pooling of samples for column extraction of RNA. One sample was collected per experimental group, each comprising eight hearts (this was found to be the minimum number of HH19 stage hearts required for successful isolation of RNA). It would have been preferable to include three samples per experimental group; however resource did not allow this. As a result, this approach does not provide enough statistical power, and data will require further validation either by subsequent addition of a further two samples per group for microarray analysis, or by QPCR validation using a minimum of three samples per group.

6.2.2 Assessment of RNA quality

High RNA purity and quality is of paramount importance in applications such as microarray expression analysis. Impurities can affect RNA labelling efficiency and the stability of the fluorescent label. RNA concentration and purity was initially measured

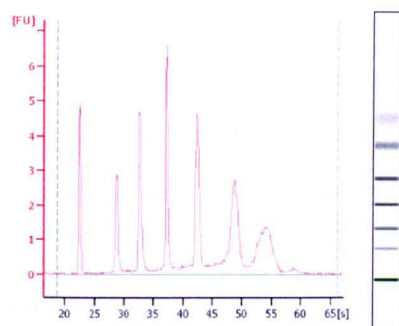
by spectrophotometry, and is displayed in Table 6.1. All samples displayed 260/280 ratios of approximately 2, and 260/230 ratios equal to or greater than 1.8, indicating high RNA purity. Samples were sent to the Nottingham Arabidopsis Stock Centre (NASC) for further QC analysis of RNA using the Agilent bioanalyser, and subsequent microarray expression profiling. Traditionally, RNA integrity is assessed by agarose gel electrophoresis, but this method requires large amounts of sample RNA (0.5 – 2.0 µg) and is not very sensitive in detecting degradation. The bioanalyser allows capillary based electrophoretic sample separation according to molecular weight, and subsequent fluorescent detection to generate electropherogram and in gel-like images. It requires far smaller quantities of RNA (as low as 200 pg) and is a more sensitive and reliable method [Imbeaud et al., 2005]. Figure 6.1 shows bioanalyser RNA profiles and in gel-like images generated using the five samples purified for microarray analysis. The two peaks represent the 18S and 28S ribosomal RNA subunits, and the absence of smaller degradation products indicates high quality RNA. RNA integrity numbers (RIN) are a means of numerically categorising sample integrity from electrophoretic traces, and range from 1 (fully degraded RNA) to 10 (intact RNA) [Schroeder et al., 2006]. RIN values generated are displayed in Table 6.1 and ranged from 9.3 to 10, indicating intact RNA.

Table 6.1: RNA concentration, purity, and integrity of samples used for microarray expression analysis

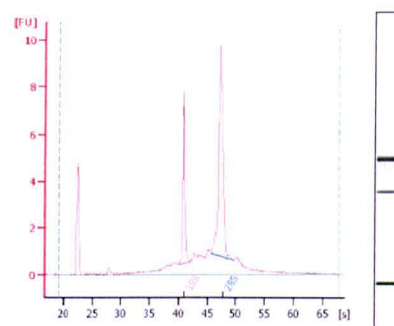
Sample	Spectrophotometry			Bioanalysis
	260 / 280	260 / 230	Concentration (ng / μ l)	RIN value
TBX5 / GATA-4 double knockdown	2.11	1.79	240.8	9.3
TBX5 knockdown (+ GATA-4 7-mis)	2.09	2.07	157.2	9.4
GATA-4 knockdown (+ TBX5 7-mis)	2.05	2.04	339.3	10
TBX5 7-mis / GATA-4 7-mis	2.09	1.99	315.8	9.9
Wild Type	2.09	2.16	282.0	9.8

RNA concentration and purity was measured by spectrophotometry, and RNA integrity numbers (RIN) assessed using a bioanalyser. All samples displayed 260/280 ratios of approximately 2, and 260/230 ratios greater than or equal to 1.8, indicating high RNA purity. RIN numbers ranged from 9.2 to 10, indicating RNA was intact.

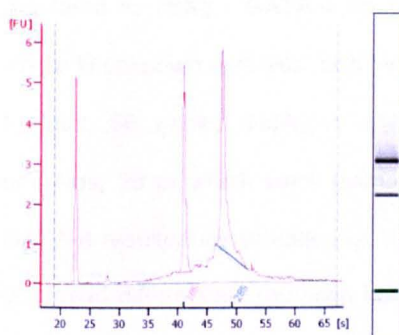
Ladder



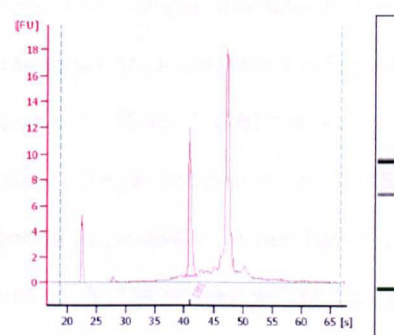
TBX5 / GATA-4 double knockdown



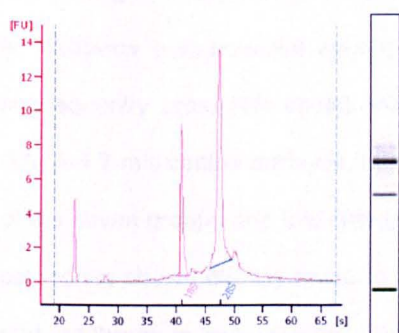
TBX5 knockdown (+ GATA-4 7-mis)



GATA-4 knockdown (+ TBX5 7-mis)



TBX5 7-mis / GATA-4 7-mis



Wild type

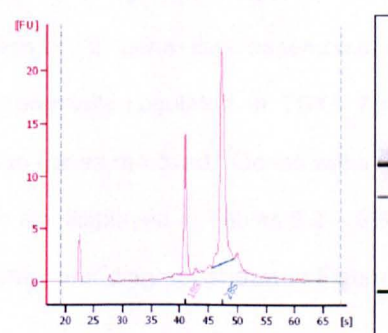


Figure 6.1 Bioanalyser analysis of RNA samples prior to microarray analysis

Bioanalyser QC analysis of RNA was performed by NASC. Electropherograms are displayed with in gel-like images to the right. The two peaks represent the 18S and 28S ribosomal RNA subunits, and the absence of smaller degradation products indicates high quality RNA. RIN values ranged from 9.3 - 10 and are displayed in Table 6.1.

6.2.3 Microarray expression study

Microarray expression profiling analysis was performed by the NASC using the Affymetrix GeneChip® Chicken Genome Array. Probe sets on this array are designed with 11 oligonucleotide pairs for specific detection of transcripts. Each array contains coverage of over 28000 chicken genes and 32773 transcripts.

6.2.3.1 Analysis of data

Data was analysed in GeneSpring software (Agilent). Genes displaying changes of 2-fold or greater in comparison to wild type embryos were selected. Genes differentially regulated in TBX5 / GATA-4 double knockdown, TBX5 single knockdown, GATA-4 single knockdown embryos, and overlapping categories are represented in Figure 6.2. In total, 56 genes displayed altered expression in TBX5 / GATA-4 knockdown embryos, 28 of which were unique to this group. Single knockdown of TBX5 and GATA-4 resulted identification of 109 and 67 genes respectively. A number of genes displayed differential regulation both in response to double knockdown of TBX5 and GATA-4, and in response to single knockdown of one or both of these transcription factors. These genes were also of relevance, particularly those displaying a greater fold change in expression in the double knockdown group compared to single knockdowns (i.e. potential synergistic activation). All gene lists assembled were subsequently cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and any common genes removed. Genes within each of the seven groups and fold changes observed are displayed in Tables 6.2 – 6.8. All categories shown directly relate to sections of the venn diagram shown in Figure 6.2, and are therefore mutually exclusive.

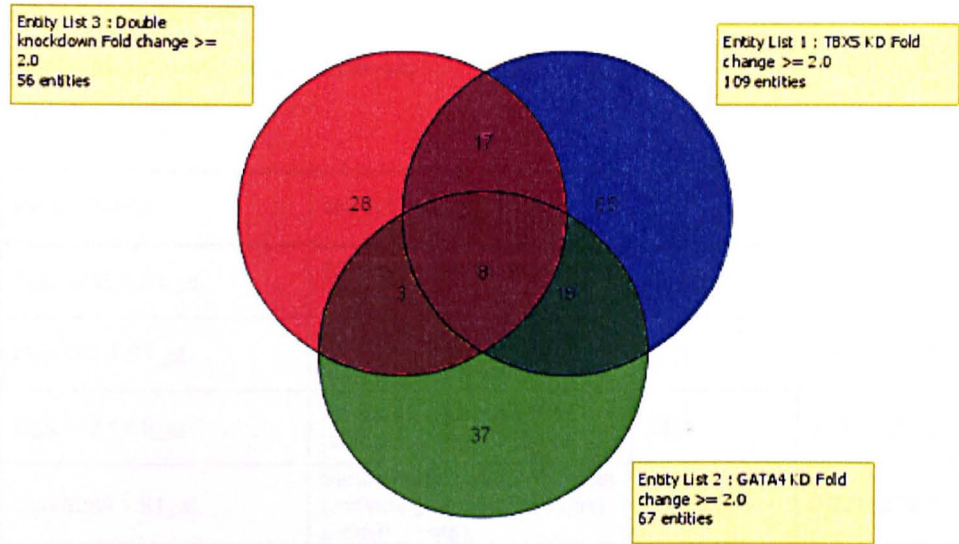


Figure 6.2 Genes differentially regulated in microarray analysis of TBX5 and GATA-4 single and double knockdown

Venn diagram displaying genes which show a ≥ 2.0 fold change in TBX5 / GATA-4 double knockdown, TBX5 single knockdown and GATA-4 single knockdown embryos in comparison to wild type animals. Gene lists created were subsequently cross-referenced genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos and common genes removed.

Table 6.2 Genes differentially regulated in microarray analysis of TBX5 / GATA-4 double knockdown chick embryos

Probe Set ID	Gene Title	Gene Symbol	Fold change
Gga.3795.1.S1_at	SRY (sex determining region Y)-box 3	SOX3	2.0196466 ↑
Gga.153.1.S1_at	Zic family member 1 (odd-paired homolog, Drosophila)	ZIC1	2.5582569 ↑
Gga.153.1.S2_at	Zic family member 1 (odd-paired homolog, Drosophila)	ZIC1	26.636639 ↑
Gga.9289.1.S1_at	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	TFAP2B	2.2718828 ↑
Gga.2977.1.S1_at	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	6.1341915 ↑
Gga.3219.1.S1_at	c-fos induced growth factor (vascular endothelial growth factor D)	FIGF	2.2266912 ↑
Gga.556.1.S1_at	paired box gene 6	PAX6	2.6883574 ↑
Gga.657.1.S1_at	forkhead box G1	FOXG1	2.8416626 ↑
Gga.3535.1.S1_at	nuclear receptor subfamily 2, group E, member 1	NR2E1	2.7937800 ↑
Gga.952.1.S1_at	NK2 homeobox 1	NKX2-1	11.427207 ↑
Gga.10.1.S1_at	orthodenticle homeobox 2	OTX2	16.048748 ↑
GgaAffx.20141.1.S1_at	orthodenticle homeobox 2	OTX2	2.7658422 ↑
GgaAffx.20545.1.S1_s_at	Finished cDNA, clone ChEST1015n20		2.0427935 ↑
Gga.1862.1.S1_at	hypothetical gene supported by CR390114	LOC420770	2.6278262 ↑
Gga.9574.1.S1_at	similar to enhancer of split related protein-7	LOC419390	2.6523864 ↑
Gga.11969.1.S1_at	cytokine-like 1	CYTL1	2.2584643 ↑
Gga.14235.1.S1_at	Finished cDNA, clone ChEST125m3		2.6366910 ↑
Gga.2646.1.S1_at	Phospholipase C, eta 1	PLCH1	2.7627573 ↑
Gga.16236.1.S1_s_at	Finished cDNA, clone ChEST928e1		2.5964615 ↑
Gga.14703.1.S1_at	SP8		2.0750513 ↑

Gga.19600.1.S1_at	Finished cDNA, clone ChEST963k5		3.6129053 ↑
Gga.19626.1.S1_s_at	suppressor of zeste 12 homolog (Drosophila)	SUZ12	2.0030265 ↑
Gga.4546.1.S1_at	cellular retinoic acid binding protein 1	CRABP1	4.9209270 ↑
GgaAffx.23845.1.S1_at	golgi autoantigen, golgin subfamily a, 4	GOLGA4	2.0230920 ↑
GgaAffx.3795.1.S1_at	glypican 3	GPC3	2.3861866 ↑
GgaAffx.24879.4.S1_at			2.2617579 ↓

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Genes displaying up-regulation are displayed in black, and down-regulated genes are displayed in red. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.3 Genes differentially regulated in microarray analysis of TBX5 single knockdown chick embryos

Probe Set ID	Gene title	Gene symbol	Fold change
Gga.919.1.S1_at	distal-less homeobox 5	DLX5	2.356541 ↑
Gga.5334.1.S1_a_at	regulator of G-protein signaling 4	RGS4	2.195912 ↑
Gga.32.1.S1_at	mab-21-like 1 (C. elegans)	MAB21L1	2.133591 ↑
Gga.8359.1.S1_at	glutathione S-transferase A3	GSTA3	4.219509 ↑
Gga.9334.1.S1_at	serum/glucocorticoid regulated kinase 1	SGK1	2.062817 ↑
Gga.635.1.S1_at	Spindling	SPIN	2.516693 ↑
Gga.827.1.S1_at	erythroid-specific transcription factor eryf1	LOC396450	2.594897 ↑
Gga.580.1.S2_at	growth factor independent 1B transcription repressor	GF11B	2.200138 ↑
GgaAffx.20420.1.S1_at	NEDD4 binding protein 2-like 1	N4BP2L1	2.208199 ↑
GgaAffx.21102.1.S1_s_at	aldolase B, fructose-bisphosphate	ALDOB	3.452897 ↑
GgaAffx.21598.1.S1_s_at	Similar to trans-Golgi protein GMx33	LOC425502	2.764642 ↑
GgaAffx.20558.1.S1_at	Chromodomain helicase DNA binding protein 1	CHD1	3.355640 ↑
Gga.4981.1.S1_s_at	hemoglobin, gamma G /// hemoglobin, gamma A	HBG1 /// HBG2	5.235574 ↑
GgaAffx.20789.1.S1_at	hemoglobin, alpha 1	HBA1	2.144276 ↑
Gga.2909.1.S1_at	hemoglobin, alpha 2	HBA2	2.369730 ↑
Gga.3078.1.S1_at	similar to general transcription factor	LOC430910	2.022142 ↑
Gga.5205.1.S1_at	benzodiazapine receptor (peripheral)-like 1	BZRPL1	2.122973 ↑
Gga.17132.1.S1_at	Finished cDNA, clone ChEST120n19		2.293467 ↑
Gga.7809.1.S1_at	Hypothetical protein LOC771069	LOC771069	3.389699 ↑
Gga.8915.1.S1_s_at	chromosome 20 open reading frame 108	C20orf108	2.639697 ↑

Gga.5139.1.S1_at	fructose-1,6-bisphosphatase 1	FBP1	2.028548 ↑
Gga.9054.1.S1_s_at	adenosine deaminase	ADA	2.316033 ↑
Gga.11474.1.S1_a_at	Rieske (Fe-S) domain containing	RFESD	2.534035 ↑
Gga.20049.1.S1_at	hypothetical gene supported by CR406891	LOC421241	2.146337 ↑
Gga.14585.1.S1_at	Finished cDNA, clone ChEST488o16		3.843172 ↑
Gga.16155.1.S1_at	Finished cDNA, clone ChEST38i14		2.132346 ↑
Gga.10658.1.S1_at	Finished cDNA, clone ChEST1007e10		2.608275 ↑
Gga.8463.1.S1_s_at	chromodomain helicase DNA binding protein 1	CHD1	2.508791 ↑
Gga.10138.1.S1_at	Finished cDNA, clone ChEST126i3		2.291931 ↑
Gga.12484.1.S1_at	Myelin oligodendrocyte glycoprotein	MOG	2.693002 ↑
Gga.16904.1.S1_at	Finished cDNA, clone ChEST749o23		2.043179 ↑
Gga.13093.1.S1_at	Finished cDNA, clone ChEST900p6		2.143198 ↑
Gga.11630.2.S1_a_at	Hypothetical LOC426615	LOC426615	2.957734 ↑
Gga.11630.2.S1_at	Hypothetical LOC426615	LOC426615	2.785122 ↑
Gga.14069.1.S1_s_at	erythrocyte membrane protein band 4.2	EPB42	2.005124 ↑
Gga.20011.1.S1_at	coiled-coil domain containing 84	CCDC84	2.052319 ↑
Gga.9566.2.S1_s_at	solute carrier family 25, member 37	SLC25A37	2.479893 ↑
Gga.11848.1.S1_at	Finished cDNA, clone ChEST394j22		2.073347 ↑
Gga.19575.1.A1_at	Finished cDNA, clone ChEST445p2		2.270295 ↑
Gga.10138.3.S1_at	Finished cDNA, clone ChEST126i3		2.569093 ↑
GgaAffx.11738.1.S1_s_at	early growth response 1	EGR1	2.099442 ↑
GgaAffx.12834.1.S1_at	cyclin D3	CCND3	2.027003 ↑
GgaAffx.23623.2.S1_at	AHNAK nucleoprotein 2	AHNAK2	2.430334 ↑

GgaAffx.23789.1.S1_at	STEAP family member 3	STEAP3	2.233264 ↑
GgaAffx.24779.1.S1_s_at	Finished cDNA, clone ChEST556p11		2.307856 ↑
Gga.6201.1.S1_at	putative ISG12-2 protein	ISG12-2	2.257 ↑
Gga.7505.1.S1_at	Acyl-CoA synthetase short-chain family member 1	ACSS1	2.126091 ↑
Gga.6481.1.S1_at	Transcribed locus		3.182043 ↑
Gga.6758.1.S1_at	Transcribed locus		2.482223 ↑
Gga.6836.1.S1_at	Transcribed locus		2.606474 ↑
Gga.6590.1.S1_at	Transcribed locus, moderately similar to NP_446096.1 cadherin 23 (otocadherin) [Rattus norvegicus]		2.269154 ↑
Gga.6209.1.A1_at			2.011379 ↓
Gga.2087.1.S1_s_at	casein kinase 1, epsilon	CSNK1E	2.123844 ↓
GgaAffx.20789.1.S1_at	Ribosomal protein, large, P1	RPLP1	2.076906 ↓
Gga.13093.1.S1_at	Finished cDNA, clone ChEST430n10		2.134106 ↓
Gga.19320.1.S1_at	hypothetical gene supported by CR406681	LOC426510	2.192061 ↓

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Genes displaying up-regulation are displayed in black, and down-regulated genes are displayed in red. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.4 Genes differentially regulated in microarray analysis of GATA-4 single knockdown chick embryos

Probe set ID	Gene Title	Gene Symbol	Fold change
Gga.2551.2.S1_s_at	lactotransferrin	LTF	2.918124 ↑
Gga.3641.1.S1_at	annexin A2	ANXA2	2.408555 ↑
Gga.3133.1.S1_a_at	collapsin response mediator protein 1	CRMP1	2.367408 ↑
Gga.16863.1.S1_s_at	Gal 10	GAL10	5.975876 ↑
Gga.3381.1.S2_at	homeobox A3	HOXA3	2.480355 ↑
Gga.2648.1.S2_at	homeobox B3	HOXB3	2.833412 ↑
GgaAffx.20083.1.S1_at	homeobox A6	HOXA6	3.819832 ↑
GgaAffx.21240.1.S1_at	Finished cDNA, clone ChEST875h18		2.303639 ↑
Gga.1546.1.S1_at	paired related homeobox 1	PRRX1	2.023035 ↑
Gga.1546.1.S2_a_at	Paired related homeobox 1	PRRX1	2.171399 ↑
Gga.8241.1.S1_at	transcription factor 21	TCF21	3.786901 ↑
Gga.4112.1.S1_at	apolipoprotein B (including Ag(x) antigen)	APOB	3.427132 ↑
Gga.12649.1.S1_at	galanin prepropeptide	GAL	2.365781 ↑
GgaAffx.22999.1.S1_s_at	collagen, type I, alpha 2	COL1A2	2.244431 ↑
Gga.496.1.S1_at	reelin	RELN	2.224683 ↑
Gga.18984.1.S1_at	Finished cDNA, clone ChEST608b15		2.356632 ↑
Gga.3676.1.S1_at	N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase	LOC423952	2.042442 ↑
Gga.18151.1.S1_at	Basonuclin 1	BNC1	3.634063 ↑
Gga.12185.1.S1_at	matrix metalloproteinase 23B	MMP23B	2.182316 ↑
Gga.12849.1.S1_at	SPARC related modular calcium binding 2	SMOC2	2.460695 ↑

Gga.13425.1.S1_at	Finished cDNA, clone ChEST130f3		2.062347 ↑
Gga.14858.1.S1_at	nuclear autoantigenic sperm protein (histone-binding)	NASP	2.072035 ↑
GgaAffx.25098.1.S1_at	propionyl Coenzyme A carboxylase, alpha polypeptide	PCCA	2.053286 ↑
GgaAffx.5758.1.S1_at	carbonic anhydrase X	CA10	2.506326 ↑
GgaAffx.1913.1.S1_at	homeobox B5	HOXB5	2.937861 ↑
Gga.5598.1.S1_at	Transcribed locus		2.189897 ↓
Gga.6873.1.S1_at	Transcribed locus		2.017625 ↓
Gga.17020.1.S1_at	Titin	TTN	2.514681 ↓
Gga.13915.1.S1_at	Titin	TTN	2.158793 ↓
Gga.12775.1.A1_at	titin	TTN	3.282518 ↓
Gga.4777.5.A1_at	Finished cDNA, clone ChEST145d21		2.097747 ↓
Gga.17680.1.S1_at	Clone cDNA3F microsatellite MCW108 sequence		2.054327 ↓

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Genes displaying up-regulation are displayed in black, and down-regulated genes are displayed in red. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.5 Genes differentially regulated in microarray analysis of TBX5 / GATA-4 double knockdown, TBX5 single knockdown, and GATA-4 single knockdown chick embryos

Probe Set ID	Gene Title	Gene Symbol	Fold change		
			TBX5 & GATA4 knockdown	TBX5 knockdown	GATA-4 knockdown
GgaAffx.21116.1.S1_s_at	oxidative stress induced growth inhibitor 1	OSGIN1	2.695851 ↑	4.087098 ↑	2.051297 ↑
Gga.16169.1.S1_at	Finished cDNA, clone ChEST45e16		2.7834854 ↑	4.394274 ↑	2.013999 ↑
Gga.19101.1.S1_at	Finished cDNA, clone ChEST926n8		3.4982224 ↑	5.769498 ↑	2.229745 ↑
Gga.10138.2.S1_a_at	Finished cDNA, clone ChEST126l3		2.3702402 ↑	2.630745 ↑	2.022664 ↑
Gga.5092.1.S1_s_at	annexin A2	ANXA2	2.1296182 ↑	2.098631 ↑	2.408555 ↑
Gga.9032.1.S1_s_at	RNA binding motif protein 35B	RBM35B	2.97302 ↑	4.49394 ↑	2.480706 ↑
GgaAffx.11525.1.S1_s_at	claudin 1	RCJMB04_2c8	3.8028853 ↑	4.62055 ↑	2.624394 ↑
GgaAffx.7796.1.S1_s_at	solute carrier family 39 (zinc transporter), member 8	SLC39A8	3.7550573 ↑	5.402943 ↑	2.87964 ↑

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.6 Genes differentially regulated in microarray analysis of TBX5 / GATA-4 double knockdown and TBX5 single knockdown and chick embryos

Probe Set ID	Gene Title	Gene Symbol	Fold change	
			TBX5 & GATA-4 knockdown	TBX5 knockdown
Gga.4842.2.S1_a_at	solute carrier family 4, anion exchanger, member 1	SLC4A1	2.0272145 ↑	3.647175 ↑
GgaAffx.21696.1.S1_at	Finished cDNA, clone ChEST747k12		2.0174587 ↑	2.58259 ↑
GgaAffx.20824.1.S1_at	Finished cDNA, clone ChEST722f15		2.6281042 ↑	4.631784 ↑
GgaAffx.21220.1.S1_s_at	Finished cDNA, clone ChEST881h18		2.0515776 ↑	3.366277 ↑
Gga.4156.1.S1_at	Finished cDNA, clone ChEST79b1		2.0187516 ↑	2.235081 ↑
Gga.8098.1.S1_at	Finished cDNA, clone ChEST811h18		3.2116323 ↑	4.168608 ↑
Gga.8089.1.S1_at	Kruppel-like factor 1 (erythroid)	KLF1	2.1141534 ↑	3.243578 ↑
Gga.7581.1.S1_at	Finished cDNA, clone ChEST885i1		18.489647 ↑	2.456972 ↑
Gga.10042.1.S1_a_at	CD200 receptor 1	CD200R1 /// RCJMB04_25i16	2.0964477 ↑	2.639697 ↑
Gga.11763.2.S1_a_at	similar to RIKEN cDNA 1110067D22	LOC423277	2.2741299 ↑	3.876498 ↑
Gga.17183.1.S1_at	Finished cDNA, clone ChEST397p8		2.011654 ↑	3.013425 ↑
Gga.13903.1.S1_at	paired-like homeodomain 1	PITX1	2.1853464 ↑	4.573031 ↑
GgaAffx.2993.1.S1_at	erythrocyte membrane protein band 4.2	EPB42	2.5261283 ↑	2.005124 ↑
GgaAffx.7796.1.S1_at	solute carrier family 39 (zinc transporter), member 8	SLC39A8	2.1189318 ↑	4.271026 ↑
Gga.5243.1.S1_at	Transcribed locus		2.2414985 ↑	2.84958 ↑
Gga.7750.1.S1_at	hypothetical LOC422459	LOC422459	2.2732491 ↑	3.474953 ↑

Gga.8578.1. S1_at	Transcribed locus		2.2378054 ↑	4.381073 ↑
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Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.7 Genes differentially regulated in microarray analysis of TBX5 / GATA-4 double knockdown and GATA-4 single knockdown and chick embryos

Probe Set ID	Gene Title	Gene Symbol	Fold change	
			TBX5 & GATA-4 knockdown	GATA-4 knockdown
Gga.2558.1.S1_a_at	collagen, type XIV, alpha 1 (undulin)	COL14A1	2.3760216 ↑	3.037223 ↑
Gga.2354.1.S1_at	Transcribed locus		2.1046243 ↑	2.661268 ↑
GgaAffx.11659.1.S1_at	sorcin	RCJMB04_3f15	2.0217292 ↓	4.004279 ↓

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Genes displaying up-regulation are displayed in black, and down-regulated genes are displayed in red. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

Table 6.8 Genes differentially regulated in microarray analysis of TBX5 single knockdown and GATA-4 single knockdown chick embryos

Probe Set ID	Gene Title	Gene Symbol	Fold change	
			TBX5 knockdown	GATA-4 knockdown
Gga.2551.2.S1_a_at	lactotransferrin	LTF	2.283916 ↑	2.918124 ↑
Gga.2620.1.S1_at	transthyretin	TTR	2.76807 ↑	4.394575 ↑
Gga.1165.1.S1_at	fibrinogen gamma chain	FGG	2.900268 ↑	2.899903 ↑
GgaAffx.21581.1.S1_s_at	chemokine (C-X-C motif) ligand 14	CXCL14	2.399975 ↑	2.832924 ↑
Gga.9481.1.S1_s_at	otokeratin	LOC395772	2.136159 ↑	2.031168 ↑
Gga.9087.1.S1_at	succinate-CoA ligase, GDP-forming, beta subunit	SUCLG2	3.021066 ↑	2.887826 ↑
GgaAffx.20374.1.S1_at	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	2.103267 ↑	2.213427 ↑
Gga.4557.1.S1_at	fibrinogen beta chain	FGB	3.862611 ↑	7.627894 ↑
GgaAffx.26441.1.S1_at	hypothetical LOC424460	LOC424460	12.39564 ↑	3.578772 ↑
Gga.6860.1.A1_at			2.082896 ↓	2.786701 ↓
Gga.14594.1.A1_at	Finished cDNA, clone ChEST240e14		2.196702 ↓	2.082312 ↓
Gga.3690.1.A1_at	Zinc finger protein 207	ZNF207	2.009688 ↓	2.287068 ↓
Gga.9087.1.S1_at	transcription factor Crx	LOC395160	2.93964 ↓	2.535343 ↓
Gga.285.1.A1_at	myomesin	LOC395524	2.096654 ↓	2.205535 ↓
GgaAffx.11133.1.A1_s_at	cyclic AMP phosphoprotein, 19 kD	RCJMB04_4f12	2.789666 ↓	2.607948 ↓

Genes displaying fold changes of 2-fold or greater in comparison to wild type embryos were selected, and fold changes observed are indicated. Genes displaying up-regulation are displayed in black, and down-regulated genes are displayed in red. Gene lists were cross-referenced with genes differentially regulated in TBX5 7-mis / GATA-4 7-mis control embryos, and common genes removed.

6.2.4 Genes identified in microarray analysis of TBX5 and GATA-4 knockdown

The purpose of this genome-wide microarray study was to identify genes co-ordinately regulated by TBX5 and GATA-4. Five sample groups were analysed, generating seven categories of genes differentially regulated in response to TBX5 / GATA-4 double knockdown, TBX5 single knockdown, GATA-4 single knockdown, and overlapping groups (represented in Figure 6.2). Of these seven categories, genes identified in the TBX5 / GATA-4 knockdown group but not other groups were of greatest interest. Secondary to this group, genes appearing in the TBX5 / GATA-4 knockdown group and one or both of the single knockdown groups were also of interest, particularly those genes which showed a greater fold change in expression in the double knockdown group compared to single knockdowns. However, time did not allow study of all of these groups, and the TBX5 / GATA-4 knockdown group were given primary focus. In total, 26 transcribed loci were identified displaying differential regulation in TBX5 / GATA-4 double-knockdown embryos (by ≥ 2 fold in comparison to wild type animals) but not in single knockdown embryos or controls. Not all loci identified corresponded to known or hypothesised genes. Eighteen of the genes of interest are 'known' genes and a full description of these is given in Appendix I.

6.2.4.1 Reliability of data and general considerations

There is always a false-discovery rate (FDR) associated with microarray gene expression profiling, which means it is unlikely that all of the genes identified by this technique are genuine targets. The FDR increases as the number of arrays per group decreases [Pawitan et al., 2005] i.e. biological replicates are very important in microarray expression profiling, exemplified in a number of studies [Kerr et al., 2000; Lee et al., 2000; Manduchi et al., 2000]. In this study one biological replicate was assayed per group which means the likelihood of obtaining false positives is very high. However, as only 18 genes of interest were identified (due to the high threshold set and large number of control groups), post-analysis elimination of false positives is not a lengthy procedure in this case.

It is surprising that knockdown of two cardiac transcription factors seems to result in misexpression of so many neurological genes and so few heart genes, particularly given that this is a heart tissue specific study. A high false positive rate due to limited biological replicates could be one contributing factor (as discussed above). There is also evidence that certain transcripts cannot be accurately detected by any technique (microarray expression analysis or QPCR) due to sequence specific effects [Larkin et al., 2005]. Also, any small levels of contaminating tissue from non-cardiac regions of the embryo may have resulted in anomalous results.

This study has resulted in the identification of a number of strong candidate genes, in particular CRABP1, GPC3, and TFAP2B. TFAP2B is arguably the most interesting as mutations in this gene are already associated with the heart-hand disorder Char syndrome [Satoda et al., 1999; Satoda et al., 2000], whose anomalies include patent ductus arteriosus (PDA), facial dysmorphism, strabismus, hearing abnormalities, and an abnormal fifth digit of the hand [Char, 1978]. The ductus arteriosus is the arterial connection between the pulmonary artery and aorta, and serves to shunt blood from the right ventricle away from the lungs, allowing blood to bypass the lungs during foetal development. The ductus normally constricts and closes shortly after birth, and failure to do so results in PDA. Structures affected in Char syndrome are all contributed to by neural crest cells [Moser et al., 1997] and fate mapping studies have shown that the medial layer of the ductus, but not the pulmonary artery and descending aorta, arises from cardiac neural crest (CNC) cells [Waldo et al., 1999]. It is likely that TFAP2B has a role in the migration or differentiation of neural crest cells, which results in a specific effect on the ductus but not surrounding arterial structures. It is interesting that this heart-hand gene may be transcriptionally regulated by another heart-hand gene, TBX5, in conjunction with GATA-4.

6.2.5 Gene expression analysis using GEISHA

Assessment of the anatomical expression pattern of genes is a useful starting point for candidate gene selection. Embryonic expression of genes was initially researched using the online GEISHA (gallus expression in situ hybridisation analysis) resource (<http://geisha.arizona.edu/geisha/index.jsp>). This is a database containing *in situ* hybridisation information for genes expressed at 0.5 - 6 days of development in chicken embryos, input via in house *in situ* hybridisation screening, review of literature, and unpublished expression information from laboratories.

The whole body expression pattern of a number of genes was described in GEISHA including SOX3, ZIC1, FOXG1, NKX2-1, PAX6, and OTX2, and is described in Table 6.9. Developmental stages HH12 – HH19 have been included where possible as these are of interest to this study. All six genes researched in GEISHA did not display obvious expression in the heart so were not considered further.

Table 6.9 Gene expression data from GEISHA

Gene	Developmental stage and region of expression
SOX3	HH7 – HH12: Ectoderm, neural plate/tube [Abu-Elmagd et al., 2001] HH13 – HH18: Ectoderm, neural plate/tube, pharyngeal arches and clefts, placode [Abu-Elmagd et al., 2001]
ZIC1	HH13 – HH18: Dermatome, myotome, neural plate/tube, sclerotome, somites, spinal cord [Sun Rhodes and Merzdorf, 2006] HH19 – HH21: Dermatome, face mesenchyme, myotome, sclerotome, somites [Sun Rhodes and Merzdorf, 2006]
FOXG1	HH7 – HH12: Forebrain, telencephalon [Bell et al., 2001] HH13 – HH15: Ear/otic placode, forebrain, pharyngeal arches and clefts (Chapman SC, 2008, unpublished) HH13 – HH19: Telencephalon [Bell et al., 2001]
NKX2-1	HH7 – HH12: Diencephalon, neural plate/tube (Chapman SC, 2008, unpublished) HH13 – HH17: Diencephalon, pharyngeal arches and clefts (Chapman SC, 2008, unpublished)
PAX6	HH7 – HH12: Diencephalon, eye, forebrain, neural plate/tube, spinal cord [Ohuchi et al., 2007] HH13 – HH24: Diencephalon, hindbrain, telencephalon [Garcia-Calero et al., 2006]
OTX2	HH7 – HH12: Forebrain, midbrain, neural plate/tube (Chapman SC, 2008, unpublished) HH13 – HH18: Eye, forebrain, midbrain, pharyngeal arches [Plouhinec et al., 2005]

Chicken whole embryo developmental expression profiles of the genes SOX3, ZIC1, FOXG1, NKX2-1, PAX6 and OTX2 have been previously characterised by *in situ* hybridisation and were found in the GEISHA database (<http://geisha.arizona.edu/geisha/index.jsp>). Expression patterns are summarised and include developmental stages HH12 – HH19 (of interest in this study) where possible.

6.2.6 RT-PCR expression analysis

In order to assess expression of other identified genes, nine of the twelve remaining genes were selected for RT-PCR expression analysis (TFAP2B, PTPRZ1, FIGF, NR2E1, LOC420770, SUZ12, CRABP1, GOLGA4, and GPC3). CYTL1, PLCH1 and LOC419390 were not studied due to limited resources and time restrictions.

RT-PCR expression analysis was performed once in chick embryonic segments at three stages of development (HH16, HH19 and HH24) as a preliminary means of temporal / spatial characterisation. Primers sequences are given in Table 2.7. Equal amounts of template were used in all reactions. PCR reactions were performed with HotStar Taq using an annealing temperature of 60 °C, extension time of 30 seconds, and 30 PCR cycles. Negative RT controls were included for each gene and did not display amplification (Appendix J). Results of expression analyses are displayed in Figure 6.3 and are described below. RT-PCR was not performed in a quantitative manner and descriptions are subjective.

SUZ12, GOLGA4 and GPC3 were expressed in all embryonic segments (head, heart, body / limbs) at all three stages of development indicating ubiquitous expression. Further studies would have to be performed to determine the exact spatial expression patterns of these genes.

Expression of TFAP2B was detected in all embryonic segments at HH16. Heart expression appeared to be lower at HH19, and was not detectable at HH24. Consistent expression was observed in other regions throughout all three stages. CRABP1 was also expressed in all regions at the stages examined. Heart expression appeared to be strongest at HH16, becoming reduced at HH19 and HH24. PTPRZ1 expression was detected in all embryonic segments at all three stages of development. Heart expression appeared to be fainter at HH24. TFAP2B, CRABP1, and PTPRZ1 follow a similar pattern, displaying expression throughout the embryo with heart expression appearing to be strongest early in development, and decreasing

in later stages. This could be indicative of a specific function in early heart development.

LOC420770 expression was detectable in the heart and body (but not head) at all three stages. Interestingly, expression at HH24 was more prominent in the limbs than body indicating a heart/limb expression profile, potentially similar to that of TBX5.

FIGF was expressed in all embryonic segments, with highest expression in the head and body and fainter expression in the heart. NR2E1 was expressed almost exclusively in the head region, with faint expression in the body (at all three stages). Heart expression could not be detected at any of the stages examined.

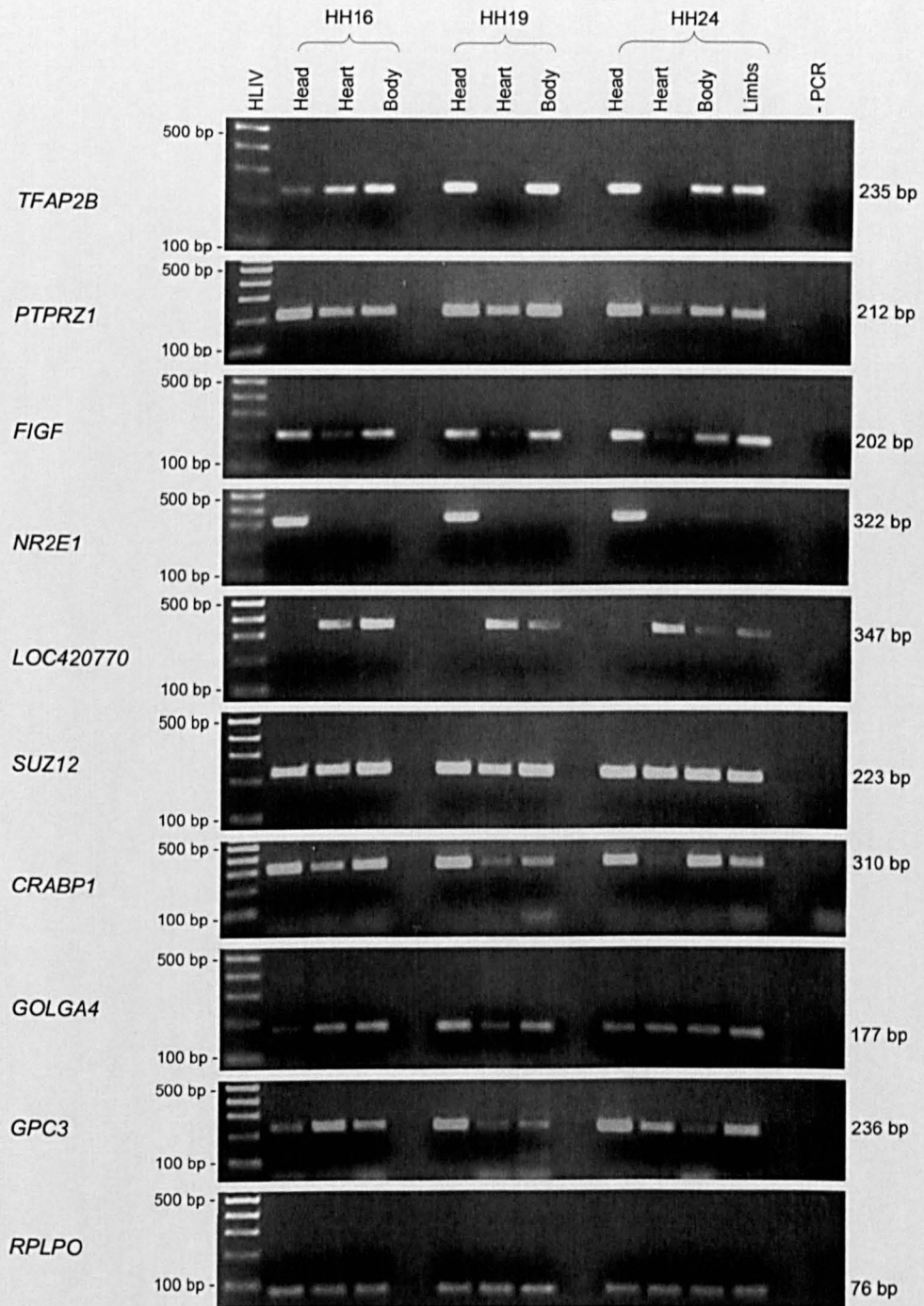


Figure 6.3 RT-PCR expression analysis of nine candidate genes

RT-PCR products were electrophoresed on 2% agarose gels using Hyperladder IV as a size marker (500 bp and 100 bp bands are indicated along with product sizes). Expression was studied in chick embryonic segments at HH16, HH19, and HH24. *RPLPO* was included as a housekeeper gene. Negative PCR controls (-PCR) are shown. Negative RT controls did not show amplification for any of the genes and are displayed in Appendix J.

CHAPTER 7

DISCUSSION

7.1 TBX5 and GATA-4 in cardiac development

Development of the heart is a highly complex and dynamic process involving a vast network of genes. These genes are diverse in function, encoding growth factors, signalling molecules, transcription factors and their co-factors and regulators, components of the extracellular matrix, and structural proteins. Defects in cardiac genes can lead to congenital heart defects, and understanding the molecular pathways involved in normal cardiogenesis will allow elucidation of the pathogenesis of disease. This in turn is the first step toward improved diagnoses and potentially novel therapies.

Heart development is a highly regulated process involving complex interplay between numerous transcription factors, which can also regulate transcription of one another. In addition, a number of transcription factors physically interact to co-ordinately regulate key genes and developmental pathways. Members of the NK2, MEF2, GATA, Tbx and Hand gene families make up a core cardiac regulatory network whose expression is initiated by inductive signals and upstream activators [Olson, 2006]. Mutations in a number of key cardiac transcription factors in this core group, including Nkx2.5, TBX5, and GATA-4, have been associated with CHDs [Garg et al., 2003; Li et al., 1997; Schott et al., 1998]. In addition, these transcription factors also interact with one another for joint regulation of targets [Garg et al., 2003; Hiroi et al., 2001; Lee et al., 1998]. TBX5 and GATA-4 are considered master regulators of cardiac development, and these two genes, in combination with Baf60c, can induce a full cardiac transcriptional programme [Takeuchi and Bruneau, 2009]. Additionally, the specific combination of TBX5, GATA-4 and MEF2C has recently been found to be able to reprogramme mouse postnatal fibroblasts into functional cardiomyocytes that

display cardiomyocyte-like global gene expression and beat spontaneously [Ieda et al.]. *TBX5* is a T-box transcription factor and a so called 'heart-hand' gene as it has an important role in heart development and specification of forelimb identity [Isaac et al., 1998]. Mutations in *TBX5* are associated with Holt-Oram syndrome [Basson et al., 1997; Fan et al., 2003; Li et al., 1997], a congenital disorder characterised by upper limb and heart abnormalities. These are usually atrial or ventricular septal defects, but can include mitral valve defects, cardiac conduction defects, tetralogy of Fallot, and hypoplastic left heart syndrome [Basson et al., 1994; Bruneau et al., 1999]. *GATA-4* is a member of the GATA family of transcription factors and has a crucial role in cardiogenesis [Molkentin and Olson, 1997; Rivera-Feliciano et al., 2006; Zeisberg et al., 2005]. Mutations in *GATA-4* are associated with atrial and ventricular septal defects [Garg et al., 2003] and tetralogy of Fallot [Nemer et al., 2006]. *TBX5* and *GATA-4* display distinct but overlapping expression patterns [Bruneau et al., 1999; Kelley et al., 1993] and form a complex that is thought to direct and synergistically regulate common cardiac pathways [Garg et al., 2003]. A number of *TBX5* mutations that are associated with cardiac septal defects abolish this biochemical interaction [Garg et al., 2003], and the *GATA-4* mutation G296S that is linked to ASDs also has this effect [Garg et al., 2003]. These data indicate the *TBX5* / *GATA-4* complex has a critical regulatory role in cardiac septation, and provide insight into why similar phenotypes are so often seen as a result of mutations in these genes. The downstream targets of *TBX5* and *GATA-4* are largely unknown, and a major aim of this thesis has been to identify and analyse these genes. This is crucial in understanding the mechanism via which mutations in these transcription factors lead to congenital heart defects.

7.2 Identification of targets of *TBX5* and *GATA-4* in the mouse P19 cell line

The research carried out in this thesis was based on a previous study that aimed to identify downstream targets of *TBX5* and *GATA-4* using the mouse P19 cell line, which provides an excellent model of cardiomyocyte differentiation [McBurney, 1993]. Microarray expression analysis of two cell lines stably transfected with *TBX5* / *GATA-4*

overexpression vectors was carried out allowing identification of a number of genes which displayed differential regulation. This was supplemented with temporal gene expression data, quantified at selected time-points of wild type cardiomyocyte differentiation. Sixteen up-regulated genes displaying wild type expression profiles mirroring wild type TBX5 and GATA-4 expression, and two down-regulated genes, were selected for further study.

7.2.1 Validation of gene expression and preliminary bioinformatic analysis

In Chapter 3 of this thesis, expression of genes of interest was validated by QPCR using the Comparative C_T method for confirmation and more accurate quantification of expression data. Genes displaying the highest fold increases in expression in the overexpression cell lines, and following the same pattern of expression as TBX5 and GATA-4 (a general increase from day 2 - day 7 of differentiation), were selected for further analysis. These included *TPM1*, *PETA-3*, *PA2.26*, *RBMS1*, *FUCA1*, and *FN*, which displayed 1.4 - 3.5 fold increases in expression in the overexpression cell lines. An additional gene (*DES*) encoding the intermediate filament protein desmin was added to the study based on a similar study where this gene showed up-regulation in response to transient overexpression of TBX5 and GATA-4 in the P19 cell line. Bioinformatic analysis of gene promoter regions was performed in Mulan software by alignment between chicken, mouse and human sequences for identification of evolutionarily conserved regions (ECRs) and consensus transcription factor binding sites within them. Putative binding site analysis revealed *RBMS1* as the best target; three potential consensus binding sites for TBX5 were identified (all within the coding region), and four GATA-4 sites were identified (two in the coding region, and two in the 3' UTR). This gene was selected for further study of its activation by TBX5 and GATA-4. Reporter assays were designed to test activation of an intronic fragment of the mouse *RBMS1* gene containing both a TBX5 and a GATA-4 site. This was thought to be a downstream enhancer or repressor element, and data showed that GATA-4 had a repressive effect on promoter activity via this region, whilst TBX5 had no effect. Attempts to study the effect of TBX5 and GATA-4 on the 5' promoter region

are ongoing. Further in depth study of RBMS1 in the developing chick was carried out and is discussed in 7.4.

7.2.2 Candidate genes

The identified genes are diverse in function, encoding muscle proteins, extracellular matrix molecules, a transcription factor and an enzyme, and this is a reflection of the role of TBX5 and GATA-4 as master regulators in the heart. The importance of sarcomeric proteins in muscle contractility is well established, and many of these genes are now also known to be important developmentally in processes such as septation. Thus, mutations in a number of genes encoding sarcomeric proteins are associated with both cardiomyopathies and congenital heart defects. *MYH6* is a well known example of such a gene whose mutations are associated with hypertrophic and dilated cardiomyopathy [Morita et al., 2005], and atrial septal defects [Ching et al., 2005]. This gene is also a known synergistic target of TBX5 and GATA-4 [Huang et al., 1995; Molkentin et al., 1994]. Other sarcomeric protein encoding genes that are associated with ASDs include *MYH7* (β -myosin heavy chain) [Budde et al., 2007] and *ACTC* (α -cardiac actin) [Matsson et al., 2008; Monserrat et al., 2007]. Mutations in *ACTA2* (α -smooth muscle actin) and *MYH11* (myosin heavy chain 11) are associated with patent ductus arteriosus [Guo et al., 2007; Zhu et al., 2006]. Initial studies by Dr Lynn Amy and Jonathan Ronksley led respectively to the identification of the muscle proteins desmin and α -tropomyosin as potential cardiac targets of TBX5 and GATA-4 [Ronksley, 2007]. Desmin is a type III intermediate filament protein with an important contractile role in muscle. In humans, numerous mutations in the desmin gene have been associated with familial cardiac and skeletal myopathy [Bergman et al., 2007; Fidzianska et al., 2005; Goldfarb et al., 1998; Goudeau et al., 2001; Olive et al., 2007; Sung et al., ; Taylor et al., 2007; Vernengo et al., ; Vrabie et al., 2005]. The *TPM1* gene encodes α -tropomyosin, which is an important contractile component of the sarcomere [Perry, 2001]. Missense mutations in *TPM1* have previously been linked with familial hypertrophic cardiomyopathy and dilated cardiomyopathy [Lakdawala et

al., ; Thierfelder et al., 1994]. To date, neither of these genes has been associated with atrial septal defects.

The extracellular matrix (ECM) consists of collagens, glycoproteins, growth factors, and proteases that form a macromolecular complex which interacts and communicates with various cardiac cell types to undergo changes in composition (remodelling) for determination of its function. The ECM plays an important role in cardiac development and growth. The ECM component fibronectin was identified in Jonathan Ronksley's study [Ronksley, 2007]. Fibronectin is an adhesive ECM glycoprotein involved in cytoskeletal organisation, cell adhesion, cell migration, and cell spreading [Waterman and Balian, 1980], and is thought to play a role in the migration of precardiac cells [Linask and Lash, 1986]. Mouse knockout of fibronectin has a pronounced effect on the heart, with heart primordia often remaining unfused [George et al., 1993]. Embryos that are not as severely affected have fused primordia with abnormal morphology. Cardiac defects such as thickening of the myocardial tissue, cardiac jelly deficiency, and abnormal endocardium morphology also occur, demonstrating the importance of fibronectin in cardiac development [George et al., 1993]. PETA-3 is a tetraspan membrane protein involved in cell-cell adhesion, cell migration, platelet adhesion, and angiogenesis [Wright et al., 2004]. PA2.26 is a small transmembrane glycoprotein and also plays a role in cell-cell adhesion and cell migration [Scholl et al., 1999]. The process of cell migration is important in formation of endocardial cushions in the heart which are essential for septa and valve formation, suggesting possible roles for fibronectin, PETA-3 and PA2.26 here.

Cardiac transcription factors play a key regulatory role in cardiac growth and patterning, and mutations in a number of cardiac transcription factors are associated with CHDs. The multifunctional gene RBMS1, which acts as both a cell cycle regulator (via binding to c-myc) and a transcription factor, was identified in the original P19 study [Ronksley, 2007]. RBMS1 has been shown to suppress transcription of α -smooth muscle actin [Kimura et al., 1998], which has important functions in the

developing heart (section 7.4), suggesting a potential mode of action here. Other downstream targets of RBMS1 are largely unknown.

This microarray study led to the identification of an interesting and diverse group of genes which may be regulated by TBX5 and GATA-4. Some of these genes are already linked to the heart, including desmin, α -tropomyosin, and fibronectin. Other genes such as PA2.26 and PETA-3 are involved in processes such as cell migration which are important in cardiac septation and valvulogenesis. RBMS1 is cell cycle regulator and transcription factor, and has not previously been implicated in cardiac development. FUCA1 encodes the exoglycosidase enzyme α -L-fucosidase, and a role for this enzyme in cardiac development has not been postulated.

7.3 Development of a model system for assessment of the responsiveness of candidate genes to TBX5 and GATA-4

At the time this part of the research was initiated (2008), there were no known *in vivo* studies looking at the effects of combined haploinsufficiency of TBX5 and GATA-4. In Chapter 4 of this thesis, the developing chick was selected as a model organism for study of candidate gene expression and responsiveness to TBX5 and GATA-4. The chick allows easy and rapid manipulation at selected time points since development occurs *in ovo* (as opposed to *in utero*), and morpholino antisense technology allows knockdown of up to two genes simultaneously. Seven genes were previously identified in the mouse P19 cell line as potential targets of TBX5 and GATA-4 and selected for further study in the developing chick embryo; PA2.26, PETA-3, FUCA1, FN, TPM1, DES, and RBMS1. As a starting point, expression of these genes was confirmed in the embryonic chick heart at HH12 - HH26.

7.3.1 Development of an *in ovo* model of TBX5 and GATA-4 knockdown

In order to assess candidate gene responsiveness to TBX5 and GATA-4 in the developing chick heart, an embryonic model of TBX5 / GATA-4 double knockdown was generated using AUG targeting morpholino antisense oligonucleotides. Five-

base mismatch morpholinos were originally tested as controls of specificity, but these were found to have an effect most likely due to retained target binding capability and were therefore subsequently replaced with 7-base mismatch controls. Morpholinos were applied at HH10 – HH16, encompassing key events such as dextral looping (HH10 – HH12), the early phase of s-looping (HH12 – HH17/18), and the initiation of atrial septation (HH14) [Anderson et al., 2003; Manner, 2000]. Previous studies have shown that complete mouse knockout of TBX5 results in embryonic lethality by E10.5, and hearts fail to undergo looping and display hypoplasia of the sinuatria and left ventricle [Bruneau et al., 2001]. Study of TBX5 heterozygous mice has demonstrated a regulatory role for TBX5 in chamber morphogenesis and cardiac septation [Bruneau et al., 2001]. In this study, *in ovo* knockdown of TBX5 resulted in abnormalities in heart shape, and a reduction in the size of the atrial septum, corresponding with the cardiac defects present in TBX5 heterozygote mice [Bruneau et al., 2001]. Mouse knockout of GATA-4 results in embryonic lethality at E7.0 – E9.5 due to defects in ventral morphogenesis and linear heart tube formation [Molkentin and Olson, 1997]. Early myocyte restricted deletion of GATA-4 by *Nkx2-5^{Cre}* results in cardiac malformations including a single predominant ventricular chamber, myocardial hypoplasia, and small atrioventricular and OFT endocardial cushions due to defects in EMT [Rivera-Feliciano et al., 2006; Zeisberg et al., 2005]. Here, chick embryonic treatment with GATA-4 targeting morpholinos resulted in heart malformations and embryos displayed indications of a reduction in the size of the atrial septum, as would be expected based on the ASDs observed in humans with GATA-4 mutation [Garg et al., 2003; Nemer et al., 2006]. In parallel with this research, *Gata4^{+/-} Tbx5^{+/-}* compound heterozygote mice were generated by another research group and full phenotypic characterisation was performed [Maitra et al., 2009]. Almost 100% of embryos suffered postnatal lethality by day 7. Embryos displayed intrauterine growth retardation, a complete lack of AV septation due to defects in endocardial cushion maturation and modelling (not EMT), and myocardial thinning affecting both ventricles at E13.5, leading to subsequent heart failure and death [Maitra et al., 2009]. In this study, although it was not possible to quantify morpholino effect on protein levels upon

chick embryonic treatment with TBX5 and GATA-4 targeting morpholinos, these embryos displayed the expected phenotypes. Combined knockdown of TBX5 together with GATA-4 resulted in more severe cardiac defects than treatment with either alone, and a wider range of defects was observed. Double knockdown embryos displayed growth retardation, severe body kinking / twisting, and general malformations in comparison to control animals. A variety of heart defects including a reduction in heart size, constriction of the ventricle, high outflow tract, and most commonly abnormal looping of the heart were also observed. These observations correlate with the phenotype of *Gata4*^{+/-} *Tbx5*^{+/-} mice, and interestingly there are also some distinct features such as the defects in cardiac looping that were observed in the chick but not mouse [Maitra et al., 2009]. This may have been for a number of reasons including differences between species such as varying physiology and response to gene dosage (discussed further in section 7.7), the level of knockdown achieved, and the developmental stage of knockdown. Overall, morpholino knockdown of TBX5 and GATA-4 individually and in combination generated the expected phenotypes based on mouse knockouts performed by others.

7.3.2 Candidate gene responsiveness to combined knockdown of TBX5 and GATA-4

In order to examine the responsiveness of candidate genes to TBX5 and GATA-4, QPCR expression analysis of these genes in double knockdown embryos was carried out using the Relative Standard Curve method. This method is ideal for the detection of small changes in expression as would be expected in an *in ovo* system, and was successfully developed and optimised for use. QPCR expression analysis showed knockdown of TBX5 and GATA-4 had no effect on expression of PETA-3, fibronectin, α -tropomyosin, desmin, and RBMS1. It was not possible to accurately quantify expression of PA2.26 and α -L-fucosidase due to low level expression. However, the level or presence of knockdown of TBX5 and GATA-4 at the protein level remains unknown. In addition, expression of MYH6 in these embryos was unaltered whilst this gene is down-regulated in *Gata4*^{+/-} *Tbx5*^{+/-} compound heterozygote mice [Maitra et al.,

2009]. If knockdown was occurring, this could be for a number of reasons; it is possible there are differences in the transcriptional regulation of MYH6 between the mouse and chick. The level of knockdown of TBX5 and GATA-4 in this system, whilst generating the expected cardiac phenotype, may not have been sufficient for down-regulation of MYH6. A low level of knockdown of TBX5 and GATA-4 may have resulted in very discrete changes in MYH6 expression which were not detected. The QPCR technique itself has limitations in its sensitivity, particularly since this study is attempting to quantify small reductions in gene expression. If this was the case, it could mean that the model generated may not have been sensitive enough to show differential regulation of candidate genes suggesting they may still be downstream targets of TBX5 and GATA-4. Establishment of knockdown and quantification of knockdown levels of TBX5 and GATA-4 knockdown achieved with these morpholinos has not been achieved and is an important factor when considering genome-wide effects. Despite the numerous technical difficulties associated with this, it is absolutely crucial for accurate interpretation of the data presented here. The research in this thesis took a two-pronged approach from this stage. One gene from the original study, RBMS1, was selected for further analysis (Chapter 5), and the *in ovo* model generated was used for identification of new synergistic targets of TBX5 and GATA-4 (Chapter 6).

7.4 Investigation of the role of RBMS1 in cardiac development

Both TBX5 and GATA-4 are involved in embryonic cardiac cell cycle progression, indicating a mechanism by which they can control cardiomyocyte proliferation [Rojas et al., 2008; Xiao et al., 2001]. RBMS1 is a cell cycle gene and transcription factor that was identified in the mouse P19 cell line as a potential downstream target of TBX5 and GATA-4. Little is currently known about the regulation of this gene other than the presence of a positive regulatory element that is necessary for transcriptional activation of RBMS1 [Fujimoto et al., 1998]. RBMS1 binds to DNA polymerase α for activation of DNA replication [Niki et al., 2000a], and can induce apoptosis, mediated at least in part by positive transcriptional regulation of the Fas gene, which has an

important apoptotic role [Iida et al., 1997; Nomura et al., 2005]. *RBMS1* also suppresses transcription of α -smooth muscle actin [Kimura et al., 1998], which is important in myofibrillogenesis [Sugi and Lough, 1992; Woodcock-Mitchell et al., 1988], EMT [Nakajima et al., 1997], and vascular contractility [Schildmeyer et al., 2000; Tomasek et al., 2006] which are all important in the heart. Mutations in α -smooth muscle actin are associated with patent ductus arteriosus, which is thought to arise due to impaired SMC contractility [Guo et al., 2007]. *In ovo* attempts to assess the transcriptional regulation of *RBMS1* in the chick embryo left the link between this gene and *TBX5* / *GATA-4* unclear due to the limitations of the study. As *RBMS1* was considered to be potentially important in cardiogenesis based on the P19 study and previous work by others, investigation of this gene in the chick embryo was continued in Chapter 5. The chromosomal location of the chicken *RBMS1* gene was identified, and this gene was found to give rise to two highly similar splice variants (confirmed by EST evidence). Multispecies sequence alignments demonstrated that this gene is highly conserved.

7.4.1 *RBMS1* expression

RBMS1 expression has been studied by northern blot analysis in the adult mouse, where expression was detected in all tissues except testis [Fujimoto et al., 2000]. However, a more detailed spatial analysis of expression has not been performed, and the developmental expression of this gene is unknown. In order to determine the spatial and temporal expression pattern of *RBMS1* during chick embryonic development, *in situ* hybridisation was performed using two individual probes designed to detect both *RBMS1* transcripts. At HH16, heart expression was weak, and other regions of the embryo including the somites displayed strong expression. The HH19 embryo displayed widespread expression, and staining to the heart was stronger at this stage. At HH24, *RBMS1* displayed expression to the heart, and was highly expressed in the developing forelimb and hind limb buds, somites, and brain. As mentioned, *RBMS1* transcriptionally regulates expression of α -smooth muscle actin [Kimura et al., 1998]. It is possible the α -actin genes share common regulatory

elements, and it was hypothesised that RBMS1 may also transcriptionally regulate expression of α -skeletal and/or α -cardiac actin.

7.4.2 Morpholino knockdown of RBMS1

Mouse knockout of RBMS1 results in growth and developmental defects which can lead to embryonic lethality [Fujimoto et al., 2001]. Deficiency of the hormone progesterone occurs in adult female mice, and this is thought to contribute to the delay in development [Fujimoto et al., 2001]. However, the effect RBMS1 knockout has on the development of the heart has not specifically been studied. In order to assess the function of RBMS1 in the developing heart, morpholino knockdown studies in the chick were performed. Due to the possibility of individual translational start sites on each RBMS1 transcript, splice-blocking morpholinos were used to allow simultaneous knockdown of both. The first morpholino was designed to bind to the exon 2-intron 2 junction (E2I2). A corresponding 5-base mismatch morpholino was also designed as a specificity control, but experimental data indicated that this morpholino may still have been able to bind to the target region, which meant it could not be used. Consultation with Gene-Tools revealed that the use of mismatch morpholinos was no longer recommended due to a high likelihood of retained target binding capability or off-target effects (as observed in this thesis). Therefore, a second experimental morpholino to the same gene was recommended, for determination of specificity upon generation of a common phenotype by both. A second experimental morpholino was designed to bind to the exon8-intron 8 boundary (E8I8). Both morpholinos would most likely result in exon skipping, or alternatively intron retention, and all outcomes were expected to cause in a premature stop and produce a truncated protein. Morpholinos were applied at HH10 - HH16. Morpholino treated embryos were examined at HH19 and displayed normal body shape and size, and cardiac malformations particularly in heart looping were apparent upon external viewing of the heart. Histological analysis of internal heart structures showed defects in atrial septation, and in some cases, a complete absence of septum formation. Similar defects are seen as a result of knockdown of TBX5 and GATA-4. This data may indicate that RBMS1 is a transcriptional regulator

of genes involved in correct septation of the heart, possibly as a downstream target of TBX5 and GATA-4. This is a novel finding and if corroborated with further evidence, will contribute to our current knowledge of the genes and transcriptional pathways regulating heart development, opening up many possibilities for future research.

7.4.3 Characterisation of morpholino effects

The effect of RBMS1 knockdown on heart development is an interesting and novel finding. The two morpholinos used for knockdown of RBMS1 gave very similar phenotypes, implying morpholino specificity. However, it was not possible to identify an effect on mRNA splicing as alternatively spliced products were not detected by RT-PCR. Splice-modified mRNA transcripts are often undetectable by RT-PCR as nonsense mediated decay (NMD) can eliminate them from the cell (personal communication with Jon Moulton, Gene-Tools). Splicing of mRNA occurs in the nucleus, and NMD is a cytosolic process, which means that pre-mRNA transcripts persist for a shorter period than transcripts undergoing NMD. Additionally, there was no reduction in the quantity of the wild type transcript (measured by QPCR) as would be expected if NMD was causing degradation of aberrant transcripts. The sensitivity of the assay was not a contributing factor since pre-mRNA transcripts, which persist for a shorter period than transcripts undergoing NMD, were detectable. Western blotting was also attempted to quantify RBMS1 protein levels in knockdown embryos, but this was not successful. However, expression of α -smooth muscle actin (a negatively regulated target of RBMS1) was up-regulated in RBMS1 E2I2 morpholino treated embryos, supporting the theory that RBMS1 knockdown was occurring. Time restrictions did not allow analysis of α -smooth muscle actin expression in embryos treated with the RBMS1 E8I8 targeting morpholino. These conflicting data raise questions as to whether the phenotypes observed could be due to non-specific effects, and whether morpholinos were in fact inducing knockdown. Morpholino antisense technology is a relatively new advance, and is a highly useful tool for studying gene function by knockdown. However, the issue of controls is extremely important and it is not clear which serves as the best control of specificity. Mismatch

morpholinos may continue to display capability to bind to target sites, but if this issue can be resolved with 7-base mismatch morpholinos (i.e. if target binding does not occur and off-targets effects are also not seen), these may be suitable when attempting to re-create an already known and characterised effect, as in Chapter 4. However, when attempting to investigate novel gene function by knockdown, specificity controls are arguably of greater importance since the expected phenotype is unknown and thus there is no reference point (from an anatomical perspective). The use of two non-overlapping targeting morpholinos is now the recommended method and has advantages over mismatch controls, but is still an indirect means of demonstrating specificity. Whilst it is unlikely in this case that an identical cardiac effect was generated by two individual morpholinos through off-target RNA binding, and the known RBMS1 target α -smooth muscle actin was up-regulated through such an interaction, the effect of morpholinos on RBMS1 needs to be investigated from new angles before a direct cause-effect relationship can be established. The mRNA rescue experiment, where mRNA coding for the protein being knocked down is co-injected with the morpholino, determines proof of specificity by restoration of the wild type phenotype. If this can be put into practice, this experiment provides good proof of principle. Alternatively, RBMS1 knockdown or knockout using an alternative model may be warranted. If the cardiac phenotype observed in this study can be confirmed to be an effect of RBMS1 knockdown, this opens up a range of possibilities for further research. Future directions may involve mutational analysis for investigation of RBMS1 as a causative gene of CHD, identification of upstream regulators and downstream targets, and functional study to determine the mechanisms by which a reduction in RBMS1 can lead to cardiac abnormalities.

7.5 Identification of new synergistic targets of TBX5 and GATA-4

Known synergistic targets of TBX5 and GATA-4 include MYH6 [Ching et al., 2005; Huang et al., 1995; Maitra et al., 2009; Molkenstein et al., 1994], ANF [Plageman and Yutzey, 2004], and connexin 40 [Linhares et al., 2004]. All three of these genes have been linked to cardiac defects; mutations in MYH6 are linked to atrial septal defects

[Ching et al., 2005], and mutations in connexin 40 and ANF are associated with atrial fibrillation [Gollob et al., 2006; Hodgson-Zingman et al., 2008]. In Chapter 6, the previously developed *in ovo* model of TBX5 and GATA-4 double knockdown was used for identification of new synergistic targets via microarray technology, allowing gene expression profiling on a genome-wide scale. Eighteen genes were identified displaying differential regulation in TBX5 / GATA-4 knockdown embryos but not in single knockdown embryos or controls (all were up-regulated by ≥ 2 fold). The majority of genes identified in the TBX5 / GATA-4 double knockdown group have known roles in embryonic development. Expression patterns of six identified genes were detailed in the online GEISHA resource (SOX3, ZIC1, FOXG1, NKX2-1, PAX6, and OTX2), and these did not display obvious expression to the heart so were not considered further. Based on current literature, some of the remaining twelve genes have been reported to display heart expression, such as the novel cytokine CYTL1 [Kim et al., 2007], and the growth factor FIGF (also called VEGFD) [Avantaggiato et al., 1998]. FIGF, in addition to displaying expression in the limb buds, ganglion, teeth, anterior pituitary, lung, kidney, derma, and vertebral column, is expressed in the presumptive endocardial cushions of the heart, potentially indicating a specific cardiac function here [Avantaggiato et al., 1998]. In addition, FIGF signalling is also important in angiogenesis [Marconcini et al., 1999; Veikkola et al., 2001]. However, the expression pattern of these twelve genes had not previously been characterised fully, and nine prioritised genes were selected for study including TFAP2B, PTPRZ1, FIGF, NR2E1, LOC420770, SUZ12, CRABP1, GOLGA4, and GPC3. Expression was examined by RT-PCR in the embryonic chick as a preliminary means of temporal and spatial characterisation of expression. With the exception of NR2E1, expression of all genes was detectable in the heart during development. LOC420770 displayed the most heart-restricted expression pattern and interestingly, once it was possible to differentiate between body and limb expression (at HH24), expression was observed to be more prominent in the limbs than body. This is suggestive of a heart/limb expression profile, similar to that of TBX5. Other genes such as TFAP2B, CRABP1 and PTPRZ1 displayed heart expression that appeared to be strongest during early

cardiac development. *TFAP2B* in particular was only detectable in the heart at HH16. Genes such as *SUZ12*, *GOLGA4*, *GPC3*, and *FIGF* displayed almost ubiquitous expression. It was not possible to study the expression patterns of *CYTL1*, *PLCH1* and *LOC419390* due to limited resources and time restrictions. Further studies need to be performed to determine the exact localisation of expression of genes of interest.

A number of the identified genes have established roles in cardiac development and disease. The small cytoplasmic protein *CRABP1* is important in the modulation of retinoic acid levels; this is very important developmentally and an excess of retinoic acid is associated with retinoic acid embryopathy (RAE), which is characterised by malformation of neural crest derived regions including conotruncal heart structures [Lammer et al., 1985]. Mutations in *CRABP1* are associated with the cerebrovascular condition Moyamoya disease [Suzuki and Takaku, 1969]. Mutations in *GPC3*, a cell surface heparan sulphate proteoglycan and component of the ECM, are linked to the overgrowth syndrome Simpson-Golabi-Behmel syndrome (SGBS) [Pilia et al., 1996]. SGBS is characterised by multiple congenital abnormalities including cardiovascular malformation such as VSDs, common AV canal, and DORV, cardiomyopathy, and conduction defects in the heart [Behmel et al., 1984; Golabi and Rosen, 1984; Lin et al., 1999; Neri et al., 1988]. *CRABP1* and *GPC3* are previously unknown targets of *TBX5* and *GATA-4*, and this is a novel finding. Mutations in the transcription factor *TFAP2B* are associated with Char syndrome, an autosomal dominant 'heart-hand' disorder whose anomalies arise in neural crest cell derived regions and include patent ductus arteriosus (PDA), facial dysmorphism and hand anomalies [Char, 1978]. It is likely that *TFAP2B* has a role in the migration or differentiation of neural crest cells. Little is currently known about the regulation of *TFAP2B*, and is interesting that this heart-hand gene may be transcriptionally co-regulated by another heart-hand gene, *TBX5*. This potentially provides a new link between cardiac transcription factors which have not previously been associated.

TBX5 and GATA-4 are known as master regulators of cardiac development, and the diverse nature of the identified genes in addition to the known roles of some of these in the heart is a demonstration of this. Further study is required to validate the link between these genes and the TBX5 / GATA-4 complex. Insight into the regulation of these genes may contribute to our current understanding of the transcriptional networks and pathways governing cardiac development, and the genetic basis of CHD. Study of identified genes was halted at this stage as a starting point for future PhD projects.

7.6 Summary of findings and implications of this research

This thesis aimed to identify and investigate genes important in cardiac development, with focus on the synergistic targets of TBX5 and GATA-4. The results of two independent microarray studies were used as a basis for this research, and a diverse group of candidate genes was identified as a result, many of which have not previously been associated with a cardiac function.

Microarray expression analysis of TBX5 / GATA-4 double overexpression P19 cell lines led to the identification of a large number of candidate genes, of which seven were selected for further study - *PA2.26*, *PETA-3*, *FUCA1*, *FN*, *TPM1*, *DES*, and *RBMS1*. *In silico* promoter analysis identified the cell cycle gene and transcription factor RBMS1 as the gene with most putative TBX5 and GATA-4 binding sites, and this gene has not previously been associated with a cardiac function. RBMS1 was therefore selected for further study of its role in cardiac development. Chick embryonic treatment with RBMS1 targeting morpholinos for knockdown of expression resulted in defects in cardiac looping and atrial septation. This was an interesting and novel finding as this gene has not previously been implicated in cardiac development, and opened up a range of possibilities for future research into cardiac transcriptional networks. However further investigation is required to confirm the effect observed was a direct result of RBMS1 knockdown. Based on the P19 study, the roles of fibronectin and α -tropomyosin in heart development were also investigated by Dr Christopher

Moore. Morpholino knockdown of fibronectin in the chick resulted in body twisting, growth retardation, abnormal looping of the heart, and a thickening of the cardiac jelly layer (unpublished data). Morpholino knockdown of α -tropomyosin caused kinking of the body, abnormal heart looping, and upon internal analysis hearts showed defects in trabeculation and atrial septum formation (unpublished data). Other genes such as *PETA-3* and *PA2.26* remain to be investigated and this may be a basis for future research projects.

Microarray expression analysis using the *in ovo* model of TBX5 and GATA-4 double knockdown led to the identification of a number of interesting putative targets, including *TFAP2B*, *GPC3*, and *CRABP1*, which have established roles in cardiac development. *TFAP2B* is arguably the most interesting as mutations in this gene are associated with the heart-hand disorder Char syndrome [Satoda et al., 1999; Satoda et al., 2000]. Research into validating the link between genes of interest and TBX5 / GATA-4 may provide new insights into regulatory networks in the heart. *LOC420770*, a novel gene of unknown function, was also identified and displayed expression potentially indicative of a heart-limb profile. Further studies to attempt elucidation of the function of this gene may provide a novel candidate gene for CHD. Investigation of these genes will form the basis of future research, adding to our current knowledge of the complex network of genes involved in heart development.

7.7 Limitations of this approach

The work in this thesis has been largely based on the chick. The many advantages of using the chick as a model of cardiac development, in terms of the structural similarity to the human heart, and ease of access and manipulation, have been discussed throughout this thesis. However, as with any model, this organism also has a number of limitations. As discussed, atrial septation in the chick differs from mammals; in the chick a single atrial septum is formed, whereas in higher vertebrates an additional septum (the septum secundum) also forms and contributes to atrial septation [Morse et al., 1984]. This means that atrial septal defects that arise from disrupted septum

secundum formation cannot be modelled in the chick. Since this study has focused on defects of the septum primum, this has not been a major concern here. Another limitation of this model is that due to the evolutionary diversity between the chick and human, homologues of candidate genes may not be present. An example of this is the gene encoding gamma filamin, which was of interest in this thesis but could not be studied in the chick. The differing physiology of organisms, and how this can manifest itself in many ways including a varied sensitivity to gene dosage, is of importance. Even within mammalian species, vast differences in the effect of equivalent levels of gene knockdown can be observed. For example, human mutations in *NKX2.5* (including those generating hypomorphic alleles) are associated with an array of congenital heart defects [Benson et al., 1999; Elliott et al., 2003; McElhinney et al., 2003; Pashmforoush et al., 2004; Schott et al., 1998]. In comparison, mouse heterozygotes of *NKX2.5* display only a mild cardiac phenotype [Biben et al., 2000]. A similar effect is seen with *TBX20*; hypomorphic mutations in this gene in humans lead to a diverse range of cardiac defects [Kirk et al., 2007], whilst in the mouse only a greater than 50% reduction will lead to heart defects [Stennard et al., 2005; Takeuchi et al., 2005]. This differing sensitivity and response to gene dosage is likely to be multi-factorial. Species-specific physiology is likely to be one contributing factor, and the genetic background, which is important in the manifestation of CHDs [Rajagopal et al., 2007], may be another. It is possible these differences are greater in the chick than mouse since the chick is more physiologically and evolutionary diverse from the human. In this thesis, knockdown of *TBX5* and *GATA-4* in the chick embryo had no effect on expression of *MYH6*. This may have been due to experimental factors, but a species-specific effect cannot be ruled out without further study. Unfortunately it is not possible to predict or quantify these effects, which means the use of any model organism has its limitations and these need to be considered when interpreting data.

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APPENDIX

APPENDIX A: An overview of cardiac development in the chick

Chick development is described from a cardiological perspective from HH 1 – HH 36. The HH 1 chick embryo is at the pre-primitive streak stage [Hamburger and Hamilton, 1992], with cardiac precursor cells identifiable in the epiblast cell layer [Yutzey and Kirby, 2002]. At HH 2 (6 – 7 hrs) cardiac progenitor cells exist in the postero-lateral epiblast and primitive streak formation begins. An intermediate primitive streak is visible at HH 3 (12 – 13 hrs). The HH 4 embryo (18 – 19 hours) displays a definitive primitive streak and precardiac cells are found either side of Hensen's node and in the anterior mesoderm. Gastrulation takes place at HH 5. At HH 5 / 6 (19 – 25 hrs) the cells that will form the heart move into two bilateral regions in the anterolateral region of the embryo, the primary heart field. Cardiac precursor cells are at a higher level of commitment at HH 6, and expression of cardiac genes is initiated at this stage [van den Hoff et al., 2004]. At HH 7 (23 – 26 hrs, 1 somite), markers of terminal myocardial differentiation can be detected in the primary heart fields [Han et al., 1992]. At HH 8 (26 – 29 hrs, 4 somites), endocardial tube formation begins. Embryonic folding leads to formation the primary heart tube at the embryonic ventral midline [Moorman et al., 2003]. Prior to looping, the heart is centrally positioned and undergoes rapid elongation by addition of myocardial cells to both ends of the heart tube along the craniocaudal axis of the embryo to form a long nearly symmetrical tube at HH9. At HH 9 (29 – 33 hrs, 7 somites), cardiac neural crest (CNC) cells undergo epithelial-to-mesenchymal transition (EMT) and migrate away from the neural tube towards the mesenchyme, outflow tract aorticopulmonary septum, and pharyngeal arch arteries [Boot et al., 2003]. The primitive tubular heart begins to beat at HH 10. Dextral looping, where the linear heart tube loops to the right to become c-shaped and asymmetric, begins at this stage and is completed at HH 12 (45 – 49 hrs, 16 somites) [Manner, 2000]. The early phase of s-looping begins at HH 13 (48 – 52 hours, 19 somites), and this phase is characterised by transformation of the heart from a c-

shape to an immature s-shaped structure. Development of the interatrial septum begins at HH 14 (50 – 53 hours, 22 somites), and the septum primum grows from the dorsal cranial wall of the common atrium into the atrial lumen, towards the endocardial cushions in the AV canal [Anderson et al., 2003]. The early phase of s-looping is completed at HH 17 – HH 18 (52 – 72 hrs, 29 – 36 somites). The late phase of s-looping occurs at HH 19 – HH 24, and ballooning of the chambers also occurs at this stage [Christoffels et al., 2000]. At HH 21 – 23 (3.5 – 4 days, 43 – 44 somites) two pairs of endocardial cushions, one proximal and one distal, are found in the outflow tract [Qayyum et al., 2001]. At HH 24 (4 days), the atrial septum becomes fused with dorsal and ventral endocardial cushions in the AV canal, and small perforations (foramina secunda) form in the septum [Morse and Hendrix, 1980]. The foramina secunda increase in size and number during days 5 and 6, creating cords of endocardium covered tissue [Morse and Hendrix, 1980]. This cord tissue thickens during days 8 to 15, reducing the size of the foramina secunda, will close to complete atrial septation by day 2 post hatching. At HH 25 – HH 26 (4.5 – 5 days), a third distal endocardial cushion (required for AV canal septation) has formed in the outflow tract. The aorticopulmonary septum, which divides the truncus into aortic and pulmonary channels, forms as a result of fusion of the left and right superior truncus swellings. Mitral and tricuspid valve primordia are present and the muscular interventricular septum, which septates the left and right ventricles, begins to grow at this stage. At HH 28 (5.5 days) division of the semilunar valve region results from formation of the aorticopulmonary septum, and AV valve development begins. At HH 34 (8.0 days), ventricular and outflow tract septation is completed. Fusion of the dorsal and ventral endocardial cushions results in septation of the AV canal. At HH 35 – HH 36 (8 – 10 days), development of AV valves is nearly complete.

Appendix B: QPCR assays designed to candidate genes in the mouse

Gene	Accession ID	Primer name	Primer sequence (5' – 3')	Position	Probe*
Neurofilament 3	NM_008691.2	Neurofilament F	GGTGCGAGGCACTAAGGAG	1024 – 1042	1
		Neurofilament R	ATTTTCCAAGTCTGGATGG	1110 – 1129	
PMM2	NM_016881.2	PMM2 F	ATGGATGGGACCCTGACTG	77 – 95	29
		PMM2 R	TCTGACCCACCTACCACTCC	164 – 183	
VEGFB	BC046303.1	VEGFB F	CAACACCAAGTCCGAATGC	498 – 516	76
		VEGFB R	GGCTTCACAGCACTCTCCTT	603 – 622	
α 2-macroglobulin	NM_175628.3	α 2-macroglobulin F	TGAGGAGGCGGTAAAAGAAG	3575 – 3594	93
		α 2-macroglobulin R	TGGCACTCTGGTTTCTGA	3620 – 3638	
Galanin R2	NM_010254.4	Galanin R2 F	GAGAGGCATTGCTTCTTGG	735 – 754	1
		Galanin R2 R	AGTACAGCTCAGTCCTAAGCCTAAA	787 – 811	
Glutaredoxin	AB013137.1	Glutaredoxin F	TGGAGCTCTGCAGTTATAAAAGG	355 – 377	13
		Glutaredoxin R	GCCATCAGCATGGTTAGACA	408 – 427	
S100A10	NM_009112.2	S100A10 F	AGTTCCTGGGTTTTTGA	228 – 246	76
		S100A10 R	CACTGGTCCAGGTCCTTCAT	281 – 300	
AP4e1	BC042530.1	AP4e1 F	TGGCATCATCGCTGTTTG	531 – 548	93
		AP4e1 R	AACATCTGCTTTTCCAACAA	578 – 598	
mRbap46	NM_009031.3	mRbap46 F	ACGCAAGATGGCGAGTAAAG	227 – 246	25
		mRbap46 R	TCCAGATTTTATACTCTTCGTTGATG	278 – 303	
Gamma Filamin	BC060276.1	Gamma Filamin F	GGGTACACACACCCTCAGGA	3698 – 3717	25
		Gamma Filamin R	TGAAGCGGATGGTATGCTTAT	3755 – 3775	
HNRPD	NM_016690.4	HNRPD F	AACCAGCAGGATGACGGTA	145 – 163	13
		HNRPD R	ATTCACTCAGATCTTCTTGCTTG	194 – 217	
Calreticulin	NM_007591.3	Calreticulin F	TGAAGCTGTTCCGAGTGGT	401 – 420	93
		Calreticulin R	GATGACATGAACCTTCTTGGTG	492 – 513	
Annexin A4	BC055871.1	Annexin A4 F	AGGCTCTACGGGAAGTCTTTG	944 – 964	25
		Annexin A4 R	GCGGCGTCTTGTTAATC	1034 – 1052	
RBMS1	BC057866.1	RBMS1 F	GAAGCAAGTGGCCAGCAG	1461 – 1478	76
		RBMS1 R	GAATCCCTTCGGTACATCTCA	1542 – 1563	
α -tropomyosin	M22479.1	α -tropomyosin F	TCGTGCTCAGGAGCGTCT	354 – 371	25
		α -tropomyosin R	TTCAATGACTTTCATGCCTCTC	426 – 447	

Fibronectin	X93167.1	Fibronectin F	TGATTGGGAGGAAGAAGACAG	3531 - 3551	93
		Fibronectin R	TGTGGAGGGAACATCCAAG	3607 - 3625	
FUCA1	NM_024243.4	FUCA1 F	GCCAGAGCTGTATGACCTTGT	640 - 660	93
		FUCA1 R	GGAGTCCAGTACGTGTCAGG	710 - 730	
PETA-3	AF033620.1	PETA-3 F	CTCCATGATAACCCACCATGT	211 - 231	76
		PETA-3 R	GCATTCCCAGGAACCTTATG	267 - 286	
PA2.26	AJ250246.1	PA2.26 F	CAGTGTGTTCTGGGTTTTGG	52 - 72	95
		PA2.26 R	TGGGGTCACAATATCATCTTCA	122 - 143	
nono	NM_023144.1	Nono F	GGTTATGCTAATGAGGCAGGA	1058 - 1078	25
		Nono R	TTCGCTTCTGAACCTCTTGG	1127 - 1146	
β -tubulin	BC003825.1	β -tubulin 5 F	GCTGGACCGAATCTCTGTGT	283 - 302	95
		β -tubulin 5 R	GACCTGAGCGAACGGAGTC	374 - 392	
Rpl13a	NM_009438.3	Rpl13a F	GCCCCACAAGACCAAGAG	285 - 302	1
		Rpl13a R	GACCACCATCCGCTTTTTC	363 - 381	
Bola2	NM_175103.3	Bola2 F	GATTACCTCCGCGAGAAGC	53 - 71	13
		Bola2 R	AACGGTTCAGAGTCGTGTCC	109 - 128	
dynactin	NM_007835.2	Dynactin F	TCCTCATCAGCACGATGAAC	2968 - 2987	76
		Dynactin R	CTGCGTCATACTCGCCTTCT	3008 - 3027	
tmem131	NM_018872.2	tmem131 F	AGTCCAGCTCCCAACC	5502 - 5519	76
		tmem131 R	GAGCTGCTGAATGGAGTGGT	5565 - 5584	

*Probe identifiers refer to pre-designed Universal ProbeLibrary probes (Roche). Each probe binds to a specific nucleotide sequence. Probe sequence information can be found online at <https://www.roche-applied-science.com>).

Appendix C: IUPAC-IUB consensus nucleotide definitions

IUPAC-IUB consensus nucleotide definitions	
IUB	Base
A	A
C	C
G	G
T	T
M	A or C
R	A or G
W	A or T
S	C or G
Y	C or T
K	G or T
V	A or C or G
H	A or C or T
D	A or G or T
B	C or G or T
N	G or A or C or T

Appendix D Cell transfection and luciferase reporter assays

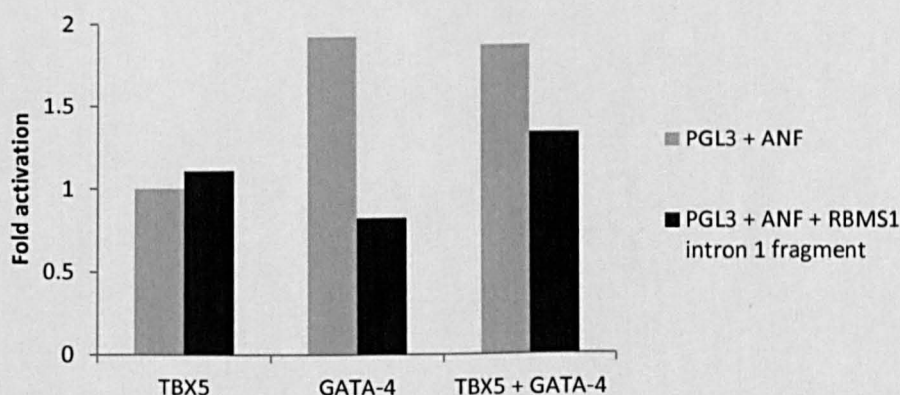
Cell transfection and luciferase reporter assays were performed by Dr Tushar Ghosh. COS7 cells were transfected (using Polyfect) with 1 µg expression plasmids (pcDNA-TBX5 and / or pcDNA-GATA-4), and 1.5 µg reporter plasmid. Cells were harvested 24 hours after transfection for measurement of cell lysate luciferase activity using the Dual Luciferase Reporter Assay system. Transfections were performed in duplicate, and each experiment carried out twice. Cells transfected with reporter plasmid but neither expression plasmid were used for normalisation.

The results of reporter assays performed in the pGL3-basic vector containing the human ANF promoter are displayed in Table 3.7 and Figure 3.9. Luciferase activity of vectors with and without the intronic fragment was very similar in the presence of TBX5. This suggests that TBX5 does not bind to this region for regulation of promoter activity. In the presence of GATA-4, luciferase activity was over 2-fold lower in the PGL3/ANF vector containing the intronic fragment compared to the PGL3/ANF vector lacking it i.e. GATA-4 has a strong repressive effect via this region. There was a 28% reduction in luciferase activity in the presence of both TBX5 and GATA-4, smaller than the effect of GATA-4 alone, ruling out synergistic repression by both transcription factors via this region. Reporter assays were also performed using the pGL3-promoter vector, but luciferase activity was too low for accurate quantification.

Effect of the RBMS1 intron 1 fragment on promoter activity

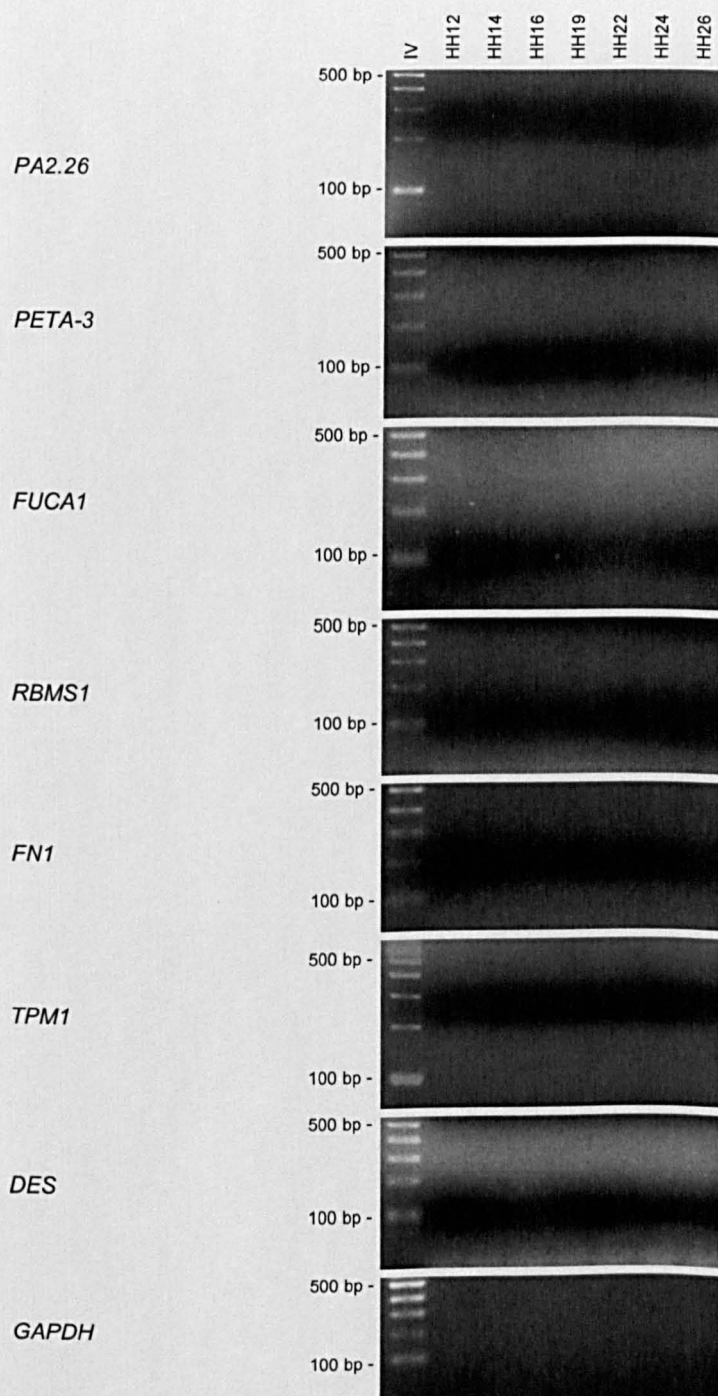
Expression plasmid	Fold Activation	
	PGL3 + ANF	PGL3 + ANF + RBMS1 intron 1 fragment
TBX5	1.00	1.11
GATA-4	1.91	0.82
TBX5 + GATA-4	1.86	1.33

COS7 cells were transfected with 1 µg expression plasmids (pcDNA-TBX5 and / or pcDNA-GATA-4), and 1.5 µg reporter plasmid. Cells were harvested 24 hours after transfection for measurement of luciferase activity. Each transfection was performed in duplicate, and each experiment carried out twice. Cells transfected with reporter plasmid, but neither expression plasmid, were used for normalisation.

**Effect of the RBMS1 intron 1 fragment on promoter activity**

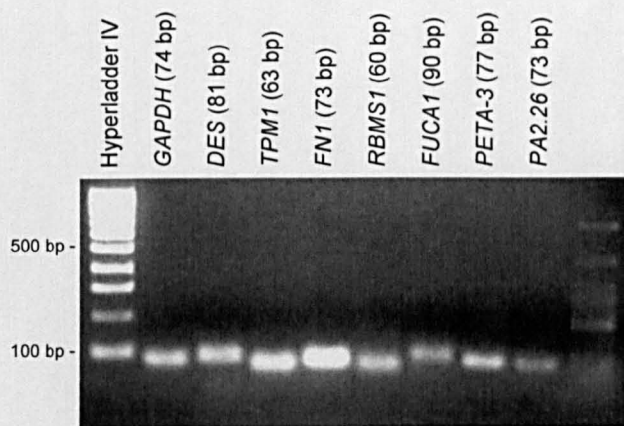
COS7 cells were transfected with 1 µg expression plasmids (pcDNA-TBX5 and / or pcDNA-GATA-4), and 1.5 µg reporter plasmid. Cells were harvested 24 hours after transfection for measurement of luciferase activity. Each transfection was performed in duplicate, and each experiment carried out twice. Cells transfected with reporter plasmid, but neither expression plasmid, were used for normalisation.

Appendix E: Negative RT controls performed in parallel to expression profiling of candidate genes in the chick heart



Samples were electrophoresed on 2% agarose gels using Hyperladder IV as a size marker (500 bp and 100 bp bands are indicated). Negative RT controls (where reverse transcriptase enzyme was not added) did not display amplification for any of the assays. All products assays spanned a 60 – 90 bp region.

Appendix F: Relative sizes of products amplified for expression profiling of candidate genes in the chick heart

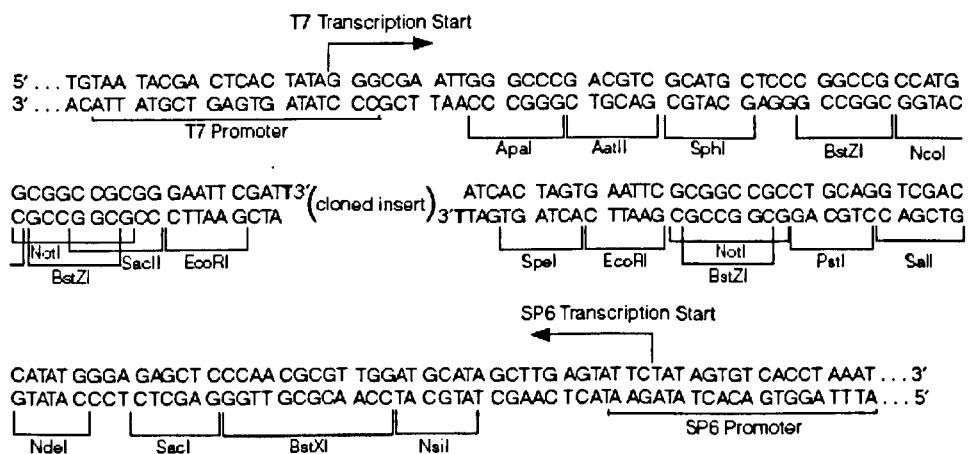
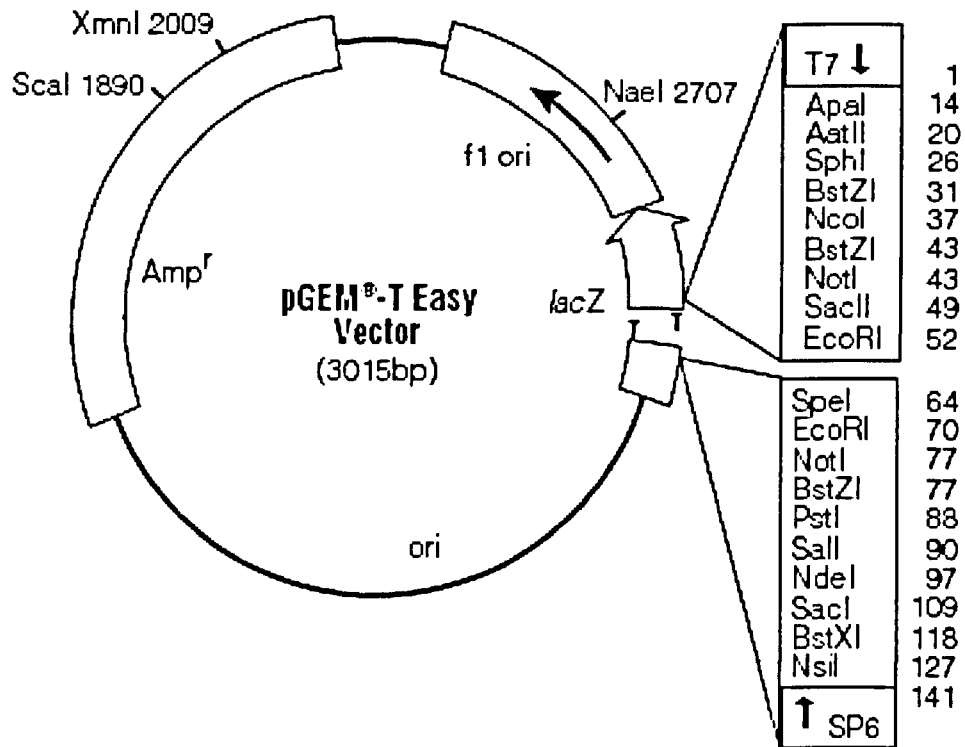


Samples were electrophoresed on 2% agarose gels using Hyperladder IV as a size marker (500 bp and 100 bp bands are indicated).

Appendix G: Standard amino acid abbreviations

Amino acid	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Selenocysteine	Sec	U
Pyrrolysine	Pyl	O

Appendix H Map of the pGEM-T Easy vector



The position of the T7 and SP6 transcription start sites are marked in addition to restriction sites. T7 RNA polymerase was used for synthesis of antisense RNA probes from SpeI linearised plasmid DNA, and SP6 RNA polymerase used for synthesis of sense control probes from SacII linearised plasmid DNA (both inserts in forward orientation).

Appendix I Genes identified in microarray analysis of TBX5 and GATA-4 knockdown

SOX3

SOX3 (SRY (sex determining region Y)-box 3) is a member of the SOX family of transcription factors that are important in embryonic development and determination of cell fate. This gene was up-regulated by 2-fold in double knockdown embryos. SOX3 null mice displayed a range of defects including craniofacial abnormalities, midline CNS defects, growth retardation, reduced fertility, and hypopituitarism [Rizzoti et al., 2004]. Human mutations in SOX3 are associated with X-linked hypopituitarism and mental retardation [Laumonnier et al., 2002].

ZIC1

ZIC1 (Zic family member 1) is a member of the conserved ZIC family of C2H2-type zinc finger transcription factors which are important in early development. Transcripts of this gene displayed 2.6 and 26.6-fold up-regulation in TBX5 / GATA-4 knockdown embryos. ZIC1 is expressed in regions that give rise to the dorsal neural tube and neural crest [Nakata et al., 1998], but not in migratory neural crest cells [Sun Rhodes and Merzdorf, 2006]. Whole mount *in situ* hybridisation expression analysis in the mouse embryo (E9.5 – E11.5) showed that ZIC1 is expressed along the dorsal midline of the cranial and spinal neural tube, and expression was not detected in the heart [Gaston-Massuet et al., 2005; Nagai et al., 1997]. ZIC1 is involved in cerebellar development, and mouse knockout of this gene results in brain and axial skeletal abnormalities [Aruga et al., 1998; Aruga et al., 1999].

PAX6

PAX6 (paired box 6) is a transcription factor which contains a paired box domain and homeodomain, with a crucial role in the development of the eyes, nose, central nervous system, and pancreas. This gene was up-regulated 2.7 fold in TBX5 / GATA-4 double knockdown embryonic hearts. PAX6 is expressed in the developing nervous

system [Krauss et al., 1991; Walther and Gruss, 1991] and eyes [Ton et al., 1991]. Mutations in this gene are linked to an array of ocular disorders including aniridia [Glaser et al., 1992], Gillespie syndrome [Glaser et al., 1994] and Peter's anomaly [Hanson et al., 1994].

OTX2

OTX2 (orthodenticle homeobox 2) is a member of the bicoid family of homeodomain transcription factors, and a homologue of the drosophila gene orthodenticle which is involved in formation of the brain and eyes [Finkelstein and Boncinelli, 1994]. OTX2 transcripts displayed 2.8 and 16 fold up-regulation in TBX5 / GATA-4 double knockdown embryos. This gene is expressed in the developing nose, ear, eyes, and brain [Martinez-Morales et al., 2001; Simeone et al., 1993]. Mouse knockout of this gene results in embryonic lethality due to defects in gastrulation which cause severe brain malformations including deletion of large regions [Acampora et al., 1995; Ang et al., 1996]. Human mutations in OTX2 are associated with a range of ocular disorders including microphthalmia syndromic type 5 (MCOPS5) [Ragge et al., 2005]. There is evidence that OTX2 interacts with other ocular transcription factors including PAX6 [Zuber et al., 2003].

FOXP1

FOXP1 (forkhead box G1) belongs to the forkhead family of transcription factors. FOXP1 displayed 2.8 fold up-regulation in TBX5 / GATA-4 double knockdown embryos. This gene is expressed in the brain and testis where it acts as a transcriptional repressor crucial in brain development [Xuan et al., 1995]. Mouse knockout of FOXP1 results in hypoplasia of the cerebral hemispheres and lethality at birth [Hanashima et al., 2004; Xuan et al., 1995]. Mutations in this gene are associated with the congenital variant of Rett syndrome, a neurodevelopmental disorder [Ariani et al., 2008].

NKX2-1

NKX2-1 (NK2 homeobox 1), also called thyroid transcription factor 1 (TTF1), is a developmentally important homeodomain transcription factor which regulates expression of thyroid specific genes. NKX2-1 displayed 11.4 fold up-regulation in response to double knockdown of TBX5 and GATA-4. NKX2-1 null mice lack thyroid glands, lungs, and pituitary glands, and display defects of the ventral forebrain [Kimura et al., 1996]. Mutations or deletions in this gene in both humans and mice are associated with neurological defects [Pohlentz et al., 2002].

CYTL1

CYTL1 (cytokine-like 1) is a secreted peptide classed as a novel cytokine based on its predicted secondary structure which indicates it contains four alpha helices, a common characteristic of cytokines [Liu et al., 2000]. This gene was up-regulated 2.3 fold in TBX5 / GATA-4 double knockdown embryos. The function of CYTL1 is not well characterised. It is expressed in the cartilage tissue of the mouse inner ear [Ficker et al., 2004] and articular cartilage in humans [Hermansson et al., 2004], and has a function in chondrogenesis [Kim et al., 2007]. RT-PCR expression studies in the adult mouse show CYTL1 expression is highest in cartilage and lungs, and is also observed in the heart, trachea, and testis [Kim et al., 2007].

PLCH1

PLCH1 (phospholipase C, eta 1) is a member of the phosphoinositide-specific phospholipase C family of enzymes which are important in intracellular signal transduction (via release of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)), intracellular Ca^{2+} release, and protein kinase C activation [Hwang et al., 2005]. PLCH1 displayed 2.8-fold up-regulation in response to TBX5 / GATA-4 double knockdown. This gene displays expression in several cell types and has a variety of functions [Stewart et al., 2005].

TFAP2B

TFAP2B (transcription factor AP-2 beta (activating enhancer binding protein 2 beta)) is a member of the AP-2 family of transcription factors which are important in retinoic acid-induced differentiation [Luscher et al., 1989; Mitchell et al., 1991]. This gene displayed 2.3-fold up-regulation in TBX5 / GATA-4 double knockdown embryos. TFAP2B is expressed in migrating neural crest cells which move into the aortic arches and contribute to the formation of a number of structures including the ductus arteriosus [Moser et al., 1997; Satoda et al., 2000]. Mutations in this gene are associated with Char syndrome in humans, an autosomal dominant disorder whose anomalies arise in neural crest cell derived regions [Satoda et al., 1999; Satoda et al., 2000]. These include patent ductus arteriosus (PDA), facial dysmorphism and hand abnormalities [Char, 1978]. Char syndrome is one of a number of 'heart-hand' disorders to include congenital heart defects and hand anomalies.

PTPRZ1

PTPRZ1 (protein tyrosine phosphatase, receptor-type, Z polypeptide 1) is a member of the receptor type protein tyrosine phosphatase family, encoding a single pass type I membrane protein. This gene displayed 6.1-fold up-regulation in response to double knockdown of TBX5 and GATA-4. PTPRZ1 expression has been reported in the brain [Kaplan et al., 1990; Krueger and Saito, 1992] and human umbilical vein cells (HUVEC) [Polykratis et al., 2005], and binds the developmentally regulated proteins midkine [Maeda et al., 1999] and pleiotrophin [Maeda et al., 1996]. These are heparin-binding growth factors with roles in neurite outgrowth [Li et al., 1990; Muramatsu and Muramatsu, 1991; Rauvala, 1989], plasminogen activity [Kojima et al., 1995], and oncogenic transformation [Chauhan et al., 1993; Kadomatsu et al., 1997]. Pleiotrophin has also been implicated in neuronal migration and EMT based on its expression pattern [Vanderwinden et al., 1992], and stimulates angiogenesis [Papadimitriou et al., 2001].

FIGF

FIGF (c-fos induced growth factor) or vascular endothelial growth factor D (VEGF-D) is a member of the PDGF/VEGF superfamily whose expression is induced by the nuclear oncogene c-fos [Orlandini et al., 1996]. In this study FIGF was up-regulated by 2.2-fold in response to double knockdown of TBX5 and GATA-4. FIGF acts via VEGFR2 and VEGFR3 receptors [Achen et al., 1998] to stimulate angiogenesis and lymphangiogenesis [Marconcini et al., 1999; Veikkola et al., 2001]. Studies of limb development in the chick have shown that FIGF is highly expressed in this region during embryogenesis [Trelles et al., 2002]. Broader studies in the developing mouse embryo have identified expression in various regions including the limbs, lungs, kidneys, liver, and presumptive endocardial cushions of the heart [Avantaggiato et al., 1998].

NR2E1

NR2E1 (nuclear receptor subfamily 2, group E, member 1) was up-regulated 2.8-fold in double knockdown embryos. This gene is a member of the orphan nuclear receptor gene superfamily and is required for cellular proliferation and differentiation of forebrain progenitor cells [Monaghan et al., 1997; Monaghan et al., 1995]. Mouse knockout of this gene results in impaired development of forebrain derived structures.

LOC420770

LOC420770 is a hypothetical chicken gene supported by EST evidence (GenBank ID: CR390114), up-regulated by 2.6 fold in response to TBX5 and GATA-4 double knockdown. The mRNA of this gene is 648 nucleotides in length and encodes a protein of 108 amino acids. A function has not yet been assigned to this gene.

LOC419390

LOC419390 is a hypothetical chicken gene supported by EST evidence (GenBank ID: CD740249), with similarity to hairy-and-enhancer-of-split related protein-7 (HES7) [Kim et al., 2009]. This gene was up-regulated by 2.7-fold in double knockdown

embryos. Hairy-and-enhancer-of-split genes belong to the basic helix-loop-helix superfamily, and the HES7 gene in humans acts as a repressor protein in Notch signalling [Davis and Turner, 2001]. Mutations in the human HES7 gene are associated with spondylocostal dysostosis (SCD) [Sparrow et al., 2008], a congenital disorder characterised by abnormal vertebral segmentation (AVS) [Turnpenny et al., 2007]. LOC419390 may be a transcriptional repressor with a developmental function in the heart.

SUZ12

SUZ12 (suppressor of zeste 12 homolog) was up-regulated by 2-fold following double knockdown of TBX5 / GATA-4. It encodes a zinc finger protein, recently classified as a member of the Polycomb group (PcG) of proteins which form multi-protein complexes that can regulate transcription through chromatin modification [Jenuwein and Allis, 2001]. SUZ12 is required for cell proliferation, and mouse knockout of this gene results in embryonic lethality [Pasini et al., 2004].

CRABP1

CRABP1 (cellular retinoic acid binding protein 1) is a small cytoplasmic protein which binds to and can modulate levels of retinoic acid [Fiorella and Napoli, 1991]. This gene displayed 4.9-fold up-regulation in response to TBX5 / GATA-4 knockdown. CRABP1 expression in the mouse embryo is observed in regions derived from the neural crest [Lyn and Giguere, 1994]. Retinoic acid is a teratogen, and an excess leads to retinoic acid embryopathy (RAE), whose abnormalities include malformation of neural crest derived regions i.e. craniofacial, thymic and CNS structures, and conotruncal heart defects [Lammer et al., 1985]. CRABP1 levels are elevated in the cerebrospinal fluid of sufferers of Moyamoya disease (MMD) [Kim et al., 2003], a cerebrovascular condition characterised by progressive occlusion of the carotid artery (as a result of fibrosis and excess proliferation of smooth muscle cells) and formation of abnormal collateral vessels at the base of the brain [Suzuki and Takaku, 1969]. Retinoid signalling has been implicated in ventricular formation [Dyson et al., 1995].

There is evidence that retinoids can modulate expression of growth factors [Boyle et al., 2000], and a number of growth factors are elevated in MMD including basic fibroblast growth factor (bFGF) [Malek et al., 1997], transforming growth factor β (TGF β) [Hojo et al., 1998], and platelet derived growth factor (PDGF) [Aoyagi et al., 1993; Aoyagi et al., 1991].

GOLGA4

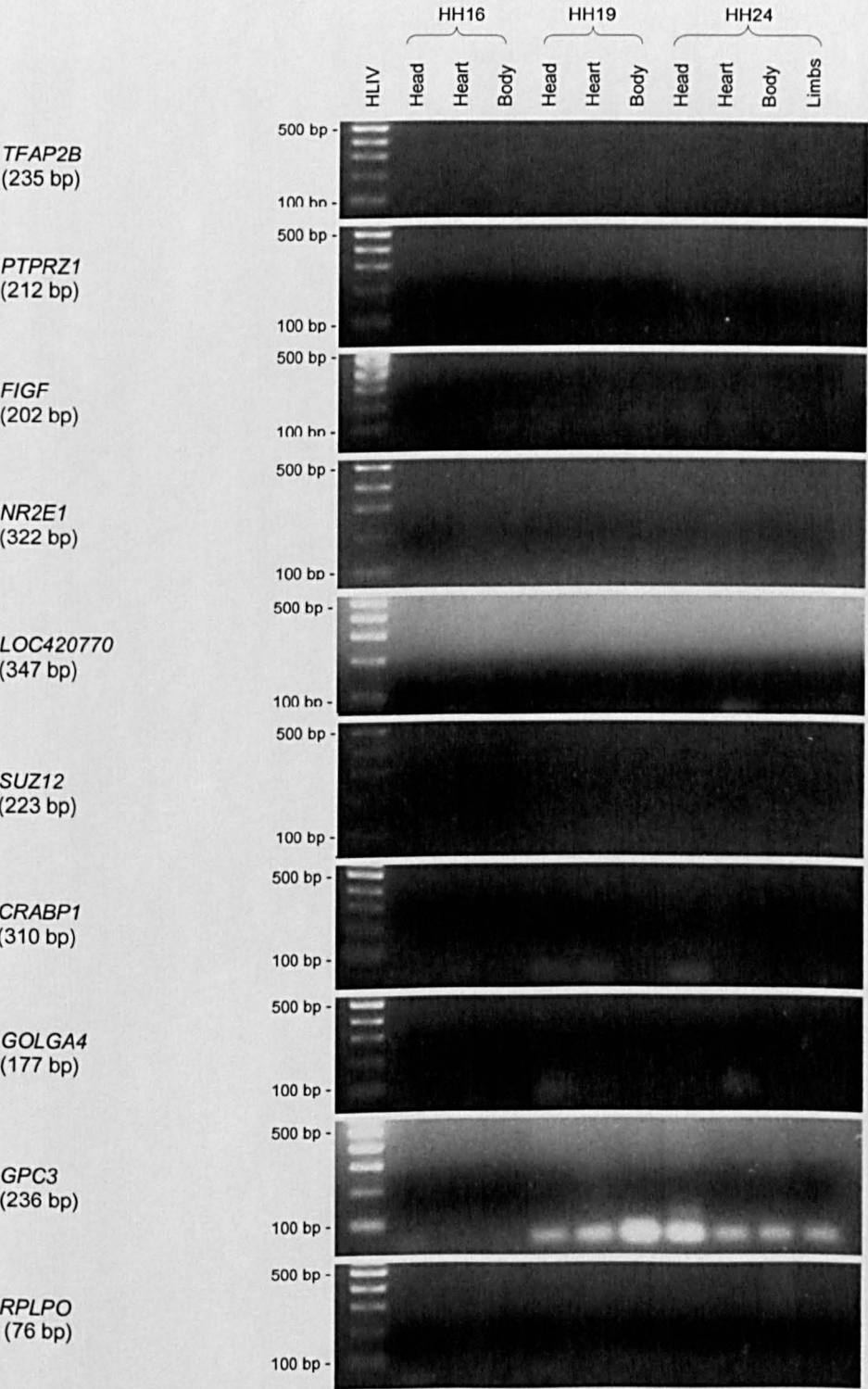
GOLGA4 (golgi autoantigen, golgin subfamily a, 4) is a member of the golgin protein family which localise with the golgi apparatus, helping to maintain its structure [Diao et al., 2003; Puthenveedu et al., 2006; Sohda et al., 2005], with an additional role in vesicle tethering [Sonnichsen et al., 1998]. GOLGA4 expression was up-regulated 2-fold in TBX5 / GATA-4 double knockdown embryos. GOLGA4 is important in protein transport [Kakinuma et al., 2004; Lieu et al., 2008].

GPC3

GPC3 (glypican 3) is a member of the glypican family of cell surface heparan sulphate proteoglycans [David, 1993], which have roles in regulation of developmental growth and signalling [Selleck, 1999]. GPC3 was up-regulated 2.4-fold in TBX5 / GATA-4 double knockdown embryos. Mutations in this gene are associated with Simpson-Golabi-Behmel syndrome (SGBS) in humans [Pilia et al., 1996], an X linked disorder characterised by pre- and post-natal overgrowth, a characteristic facial appearance, and multiple congenital abnormalities including cardiac defects [Behmel et al., 1984; Golabi and Rosen, 1984; Neri et al., 1988]. Cardiac anomalies occur in approximately 50% of SGBS cases and include cardiovascular malformation, cardiomyopathy, and conduction defects [Lin et al., 1999]. Developmental expression of GPC3 in the mouse is observed in the organs that overgrow in SGBS, correlating with the phenotype of this disorder [Pellegrini et al., 1998]. GPC3 null mice display several of the clinical features of SGBS including developmental overgrowth, perinatal death, kidney malformations, and lung abnormalities [Cano-Gauci et al., 1999]. Congenital cardiac malformations such as VSDs, common AV canal, and DORV are also

observed, along with coronary vasculature defects [Ng et al., 2009]. There is evidence that GPC3 acts as a receptor for FGF9, a growth factor with a crucial role in mesenchymal differentiation [Colvin et al., 2001].

Appendix J: Negative RT controls performed in parallel to expression profiling of candidate genes chick embryonic segments at HH16, HH19 and HH24



Samples were electrophoresed on 2% agarose gels using Hyperladder IV as a size marker (500 bp and 100 bp bands are indicated). Negative RT controls (where reverse transcriptase enzyme was not added) did not display amplification for any of the assays.