1 Introduction

Congenital heart defects (CHDs) occurring in 1% of all live births are the most common birth defect and the leading non-infectious cause of mortality in newborns. Only a minority of CHDs are caused by single-gene defects and follow a clear Mendelian inheritance, whereas most malformations are thought to be multigenetic disorders. Moreover, the heterogeneity of CHDs associated with single-gene defects, as shown for *NKX2.5* and *TBX5* mutations (Basson et al. 1997; Basson et al. 1999) points to a complex genetic network with modifier genes, genetic polymorphisms and the influence of environmental factors (Srivastava 2001; Solloway et al. 2003; Olson 2004).

During embryogenesis, the heart is the first organ to be formed. In human cardiac development occurs between the third and seventh week of gestation and is terminated by the formation of the 4-chambered heart, defining the basis of normal cardiac physiology. The right heart belongs to the low-pressure pulmonary system and the left heart to the high-pressure body circulation. As a consequence of heart malformations, abnormal hemodynamic features can occur because of improper volume or pressure load and lead to adaptation processes of the heart. To date, however, very little is known about these adaptation processes and knowledge about molecular pathways involved in this process has been mainly gained by animal studies (Bauer et al. 1998; Nediani et al. 2000; Baumgarten et al. 2002).

1.1 The Normal Heart

The normal heart is composed of four chambers and is surrounded by a space called the pericardial cavity. It is formed by the pericardium, a closed double-layered sac that surrounds the heart and anchors it within the mediastinum. From the inside, the heart wall is composed of three layers of tissues: the epicardium, the myocardium and the endocardium. The epicardium, also called the visceral pericardium, is a thin serous membrane forming the smooth outer surface of the heart. The thick middle layer of the heart, the myocardium is composed of cardiac muscle cells and is responsible for the ability of the heart to contract. The smooth inner surface of the heart chambers is the endocardium, which consists of simple squamous epithelium covering a layer of connective tissue.

The two atria collect the blood returning from the veins before contraction of the atria forces the blood into the ventricles. The right atrium has two major openings, called inferior and superior vena cava, collecting the blood from various parts of the body. In addition the coronary sinus enters the right atrium from the wall of the heart. The left atrium has four openings that receive the four pulmonary veins from the lungs. The two atria are separated from each other by the interatrial septum.

The ventricles are the major pumping chambers of the heart. They eject the blood into the arteries and force it to flow through the circulatory system, each ventricle harbouring one large outflow root located superiorly near the midline of the heart. The right ventricle opens into the pulmonary trunk, while the left ventricle ejects into the aorta. The two ventricles are separated from each other by the interventricular septum.

The atrioventricular valve separating the right atrium and the right ventricle is called "tricuspid valve", the one located between the left atrium and the left ventricle "mitral valve". These valves allow blood flow from the atria into the ventricles but prevent the blood from running back into the atria. The aorta and the pulmonary trunk possess aortic and pulmonary semilunar valves, preventing the back flow into the ventricles (Figure 1.1A).

1.2 Congenital Heart Defects

1.2.1 Atrial Septal Defect

Septation defects permitting an interatrial shunt are at first sight among the simplest of congenital cardiac malformations. An opening within the atrial septum is an integral part of the foetal circulation, allowing the blood to bypass the lung and usually closes about the time the baby is born. Most interatrial communications represent persistence of this foetal arrangement and can be divided into three groups. The most common type atrial septal defect (ASD) is the secundum ASD (50-70% of all ASDs), localised in the central portion of the septum, whereas a defect in the lower part of the septum is called primum ASD (30% of all ASDs). A rare type of ASD is the sinus venosus defect (10% of all ASDs), localised near the entrance of the superior or inferior vena cava into the right atrium. Depending on the size of the opening ASDs lead to a left-right shunt, with a volume overload to the right atrium and ventricle and an increased pulmonary blood flow frequently leading to pulmonary hypertension in adult life (Figure 1.1B).

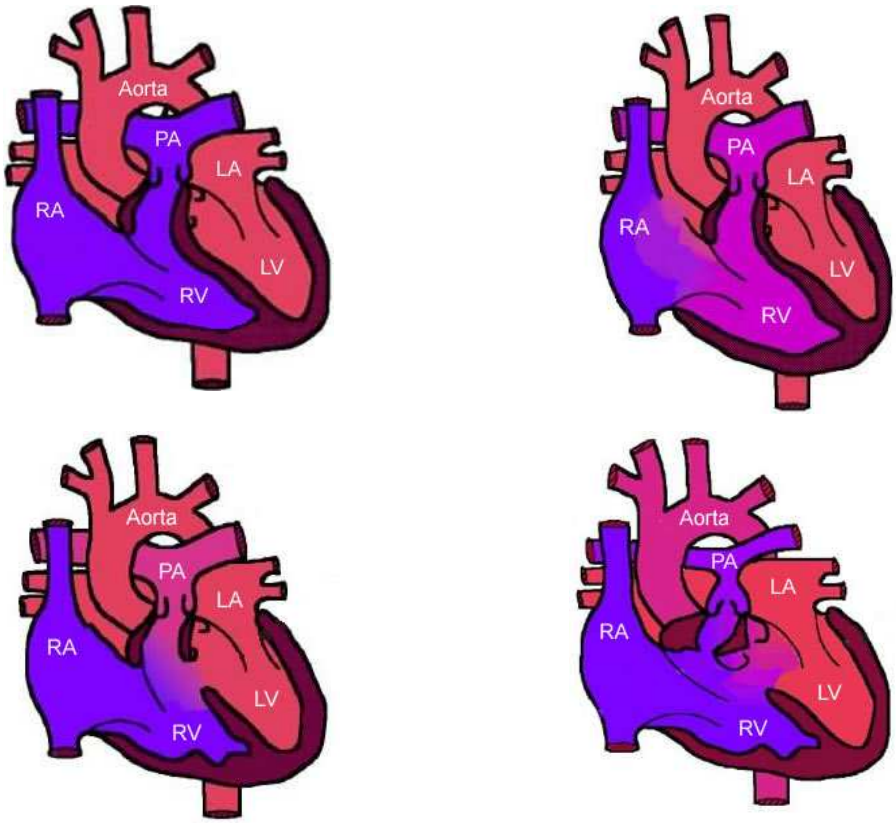

Figure 1.1. Anatomical comparison of normal and malformed hearts. A-Normal heart, B-Atrial Septal Defect (ASD), C-Ventricular Septal Defect (VSD), D-Tetralogy of Fallot (TOF). RA-Right Atrium, RV-Right Ventricle, LA-Left Atrium, LV-Left Ventricle, PA-Pulmonary artery. Colours: -high oxygen, -low oxygen.

1.2.2 Ventricular Septal Defect

The most common form of CHDs are ventricular septal defects (VSD, 25% of all CHDs). The normal ventricular septum consists of a small membranous septum and a larger muscular septum. The muscular septum itself can be divided into the inlet part close to the tricuspid valve, the infundibular and the trabecular (or simply muscular) septum. The most common type of VSD is a membranous defect involving a varying amount of muscular septum adjacent to it, called “perimembranous” VSD (70% of all VSD). The defects vary in size from small defects without hemodynamic significance to large defects with massive left-right shunts, pulmonary hypertension and congestive heart failure. The absence of the interventricular septum results in a common ventricle (Figure 1.1C).

1.2.3 Tetralogy of Fallot

Tetralogy of Fallot (TOF) is the most common cyanotic CHD beyond infancy and occurs in 10% of all CHD patients. Although it had been described much earlier, it was Etiene-Louis Arthur Fallot, who in 1988, separated this malformation from other anatomical lesions responsible for the “maladie blue”. The original description of TOF includes four abnormalities: a large VSD, a right ventricular outflow tract obstruction, a biventricular origin of the aortic valve (overriding aorta) and a hypertrophy of the right ventricle. The VSD is a perimembranous defect with extension into the infundibular septum, usually large enough to equalise pressures in both ventricles. The right ventricular outflow tract obstruction can an infundibular stenosis and or a pulmonary valve stenosis, in the most severe form of the anomaly the pulmonary valve is atretic. Due to the nonrestrictive VSD and the resulting identical pressure in the right and left ventricle the direction of the interventricular shunt depends on the on the degree of the right ventricular outflow tract obstruction (Figure 1.1D). A more severe degree of pulmonary stenosis leads to a predominant right-left shunt and cyanosis (Myung 1997) .

1.3 Heart Development

Heart development is the result of complex morphogenetic changes in orchestration with various molecular pathways. It varies between species but can generally be classified into the steps of cardiomycoyte determination and specification, formation of the heart tube, looping, chamber development and growth, and endocardial cushion, valve, and septal morphogenesis (Figure 1.2).

The heart develops from two primordia that are derivatives of the lateral plate mesoderm (Raffin et al. 2000; Redkar et al. 2002; Hochgreb et al. 2003). These heart primordia can be defined by fate mapping in E7.25 mouse embryos and migrate anteriorly as a sheet. The myocardial and endocardial cell layers form and are separated by a layer of extracellular matrix, the cardiac-jelly (Linask et al. 1993). The two heart fields fuse in the ventral midline and form the heart tube that shortly thereafter starts to beat (DeRuiter et al. 1992). Subsequently, the heart tube loops into the S-shaped heart and becomes displaced towards the right side of the body (Manner 2000), the first morphological sign of the left-right axis. The outflow tract and almost the entire right ventricle are derivatives of a secondary or anterior heart field (Mjaatvedt et al. 2001). The mesoderm that forms the anterior heart field is initially present medially to the

primary heart field. After tubular heart formation it becomes positioned dorsoanterior to the heart tube. For an extended period of time mesodermal precursors are continuously added to the anterior end of the developing heart. Moreover, the anterior heart field also contributes cells to the left ventricle and atria (Cai et al. 2003). The S-shaped heart develops into a four-chambered heart by growth of the cardiac chambers, a process called ballooning, and the formation of the cardiac septa (Christoffels et al. 2000). A further process that is important for the correct connection of the ventricles with the developing great arteries is the remodelling of the inner curvature of the heart loop (Harvey 1999). In the atrioventricular canal and in the outflow tract, cushions are formed that contain large amounts of extracellular matrix and are populated by endocardial cells after epithelial mesenchymal transition (Eisenberg et al. 1995). The cushions are primitive valve-like structures leading to a unidirectional blood-flow. Subsequently several cell populations migrate into the cardiac cushions which develop into the mature valves (van den Hoff et al. 2001). Neural crest cells migrate into the outflow tract and participate in the formation of smooth muscle cells that substitute for the myocardial wall of the outflow tract (Kirby et al. 1995). In addition, cardiac neural crest cells are necessary for the development of the aortico-pulmonary septum, which divides the distal outflow tract into the aortic and pulmonary flow pathways. Another cell type important in myocardial development is the proepicardial serosa (Manner et al. 2001). It develops from the coelomic mesothelium adjacent to the sinus venosus and migrates onto the surface of the heart forming the epicardium and subepicardial connective tissue. Moreover, some cells invade the myocardial layer and differentiate into cells of the coronary vascular system, connective tissue and endocardial cells (Mikawa et al. 1996). The epicardium and the endocardium seem to be signalling centers for the development of the myocardium of the ventricular wall (Chen et al. 2002; Stuckmann et al. 2003). The primitive ventricular chamber contains two different cell populations: the compact layer, consisting of immature myocytes with a high level of proliferative activity that depends on signals from the adjacent epicardium (Kastner et al. 1997) and the trabecular layer formed by differentiated cardiac myocytes (Sedmera et al. 2000). The trabecular cardiac myocytes depend on signals from the adjacent endocardium (Gassmann et al. 1995; Lee et al. 1995; Meyer et al. 1995). This short summary of the major steps in heart development points to a complicated developmental process prone to disturbances that result in congenital heart malformations.

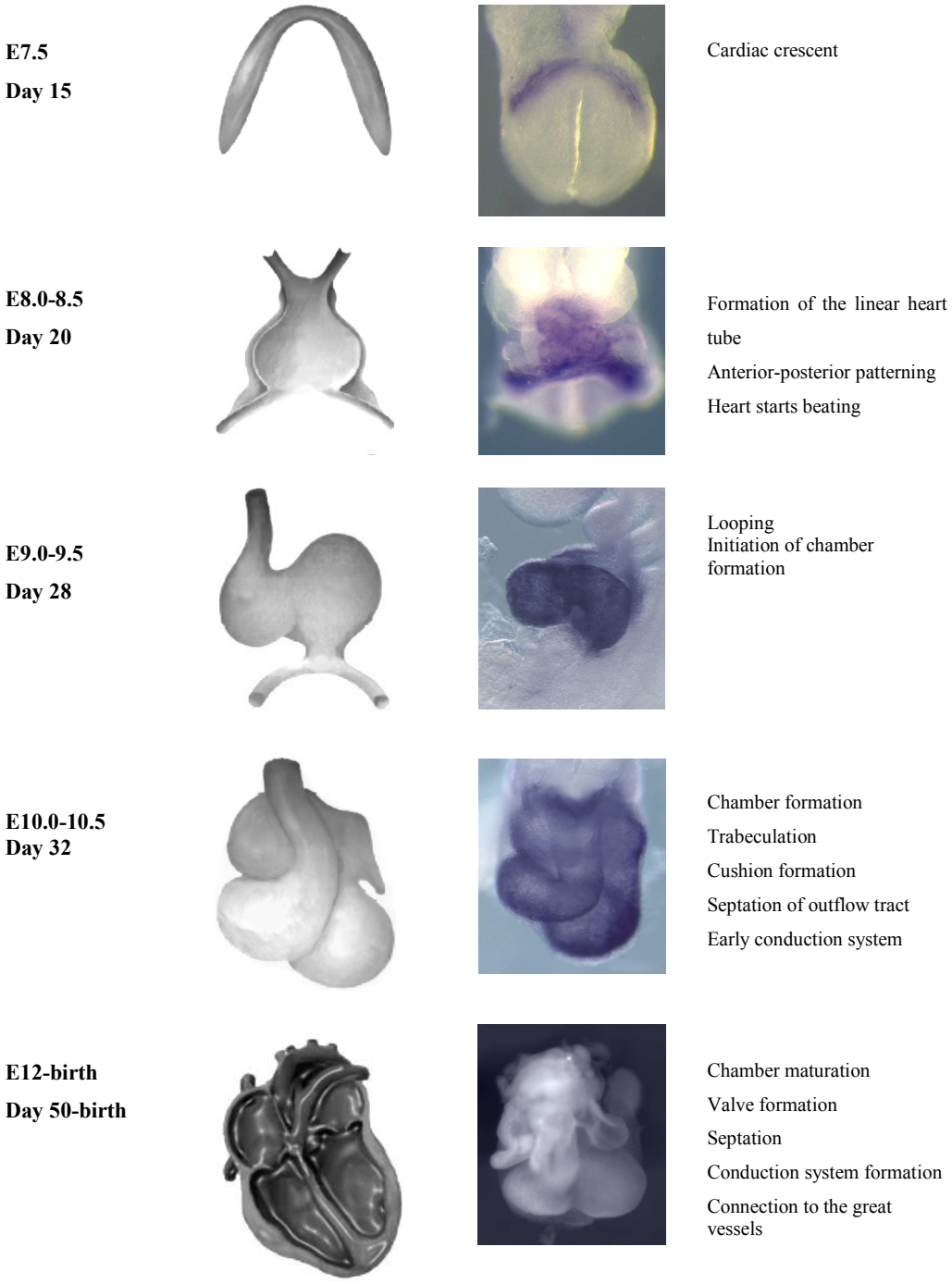

Figure 1.2. Overview of morphological events during the early development of the heart. In the first column the mouse stages (post coitum, e.g. E7) as well as the corresponding human stages in days of gestation are indicated. The second column depicts the schematic development of the heart (modified after Bruneau, 2002). Frontal views are given in rows one to four; row five shows an opened heart.
The third column shows the developing heart of mouse embryos. The fourth column shows the RNA *in situ* hybridisations of whole mount embryos using; row one to two Dpf3 antisense riboprobe and row three to four Nkx2.5 antisense riboprobe. Row five is a dissected mouse heart. Row one, two, four and five are frontal views, row three shows the heart from the left side.

Mouse **Human**	**E7.5** **Day 15**	**E8.0** **Day 20**	**E9.0** **Day28**	**E10.0** **Day 32**	**E12-birth** **Day 50-birth**
CHDs	?	*Cardia bifida *Laterality defects	*Laterality defects *Hypoplastic left heart *Hypoplastic right heart	*Conotruncal defects *Defective valves *DORV *AV canal defects *Tetralogy of Fallot	*ASDs/VSDs *DORV *AV canal defects *Tetralogy of Fallot *Conduction defects

Table 1.1. A brief overview of CHDs that might occur during embryonic development. The mouse stages (post coitum, e.g. E7) as well as the corresponding human stages are indicated.

1.4 Strategies to Identify Disease Causing Genes

Less than 10 % of congenital heart malformations exhibit a Mendelian transmission. However, the analysis of the offspring of adults with sporadic heart malformations revealed a 3 % recurrence rate (Romano-Zelekha et al. 2001), pointing to a contribution of genetic factors in the etiology of CHD.

Traditional approaches aiming to identify disease causing genes are based on chromosome analysis and linkage analysis in syndromes and affected families and have led to the identification of e.g. TBX5, GATA4, NKX2.5, MYH6 (Basson et al. 1997; Li et al. 1997; Schott et al. 1998; Garg et al. 2003; Ching et al. 2005).

Important contributions for the comprehension of congenital heart malformations as well as normal heart development were also provided by the use of model systems such as mouse, zebra fish and chicken. Genes shown to affect development in other species provide a source of candidates for genetic studies in humans.

Another approach is to study the transcriptome of the myocardium in samples collected from individuals undergoing surgical procedures to repair complex heart malformations. The comparison of the transcriptomes of normal and malformed human hearts may point to interesting candidate genes and genetic pathways involved in normal and aberrant development of the heart. Analyses of this type became feasible with the development of the array-technology some years ago. Transcriptional profiling with microarrays offers simultaneous expression analysis of thousands of genes, revealing unique biological insights through patterns of expression and suggesting function of unknown genes. Generally, cDNA arrays are a measurement tool with cDNA (the *probes*) of known sequence immobilised in an orderly arrangement of tens to hundreds of thousands of unique DNA molecules. Labelled cDNA samples (the *targets*) are

simultaneously hybridised to the array and the signal intensity from the bound target is quantified. For a given sequence spotted on the array, the quantity of the corresponding transcript of the sample hybridised is measured using the intensity of the fluorescence or radioactive signal, which should be proportional to the quantity of the corresponding transcript. Finally, the obtained transcript quantities for each gene within each sample are bioinformatically and statistically analysed with respect to the hypothesis that drive the experiment.

A RNA *in situ* hybridisation screen enables the spatio-temporal expression analysis of distinct genes. Here the distinct expression pattern throughout the embryo at different developmental stages can be analysed. Comparing the transcriptomes of normal and malformed human hearts combined with a RNA *in situ* hybridisation screen in mouse embryos provides a powerful tool towards the identification of new genes involved in heart development. Genes differentially expressed when comparing normal and malformed human hearts and expressed during early heart development in mouse are particularly interesting candidates potentially involved in heart development.

1.5 Purpose of the Study

Although clinical diagnosis of CHDs has been defined for more than a decade, the genetic basis of most forms of CHD remains unknown, especially as the complex genetic network that leads together with environmental factors and adaptation processes to the final phenotypes of CHDs makes the mechanistic understanding of disease causing genes and their function challenging.

Recent advances in microarray technology enable the analysis of global gene expression profiles for various disease phenotypes. Despite the usefulness of this technology, only a few studies have been carried out for human CHD so far. Most of the studies available to date either used very limited samples or were based on animal models.

In the study presented here we attempted to identify disease causing signalling pathways in the onset of CHD in human as a multigenetic and multifactorial disorder. Therefore we applied the following strategy:

- In a genome wide approach we aimed to establish molecular signatures of different CHD phenotypes as well as normal hearts by performing array hybridisations using cardiac samples of patients with well characterised phenotypes of ASD, VSD, TOF and RVdis. Special attention was given to careful statistical analysis of these

experiments to extract the different molecular fingerprints. Moreover, the array results needed verification by quantitative Real-time PCR.

- To elucidate the involvement of identified candidate genes in cardiac development investigation of the corresponding spatial-temporal expression patterns during development in model organisms is of great interest. Therefore novel candidate genes were taken from the human study and RNA *in situ* hybridisations of mouse, chicken and zebrafish embryos were performed.
- Finally, the elucidation of a new potential key player in the heart developmental process was envisaged.