4 Discussion

Congenital heart diseases (CHD) are the clinical manifestation of anomalies in heart development, a complex process requiring the precise integration of cell type-specific gene expression and morphological development; both are intertwined in their regulation by transcription factors. Although studies of heart development in fish, frog, mice, chicken, flies and worms have begun to unravel how morphogenesis and hierarchies of developmental control are exerted, the basic mechanism for CHD in human is still incompletely defined. The rarity of large CHD human families and the incomplete penetrance of CHD as a phenotype have limited the usefulness of linkage analysis in identifying causative genes. The genetics of CHD points to the existence of powerful disease modifiers as wide phenotypic spectra are seen in patients harbouring identical disease alleles.

4.1 Genome-wide Array Analysis of Human Heart

The application of cDNA arrays as a large-scale technology for the simultaneous analysis of thousands of genes permits the identification of specific gene expression profiles of the various stages of cardiac development. Moreover, the comparison of gene expression patterns between human normal and congenital malformed hearts or among different types of CHDs will identify differentially expressed genes that may point as markers to the molecular mechanisms involved in normal and CHD states, and perhaps provide new insights for the discovery of novel molecular targets for diagnostics and therapeutics.

In the present study “Human Unigene Set-RZPD 2” cDNA arrays (nylon membrane arrays) from the German Resource Centre for Genome Research were used, which had been the most comprehensive cDNA array resource at that time and enabled us to analyse over 2/3 of the entire set of human transcripts with samples as small as 8µg of total RNA for one assay. Over the last years several further sources of full-genome arrays for human have been established, where GeneChips (Affymetrix Inc., Santa Clara, CA, USA) are the most widely used. Those arrays are made of oligonucleotide stretches directly synthesised on the glass array surface.

Facing the statistical problems of array analysis, the presented study was initiated by the collection of a large amount of samples, enabling a final selection of a patient population that is as balanced as possible with respect to known confounding factors.

After visual inspection of the individual expression profiles using of commonly used *t* test analysis, we observed age and tissue to be major confounding factors and therefore chose a linear model incorporating these factors as our statistical framework. Thus, the final selection of differentially expressed genes was based on statistical relevance (*P* values).

4.1.1 Atrium versus Ventricle

Comparing the two most distinct cardiac structures, namely the atria and ventricles a fraction of genes were found to be differentially expressed. The atria were characterised by higher expression of genes encoding proteins associated with extracellular matrix or actin modulation proteins, such as CST3 and PCOLCE. DNA helicase REQL4 and the translation factor EEF1A were significantly upregulated in atria. For instance, EEF1A-2/S1 protein is known to be activated on myogenic differentiation and delays myotube death subsequently to the apoptotic stress induction (Chambers et al. 1998). KCNIP2 is a likely to be a target for ventricular tachycardia (Kuo et al. 2001; Guo et al. 2002) is even more highly expressed in the human atria than in the ventricle (P=0.01), pointing to a potential role also in atrial arrhythmia.

Genes with expression levels higher in ventricle than in atria belong namely to three major functional classes: cytoskeleton-contraction, metabolism–energy turnover, and cell cycle–growth. Several of these genes are involved in ventricular myocardial disorders: TMP1 (Karibe et al. 2001) is mutated in human cardiomyopathies, FHL1 is downregulated in failing human hearts (Yang et al. 2000), and ANKRD2 is involved in the process of cardiac hypertrophy (Pallavicini et al. 2001).

Another genome wide array study performed with normal mouse hearts showed a similar tendency of expression and confirmed the present study (Tabibiazar et al. 2003). In their work, Dkk3, which acts as a potent inhibitor of Wnt signalling, is expressed in cells of the bulbus cordis and sinus venosus in the 9-day-old embryo. However, its expression becomes restricted to the atria and endocardial cushions by embryonic day 12, suggesting that it plays a role in the establishment of atrial identity(Monaghan et al. 1999). Dkk3 maintains a highly localised expression in the atrium throughout adulthood, whereas it shows little ventricular abundancy, confirming our results and suggesting that it may also be important for the maintenance of atrial identity by antagonizing Wnt signalling (Zhao et al. 2002). Keeping these findings in mind, it has been observed that dishevelled-1 (Dvl1) is significantly more highly expressed in the

ventricles than in the atria of the mouse. An appealing hypothesis is suggested, such that Wnt signalling is inhibited by Dkk3 in the atria and not inhibited in the ventricles as evidenced by high levels of Dvl1 transcripts. Wnt mediators often operate in combination with other pathways such as LIM proteins. Recent studies have shown the four and a half LIM-only protein 2 (FHL2), a novel ß-catenin–interacting protein can function as a Wnt-coactivator (Wei et al. 2003). In the present study in human FHL2 was significantly upregulated in the ventricle which could be confirmed by Tabibiazar.

4.1.2 Comparison of Left, Right Ventricle and Interventricular Septum

The left ventricle (LV) represents the high-pressure system and the right ventricle (RV) represents the low-pressure compartment of normal human heart. These physiological differences were confirmed by the expression profiles, such that, genes encoding proteins involved in cell cycle, cell differentiation, and energy metabolism were found to be downregulated in the RV compared to the LV. For example, Unigene cluster Hs.323099 sharing a significant sequence similarity to ALK3 that is essential for heart development and it is derived from the LV-specific expression analysis.

Comparing LV with the interventricular septum (IVS) revealed similar expression profiles for both, which are concordant with their similar contractile function. This finding could be confirmed in a complementary study in mouse, (Tabibiazar et al. 2003).

4.1.3 Tetralogy of Fallot

Tetralogy of Fallot (TOF) and right ventricular hypertrophy (RVH) exhibited distinct molecular portraits with genes of various functional classes. Even though the right ventricular hypertrophy is part of TOF, two gene expression profiles could clearly be distinguished by comparing once TOF versus normal hearts and once TOF combined with the right ventricular dystrophy (RVdis) as one phenotype (RVH) versus normal heart. Therefore, TOF reveals the molecular signature of the malformation in addition to the adaptation portrait.

Apart from genes involved in cell cycle, a characteristic feature of the TOF signature is the upregulation of several ribosomal proteins. A specific role of ribosomal proteins during cardiac development has been only described for the chicken ribosomal protein L10, which is downregulated in the cardiac outflow tract of chicken embryos with ablated neural crest cells (Kirby et al. 1995).

Expression data reveal a TOF-specific dysregulation of potential targets that could be involved in pathways leading to cardiac dysdevelopment; for example, SNIP1, A2BP1, KIAA1437 and CERD4 (DPF3), which was previously associated to nervous system, are upregulated. SNIP1 interacts with Smad4, a mediator of TGF-ß, activin, and BMP signalling, which are essential for normal cardiac development (Kiehl et al. 2001). A2BP1 belongs to a novel gene family sharing RNA-binding motifs expressed at the developing heart during mouse embryogenesis. KIAA1437 binds k-ras, where k-ras–deficient mice develop a thin ventricular wall and die in early stages of the embryogenesis (Garcia et al. 2000). STK, BRDG1 and TEKT2 were significantly downregulated in TOF. STK33 and its phylogenetic analysis suggests that STK33 may belong to the calcium/calmodulin-dependent protein kinase group, even though, like several other members of the group, it lacks the calcium/calmodulin binding domain and its one of its strongest expression is found in foetal and adult heart (Mujica et al. 2001). BRDG1 is also called “Stem cell adaptor protein 1” and structurally related to STAP-1/ STAP-2. STAP-2 inhibits NF-kappaB transcriptional activation and it is suggested to be involved in potentiation of MAP kinase activation in response to EGF (Minoguchi et al. 2003). TEKT2 is linked to microtubule cytoskeleton organisation, which is necessary for development of the heart.

4.1.4 Right Ventricular Hypertrophy

In RVH, a hypertrophy-specific gene expression pattern was observed of genes mainly involved in stress response, cell proliferation, and metabolism. Intriguing is the upregulation of ADD2, whose homologue ADD1 had been shown to be associated with hypertension in human (Morrison et al. 2002). Another example is HIRIP3, which is a novel histone binding protein that belongs to the HIRA family that was upregulated in RVH. HIRA-HIRP3 containing complex might function in some aspects of chromatin and histone metabolism during development (Lorain et al. 1998). And HIRA is already known for its involvement in heart defects. Since the expression of several genes of the RVH signature was found to be similar to expression levels in LV, the next analysis focused on whether the molecular adaptation to pressure overload could lead to a molecular transition from right to left ventricular characteristics. The expression difference between each gene in RVH and that in normal RV samples was compared to normal LV samples. The significant positive correlation indicated that the genes

dysregulated in RVH have a tendency to behave similar in the disease state as in normal LV tissue.

4.1.5 Tetralogy of Fallot and Right Ventricular Hypertrophy

To separate in TOF the malformation from the adaptation-specific molecular signature, RVH genes were subtracted from genes belonging to TOF molecular portrait. A conservative subtraction was enabled by a 10 times lower P-value threshold for RVH associated genes compared to TOF. Data revealed 88 clones including functionally uncharacterised ESTs beside known genes. For example, TMOD2 previously related to neuronal system and TMOD4 causative for dominantly inherited muscular dystrophy associated with atrioventricular conduction disturbances and dilated cardiomyopathy (Cox et al. 2000). Another example is yeast vacuolar protein sorting 35 (VPS) required for endosome-to-Golgi trafficking. Knock-out mice of its homologs SNX1 and SNX2 show a severe developmental delay phenotype including heart defects and unfused neural folds and heart (Haft et al. 2000; Schwarz et al. 2002).

4.1.6 Ventricular Septal Defect

To obtain a molecular portrait that is not influenced by biomechanical adaptation processes, we studied RA samples of patients with VSD, intact tricuspid valve, and normal RA pressure. We observed a molecular signature dominated by downregulated genes with respect to the other RA samples. As seen in TOF, several ribosomal proteins are differentially expressed, but here they are downregulated. Other VSD-specific genes encode ion transporters or function during vertebrate development. The differential expression of ion channels was restricted to solute and potassium channels. During looping of the embryonic heart it is assumed that the inner and outer curvature of the embryonic heart express solute channel proteins at different levels (Moorman et al. 2003). Through an imbalance in the expression levels of those solute channels could potentially lead to the disease state in VSD. A literature study of the genes markedly downregulated in VSD, pointed to functional classes such as cell proliferation, differentiation during embryogenesis and apoptosis.

The global analysis of the VSD molecular signature showed a general transcriptional downregulation compared with TOF as well as with all other sample categories. The question of whether the observed downregulated transcription mirrors a reduction of

essential proteins leading to incomplete fusion, an underdeveloped atrium, or an unknown physiological process will have to be elucidated in the future studies.

4.2 Characterisation of Dpf3

Development of the heart is characterised by complex morphogenetic events that are in part directed by complex patterns of gene expression. It is also considered to be an additive process, in which additional layers of complexity are added in form of modules (atria, ventricles, septa, and valves). Each module confers an added capacity to the heart and can be identified as individual structures patterned in a specific way. An understanding of individual modular steps in cardiac morphogenesis is particularly relevant to discover the causes of CHDs, which usually involves defects in specific structural components of the developing heart. Organ formation requires integration of cell type-specific gene expression and morphological development; both are orchestrated in their regulation by transcription factors. Even though many transcription factors were identified as regulators of cardiac gene expression, the transcriptional regulation of cardiac morphogenesis is still not well understood. For a transcription factor to be considered directly involved in heart development, it must be expressed in the developing heart tissues and show an influence on processes that impact the morphogenesis of the heart (Bruneau 2002).

In this respect Dpf3 was selected for further detailed characterisation and approached with *in situ* hybridisation of mouse, chicken embryos, zebrafish, Northern blot analysis and all-*trans*-retinoic acid treatment in chick.

4.2.1 Dpf3 in Species Comparison

Dpf3 belongs to the D4 gene family representing transcription factors previously associated to the nervous system. The D4 gene family is characterised by a C2H2 type zinc finger and a double PHD finger domain (Ninkina et al. 2001). Sequence analysis of the completed fruit fly genome also demonstrated that D4 family is present in invertebrates. In an evolutionary perspective of all higher vertebrates, apart from the fruit fly, DPF3 is represented by two splice forms, namely DPF3/1 and DPF3/2 with differences at the protein level (Nabirochkina et al. 2002). Apart from the known two transcripts of DPF3 in mammals, chicken has two additional splice forms (DPF3/1a and DPF3/2a) and in zebrafish Dpf3/2 is represented by three additional variants. The

functional relevance of those additional splice forms still remains to be explored and might be redundant. Although vertical and horizontal transitions may occur during evolution, it has not been proven so far for Dpf3. One hint could be that the majority of the DPF3 introns are substantially larger compared to introns of the other two D4 family members (Mertsalov et al. 2000).

Functional studies of Dpf1 and Dpf2 suggest that members of the D4 family participate in regulation of cell survival. Overexpression of at least one isoform of Dpf1 protein in NGF-dependent (nerve growth factor) SCG neurons rescues these neurons from death after withdrawal of NGF. Further Dpf2 is crucial for the apoptotic cell death caused by IL-3 deprivation of IL-3 dependent myeloid cell line (Gabig et al. 1994)

4.2.2 C2H2 Type Zinc Finger and Double PHD Domain

The abundance and diverse sequences of PHD motifs found in nature suggest that this domain is able to mediate many different interactions and may fulfill a variety of cellular roles. The PHD motifs identified to date are between 50 and 100 residues in length and share a $\underline{C}\text{-}X_{1\text{-}2}\text{-}\underline{C}\text{-}X_{9\text{–}21}\text{-}\underline{C}\text{-}X_{2\text{–}4}\text{-}\underline{C}\text{-}X_{4\text{-}5}\text{-}\underline{H}\text{-}X_{2}\text{-}\underline{C}\text{-}X_{12\text{–}46}\text{-}\underline{C}\text{-}X_{2}\text{-}\underline{C}$ consensus sequence, where the 8 underlined residues ligate two zinc ions (Pascual et al. 2000; Capili et al. 2001). The sequence can be considered to comprise four sequential pairs of zinc ligands, separated by three loops (X9–21, X4-5, and X12–46; or L1, L2, and L3, respectively). The alignment of PHD sequences (Aasland et al. 1995) from different proteins shows that little conservation exists in the L1 and L3 sequences. This variation suggests that the L1 and L3 regions may be responsible for specifying the binding properties of individual PHDs (Pascual et al. 2000; Capili et al. 2001; Kwan et al. 2003). Apart from the general structure of proteins within the PHD family, the D4 family has a cysteine/histidine-rich region on the C-terminus, which is called the d4-domain; a double-paired finger motif consisting of two tandemly arranged PHD finger domains. PHD fingers are hallmarks of many proteins implicated in chromatin-mediated transcriptional control (Aasland et al. 1995; Saha et al. 1995; 1997; Gibbons et al. 1997) and have some structural similarity with the LIM domain and RING fingers, two well studied types of paired zinc fingers (Sanchez-Garcia et al. 1994; Schmeichel et al. 1994; Saurin et al. 1996). A single Krüppel-type zinc finger, which has nucleic acid binding capacity, and a novel domain, domain 2/3, which has no sequence similarity to any other known protein sequences, are localised in the N-terminal part of the DPF1 protein (Buchman et al. 1992; Mertsalov et al. 2000). However, some species lack these domains along with a nuclear localisation

signal and a stretch of negatively charged amino acids in the DPF1 proteins. The second member of the d4 family, named DPF2 (Chestkov et al. 1996; Mertsalov et al. 2000) or Requiem (Gabig et al. 1994; Gabig et al. 1998), has the same domain organisation of the encoded protein but does not have isoforms generated by differential splicing and is expressed in all tissues and cells studied so far. DPF3 is characterised by two splice variants in human and mouse with DPF3/1 encoding only one incomplete PHD finger and DPF3/2 encoding the double PHD finger. Thus, it can be suggested that different functions of DPF3 are encoded in the full or partial structure of the PHD domain, and the zinc finger part may facilitate the other functions associated with other domains, such as the newly discovered TAZ (transcriptional adaptor ZnF) domains, which are present to the CBP/p300 family. In addition to its TAZ domain CBP contains a PHD domain which mediate protein-protein interactions in multi-protein complexes and might be involved in chromatin remodelling and ubiquitination (Aasland et al. 1995; Liew et al. 2000; Vo et al. 2001; Matthews et al. 2002). The three Znf domains in CBP/p300 proteins are crucial for the interaction of these widely expressed tumour suppressor proteins with a surprisingly large number of transcription factors (Vo et al. 2001).

A further characteristic Znf-PHD domain containing gene is WHSC1, which has causative impact on the development of the Wolf-Hirschhorn syndrome (WHS). This partial deletion syndrom is characterised by various disorders such as asymmetry, mental retardation, a variety of skeletal anomalies, uropathy, hearing defects, optic nerve defect and including congenital heart malformations such as patent ductus arteriosus associated with ventricular septal defect or aortic insufficiency, isolated atrial septal defect, pulmonary stenosis as well as various severe neural disorders (Battaglia et al. 1999).

4.2.3 Dpf3 Splice Variants

Northern blot analysis showed two splice forms of DPF3 in humans corresponding to Dpf3/1 and DPF3/2 homologs of mouse transcripts. A previously presented additional splice variant in human could not be confirmed in our study. Quantitative analysis of DPF3 in adult human hearts as well as embryonic mouse hearts revealed higher expression levels of DPF3/1 than DPF3/2. These findings suggest that DPF3/1 plays the major role during heart development and maintenance. Even both splice variants are significantly upregulated in the right ventricular tissue of patients with TOF compared

to healthy individuals, this effect is several magnitudes of order higher for DPF3/1. These findings suggest that upregulation of DPF3/1 may have an impact on the development of TOF. The impact of the titration of transcription factors has recently been shown for several cardiac specific transcription factors, like BAF chromatin remodelling complex in Tbox proteins and Gata-4 in mouse models for CHD (Lickert et al. 2004; Pu et al. 2004).

4.2.4 Dpf3 Expression in Comparison to Markers of Heart Development

During early embryogenesis, mouse embryonic day E7.0-7.5, restricted expression of Dpf3/1 was observed in cardiac crescent where the heart is derived from the anterior splanchnic mesoderm and forms from two crescent-like cardiogenic plates. (Gittenberger-de Groot et al. 2005). Dpf3/1 partially shares this expression pattern with Nkx-2.5, Gata-4 and Tbx5 (Bruneau et al. 1999), which are well known markers for the cardiac crescent. Moreover, the expression pattern of CoupTFII (Pereira et al. 1999) was observed to be overlapping with Dpf3/1 in the caudal part of the cardiac crescent. At E8.0-E8.5 the linear heart tube is formed with cardiac mesodermal bands merging in the midline and the anterior intestinal portal (AIP) forms. At this stage Dpf3/1 expression was detected in the cardiogenic region and also in allantois. Moreover, Dpf3/1 expression pattern was observed in the presomitic mesodermal stripes localised in the anterior compartment of the condensing and newly formed somites overlapping with the expression pattern of Jade-1 (Tzouanacou et al. 2003).

At E8.5 embryonic hearts start to beat and primary myocardium is developed. At this stage Dpf3/1 was expressed from cranial to caudal in the complete heart tube, characterised by the outflow tract, the common ventricle and the inflow region (primitive atrial pool and sinus venosus). In addition to known cardiogenic markers, Dpf3/1 expression overlapped with structural genes such as Mlc2a in the atrial region, Mlc2v in the ventricle specific region, and Mhc in the complete heart tube, respectively. Moreover, Dpf3/1 expression was overlapping with Bmp2 in both, outflow and inflow tract, and Bmp4 in the outflow region.

From embryonic stage E9.0-E9.5 onwards cardiac looping, one of the most important events of morphogenesis takes place. The heart starts to loop rightwards, and this is the first time, where the morphological boundaries of the chambers can be observed. At this stage, Dpf3/1 expression was detected in the outflow tract, the common ventricle and the common atrium, the sinus venosus overlapping partially with Nkx-2.5 and Gata-4

expression domains. Detailed analysis showed Dpf3/1 expression in the initial chamber myocardium. Moreover, expression was detected in the somites as well as in the developing midbrain and in the liver at this stage. Starting from E10.5 expression of Dpf3/1 in the cardiac region decreases, whereas expression in the developing brain and liver is getting more pronounced. The Dpf3/1 signal detected in the developing midbrain was overlapping with the expression pattern of the other D4 family member Dpf1. Weak cardiac Dpf3/1 expression persists throughout all further embryonic stages investigated in this study (until E13.5 embryos).

Dpf3/2 expression was first detected at E9.5 in the developing liver overlapping with Dpf3/1, but it did not show any cardiac specific expression during development. Furthermore, form E10.5 onward Dpf3/2 signals were observed in the developing brain, consistent with the expression of Dpf3/1 and Dpf1.

Next, chicken *in situ* hybridisation with Dpf3 probes gave some additional insights into the possible roles of the gene, as in chicken distinguishing early structures is easier. At chick embryonic stage HH7, Dpf3 expression pattern overlapped completely with Bmp2 and partially with Nkx-2.5, Gata-6 and Isl1. At HH10, Dpf3 expression was restricted to the sinoatrial node and the mesodermal stripes, which was consistent with Bmp2 and Gata-6 expression. In addition, Bmp4 expression was overlapping with Dpf3 on the anterior part of the sinoatrial node but not in the anterior intestinal portal. The markers Gata-4, Gata-5 and Nkx2.5 cover almost the whole cardiac region, therefore overlapping with Dpf3 expression in the sinoatrial node and mesodermal stripes.

With the looping at HH12, Dpf3 expression was observed mainly in the anterior intestinal portal, the inflow region, the primitive atrium and the atrioventricular groove, a weaker expression was detected in the ventricle. This expression pattern shows similarities with the results from mouse embryos at the correlative stage E9.5. In this stage, while the expression of Dpf3 was covered by Gata-4 and Nkx-2.5 transcription factors and Bmp5 and Bmp6 signalling peptides, Gata-6 showed partial overlapping in the mesodermal stripes. At HH12 Bmp2 is restricted to the outflow tract, the atrioventricular groove, the outer edges of the sinoatrial node and the anterior intestinal portal. In contrast no Bmp2 expression is observed within the common atrium and the ventricle. Moreover, expression of Dpf3 overlapped with Bmp4 in the atrioventricular groove. At stage HH16 embryos showed a restricted expression of Dpf3 in the atrium and sinus venosus. In addition, from HH25 onwards, section hybridisations showed that

expression of Dpf3 is decreasing, but remaining in lower levels in the developing heart resembling its expression in the mouse.

In zebrafish embryos, expression of Dpf3 was observed at the complete head region of the embryo, however, because of this strong expression in the nervous system it was impossible to differentiate a possible expression at the developing heart.

4.2.5 Possible Roles of Dpf3 in Developmental Regulatory Pathways

The heart forms with complex interactions of different germ layers under the influence of several positive and negative signalling inputs (Gittenberger-de Groot et al. 2005). These inputs can activate and/or deactivate cardiac transcription factors. Heart lineages are induced at lateral field margins of the head precursor zone, where Wnt signalling is inhibited but BMP signalling is maintained (Marvin et al. 2001; Schneider et al. 2001). BMPs show their effect by regulating the Smad proteins Smad1, Smad3 and Smad5 during cardiac induction. This signalling cascade directly activates several cardiac transcription factor genes, including Nkx2.5 (Sparrow et al. 2000; Liberatore et al. 2002; Lien et al. 2002; Solloway et al. 2003). Interestingly the DPF3 protein was upregulated in TGFβ-1 treated human microvascular endothelial cells (Lomnytska et al. 2004). At this moment it is also possible to mention the importance of the TGFβ pathway in congenital heart diseases. Allen SP and colleagues demonstrated the congenital defects ranging from outflow tract to ventricular septum anomalies via dysregulation of Bmp2 and Bmp4 (Allen et al. 2001). Although, the roles of BMPs are not easy to distinguish due to the redundancy in vertebrates, interestingly, expression patterns obtained from chicken *in situ* hybridisation with Dpf3 showed a concordant result and overlapping expression patterns with chicken Bmp2 that was demonstrated previously (Somi et al. 2004).

There is also another possibility for involvement of the TGFβ family in heart development. It has been suggested that members of the TGFβ family are downstream effectors of the retinoic acid signalling pathway (Harvey et al. 1999), which has already been described to be involved in the development of the heart. The specific role of TGFβ in the RA signalling pathway, however, remains to be elucidated (Zile 2004). Moreover, migratory neural crest cells contribute to the outflow tract, which might explain the outflow expression of Dpf3/1 starting at the looping heart stage.These migratory neural crest cells have previously reported to be regulated by the TGFβ pathway (Allen et al. 2001).

The cardiac crescent in amniotes is shaped not only by the positive influences provided by BMPs and other inducers, but also through repressive signals emanating from axial mesoderm and neural plate. At this point, canonical "Wnt/β-catenin" signalling and the non-canonical "Wnt/JNK" pathway are necessary to form the cardiac fields. The balance of Wnts and their antagonists plays a critical role in cardiogenesis (Solloway et al. 2003), but at the moment there is no functional data available connecting Dpf3 directly to Wnt pathways via BMPs.

There are two known origins of the initial heart field called primary and secondary (anterior) heart fields. Early expression of Dpf3 might be under indirect control of heart forming transcription factors, like Gata-4, -5, -6, Nkx-2.5, Tbx-2, 5 and Isl1 orchestrating the cardiogenic region with their key expression during embryogenesis (Cai et al. 2003). Gata-4 and Nkx-2.5 were shown to be mutual co-factors in their synergistic activation (Durocher et al. 1997) and Gata-4 is expressed in anterior endoderm and mesoderm and may help to restrict the cardiac fate (Alsan et al. 2002). Nkx-2.5, encoding a homeodomain factor, is also expressed early in the cardiogenic program and is often used to delineate cardiac progenitors. Dpf3/1 showed an overlapping expression pattern with Nkx2.5 from cranial to caudal of the cardiac crescent in mouse. Nkx-2.5 may also play a role in the Isl1 expressing secondary heart field, although it clearly is not as crucial as Isl1 for the secondary heart field, given the contrasting phenotypes of Isl1 and Nkx-2.5 (Cai et al. 2003). Dpf3/1 was observed in presomitic mesoderm and in later stages in the first somites like Jade1, which is another member of the Znf-PHD finger proteins (Tzouanacou et al. 2003). This pattern is reminiscent of genes involved in segmentation of the paraxial mesoderm and determination of the anterior/posterior identity (Saga et al. 2001; Zakany et al. 2001; Pourquie 2002; Tzouanacou et al. 2003). It reveals that during early development, Dpf3 may play different roles in a tissue specific manner. Dpf3/1 expression was not clearly observed at the anterior heart field although both fields are not easy to distinguish. During the tubular heart formation, the secondary heart field situates anterodorsal to the tubular heart tube and contributes to the outflow region and future RV. Isl1, Fgf8 and Fgf10 are known markers for that region (Waldo et al. 2001; Cai et al. 2003). Since the expression of Dpf3/1 was not overlapping with these markers from stage E10.5 onward, possible roles of Dpf3 might not be related to the anterior (secondary) heart field.

As the specification is getting more complex after E9.5, previously defined transcriptional domains might control different programs in different stages of heart

development. In this respect expression of Dpf3 showed similar expression to Nkx-2.5, nearly covers the complete heart forming region. Interestingly, the expression pattern observed for CoupTFII (Pereira et al. 1999) is overlapping with the Dpf3/1 specific signals.

In zebrafish it was impossible to differentiate a possible Dpf3 expression at the developing heart, because of its strong expression in the nervous system. However, there is a potential expression at the dorsal aorta, which might indicate a role for Dpf3 in angiogenesis. This is supported by a recent study in mouse, showed an involvement of CoupTFII in angiogenesis, where CoupTFII shares the sinus venosus expression pattern with Dpf3/1.

From E10.5 onwards, the expression of Dpf3 became weaker in the heart compared to previous stages. This result may be explained with a previously suggested model (Christoffels et al. 2000). After the crescent stage, development of the heart can be divided into two levels, the primary program that is mainly covering the linear heart tube and the looping heart characterised by primary myocardium. The secondary program is involved in the ventral-dorsal enlargement of the chamber myocardium. Formation of atrial and ventricular compartments in their correct A/P and D/V positions in the linear heart tube presumably requires positional information encoded by A/P and D/V signals, and this may involve secreted peptides and transcription factors (Xavier-Neto et al. 1999; Christoffels et al. 2000). An additional candidate for an A/P polarity-defining pathway is the retinoic acid signalling system, which has been shown to be dynamically involved in specification of posterior (sinoatrial) structures and the regulation of gene expression along the A/P axis (Xavier-Neto et al. 1999). For example, the Irx4 expression pattern may demarcate the limits of the A/P region in the linear heart where the ventricular chamber myocardium is formed. This implies that the combinatorial action of A/P signalling such as retinoic acid and/or Irx4 and maybe Dpf3 together with a second signal along the D/V axis defines the sites of chamber myocardium within the linear and looping heart (Christoffels et al. 2000). Once the sites were defined, expression of Dpf3 might not be needed in later stages, thus the expression of the gene decreases compared to previous stages but never disappears as it is common to most of the A/P patterning genes. Also the results of section *in situ* hybridisations in chicken confirm that Dpf3 expression remains in lower levels in the myocardium during stage HH25 and HH30.

Interestingly, there is evidence that Dpf3 may have an active role within the retinoic acid signalling pathway. A recent publication showed that liver specific HNF4α binds to the DPF3 promoter, which could explain the expression of Dpf3 in the septum transversum and later on at the liver (Odom et al. 2004). This binding site is shared by HNF4α and COUPTFs (I and II). Upon close examination of this binding site, it became evident that the binding site is also a retinoic acid response element type (RARE) DR1 (Kurokawa et al. 1994; Forman et al. 1995). RAREs/DR1 are specially recognised by RAR/RXRs. CoupTFII was shown to be expressed at the sinus venosus and Dpf3 is also strongly expressed at the sinus venosus and throughout the atrium from chicken embryonic stage HH10 onwards. It has already been reported that retinoic acid is crucial for chicken embryogenesis from stage HH8 onward for a correct determination of cell fate (Zile 2004).

Retinoids regulate gene expression through binding to specific nuclear receptors that in the following bind their DNA response elements and up- or downregulate transcription. RAR is the receptor for all-*trans*-RA. RXR is the receptor for 9-*cis*-RA. RXR and RAR belong to a large super family of related nuclear hormone receptors, which share a common architecture. There is a conserved DNA binding domain (DBD) and a ligand-binding domain (LBD). These factors use their LBD to bind to their cognate retinoids and their DBDs to bind to response sites from which they regulate the genes. RAR and RXR are related to each other. Cross interactions occur in heterodimer form between RXR-RXR, RXR-RAR, RXR and other nuclear receptors (Zile 2004) including the orphan nuclear receptors CoupTFI and CoupTFII (Barger et al. 1997; Malpel et al. 2000). The relationship between CoupTFs and retinoic acid signalling was shown in several publications (Lutz et al. 1994; van der Wees et al. 1996; Huggins et al. 2001). Also, CoupTFI or CoupTFII is known to bind to promoters in a tissue specific manner. Two mechanisms might be suggested according to previously drawn models (Tsai et al. 1997) (Malpel et al. 2000; Park et al. 2003). In the first one retinoic acid induction can lead to activation of RARs at protein level where RAR and RXR forms heterodimers and the induction RA diminishes the COUPTFs in transcriptional level. Hence, the amount of RXR/COUPTF complex will be less than the amount of RAR/RXR complex and, therefore less RXR/COUPTF will bind to the RARE/DR1 site of the Dpf3 promoter and the transcription of Dpf3 will decrease. In the second scenario, retinoic acid will activate the CoupTFs in transcriptional level and the COUPTF/RXR complex will bind to the RARE/DR1 site of Dpf3 promoter and activate its transcription.

To test these hypothesis in vivo, and to find out the possible interaction between retinoic acid and Dpf3, chicken embryos were cultured and treated with all-*trans*-retinoic acid and subsequently hybridised with a chicken Dpf3. Apart from the rearrangements in development of the body axis, Dpf3 expression was increased at the sinus venosus, anterior intestinal portal and cardiac mesodermal bands compared to untreated ones. Furthermore, treatment of RA lead to altered neural tube expression of Dpf3.

These results suggest an appealing hypothesis for the regulation of Dpf3 and its connection with CoupTFII in the retinoic acid-signalling pathway during embryonic development. Transcriptional activation of CoupTFII in sinoatrial region of the embryonic heart increased the expression of Dpf3/1. The second effect of all-*trans*-retinoic acid was observed at the nervous system. In this case, application of retinoic acid activated the RARs and decreased of CoupTFs via activation of RAR in protein level. Thus the competition of RAR-RXR and RXR-CoupTF complexes for DNA binding sites was shifted to increased binding of RAR/RXR to the RARE/DR1 resulting in decreased expression of Dpf3 in the neural tube.

4.3 Outlook

Additional studies will be necessary to elucidate the role of the identified genes in normal and diseased human hearts. For further prospect, several important points need to be explored in the future to elucidate the mechanisms regulating combinatorial interactions at different stages of cardiac development and their connection to CHDs. One involves analysis of post-translational regulation of each protein in a given complex and its effect on complex assembly and function: acetylation/deacetylation as well as phosphorylation/dephosphorylation events are probable key regulatory mechanisms that modulate activity of many transcription factors in different cellular context such as proliferation, differentiation, and apoptosis (Nemer et al. 2001).

Many of the genes found to be involved in cardiac development as well as CHD pathology are remaining unknown in terms of their functional roles. Apart from the expression analysis, further studies will have to elucidate the molecular pathways of CHDs.