Molecular and genetic basis of congenital conotruncal heart defects
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Summary

Congenital anomalies of the heart are the leading cause of birth defect-related infant morbidity and mortality, with a prevalence of up to 1% in newborns and 10-20% in still births. Certain defects are rooted in genetic or environmental disturbances affecting the developing heart. Our understanding of cardiac development has increased significantly in the last fifteen years. Advances in molecular biology have allowed the generation of animal models of heart disease and have enforced the study of gene disruptions restricted to organs and specific cell types. Of particular interest in this context is the upcoming field of progenitor cell biology, which aims to unravel the genetic, epigenetic and molecular mechanisms that control the fate of cells.

The heart is the first organ to form in the developing embryo, and is vital for the distribution of oxygen and nutrients and the disposal of metabolic waste products. Heart formation is initiated between 17 and 19 days of human development, corresponding to 7.5 days of mouse development. At these stages, mesodermal cardiogenic precursors in the heart-forming fields respond to cues originating from different but adjacent tissues and start to activate a number of transcriptional programs that direct the differentiation of these precursors into cardiomyocytes. Subsequent to the migration of the precursors to the midline, cardiomyocytes differentiate forming the cardiac crescent, which, in turn, transforms into a bowl-shaped layer of myocardium that fuses at the dorsal side to form the cardiac tube. The tubular heart detaches from the ventral pericardial wall but remains attached to the pericardial wall at the arterial and venous poles. Localized differentiation and proliferation processes will subsequently transform the human embryonic tube-shaped heart into a fully septated four-chambered heart, comprised of myocardial and non-myocardial cells.

The cells that form the embryonic ventricle of the heart are the first cardiac precursors to differentiate, and are thus collectively referred to as the primary or first heart field (FHF). A large number of cardiac progenitor cells remain present as an undifferentiated subpopulation medially and caudally to the cardiac crescent and tubular heart, and maintain their proliferative level before being added to the heart. These cardiac precursors are added after the formation of the tubular heart, and therefore have been dubbed the second heart field (SHF). The SHF pool of cardiac progenitors was first described several decades ago in chicken embryos, by demonstrating the dynamic contribution of cells after the formation of the primary heart tube. More recently, retrospective clonal analysis in the early developing mouse heart as well as several mouse studies have revealed that the onset of differentiation correlates with the addition of cells to the heart. These findings have led to the current paradigm that the heart tube elongates by recruitment of SHF cells that give rise to the outflow tract, right ventricle and ventricular septum and to the remaining part of the left ventricle and the atria. Taken together, the FHF represents the cardiac precursors of the primitive myocardial crescent or bowl, and is important for the developing primitive ventricle of the tubular heart, whereas the remainder of the heart requires the ongoing contribution of SHF cells. Impaired development of the SHF particularly impacts on the arterial pole, accounting for approximately one third of all congenital heart disease cases. Unravelling the regulatory networks that guide the transition of progenitors into distinct cell
types is of particular importance for gene and stem cell-based therapies and may provide opportunities to restore the function of malformed or failing hearts.

The work described in this thesis focuses on the developmental mechanisms involved in the process of SHF and arterial pole development, and aims to increase our insight into the genetic basis of congenital defects of the arterial pole of the heart. In Chapter 1, we review the research that has led to the current paradigm of heart development, with a primary focus on the developing arterial pole and second heart field progenitor pool.

In Chapter 2, we focus on the developmental remodelling of the chicken cardiac outflow tract. Right ventricular precursors in the chicken heart were thought to be present in the linear heart tube and not to reside as cardiac progenitor cells outside the heart until being recruited to the arterial pole. The myocardial wall of the mouse outflow tract is known to become part of the right ventricle, and non-myocardial cells are continuously added to form the great vessels and branching arteries. As a first step to elucidate this issue in the chicken heart, we visualized the myocardial cells of the chicken outflow tract by fluorescent immunohistochemistry and determined the changing distal myocardial border by measuring the length of the myocardial and non-myocardial cells of the outflow tract. We found that a non-myocardial component is only evident after stage Hamilton/Hamburger (HH) 22 between the distal myocardial border and the pericardial reflection and that the non-myocardial portion elongates, whereas the myocardial component shortens. To address the issue of shortening, we labelled small groups of outflow tract cells using the lipophilic fluorescent marker DiI and labelled hearts at HH16 and HH22 in ovo, and traced the fate of the DiI-labels 96 hours following re-incubation. We observed that a significant part of the myocardial outflow tract was incorporated into the right ventricle, and that programmed cell death played a minor role in the disappearance of the myocardial cells. We demonstrate that the avian trabeculated free wall of the right ventricle is derived from SHF myocardium and that the developmental origin of the right ventricle is therefore evolutionarily conserved in birds and mammals.

In Chapter 3, we re-evaluated the development of the human aortic arch system, because it was unclear whether the text book representations were based on original observations in human embryos, or on the configuration found in lower vertebrates. Anomalous patterning and remodeling of the embryonic arterial pole can result in anomalies of the aortic arch and great vessels, representing approximately one-third of all congenital cardiovascular defects. In contrast to recent mouse and chicken studies showing the developing pharyngeal arch arteries in high detail, the most accurate overview of human aortic arch system development is almost a century old and is unfortunately often incorrectly interpreted. In addition, molecular data relevant for the development of the human aortic arch system are entirely lacking. We aimed to determine whether current animal-derived insights are applicable to the human situation by generating interactive three-dimensional reconstructions of the pharyngeal arch arteries and related structures, and by studying the expression patterns of developmental regulators in human embryos. This would also facilitate unbiased interpretations and comparisons with previously generated data. Our findings provide an accurate overview of the remodeling processes that transform the pharyngeal arch arteries into the distinct components of the human aortic
arch and tributaries. Unfortunately, the developmental origin of these components has largely been theorized by observing congenital malformations of the outflow tract and great arteries. Lineage and cell-labeling experiments, demarcating distinct parts of the arch arteries, are therefore critical to unambiguously assess the developmental origin of the distinct components of the human arterial pole. We submit that the mechanisms underlying human and mouse aortic arch system development are largely conserved and, as such, support future genetic and molecular analyses using animal models of congenital heart disease. The interactive presentation of this remodeling process of the arterial pole and great arteries is of educational and clinical value and may further increase our understanding of the etiology of a variety of aortic arch abnormalities. Future genetic and molecular analyses of the developing vasculature using animal model systems will aid in unraveling the underlying pathogenesis of common aortic arch malformations.

In Chapters 4 and 5, we elaborate on the genetic and molecular underpinning of the development and deployment of the SHF. We focus on three members of the T-box family of transcription factor, Tbx1, Tbx2 and Tbx3. In Chapter 4, we explored the underlying mechanisms for the loss of SHF cells, and subpulmonary myocardial precursors in particular, in mice lacking Tbx1, an important regulator of SHF and outflow tract development. In human, Tbx1 has been implicated in 22q11.2 Deletion Syndrome (22q11.2DS), caused by an microdeletion of up to 3 Mb in chromosome 22. Recent findings have revealed that the subpulmonary myocardium shares a lineage relationship with venous pole precursors, albeit the specification and segregation of these progenitors is not fully clear. We first identified the transcriptional features of the outflow tract wall by microarray-based gene expression profiling of the inferior and superior wall of the distal outflow tract. A number of transcripts were particularly enriched in the inferior outflow tract, including Sema3c, EFHD1, BARX1, DLX2 and NRP2. Interestingly, these proteins are involved in neural crest cell migration, differentiation, and development, and are required for the septation of the outflow tract into the great vessels, demonstrating that future subpulmonary myocardial cells in the inferior outflow tract have a distinct gene expression profile that is expected to influence neural crest influx to facilitate outflow tract septation. Using genetic tracing experiments, we furthermore show that loss of Tbx1 reduces the number of precursors of the subpulmonary myocardium, explaining the septation defects observed in Tbx1-deficient foetuses. Additional genetic and Dil tracing experiments and three-dimensional reconstruction tools were deployed to determine what happened with the subpulmonary myocardial precursors. We observed that proliferation and anterior movement of caudal SHF cells were affected by loss of Tbx1, and that anterior SHF cells acquired a posterior SHF fate, identifying a crucial role for Tbx1 in prepatterning the cardiac progenitor pool. We demonstrate that Tbx1 controls venous as well as arterial pole development by regulating the segregation of a common cardiac progenitor pool into different arterial and venous sublineages, providing new insights into the etiology of CHD and 22q11.2DS phenotypes and how arterial and atrioventricular septal defects can coexist.

The T-box family members Tbx2 and Tbx3 are also known to be required during OFT development. We used mouse genetics and gene expression analyses to study the combinatorial requirements for Tbx1, Tbx2, and Tbx3 in the arterial pole and pharyngeal region (Chapter 5). Our study indicates that Tbx1, Tbx2, and Tbx3 constitute a T-box regulatory network that controls OFT and pharyngeal development. Tbx1 was found to be
required for the expression of Tbx2 and Tbx3 in pharyngeal and neural crest-derived mesenchyme. As Tbx1 is not expressed in neural crest cells the effect on Tbx2 and Tbx3 in this cell type is likely to be indirect, mediated by altered intercellular signaling between Tbx1 expressing mesoderm or pharyngeal epithelia and neural crest cells. In addition, loss of both Tbx2 and Tbx3 causes overall growth impairment and hypoplasia of the OFT and right ventricle suggesting defective SHF deployment. Tbx1 and Bmp4 were up-regulated and Shh was decreased in the ventral endoderm in the absence of Tbx2 and Tbx3, suggesting that Tbx2 and Tbx3 directly regulate the expression of these genes in this domain. We provide evidence for the functional importance of a cross-regulating Tbx1/Tbx2/Tbx3 network, as embryos lacking Tbx1/Tbx2 or Tbx1/Tbx3 present similar severe pharyngeal and heart tube elongation phenotypes. Furthermore we found that in compound mutant embryos, but not in single mutant embryos, combinatorial defects are observed such that proximal to the elongating heart tube FGF signaling is elevated and BMP signaling downregulated. Our findings highlight the central roles of Tbx1/Tbx2/Tbx3 in conotruncal morphogenesis and identify Tbx2 and Tbx3 as candidate modifier genes of the cardiopharyngeal phenotypes in TBX1 haploinsufficient 22q11.2DS patients.

We next set out to identify novel candidate genes underlying tetralogy of Fallot (ToF), the most common cyanotic congenital arterial pole defect (Chapter 6). The multigenic etiology of this common anomaly was investigated by deploying a microfluidic PCR-based amplicon-tagging and enrichment strategy followed by next-generation sequencing in 480 unrelated patients with isolated non-syndromic ToF. Mutational screening of the ToF candidate genes CRKL, FRS2 or GATA6 unveiled that the majority of the sequence variants were present in non-coding sequences, including intronic and intergenic regions and the 3′-UTR. Our approach is of use to rapidly screen a large number of suspected candidate genes in hundreds of DNA samples, as it is cost-efficient, easily adjustable and allows the generation of a patient-specific amplicon library. Our results are in agreement with the notion that synonymous coding variants are relatively rare in congenital heart defect patients. We propose that non-coding regions, known to be pivotal in the regulation of gene expression and mRNA translation, should be more frequently interrogated for variants and compared with accumulating databases on regulatory elements in order to fully unveil the multigenic etiology of ToF.