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Irina I. Nagy

WNT-11 SIGNALING ROLES DURING HEART AND KIDNEY DEVELOPMENT

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Abstract

Organogenesis involves precursor cells proliferation, differentiation along with their coordinated organization into precise multicellular arrangements by planar cell polarity (PCP) pathways. The beta-catenin independent/non-canonical type of Wnt-11 signaling has been known as a PCP modulator during development. In this thesis were analyzed the roles of Wnt-11 in heart and kidney development by using in vivo functional genomics technologies.

We show that the *Wnt-11* gene is important for murine ventricular myocardium development, since *Wnt-11* deficiency in early cardiogenesis leads to impaired organization and maturation of mouse ventricular cardiomyocytes, causing primary cardiomyopathy with in utero lethality. Wnt-11 coordinates the co-localized expression of the cell adhesion molecules N-cadherin and β-catenin, which are critical for the spatially specific organization of cardiomyocytes. We show that *Wnt-11* deficiency causes primary hypertrophic and noncompaction cardiomyopathy in adult mice, with consequences for regional myocardium function.

The Wnt family of secreted signals has been implicated in kidney tubule development and tubular cystic diseases such as polycystic kidney disease. We show here that Wnt-11 is expressed in mature nephrons and is involved in late steps of nephrogenesis, since the kidney tubule organization is deregulated in *Wnt-11* deficient kidneys, to enlarged lumen with increased convolution. These tubule abnormalities are associated with glomerular microcyst formation and kidney failure. *Wnt-11* deficiency reduced significantly *Wnt-9b* expression, a critical signal for PCP-mediated kidney tubule elongation. In the cortical region this associated with reduced expression of nephron and stromal progenitor cell marker.

The results in this thesis point out that Wnt-11 function is required for proper myocardium organization and maturation as well as proper morphogenesis of the kidney tubules during the embryonic and postnatal developmental stages. *Wnt-11* knockout phenotypes depend on the genetic background, similarly to human congenital disease. This data may be relevant for human congenital cardiomyopathy and glomerulocystic kidney disease studies.

Keywords: cardiomyopathy, convolution, glomerular cyst, human congenital diseases, kidney tubule, lumen, myocardium, organogenesis, planar cell polarity, Wnt-11

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Tiivistelmä

Alkion sisäelinten kehityksen aikana esisolut lisääntyvät ja erilaistuvat muodostaen tarkoin määriteltyjä monisoluisia rakenteita. Muodostuvan kudosrakenteen määrittelyssä erilaiset solusignaalit ovat keskeisessä asemassa. Yksi näistä on nk. Wnt signaali perhe. Wnt perheeen jäsen Wnt-11 tehtävät on huonosti tunnettu. Wnt-11 viestittää ilmeisesti nk. planaaristen solupolariteettireittien (PCP) avulla, joka on beeta-kateniinista riippumattoman nk. ei-kanonisen Wnt signaali. Väitöskirjatyössä selvitettiin Wnt-11:n vaikutuksia sydämen ja munuaisten kehitykseen *in vivo* funktionaalisten genomisten menetelmien avulla.

Ihmisen synnynnäiset kardiomyopatiat ovat sydänlihaksen ensisijaisia vaurioita, joiden taustalla on sydänlihaksen kehityshäiriö. Tutkimuksessa osoitetaan, että *Wnt-11*-geenillä on tärkeä merkitys hiiren sydänkammion kehitykselle, koska *Wnt-11*-geenin puute sydämen varhaisen kehityksen vaiheessa johtaa sydänlihassolujen järjestäytymisen ja kypsymisen häiriintymiseen, jolloin seurauksena on ensisijaisesta kardiomyopatiasta johtuva sikiökuolema. *Wnt-11* koordinoi kahden solukiinnitysmolekyylin, N-kadheriinin ja β-kateniinin, samanaikasta ilmentymistä. Kyseiset molekyylit ovat keskeisen tärkeitä sydänlihasssolujen spatiaalisen järjestäytymisen kannalta. Tutkimuksessa osoitetaan, että *Wnt-11*-puutos aiheuttaa aikuisilla hiirillä ensisijaista sydänlihaksen liikakasvua ja trabekuloivaa kardiomyopatiaa, mikä vaikuttaa sydänlihaksen toimintaan. Tuloksilla voi olla merkitystä tutkittaessa ihmisen synnynnäisiä kardiomyopatioita.

Wnt-signaaliperheen on osoitettu olevan yhteydessä munuaisputken kehitykseen ja sen sairauksiin, kuten munuaisten monirakkulatautiin. Väitöstutkimuksessa osoitetaan, että Wnt-11 ilmentyy kypsissä nefroneissa ja että se osallistuu nefrogeneesiin myöhempiin vaiheisiin, koska munuaisputken kehityksen säätely on poikkeavaa niissä munuaisissa, joista Wnt-11 puuttuu. Seurauksena on laajentunut, normaalia poimuttuneempi luumen. Munuaisputken poikkeavuuksilla oli yhteyttä munuaiskerästen mikrokystien muodostumiseen sekä munuaisten vajaatoimintaan. *Wnt-11* -puute vähensi huomattavasti *Wnt-9b*-ilmentymistä, joka on PCP-välitteisen munuaisputken pidentymisen kannalta keskeisen tärkeä signaali. Kortikaalialueella *Wnt9b* vaimennussäätely liittyi poikkeavaan solujen lisääntymiseen, apoptoosiin ja kypsymiseen sekä vähentyneeseen nefroni- ja stroomakantasolujen merkkiaineen ilmentymiseen.

Väitöskirjatutkimuksen tulokset viittaavat siihen, että Wnt-11 -toiminto on välttämätön sydänlihaksen normaalin muodostumisen ja kypsymisen sekä munuaisputken normaalin morfogeneesin kannalta sikiövaiheen ja syntymän jälkeisen kehityksen aikana. *Wnt-11* -poistogeenisen hiiren fenotyypi riippuu geneettisestä tausta, samaan tapaan kuin ihmisen synnynnäisissä sairauksissa. Väitöstutkimuksesta saatavalla tiedolla voi olla merkitystä tutkittaessa ihmisen synnynnnäistä kardiomyopatiaa ja munuaisten monirakkulatautia.

Asiasanat: glomerulaarinen kysta, ihmisen synnynnäiset sairaudet, kardiomyopatia, luumen, munuaisputki, organogeneesi, planaarinen solupolariteetti, poimuuntuminen, sydänlihas, Wnt-11

To my family

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Oulu, april 2014 Irina I. Nagy

Abbreviations

AO	aorta
AV	atrioventricular
ANP	atrial natriuretic peptide
Bmp	bone morphogenetic protein
Brg	LysR family transcriptional regulator
BUN	blood urea nitrogen
Ccr	creatinine clearance
CD	collecting duct
CHD	congenital heart disease
Cl	chlorine
CM	cap mesenchyme
CMP	cardiomyopathy
cCMP	congenital cardiomyopathy
cRet	receptor tyrosine kinase to Gdnf
DCM	dilated cardiomyopathy
DORV	double outlet right ventricle
Dkk	dickkopf
Dvl	disheveled
EB	embryonic body
Egf	epithelial growth factor
Erbb	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
Ffg	fibroblast growth factor
Fox	forkhead box
EMT	epithelial to mesenchymal transformation
FHF	first heart field
Fz	Frizzled homolog receptor
Gata	Gata-Zinc finger DNA binding factor
GCK	glomerular cystic kidney
Gdnf	glial cell-derived neurotrophic factor
GFR	glomerular filtration rate
GTP	guanidine triphosphate
HCM	hypertrophic cardiomyopathy
HL	Henle loop
Hnf	hepatocyte nuclear factor
Hox	homeobox factor

HR	heart rate
ICD	intercalated disc structure
IRT	isovolumetric relaxation time
Isl	Lim-type homeobox gene islet factor
JNK	c-Jun NH2-termianl kinase
K	potassium
LV	left ventricle
LVNC	left ventricular noncompaction
Mef	myocyte enhancer factor
Mesp	mesoderm posterior homologue factor
MET	mesenchyme-to-epithelial transformation
Nkx	Nk homeobox-containing transcription factor
MM	metanephric mesenchyme
Na	natrium
Notch	neurogenic locus notch homolog factor
OFT	outflow tract
PA	for kidney section - pretubular aggregate
PA	for heart section - pulmonary artery
PEO	proepicardial organ
Pitx	paired-like homeodomain factor
PT	proximal tubule
PTA	persistent truncus arteriousus
PCNA	proliferation cell nucleic antigen
PCP	planar cell polarity
PKD	polycystic kidney disease
Pkd	polycystin
RA	retinoic acid
Rac	Ras-related C3 botulinum toxin substrate protein
Rho	Ras homologue factor
RV	for kidney section - renal vesicle
RV	for heart section - right ventricle
Shh	sonic hedgehog
SHF	second heart field
Scribble	Scribbled homolog (Drosophila) protein-Circletail homolog
Spry	Sprouty
UB	ureteric bud
Vangl	Van Gogh/strabismus homolog (Drosophila)-Loop-Tail homolog

Vegf	vascular endothelial growth factor
VNC	ventricular noncompaction
VSD	ventricular septum defect
Wnt	wingless-related MMTV integration site factor
Tbx	t-box factor
TGA	transposition of big arteries
Tgf	transforming growth factor

List of original articles

The thesis is based on the following articles, which are referred to in the text by their roman numerals:

I Nagy II, Railo A, Rapila R, Hast T, Sormunen R, Tavi P, Räsänen J & Vainio SJ (2010) Wnt-11 signaling controls ventricular myocardium development by patterning N-cadherin and beta-catenin expression. Cardiovasc Res 85(1): 100–109.

II Nagy II, Szabo Z, Kerkelä R & Vainio SJ (2014) *Wnt-11* deficiency causes primary cardiomyopathy in *C57Bl6* mice. Manuscript.

III Nagy II, Naillat F, Jokela T, Miinalainen I, Sormunen R & Vainio SJ (2014) Wnt-11 signaling contributes to kidney tubular system development during nephrogenesis. Manuscript.

Table of contents

1 Introduction

In development, organogenesis is the process by which precursor cells form mature functional organs. This is achieved by complex cellular inductive interactions (Glibert 2006). Organ development involves not only the proliferation and differentiation of cells, but also their coordinated organization into precise multicellular arrangements by cell polarity pathways. This precise cell arrangement in an organ cannot be disturbed without impairing its function. The ability to regenerate damaged human organs constitutes the "holy grail" of medicine and researchers are attempting to find ways of awakening in the adult developmental programs that were used during organogenesis (Gilbert 2006).

Congenital malformations are defects in organogenesis "present at birth". For most congenital anomalies (50–60%) the etiology is unknown; gene mutations are thought to account for 7–8% of human birth defects, while 20–15% are caused by a combination of genetic and environmental factors (Moon & Persaud 2003).

The Wnt gene family encodes secreted signaling molecules and is critical for the developmental process of organogenesis, including specifically cardiac and kidney development.

The mammalian heart develops through a series of sequential morphogenetic steps during which the cardiogenic precursor cells in the mesoderm differentiate into cardiomyocytes. During the early stages of cardiogenesis the ventricular cardiomyocytes become polarized, although the cues that regulate the spatial organization of the cardiomyocytes during heart ontogeny are still poorly characterized. Moreover, very little is known about possible consequences for heart function *in utero* or later adult life in case of impaired processes of embryonic cardiomyocyte polarization.

The Wnts family of secreted signals and Wnt signal transduction pathways have been implicated in kidney development and in kidney tubular diseases, which include cystogenesis. The underlying mechanism correlates with the main roles of the Wnts in controlling tubular diameter and length via the cell polarity pathways (Merkel *et al.* 2007, Karner *et al.* 2009, Yu *et al.* 2009). The latter Wnt/PCP signaling has risen as a novel participant in PKD etiology and pathogenesis, as shown by human and mouse studies (Karner *et al.* 2009, Yu *et al.* 2009, Bagherie-Lachidan & McNeill 2010, Lancaster & Gleeson 2010, Luyten *et al.* 2010, Wuebken & Schmidt-Ott 2011). Consistent with these, Wnt pathway-related proteins are involved in human and mouse PKD (Romaker *et al.* 2009, Torres & Harris 2009).

Based on previous experiments, it was shown that Wnt-11 has an important role in outflow tract during cardiac development (Zhou *et al.* 2008) and in ureter bud branching during kidney development (Majumdar *et al.* 2003).

The overall goal of this thesis is to gain an understanding of how Wnt-11 participates in cardiomyocyte polarization during myocardium development and epithelium organization during kidney tubule morphogenesis, including identifying Wnt-11 downstream effectors. By using a *Wnt-11* knockout model, this thesis aimed to understand the consequences of Wnt-11 loss of function for heart and kidney function during the embryonic stage but also later in adult life.

2 Review of the literature

2.1 Heart organogenesis

The heart is the first organ to form in the embryo and its pumping function is essential from early embryonic life, permitting the exchange of gases, nutrients and waste between tissue and vessel circulation. The majority of the heart cells have their origin in mesodermal cardiac progenitor cells, migrating from the middle ventral region of the primitive streak, during embryonic gastrulation (see Fig 1 A) (Vincent & Buckingham 2010). Specific events lead to demarcation and separation of cardiac progenitor cells as a separate cellular compartment within the homogenous population of the mesoderm. Formation of the pericardial coelom is a visible event in the mesoderm splitting, with subsequent cellular sorting into ventral-cardiac and dorsal-somatic layers. Mesodermal splitting involves N-cadherin/β-catenin complex patterning (Linask *et al.* 1997).

2.1.1 Cell behavior in cardiac mesoderm – cardiac heart fields and linear heart tube

Mesodermal cardiac progenitor cell specification is modeled by a complex network of positive and negative intercellular signal events (Evans *et al.* 2010). Pro-differentiating factors, including bone morphogenetic protein and fibroblasts growth factors, come from the endoderm, whereas negative signals arrive from the neural tube as beta-catenin dependent-Wnt, which is modulated by endodermal procardiogenic Wnt antagonists, such as dickkopf (Fig. 1) (Cohen *et al.* 2008, Vincent & Buckingham 2010).

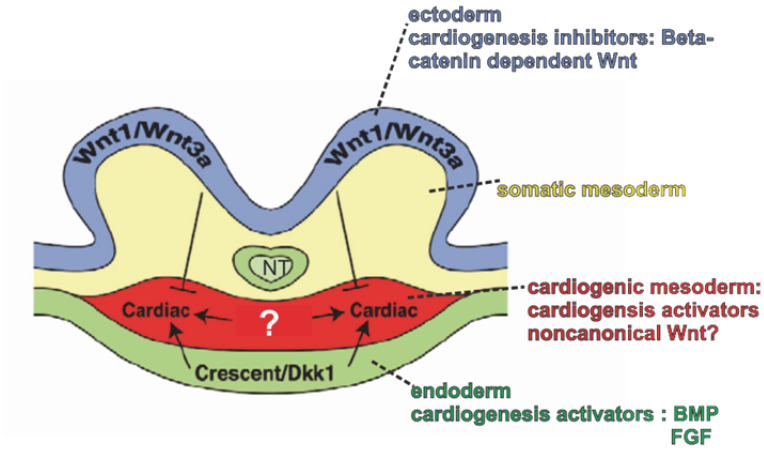

Fig. 1. Cross section through post-gastrulation embryo depicting agonist and antagonist signals in early cardiac specification. Modified from Cohen *et al.* 2008.

When leaving the streak, cells expressing the *Mesp1* mesodermal cardiac progenitor marker migrate initially in an anterior-lateral direction, then reposition themselves in a medial/lateral direction, under the head-folds (Kitajima *et al.* 2000) (Figure 2 A and B red cells). During their medial migration these early cardiomyocytes form cellular junctions adopting characteristics of coherent polarized epithelia (Linask *et al.* 1997, Glickman & Yelon 2004). The lateral group of cells constitutes the first heart field (FHF) or cardiac crescent (CC) (Fig. 2 B) expressing cardiac fate transcription factors *Nkx2-5*, *Gata-4*, *Mef2C*, *Tbx-5* in the presence of the chromatin remodeling component Baf60c/Smarcd3 (Bruneau 2008). Once CC fuses on the midline to form the primary heart tube it incorporates through arterial poles cells migrating from the second heart field (SHF), positioned medially (fig 2 C) and expressing *Isl1*, *Tbx1*, *Fgf8* and *Fgf10* factors.

These early cardiomyocyte morphogenetic processes seem to be regulated by N-cadherin/β-catenin, extracellular matrix proteins such as fibronectin, as well as non-canonical type of Wnt signaling modulating cell movement and behavior (Linask & Lash 1988a, Linask & Lash 1988b, Linask *et al.* 1997, Glickman & Yelon 2004, Matsui *et al.* 2005, Henderson & Chaudhry 2011). After epithelial polarization, cardiomyocyte phenotype is marked with the initiation of electrical activity and myofibrillogenesis (Linask & Linask 2010). Altogether, the formed

primitive heart tube provides an essential structural scaffold (van de Schans *et al.* 2007, van de Schans *et al.* 2007) for the subsequent cardiac progenitor incorporation and chamber morphogenetic processes of looping, septation and myocardium growth. (For reviews see (Sirbu *et al.* 2008, Gessert & Kuhl 2010, Vincent & Buckingham 2010, Miquerol & Kelly 2013, Pandur *et al.* 2013).

The primary heart tube thus formed has a simple structure, with one or two outer layers of cardiomyocytes and a luminal epithelial layer, the endocardium, (Figure 3 A), emerging from a hematopoietic vascular lineage. Close to the venous pole of the heart tube, a group of cells named the proepicardial organ (PEO), generates the epicardium, the outer epithelial layer, and the smooth muscles cells of the coronary vasculature (Fig. 2 E). Although considered to be of SHF origin, marker analysis suggested that endocardium and PEO contain cells originating from both heart fields. In mouse embryo, retrospective clonal analysis showed that the FHF generates most cells which form the left ventricle (LV), while SHF contributes mainly to outflow tract (OFT) and right ventricle (RV) cells. Atria have contributions from both lineages. The OFT structure also incorporates a population of neuroectodermal origin, neural crest cells migrating from the dorsal neural tube (Fig. 2 D-F) (Vincent & Buckingham 2010).

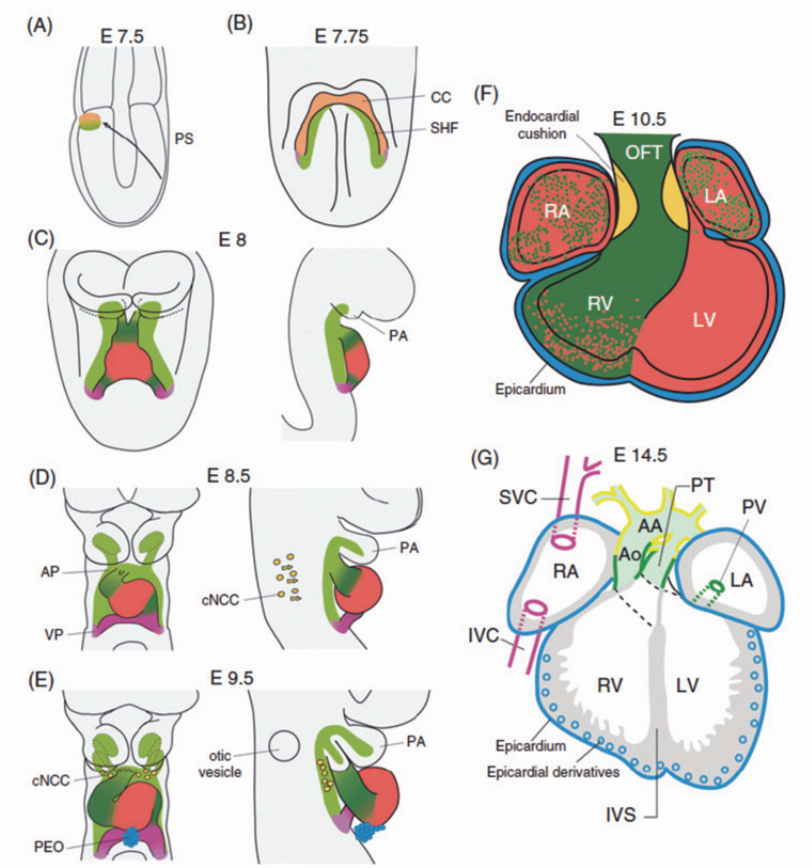

Fig. 2. Heart organogenesis stages: (A) cardiac mesodermal progenitor migration anteriorly from the primitive streak (PS). (B) Formation of the cardiac crescent (CC), with the second heart field (SHF). (C–E) Front (left) and lateral (right) views of the heart tube as it begins to loop with contributions of cardiac neural crest cells (cNCC), which migrate from the pharyngeal arches (PA) to the arterial pole (AP). The proepicardial organ (PEO) is close to the venous pole (VP). (F) The looped heart tube, with the cardiac compartments—OFT, outflow tract; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. (G) The mature heart which has undergone septation—IVS, interventricular septum; AA, aortic arch; Ao, aorta; PT, pulmonary trunk; PV, pulmonary vein; SVC, superior caval vein; IVC, inferior caval vein. The first heart field (FHF) and its myocardial contribution are shown in red, the SHF and its derivatives in dark green (myocardium) and pale green (vascular endothelial cells), cNCC in yellow (vascular smooth muscle of the AA, endocardial cushions), and PEO derivatives in blue. Modified from Vincent & Buckingham 2010. Used with permission from Elsevier.

Our understanding of heart progenitor origins has changed dramatically with the novel finding of the SHF and its complex cell behavior regulation (Buckingham *et al.* 2005). Initially, it was thought that certain transcription factors are specific to certain heart field progenitors; however, later retrospective clonal analysis showed that several transcription factors mark cells from both FHF and SHF (*Nkx2-5*, *Gata-4*, *Mef2C*, *Isl-1*, *Tbx1*), with the two heart fields being clearly juxtaposed in the mouse at E7.5 (Abu-Issa & Kirby 2007, Sirbu *et al.* 2008, Pandur *et al.* 2013). Furthermore, cell tracing experiments were able to characterize subdomains in SHF, but their spatiotemporal significance is poorly understood (Vincent & Buckingham 2010). Mutant phenotypes indicate that many of these cardiac regulators are mainly functional in the SHF progenitor population although also present in cells of FHF (Buckingham *et al.* 2005, Cohen *et al.* 2012). These cardiac factors might be redundant in their functions, which would explain why myocardial cell differentiation is not completely abolished by any single mutation in a cardiac regulatory gene. The network of interacting cardiac factors has continued to grow in complexity; for example, Tbx5, Gata-4, and Nkx2-5 associate physically to promote genes of cardiomyocyte differentiation, while Tbx5 and Sall4 interact to either promote or repress these genes. These factors are all dose-sensitive regulators of cardiogenesis, where the level required for progenitor survival differs from the level required for progenitor differentiation.

SHF size and anterior to posterior axis patterning is controlled by retinoic acid, (Sirbu *et al.* 2008), while left to right axis is patterned by Sonic hedgehog (Hildreth *et al.* 2009) and Nodal-Pitx2 (Linask *et al.* 2002, Tessari *et al.* 2008). Regarding heart field progenitor proliferation and maintenance, SHF seems to maintain more active cell proliferation and lower differentiation than primary heart tube cells. Early heart field cell proliferation involves also a complex spatial control in clonal proliferation, but cell field expansion is also achieved by changes in cell size (Meilhac *et al.* 2003). Maintenance and proliferation in SHF is controlled through Fgf, Shh and beta-catenin-dependent Wnt signaling, while differentiation of SHF is controlled through Shh, Notch, Bmp and non-canonical Wnt signaling (Cohen *et al.* 2008). For reviews see (Henderson & Anderson 2009, Vincent & Buckingham 2010, Miquerol & Kelly 2013).

2.1.2 Development of the ventricles, looping and ballooning

To shape the heart into its final form the primary heart tube follows complex processes of looping and ballooning.

At first the looping process generates an S-shape heart configuration, with definition of the inflow and outflow-ventricular segments (fig 2 D-F), mouse embryonic day 9–10 and human Carnegie 12/26 days (Henderson & Anderson 2009). During cardiac looping the cardiac jelly disappears from the atria and ventricle walls, while accumulating in the junction between the chambers and developing OFT, forming the endocardial cushions, the primary structure which generates the cardiac septum and valve (Figure 2 F) (Snarr *et al.* 2008). Endocardial cushion morphogenesis is mediated by epithelial-to-mesenchymal transformation, regulated by intrinsic and extrinsic factors secreted from the nearby myocardium, such as Gata-4, EGF, Tgfβ and Notch (Wessels & Sedmera 2003, Niessen & Karsan 2008, Snarr *et al.* 2008).

Next, the heart chamber atria and ventricles are formed by serial ballooning processes from the cavity of the primary heart tube. The process involves regional differences in the rate of proliferation, with lower levels in the heart loop inner curvature and complex proliferation patterns of clonal growth (Meilhac *et al.* 2003, Sedmera & Thompson 2011). Each ventricular chamber is defined by its own inlet/inflow, outlet/outflow and apex components, and the interventricular communication is remodeled as well, bringing in direct communication right chambers (RA and RV) and left chambers (LA and LV), changes essential in generating the AV conduction axis (Moorman & Christoffels 2003). Finally, twisting of OFT accompanied by fusion and muscularization of the proximal outflow cushions permits separation of pulmonary and aortic-systemic circulations, by closing interventricular communication and connecting the aorta to LV and the pulmonary artery to RV (fig 2 G). Heart gross morphology is complete in mouse by embryonic day 14.5, human Carnegie stage 23/end of week 7), although the heart continues to mature throughout the remainder of *in utero* life (Meilhac *et al.* 2003, Henderson & Anderson 2009, Savolainen *et al.* 2009, Sedmera 2011).

2.1.3 Myocardium patterning during development

During cardiac development the ventricular myocardium follows complex patterning with specification into compact and trabeculated type of myocardium. Cytoarchitectural changes involve distinct arrangement of cardiomyocytes layers within the ventricular wall, with increasing cardiomyocytes differentiation levels from the epicardium towards the endocardium. In regulation of patterning

processes a prime candidate has arisen, the non-canonical type of Wnt signaling through planar cell polarity pathway (Henderson & Chaudhry 2011)

Before the looping stage there is quite homogenous cellular architecture along the cardiac tube, with one or two layers of cuboidal-shaped cardiomyocytes and circumferential arrangement of undifferentiated myofibrils (fig 3 A) (Hirschy *et al.* 2006). The early patterning of cytoskeleton, myofibrils and cellular junctional N-cadherin seems to be play a crucial role in heart looping morphogenesis of different species (Linask *et al.* 1997, Linask *et al.* 2002, Linask & Vanauker 2007). However, the degree of organelle differentiation in these early cardiomyocytes is dependent on genetic background, for example in mice strain (Kastner *et al.* 1997).

Clear regional differences in myocardial cell myofibrillar pattern were observed after looping stage, when trabeculations emerge next to the maximum curvature areas (Fig. 3 B)(Hirschy *et al.* 2006). The trabeculation morphogenesis includes important exchange of inductive and patterning intercellular signals between the endocardium, myocardium and the myocardium secreted cardiac jelly. These signals include endocardial neuregulin binding on myocardial receptor Erbb, endocardial Brg1 repression on endocardial matrix metalloproteinases, secreted Vegf and angiopoietin from the myocardium acting on the endocardium (Stankunas *et al.* 2008).

Before heart septation, compact myocardium proliferative activity is higher and its differentiation is lower than in trabeculated myocardium, while the latter has the main responsibility for the heart pumping activity at this stage (Sedmera & Thompson 2011). The patterns of early trabeculations are similar in RV and LV, but by the completion of septation (mouse embryo day 14.5 and human Carnegie 22/8 weeks) ventricles obtain a more specific morphological patterning, with certain differences between species. Through these processes the radial trabecular myocardium is rearranged into apico-basal orientation. Coalescence of the trabecular myocardium leads to formation of the papillary muscles supporting the leaflets of the AV valves (Sedmera *et al.* 2000).

Once heart septation is complete, the compact myocardium increases in proportion and thickness over the trabeculated myocardium, with its contribution to the total myocardial mass and pressure pump function becoming significant especially in the LV (Sedmera *et al.* 2000). This process called myocardial compaction is at present poorly understood (Henderson & Anderson 2009). It has been hypothesized to be generated by compression/consolidation of trabeculations within the ventricular wall (Sedmera *et al.* 2000); however, no evidence for this

exists so far except for the formation of papillary muscles. Major thickening of ventricular wall compact myocardium is currently thought to be brought on by its proliferation and myoarchitecture remodeling (Henderson & Anderson 2009). *In utero* proliferation and differentiation of the compact myocardium are regulated by epicardial-derived signals such as Fgf, retinoic acid, erythropoietin and Wnts (Sedmera *et al.* 2000, Henderson & Anderson 2009). Cardiomyocyte differentiation involves cell size increase and shape changes, together with increasing abundance in mature specialized cardiomyocytes organelles such as Z bands defining sarcomeres in myofibrillar bundles and intercalated disc (ICD) cell-cell contacts (Kastner *et al.* 1997, Du *et al.* 2003). ICD are specific to cardiomyocytes and their complex structure distinguishes three types of cell contact: adherens junctions anchoring actin filaments (cadherins, catenins, vinculin, alpha-actinin), desmosomes anchoring intermediate filaments (desmoplakins) and gap junctions mediating ion transfer (connexins). In adult myocardium ICD become exclusively located at bipolar ends of rod-shaped cardiomyocytes; however, this is not the case *in utero* when these structures are found surrounding the cardiomyocytes' lateral membranes (Peters *et al.* 1994, Hirschy *et al.* 2006). Maturation of cardiomyocytes cell-cell contacts is a slow process coordinated intimately to the changes in cellular dimensions and cardiomyocyte-polarized orientation into the growing myocardial wall. At neonatal stage cardiomyocyte adherens junctions no longer surround the whole cells and become restricted to myofibrillar attachment sites; however, electrical and mechanical coupling still continues to be fine-tuned and remodeled in humans up to about 6 years of age. An essential adaptation to the almost 16-fold increase in heart weight in the first years of life, conduction velocities increase through increased association between gap junctions and adherens junctions (Peters *et al.* 1994, Hirschy *et al.* 2006).

Substantial further myocardial growth and compaction continues postnatally, and this process involves further improvement in muscle organization complexity. The adult ventricular myocardium has a three-layered spiral/helical system of myocardial fibers, correlating to the twisting pattern of contraction existing from early heart development, after initiation of trabeculation (fig 4 D). This complex adult architecture is behind the observed regional heterogeneity in myocardium shortening and stretching during cardiac cycle (Sedmera *et al.* 2000, Henderson & Anderson 2009, Sedmera 2011).

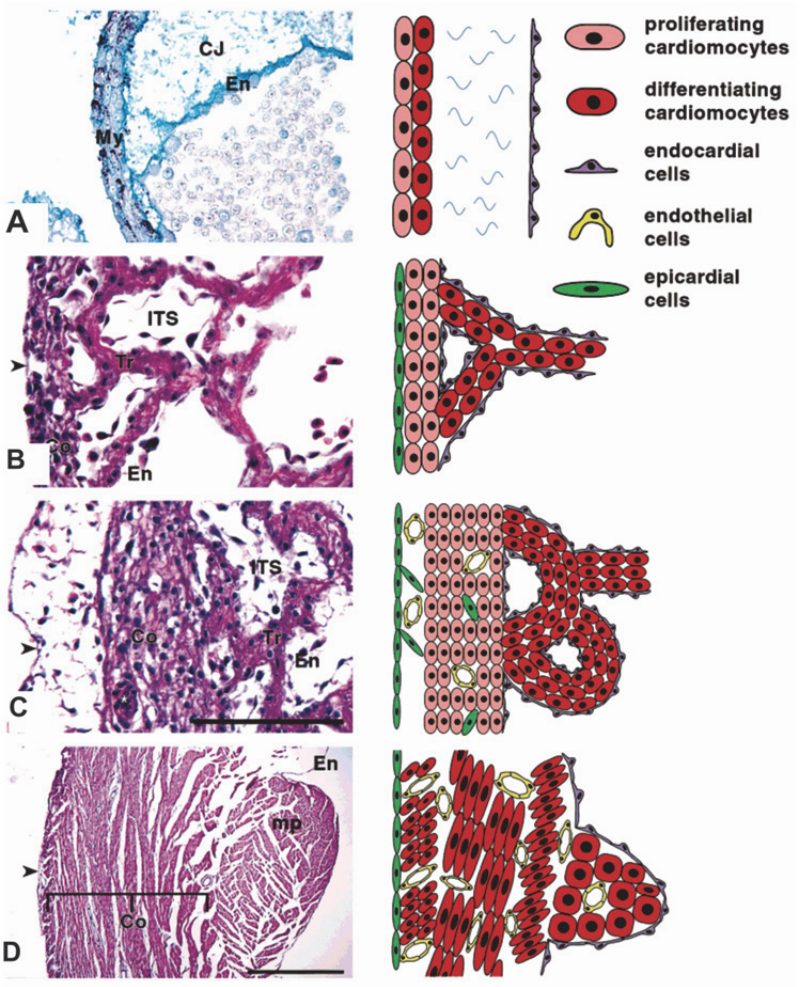

Fig. 3. Development of the myocardial wall. (A) The tubular myocardium (My) with acellular cardiac jelly (CJ) composed of proteoglycans, collagens and other fibrillar extracellular matrix components. (B) After looping most of the myocardial mass is made up by the trabeculations (Tr), Arrowhead epicardium. (C) At mouse midgestation the compact layer has thickened and become invaded by developing coronaries (star). (D) Neonatal multilayered architecture of left ventricle layer (outer longitudinal, middle circular, and inner longitudinal). On the right side a schematic drawing of the major steps in development of ventricular myoarchitecture. BARS A, B, C 100 mm; D, 500 mm. Modified from Sedmera *et al.* 2000. Used with permission from John Wiley and Sons.

Cell polarity pathways and myocardium patterning

The dynamics of organogenesis does not involve only proliferation and differentiation. Organs shape and dimensions are modeled in a particular axis though regulation of cell division orientation (OCD) and convergent extension (CE-cellular rearrangement through intercalation with each other in order to narrow and extend a tissue), both regulated by the planar cell polarity pathways (PCP). In these dynamic processes cells are only transiently polarized in a certain plane, and afterwards move and/or divide in another orientation. Many of the components of PCP pathways are conserved between flies and vertebrates, but the degree of mechanistic conservation is still unclear. However, certain similarities have been found between gastrulation convergent extension-like movements and the behavior of special migrating cell types, such as neural crest cells and epithelial cells in kidney tubule elongation. (Goodrich & Strutt 2011).

One compelling model for cell polarity during organogenesis holds that cells regulate their proliferation, orientation (polarization) and movement (cell intercalation) in response to a specific morphogen gradient. Beta-catenin independent Wnt ligands, including Wnt-11, which bind to frizzled (Fz) receptors, are involved in PCP pathway activation. However, there is poor evidence to clarify whether these signals are permissive or inductive. Fz receptors are part of the so-called core group in PCP signaling molecules, together with Dishevelled, Prickle, Van Gogh/strabismus, Diego, Flamingo. These molecules are characterized by asymmetric cell membrane or subcellular organization, which act to amplify and stabilize polarization signal by communicating to the boundary of adjacent cells. Their asymmetric cellular organization is thought to result from intracellular dynamic interactions between cellular proximal and distal components. Recruited cellular effectors such as JNK kinase and small GTP-ases (RhoA and Rac) modulate the cell shape and behavior by affecting cytoskeleton and possibly also cell-to-cell junctional molecules (For reviews see (Karner *et al.* 2006, Simons & Mlodzik 2008, Goodrich & Strutt 2011).

Disruption of PCP may play a causal role in several human congenital diseases. The original PCP pathways have been expanded to play morphogenetic roles in tissues that do not show obvious planar organizations, which is why in these contexts PCP organization becomes harder to understand (Goodrich & Strutt 2011).

For normal morphogenesis of the primitive heart tube (E8.5) establishing an apical-basal cell polarity as well as planar cell polarity seems to be important.

Disruption in these processes causes cardia bifida in *Diversin* and *dominant Dsh* in zebrafish mutants in addition to disrupted organization of cardiomyocytes in mouse *Scrib* and *Vangl2* mutants (Phillips *et al.* 2005, Phillips *et al.* 2007, Henderson & Chaudhry 2011). Perturbation in the early epithelialization of cardiomyocytes affects their coherent migration and may disrupt severely heart formation at this developmental stage, resulting in cardiac phenotypes such as abnormal heart tube formation (Phillips *et al.* 2007, Henderson & Chaudhry 2011), *cardia bifida* (Pandur *et al.* 2002, Garriock *et al.* 2005, Matsui *et al.* 2005) or abolishment of heart formation in chick (Linask *et al.* 1997). These phenotypes associated with abnormal apico-basal polarity and improper junctional complexes as shown by antibody blocking N-cadherin function in avians (Linask *et al.* 1997), fibronectin mutation disrupting interaction with extracellular matrix in chick (Linask & Lash 1988a, Linask & Lash 1988b), zebrafish and mouse (Glickman & Yelon 2004) and also impaired Wnt-11 signaling by morpholino in *Xenopus* (Pandur *et al.* 2002, Garriock *et al.* 2005) and zebrafish (Matsui *et al.* 2005).

Impaired PCP with causal role in cardiac anomalies is linked to defects in early heart tube looping, outflow tract morphogenesis and septation. Little data is available on cardiomyocyte cell polarity in ventricular walls, due to the fact that aberrations from normal ventricular development do not resemble classic PCP phenotypes (Davis & Katsanis 2007, Simons & Mlodzik 2008, Henderson & Chaudhry 2011). In the tubular heart shape cardiomyocytes are round with no polarized cell-cell junctional proteins, junctional components are distributed around the membrane, and first polarization behavior can be observed in mice in midgestation when cardiomyocytes change their shape and elongate (Hirschy *et al.* 2006, Henderson & Chaudhry 2011). Adult cardiomyocytes have a rod-like shape and junctional components are tightly polarized as ICD on the cardiomyocyte long axis membranes while gap junctions, ion channels, beta –adrenergic receptors become polarized on lateral membranes (Peters *et al.* 1994, Hirschy *et al.* 2006). In mouse, mutation in PCP core proteins causes congenital heart disease with OFT defects (*Vangl2-Lp* mutant, *Scrib-Crc* mutant), but these mice have also a clear myocardium phenotype with defects in cell shape, proliferation and patterning of myocardium (Phillips *et al.* 2005, Henderson *et al.* 2006, Phillips *et al.* 2007). By late gestation myocardial defects in core PCP mutants resemble ventricular noncompaction (VNC) observed in humans (Henderson & Chaudhry 2011). Wnt-11 stands as a good candidate for PCP control of heart organogenesis; mutation in homologous *Wnt-11 R* zebrafish causes possibly a similar phenotype of disorganized cells in ventricular myocardium, and a line of

evidence that Wnt-11 acts as a directional cue to organize muscle has been shown in *Xenopus* elongation of early skeletal muscle fibers (Garriock *et al.* 2005, Gros *et al.* 2008).

Many of the core proteins are expressed in the myocardium from early embryo to adulthood, suggesting a role in myocardium assembly but also maintenance. Altogether PCP pathway components have been shown to cause changes in mammalian cardiomyocyte shape and junctional organization, but these might not be analogous to classic PCP pathway, pointing out that myocardium cell polarity is at present poorly understood (Henderson & Chaudhry 2011).

2.1.4 Abnormal heart organogenesis and consequences for cardiac functional development

Although extremely simple and lacking a differentiated cellular structure, the primitive tubular heart becomes a functional organ. First signs of electrical activity generate peristaltic-like movements and have been recorded as early as mouse embryonic day 8.5, equivalent to human Carnegie stage 10, 21–23 days of gestation (Sedmera *et al.* 2000). As the embryo grows, there is a developmental relationship between heart regional architecture, myofiber alignment and cardiac function (Tobita *et al.* 2005). Improvement in ventricular function, especially diastolic function, correlates with changes in ventricular myoarchitecture during cardiac developmental (Ishiwata *et al.* 2003). However, changing from normal hemodynamic conditions (pressure and volume load) affected normal morphogenesis in experimental chick embryos, by accelerating development in increased loading conditions and delaying development in decreased loading conditions (Tobita *et al.* 2005).

The heart exhibits remarkable adaptive responses to a wide array of genetic and extrinsic factors to maintain cardiac function; however, persistent activation of these pathways leads eventually to cardiac dysfunction and apoptotic loss of cardiomyocytes (Harvey & Leinwand 2011). After apoptosis the ability of mature adult myocardium to regenerate is reduced, adaptive response to maintain cardiac workload is achieved mainly by ventricular hypertrophy or dilation, with changes in extracellular matrix composition, individual cardiomyocyte volume and organelle content (Fogel *et al.* 2005, Harvey & Leinwand 2011). Recent evidence suggests that cardiomyocyte proliferation may play an unrecognized role during the period of developmental heart growth between birth and adolescence; this

evidence came combined with proof for cardiomyocyte turnover in adult humans (Mollova *et al.* 2012). In contrast, during development the heart can also respond robustly to changes in loading conditions by cardiomyocyte hyperplasia, or to decreased load by hypoplasia, as shown in chick models (Sedmera *et al.* 1999, Tobita *et al.* 2005). In the neonatal period there is a shift from a fetal isoform gene-expression profile to adult isoforms, especially in genes concerning contractile apparatus components (Epstein 2010). Both hyperplastic and hypertrophic responses to an increased pressure load are normal characteristics of fetal to postnatal heart development; however, the exact time point and factors involved in switching from hyperplastic to pure hypertrophic response are not yet fully understood. Epigenetic control of chromatin structure is recently believed to regulate the transition from fetal gene program to adult gene program (Epstein 2010).

Cardiac functional evaluation – global cardiac function by conventional Doppler echography

Cardiovascular functions involve broad aspects of cardiac physiology, including contraction and relaxation during cardiac cycle systole and diastole, and distribution of blood flow over specific vascular beds. The conventional approach is to assess the global cardiac function by (2D) Doppler ultrasonography, and so far multiparameter analysis is required because of the limitations of any single parameter.

Fetal echocardiography is a well-established tool for prenatal diagnosis of structural, but also functional heart disease. Studies of human fetuses and mouse models agree that developmental changes in cardiac function must meet the requirements of the growing embryo, in particular diastolic function parameters, which may provide a key prognostic factor in cardiomyopathies (Pedra *et al.* 2002, Ishiwata *et al.* 2003). In human fetal cardiomyopathies the diastolic dysfunction causes secondarily an increase in systemic venous pressure and associates with greatest risk in fetal mortality (Pedra *et al.* 2002).

Pulsatility index values of vessels stands for PI = peak systolic velocity-end-diastolic velocity/time-averaged maximum velocity in cardiac cycle. This correlate to intrinsic vascular wall properties and downstream vascular resistance resistance/impedance = pressure/volume. Pulsatility index values of vessels proved to be relevant for both systolic and diastolic cardiac functions (Pedra *et al.* 2002. Indeed, increased PIs in umbilical artery and aorta are observed in human

placental insufficiency, and increased PI in ductus venosus implies an increase in systemic venous pressure as a sign of diastolic or congestive heart failure. A specific diastolic parameter is ventricular isovolumetric relaxation time (IRT%), which describes the time needed for the ventricle to drop its pressure from a systemic to an atrial level (Makikallio *et al.* 2005). IRT% is sensitive to the rate of ventricular relaxation, which depends on the passive properties of the myocardium, but is also load-dependent by aortic diastolic pressure and atrial pressure, and mouse studies showed that improvement in active relaxation correlates directly to maturation in compact myocardium architecture (Ishiwata *et al.* 2003). Improvement in cardiac diastole in early development might be important for the fetal heart to adapt to the increased volume of blood flow (Makikallio *et al.* 2005).

Systolic function is evaluated by isovolumetric contraction time (ICT%), which describes the time needed for the ventricle to increase its pressure to the systemic blood flow level, directly correlating with ventricular contractility and pressure generation (Makikallio *et al.* 2005). ICT% decreases in early embryonic development according to the improvement in ventricular pressure generation. Inflow and outflow velocities are proportional to volume blood flows (Vmean = fetal heart rate HR × time velocity integrals), and umbilical artery velocity correlates directly to umbilico-placental blood flow. The cardiac output parameter describes the cardiac hemodynamics at one point in time, being the volume of blood being pumped by the heart in one minute. Cardiac output depends on heart rate and correlates with the conventional systolic parameter in adult cardiac echocardiography: ejection fraction (EF%). EF is the fraction of blood ejected by the ventricle during the contraction or ejection phase of the Systole in cardiac cycle: EF% = (end diastolic volume-end systolic volume)/end diastolic volume) × 100%. In practice, however, ejection fraction has weak inter-assay reproducibility, being dependent on preload, afterload and heart rate. EF assesses systolic global ventricular performance and does not take into consideration myocardial regional contractile dysfunction (Abraham *et al.* 2007, Hoit 2011).

Cardiac functional evaluation - regional myocardial function by Speckle tracking echography

Speckle tracking echography (STE) as tissue strain imaging is a relatively new echocardiography method, yet proving to be difficult to standardize. The

mechanical properties of tissue have long been recognized as a useful indicator of disease and STE looks directly into the myocardium and measures myocardial deformation in strain (Abraham *et al.* 2007, Hoit 2011). While standard Doppler echography captures global changes in blood flow showing global cardiac function, tissue strain is a non-Doppler technology that evaluates cardiac regional function by detecting complex myocardial line motion (Thomas 2004). Imaging tracking technology is fast advancing and numerous studies are clarifying the limitations and clinical applicability of the methods. STE is automated, angle-independent, less noise sensitive with better intra- and inter-observed variability than standard Doppler technique (Hoit 2010).

STE measures velocity and strain parameters of myocardium performance in 2D longitudinal, radial and circumferential directions during systole and diastole. These may reflect closely the regional functional impact of any changes in regional myocardium architecture caused by cardiac diseases. Strain technology uses continuum mechanism Lagrangian strain principles, where strain represents the magnitude and rate of deformation between two points in the myocardium, and the rate of deformation is referred to as strain rate (SR; in seconds−1). SR is noisier, less reproducible, but less dependent on load conditions compared to strain.

In measuring the tissue motion, for displacement and velocity parameters a reference point from the transducer is used; this is biased by translational movement and tethering, as it cannot distinguish well between passive or active tissue movements. The myocardium deformations are complex; these occur not only perpendicular to a given plane (normal strain) but also in between planes, or as shear strain. 3D ultrasound techniques are expected to improve and expand the 2D ecography diagnostic capabilities.

Mathematically, strain is the integral of SR, with compression expressed in a negative value and expansion in a positive value (Dandel *et al.* 2009, Hoit 2011). Lower values in tissue velocities and SR have been reported in ischemic myocardium, where changes in SR may even precede changes in velocity (Abraham *et al.* 2007, Hoit 2011). Lower longitudinal strain and SR have been observed in hypertrophic cardiomyopathy versus physiological athletic hypertrophic cardiomyopthay; moreover, in a study of patients with suspected congestive heart failure of different etiologies it was also shown that mean longitudinal LV strain is closely related to plasma brain-type natriuretic peptide (BNP) levels in patients with both systolic and diastolic heart failure. (Dandel *et*

al. 2009, Wang & Nagueh 2009, Hoit 2011) These techniques are promising in terms of potential clinical value especially in sub-clinical cases.

2.1.5 Developmental cardiac defects: Congenital heart diseases and Cardiomyopathies

Congenital heart defects (CHD) have worldwide prevalence approaching 1:100 births and refer to structural or functional cardiac abnormalities that arise before birth (Bruneau 2008, Houyel *et al.* 2011, Garne *et al.* 2012). Remarkably, excluded from this number are congenital cardiomyopathies (cCMP), inherited primary defects of the myocardium, which are defined as an isolated group of cardiac anomalies in the absence of congenital structural heart disease (Maron *et al.* 2006, Elliott *et al.* 2008), although it has long been known that these phenotypes can coexist (Somerville & Becu 1978, Eidem *et al.* 2000). A more exact prevalence of primary or congenital cardiomyopathies is currently unknown, varying from 1:500 for the hypertrophic form of cardiomyopathy to 1:2000 for dilated cardiomyopathy, and to 1:5000 for the more uncommon unclassified cardiomyopathy, ventricular noncompaction (VNC) (Seidman & Seidman 2001, Maron *et al.* 2006, Elliott *et al.* 2008). The separation of the developmental heart defects into the two groups, CHD and CMP, has been sustained through differences in clinical presentation and treatment approaches (Bruneau 2008). It is increasingly realized that the current nomenclature fails to adequately describe a substantial overlap between the classic cardiomyopathy syndromes (Klaassen *et al.* 2008), or fails to describe how the same/similar developmental abnormalities can underlie anatomically distinct heart defects (Epstein 2010). It is also important for understanding heart morphogenesis in the context of heart functional development, as heart function is required for life from very early heart morphogenesis on; hemodynamics alterations may have consequences for heart morphogenesis. This may explain why defects in myosin contractile proteins encoding genes cause inherited atrial septum defects (for review see (Bruneau 2008).

Among the major forms of cardiomyopathies hypertrophic CMP (HCM) is characterized by increased ventricular chamber size, increased contractility but impaired diastolic relaxation, while dilated cardiomyopathy (DCM) is characterized by increased ventricular chamber size and reduced contractility (Seidman & Seidman 2001). Ventricular noncompaction (VNC), a distinct form of CMP, has only recently been added to the primary CMP group with genetic

origins. It is often found associated with CHD and other forms of CMP (Tsai *et al.* 2009, Oechslin & Jenni 2011). VNC is characterized by abnormal trabeculations associated with deep intertrabecular recesses and thin compact myocardium. Based on elucidations of its genetic causes, the human inherited cardiomyopathies show mutations in sarcomeric proteins and sarcomeric-related proteins, although 50% of the HCM patients have no mutations in these proteins (Seidman & Seidman 2001, Alcalai *et al.* 2008).

Cardiac disease studies raise the question whether each CMP form is a distinct program or just a different morphologic phenotype reflecting gradation steps in a single disease model (Seidman & Seidman 2001, Ahmad *et al.* 2005). Important to remember here, all CMP forms are affected by modifier genes: the prime candidates are variants in renin-angiotensin-aldosterone system, transforming growth factor and insulin-like growth factor, endothelin-1 and tumor necrosis factor, calcium regulations and homeostasis (Seidman & Seidman 2001, Towbin & Bowles 2002, Alcalai *et al.* 2008).

2.2 Kidney organogenesis

The kidney derives from the intermediate mesoderm, which forms in temporal and spatial sequences transient structures on the right and left side of the body: first forming is the pronephros, second the mesonephros and third and last, the metanephros. In higher vertebrates only metanephros differentiates into permanent kidney, while the others participate in adrenal gland and gonad organogenesis.

2.2.1 Cortical nephrogenesis and ureter branching

Kidney organogenesis from the metanephros comprises complex reciprocal inductive interactions between two primordial tissues: epithelial structured ureteric bud (UB) and surrounding metanephric mesenchyme (MM). While we have been aware of these reciprocal inductive interactions for almost 50 years since their discovery by Grobstein (Grobstein 1956, Saxen & Sariola 1987), the clues towards understanding morphogenesis and its molecular basis in detail have increased dramatically during the last decade (Little & McMahon 2012).

In vitro experiments proved that these tissues must be in close contact to each other to activate reciprocal signals for kidney induction (Saxen & Sariola 1987). The ureteric bud grows and branches in a certain bifurcation or trifurcation

pattern in response to mesenchymal signals, while mesenchymal tissue forms new early nephron structures, renal vesicles (RV), through mesenchyme-to-epithelial transformation (MET) in response to signals from branching ureter tips. Before committing towards RV formation, the mesenchymal cells go through two intermediary phases, cap mesenchyme (CM) and pretubular aggregate (PA). The formation of RV represents the "birth" of a single nephron, RV being the precursor for most segments of the nephron from the glomerular podocytes to the connecting segment to collecting ducts system, while collecting duct cells originate from the ureteric bud. Eventually, these small renal vesicles begin to pattern as they proliferate and elongate to form tubular nephrons. In addition to the nephrogenic MM and ureter epithelium, a third cell type has been described in the nephrogenic cortex, i.e., cortical stromal cells, which surround the CM domain (Cullen-McEwen *et al.* 2005, Yallowitz *et al.* 2011). For reviews see (Hendry *et al.* 2011, Little 2011, Little & McMahon 2012).

Self-renewal and progenitor survival within MM, UB and stroma are essential for generating normal nephron number. This is defined by complex reiterative positive loops between these cells. A subpopulation of MM/CM expressing *Six2* represents a self-renewing multipotent nephron progenitor population throughout kidney organogenesis (Kobayashi *et al.* 2008). Survival of these MM progenitor cells seems to be a separate attribute to self-renewing and depends on ureteric, not fully known UB signals, but also on intrinsic MM signals including *WT1*, *Eya1*, *Hox11* paralogs. Signaling from stromal cells *Hox10* paralog, *Foxd1*, is important for maintaining a stroma progenitor population forming the kidney capsule, but also seems to play critical roles in nephron differentiation, ureter branching and mesenchyme renewal (Yallowitz *et al.* 2011, Little & McMahon 2012). Mesenchymal Six2 cooperates closely with epithelial Wnt-9b, which seems to be critical in balancing this mesenchymal progenitor self-renewal versus differentiation (Carroll *et al.* 2005, Karner *et al.* 2011). This could be explained by different levels in Wnt-9b signaling reaching the different cellular compartments of CM (Little & McMahon 2012). Besides this important role in maintaining mesenchymal Six2+ progenitor population, Wnt-9b has a crucial inductive role in the subsequent requirement for *Wnt-4* expression in initiation of MET and RV formation (Thiagarajan *et al.* 2011, Hendry & Little 2012, Little & McMahon 2012).

Wnt-9b localizes in the ureter epithelium together with other Wnts, *Wnt-11* and *Wnt-7b*, although in different subdomains, *Wnt-11* more proximal at the tips, *Wnt-9b* and *Wnt-7b* more distal in the stalk (Kispert *et al.* 1996, Carroll *et al.*

2005, Yu *et al.* 2009). Ureter bud also expresses *Pax2*, which is essential for ureter bud survival and growth. The *Wnt-11* expression at the tips of the branching UB is required in a positive feedback loop that promotes mesenchymal secreted glial-derived growth factor (Gdnf) essential for survival and branching of the ureter bud, to bind its receptor cRet receptor in UB (Kispert *et al.* 1996, Majumdar *et al.* 2003). Gdnf/cRet signaling lies at the signaling core of epithelial branching and determines the kidney nephron number as shown in *Wnt-11* and *cRet* deficient hypoplastic kidneys (Majumdar *et al.* 2003), while *Gdnf* null mice show a more extreme phenotype as renal agenesis due to lack of ureter induction. The UB branching is maintained by multiple and complex positive and negative reiterative loops between the mesenchyme, epithelium and also stroma maintaining optimal signaling in the Wnt-11/Gdnf/cRet loop. Stromal Fgf7 promotes UB branching (Cullen-McEwen *et al.* 2005) having possibly redundant function to the upper loop. Stromal Bmp4 has inhibitory function on UB branching (Raatikainen-Ahokas *et al.* 2000), but is finely tuned by its inhibitor Gremlin (Michos 2009), both acting downstream of cRet. Epithelial Wnt-9b and mesenchymal Hox11 are reinforcing levels of mesenchymal *Six2* upstream of Gdnf (Kobayashi *et al.* 2008, Karner *et al.* 2011). GLI3 repressor restricts hedgehog signaling maintaining normal levels of *Wnt-11* and *cRet* in UB (Cain *et al.* 2009); stroma Foxd1 and retinoic acid-related molecule (Batourina *et al.* 2001) and epithelial Gata3 cooperate with Pax2/8 to maintain optimal levels and correct topography of *cRet* and *Wnt-11* in the epithelium. For reviews see (Cullen-McEwen *et al.* 2005, Grote *et al.* 2006, Costantini & Kopan 2010, Costantini 2010, Hendry *et al.* 2011, Hendry & Little 2012).

Downstream of Gdnf/cRet signaling lies, among others, Etv4/5 from the Pea3 family of ETS transcription factors, which control genes involved in cell proliferation, migration and extracellular matrix during UB branching (Kuure *et al.* 2010a, Kuure *et al.* 2010b). Within important feedback inhibitor of ureter branching lies Sprouty 1 (Spry 1), also downstream of Gdnf/cRet. MM marker Fgf10 is able to rescue ureter branching in the combined absence of Gdnf/cRet signaling and Spry1 negative regulation; its pattern of branching is, however, severely perturbed, pointing to a unique function of Gdnf in ureteric bud patterning (Michos *et al.* 2010).

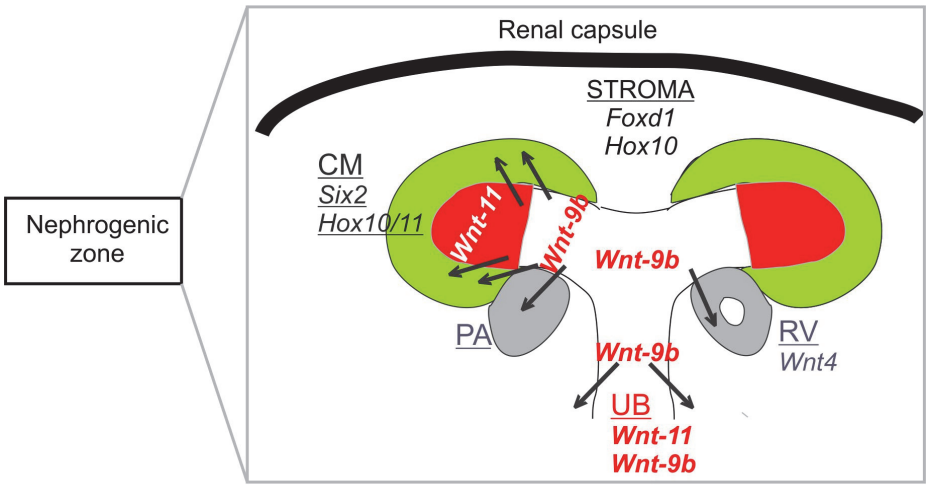

Fig. 4. Kidney nephrogenic zone progenitor gene expression. Diagram is representing ureter bud (UB) branching within cortical nephrogenic zone progenitor niche, with ureter tip (red) surrounded by cap mesenchyme (CM) (green). Nascent nephrons go through pretubular aggregate (PA) phase and next renal vesicle (RV) phase. CM expresses progenitor genes *Six2*, *Hox10/11*, *Pax2*, *Eya1*, *Wt1*, *Sall1*, PA expresses *Six2*, *Fgf8*, *Fgfrl1*, *Pax8*; while RV expresses *Wnt4*, *Fgf8*, *Gfgrl1*, *Pax8* (Hendry *et al.* 2011). Note the localization of *Wnt-11* and *Wnt-9b* in the ureteric tree.

Cessation of nephrogenesis and branching

After invading the MM, the UB undergoes about ten generations of repeated branching, followed by a period of more intense tubule elongation with decreased branching. The branching number and pattern determines the kidney size and shape (al-Awqati & Goldberg 1998). The progenitor population decreases postnatally and is exhausted in mouse by postnatal day 5 through final waves of accelerated nephrogenesis compared to early kidney development. Inductive signals such as *Wnt-9b* expression continue in ureter branches (Hartman *et al.* 2007, Karner *et al.* 2011, Little & McMahon 2012). The last waves of nephrogenesis and branching seem to differ as until birth, the nephron to ureter branch is one-to-one with few exceptions, while after birth multiple new nephrons jointly attach to one ureter tip forming arcades (al-Awqati & Goldberg 1998, Hartman *et al.* 2007, Rumballe *et al.* 2011). The process coincides with loss of *Wnt-11* in ureter tips as marker of ureter tip branching. The exact trigger and processes involved in cessation of nephrogenesis and branching morphogenesis

are poorly known. It is thought to be initiated by a shift in mesenchyme fate through presumptive increased inductive signals, possibly in combination with decreased sensitivity to growth factors and inhibitor of differentiation. As a result *Six2* progenitor gene expression in cap mesenchyme is lost together with *Foxd1* progenitor gene expression in stroma, but surprisingly, some mesenchymal cells continue to proliferate with no dramatic change in apoptosis (Hartman *et al.* 2007, Karner *et al.* 2011). In humans, nephron formation is regarded as complete by 36 weeks of gestation while in mice it is not regarded as complete until postnatal day 3–4 (Rumballe *et al.* 2011). Once nephrogenesis has ceased renal development concentrates around tubule elongation and nephron segment maturation (Costantini 2010).

2.2.2 Medulla formation and kidney tubule maturation and elongation

Kidney tubule maturation and elongation are essential to kidney medulla formation during prenatal, but also postnatal kidney development; after cessation of glomerulogenesis, these processes seem to be governed by PCP pathways and Wnts (Yu 2011).

Epithelial RV representing stage I nephron undergoes shape changes through stage II-S shape and III-capillary loop until the final stage IV, the mature nephron. The nephron fuses immediately after MET event to ureter tip and proximal and distal gene expression becomes polarized. Proximal RV gives rise to glomerulus podocytes and Bowman capsule while distal RV generates from a specific subset of cells the proximal tubule (PT) together with Henle's loop, then distal tubules and respectively connecting tubules. Each nephron forms a spatial cortico-medullary pyramid, named also the malpighian pyramid, which is well conserved in mammals, the broad base adjacent to the renal cortex and the narrow apex as kidney papilla. However, the number of these structures and therefore the number of papillae differs from species to species: humans have eight papillae while mice only have one (Yu 2011, Song & Yosypiv 2012).

Collecting ducts (CD), which form from ureter epithelium, undergo distinct morphogenesis through dichotomous branching, tubular growth and elongation within a fan-like linear array patterning leading to formation of kidney medullary collecting ducts. The latter converge centrally also in a dichotomous fashion to form larger tubules, papillary tubules whose terminal merger gives rise to the ducts of Bellini (Dwyer & Schmidt-Nielsen 2003, Costantini & Kopan 2010, Song & Yosypiv 2012) (Fig. 5).

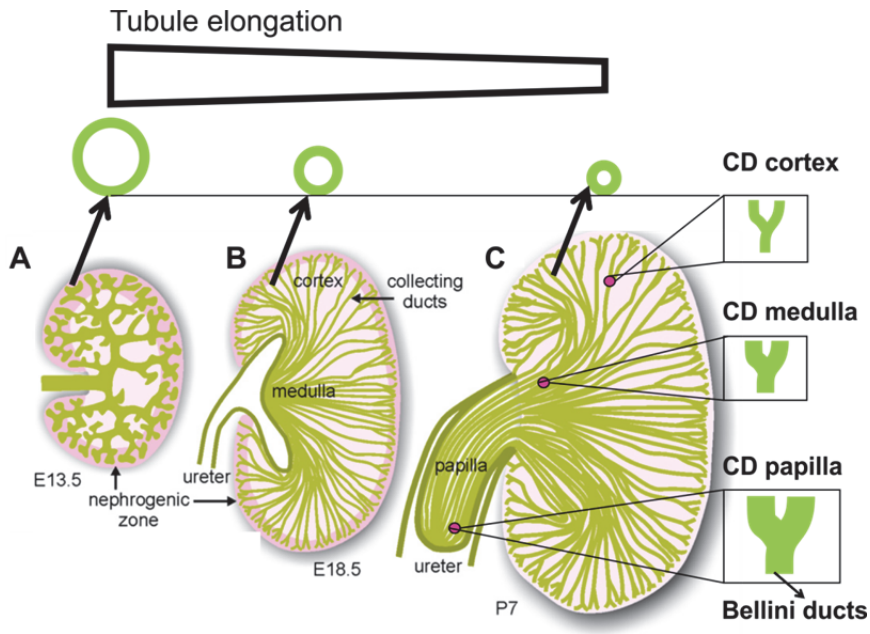

Fig. 5. Diagram of kidney medulla and papilla development showing CD tubule elongation. Note the smaller cross section tubules as development advances from embryonic day E13.5 (A), to E18.5 (B) to postnatal day 7 P7 (C). Observe in postnatal development how successive CD merging from cortex to papilla forms wider tubules. Modified from Costantini & Kopan 2010.

Kidney papilla grows in length through proliferating CD cells in a distal-to-proximal fashion; cell proliferation is arrested in the distal mouse papilla before E17.5, a time when extensive proliferative growth continues in the proximal papilla (Adams & Oxburgh 2009). Stromal mesenchymal cells surrounding these nascent kidney tubules give rise to renal medulla interstitium. Altogether kidney tubules and renal interstitium play essential roles in regulation of body water, electrolyte homeostasis and systemic blood pressure regulation, the main function of the medulla and papilla being the regulation of urine concentration (Dwyer & Schmidt-Nielsen 2003, Schmidt-Nielsen & Schmidt-Nielsen 2011). This capacity to concentrate urine increases during development while mouse kidney medulla

becomes identifiable morphologically only at E15.5, increases 4.5-fold up to birth and continues to grow during postnatal development (Song & Yosypiv 2012).

The main process in medulla and papilla growth seems to be tubular elongation, which generates narrower tubular diameter as development advances (see Fig. 5) (Yu 2011, Song & Yosypiv 2012). Elongation of tubules seems to happen through two planar cell polarity processes: orientated cell division (OCD), which aligns the cells on the long axis of the tube, and orientated cell migration or convergent extension-like processes, which drive specific migration of polarized cells (Karner *et al.* 2009) on cell polarity see also section 2.1.3). It was recently shown by live imaging technique that the PCP convergent extension-like movement during tubule elongation is driven in large part by a myosin-dependent, multicellular rosette-based mechanism for mediolateral cell intercalation, a deeply conserved cellular engine for epithelial morphogenesis. Disturbance in any of these two processes has been shown to cause renal cyst formation; increased rosette-based intercalation can restore normal tubule elongation if OCD is impaired (Lienkamp *et al.* 2012).

These planar cell polarity processes start in embryonic kidney development but are mainly responsible for the significant medullary and papillary postnatal growth. Two Wnts have been described so far to modulate these processes; *Wnt-9b* and *Wnt-7b* (Karner *et al.* 2009, Yu *et al.* 2009). *Wnt-9b* seems to regulate both tubular elongation processes but at different stages: in embryonic stage influencing the convergent extension-like movements, and postnatally regulating OCD. In hypomorph *Wnt-9b* kidney the tubules' cellular orientation is randomized, resulting in larger diameter tubules which consequently develop into tubular cysts. OCD is more tightly regulated during postnatal tubular elongation but also seems to be of crucial importance in the embryo during initiation of renal medulla at E15.5, as *Wnt-7b* mutants orientate their cells in a plane opposite to wild type and undergo increased apoptosis, which leads to failure in medulla formation. Moreover, *Wnt-7b* also seems to have survival properties on tubular cells as inactivation of *Dickkopf1* (*Dkk1*), a Wnt inhibitor on UB lineage, leads to a phenotype with overgrown kidney papilla with increased *Wnt-7b* signal (Pietila *et al.* 2011). These two Wnts seem to act in both autocrine and paracrine fashion, autocrine in collecting ducts (*Wnt-9b* and *Wnt-7b*) and paracrine in proximal tubules (*Wnt-9b*) and Henle's loop (*Wnt-7b*), the paracrine processes taking place in close relationship to medullary and papillary stromal cells (Karner *et al.* 2009, Yu *et al.* 2009, Costantini & Kopan 2010). Both of these Wnt signals implicated in cortical UB branching and determining nephron number endowment are also

important for tubule elongation in the medulla and papilla. Loss of these Wnts causes phenotypes of hypoplasia and dysplasia, hypodysplasia. Medulla and papilla hypodysplasia defects may indeed be secondary also to UB branching defects (Song *et al.* 2012), but in most mouse models these seem to be caused by an intrinsic role of these signals in medulla-papilla morphogenesis (Song & Yosypiv 2012).

Tubule maturation is achieved through establishing specialized cell types with a specific segmental cell type ratio (Costantini & Kopan 2010). CD include intercalated cells, which regulate pH homeostasis by secretion of H+ and HCO_3- into the urine, and principal cells, which concentrate the urine by absorbing water and regulating Na+ homeostasis. Towards the medulla there is a gradual increase in principal cells in relationship to the number of intercalated cells. Loss of principal cells and consequently their marker water channel aquaporin 2 (Aqp2) causes impairment in urine concentration, diabetes insipidus, while loss of intercalated cells causes distal renal tubular acidosis (Costantini & Kopan 2010). Cells in medulla, but mostly papilla, are able to sustain extreme osmolarity stress; this is mainly achieved through osmoprotective control in the ability to change cell volume through cytoskeleton rearrangement, aquaporin water channel control and osmolyte transport (Dwyer & Schmidt-Nielsen 2003, Gabert & Kultz 2011).

If our current understanding of tubule elongation is new and limited, even less is known about the complex tri-dimensional kidney tissue architecture within different organ axis, about tubular shape patterning, how a kidney tubular segment is confined to be straight or convoluted, or why the nephrons in the rostro-caudal poles of the kidney have more extensive curvature than the central ones (al-Awqati & Goldberg 1998, Yu 2011).

2.2.3 Functional evaluation of kidney disease

The role of a healthy kidney is to maintain the fluid and electrolyte composition, but it also plays key roles in several hormonal systems. This is accomplished by glomerular filtration, tubular reabsorption and secretion of electrolytes, solutes and water mediated by various hormones. In evaluation of kidney function urine analysis remains the most important screening tool for renal disease. In the mouse, daily 24-hour urine collections are possible in metabolic cages; however, they are still prone to bias, which is why calculation of concentration ratio of the analytes to urinary creatinine is necessary. Renal function and urinary excretion rate should also be related to body size, surface area or caloric expenditure.

Glomerular filtration rate (GFR) remains the single most useful quantitative measure of renal function in both health and disease and creatinine the filtration marker of choice for estimating GFR. Creatinine is an endogenous product of skeletal muscle metabolism, and the rate of production may vary depending on the muscle. It is freely filtered by the glomeruli but approximately 10–30% is secreted by the proximal tubule (PT). Under normal circumstances the rates of creatinine production and excretion are fairly constant.

Creatinine clearance (Ccr) can be used to estimate GFR, even though it slightly overestimates the result because of PT secretion of creatinine. It is an excellent late marker for renal damage but not useful in acute kidney injury when tubular secretion of creatinine can increase twofold with a very slow increase in plasma creatinine, both underestimating the severity of renal injury. The formula used to calculate Ccr (mL/min) = (Ucr × V)/Pcr, where Ucr is urine creatinine, V is the urine volume in mL per minute and Pcr is the plasma creatinine concentration.

Blood urea nitrogen (BUN) is another marker used in conjunction with creatinine and evaluates the capacity of the kidney to excrete toxic nitrogen recycled as urea BUN (mg/dL) = urea (mmol/L) × 2.8. Urea is synthetized in the liver cells from ammonia by the urea cycle and more than 90% is excreted by kidney. Because of some tubular reabsorption, especially in the presence of oliguria, measurement of BUN underestimates GFR. Increased BUN also suggests obstructive renal disease with stagnation of urine in proximal tubules and increased passive reabsorption of urea. BUN over plasma creatinine ratio gives an important direction in renal disease diagnostics (prerenal, renal, postrenal-obstructive); however, interpretation must be careful as creatinine values might be lower due to lower body mass. A complex situation is chronic obstructive disease, which would be characterized by postrenal azotemia as elevated BUN and plasma creatinine. The obstruction leads to slow renal flow, and therefore to increased passive reabsorption of urea, but also to increased pressure in urinary space and therefore decreased glomerular filtration. Chronic obstruction will lead to gradual loss of nephrons (glomerular and tubular function loss) and to renal azotemia features (Dennis J. Dietzen and Michael J. Bennett, 4th edition).

2.2.4 Congenital kidney disease, cystogenesis and impaired prenatal and postnatal kidney development

The term glomerulocystic kidney (GCK) is not currently in frequent use and it is mainly accepted as a phenotypic characterization of the two major forms of congenital polycystic kidney diseases (PKD), autosomal dominant and recessive. The GCK is defined as a kidney with more than 5% cystic glomeruli, glomerular cysts comprising Bowman space dilatations greater than 2 to 3 times normal size. GCK has variable clinical presentation; however, even though kidney function can be stable for years, most cases progress eventually to end-stage kidney failure (Torres & Harris 2009, Bissler *et al.* 2010, Lennerz *et al.* 2010, Torres & Harris 2011). For a long time, the glomerular cyst formation was seen as a secondary consequence to gross urine reflux into kidney parenchyma, caused by lower urinary tract obstructions. A few mouse models have shown that primary defects in glomerulus can cause glomerulocystic phenotypes, but there is little evidence from human disease genetic studies. Recent investigations postulate that kidney tubular abnormalities may play a role in glomerular cystogenesis; however, this aspect has been poorly investigated so far.

Disturbances in kidney tubule patterning play a major the role in the etiology of polycystic kidney disease (PKD) in general. Human disease studies and their targeted genetic models showed that PKDs are mainly ciliopathies, caused by mutations in proteins located in primary cilia and associated with abnormal rates in tubular epithelial proliferation and apoptosis (Torres & Harris 2009, Bagherie-Lachidan & McNeill 2010, Luyten *et al.* 2010). Advanced research studies in ciliary protein mouse models introduced planar cell polarity (PCP) as fundamental to the pathogenesis of PKD (Bagherie-Lachidan & McNeill 2010, Luyten *et al.* 2010). An earlier study in a Xenopus laevis PKD model proposed that the ciliary protein inversin regulates the switch between canonical and noncanonical Wnt in kidney tubulogenesis (Simons *et al.* 2005, Simons & Mlodzik 2008). However, the inversin mouse study which failed to support the cystogenesis Wnt switching hypothesis showed a loss of orientated mitotic spindle, considered to be managed by non-canonical Wnt/PCP signaling (Sugiyama *et al.* 2011). In recent years the Wnt and Wnt pathway related proteins, well known as cell polarity tissue organizers, have emerged as stronger candidates in mouse PKD pathogenesis through PCP control in kidney tubule lumen size and elongation (Karner *et al.* 2009, Yu *et al.* 2009, Bagherie-Lachidan & McNeill 2010, Lancaster & Gleeson 2010, Wuebken & Schmidt-Ott 2011).

Changes in Wnt pathway related proteins have now been noted in studies of human PKD disease and different mouse models of PKD (Romaker *et al.* 2009, Torres & Harris 2009). Cilia proteins, PCP signaling proteins and Wnts are suggested to be part of a complex network that controls and maintains tubule diameter and elongation; if impaired, any of these may initiate cystogenesis (Wallingford & Mitchell 2011).

Renal hypoplasia is a common, yet often misused term describing congenital kidney diseases. (Cain *et al.* 2010). Renal hypoplasia is defined as smaller kidney with reduced nephron number but otherwise normal morphology. The exact incidence of pure renal hypoplasia is unknown because congenital kidney diseases exhibit evidence of kidney tissue abnormality, defined as dysplasia. Confusion and term misuse is mostly due to limitation of noninvasive tools (ultrasound) to discriminate between pure hypoplasia and hypodysplasia. Renal hypoplasia/dysplasia are the leading cause for childhood end-stage renal disease. Subtle changes in normal nephron number, such as mild bilateral hypoplasia, are associated with adult onset hypertension disease and chronic kidney disease. Mouse mutant models, but also studies of human congenital diseases, provide insights into molecular pathogenesis of renal hypoplasia. Any of the nephrogenesis processes involving MM, UB and stroma can cause kidney hypoplasia with a lower number of nephrons: defects in induction, growth and branching, survival and differentiation (Cain *et al.* 2010).

2.3 Wnt-11 gene, protein and signaling pathways in mammalian organogenesis with emphasis on heart and kidney development

Wnt-11 is a unique member of the Wnt family, with multiple expression sites during embryonic development and possible action in specific tissue and cellular context through several distinct pathway networks (Uysal-Onganer & Kypta 2012).

Initially, Wnt-11 was classified as a non-canonical Wnt, a subclass of Wnts such as Wnt-4, and Wnt-5, which are not able to stabilize intracellular β-catenin, but activate RhoA, JNK or calcium-dependent pathways (Fig 6). However, the initial classification became extremely controversial as further studies showed that most Wnts, including Wnt-11, have the potential to activate more than one type of Wnt pathway in what might be a complex Wnt signaling network (Kestler & Kuhl 2008). This fresh view considers that specific Wnt activity is conferred by

the expression profile of receptors and signal transducers rather than by an intrinsic protein sequence (Kestler & Kuhl 2008, Willert & Nusse 2012). Canonical and noncanonical Wnts are capable of using common mechanisms, including Dvl and Gsk-3β, to activate completely unrelated coreceptors in murine multipotent cells (Grumolato *et al.* 2010).

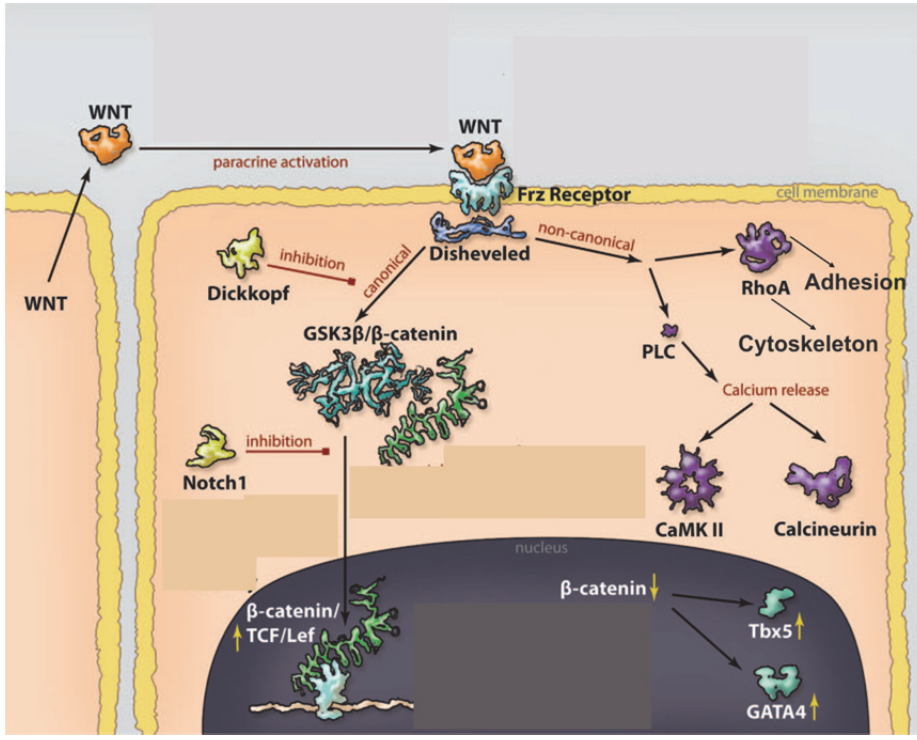

Fig. 6. Summary of Wnt pathways in cardiac compartment: canonical β-catenin-dependent Wnt signaling and non-canonical β-catenin independent/cell polarity Wnt signalling. Modified from Bergman 2010. Used with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health.

Indeed, Wnts share high sequence identity with a Wnt family-specific invariant positioning of 22 cysteine residues. However, prediction studies have suggested isoforms and specific alternative splicing together with distinct post-translational modifications profiles of glycosylation and acylation (Willert & Nusse 2012). Difficulties in studying Wnt proteins also arise from their poor solubility and strong association with cell surface and extracellular matrix (Kispert *et al.* 1996,

Eisenberg & Eisenberg 1999). Particularly Wnt-11 has more predicted glycosylation sites than other Wnt members, and in certain biological system it is subjected to additional post-transcriptional modifications. During Xenopus axis formation this involves tyrosine sulphation hetero-oligomerization with Wnt-5a forming a multimer Wnt with higher Wnt signaling activity than either individual (Cha *et al.* 2009). *Wnt-5a* and *Wnt-11* cooperation seem to go beyond lower vertebrates in heart development in mammals (Cohen *et al.* 2012).

The *Wnt-11* gene is highly conserved during evolution; however, some differences have been discovered between mammalian *Wnt-11* and other vertebrates' *Wnt-11*. In many vertebrate species there exists more than one *Wnt-11* gene, also called Wnt-11-related genes, but Xenopus (*Wnt-11R*), zebrafish (*Wnt-11r*) and chick (*Wnt-11b*) are true orthologs to human *Wnt-11*, sharing higher sequence identity than their Wnt-11 relatives. Therefore care should be taken when interpreting studies from Xenopus and zebrafish relative to mammals (Uysal-Onganer & Kypta 2012).

Expression patterns have been reported for *Wnt-11* during mammalian development in morula-blastocyst cells, kidney ureter buds and other areas of the urogenital system, somite, limb, perichondrium of developing skeleton, lung mesenchyme, cortex of adrenal gland, somites, limb, skin, development, digestive tract and pancreatic epithelium (Uysal-Onganer & Kypta 2012). Among these, maybe the most commonly known have been the roles of Wnt-11 in heart and kidney development.

2.3.1 Wnt-11 during heart development

Wnts are required throughout cardiogenesis and seem to play temporally distinct roles (Fig. 3). In the first phase of cardiogenesis, β-catenin-dependent canonical Wnt (Wnt/β-catenin) signaling promotes formation of mesoderm and proliferation of cardiac progenitors in precardiac mesodermal cells, while inhibiting their cardiac specification. In later stages, Wnt/β-catenin inhibits cardiomyocyte differentiation (Fig. 7, Naito *et al.* 2006, Ueno *et al.* 2007, Gessert & Kuhl 2010). In contrast, cardiogenesis requires β-catenin-independent (non-canonical) Wnt activity of *Wnt-5a* and *Wnt-11*, which have the dual role of promoting cardiomyocyte specification and terminal differentiation while restraining the inhibitory effect of canonical Wnt (Abdul-Ghani *et al.* 2011, Cohen *et al.* 2012). Besides promoting cardiomyocyte differentiation, β-catenin-independent Wnts seem to modulate cardiomyocyte proliferation/survival, an overexpression of

Wnt-11 signaling resulting in a bigger heart through hyperplasic mechanisms (Abdul-Ghani *et al.* 2011).

The postnatal roles of Wnt signaling have mainly been studied in the context of adult heart remodeling. Loss of β-catenin or inhibition of GSK3β/ β-catenin in Dvl knockout mouse attenuated stress-induced hypertrophic response, while *in vitro* or *in vivo* stabilization of β-catenin in cardiomyocytes triggered hypertrophic response, with or without stimuli (Bergmann 2010). Dvl lies upstream of both β-catenin and β-catenin-independent Wnt signaling (Grumolato *et al.* 2010). These strongly suggest that Wnt pathways have a potential role in adult cardiac hypertrophy and remodeling, a role that needs to be investigated in more detail. For reviews see (Brade *et al.* 2006, Gessert & Kuhl 2010, Vincent & Buckingham 2010, Miquerol & Kelly 2013).

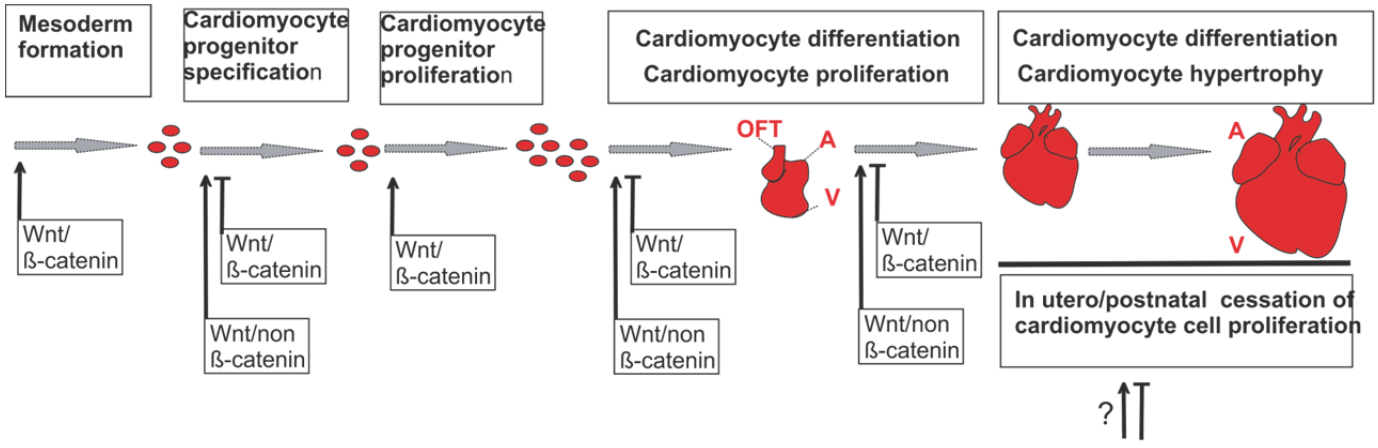

Fig. 7. The multiple phases of Wnt signaling during cardiogenesis: Wnt/β-catenin signaling is required for early cardiac mesodermal progenitor formation (see text). A atrium; V ventricle; OFT outflow tract. Modified from Gessert & Kuhl 2010. Used with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health.

Wnt-11 expression during heart development

Wnt-11 activity during mammalian gastrulation within or in close proximity to the cardiogenic mesoderm remains poorly understood. In zebrafish, *Xenopus* and avians *Wnt-11* is active in the cardiogenic mesoderm where it seeems to be important in early cardiomyocyte coherent migration (Pandur *et al.* 2002, Garriock *et al.* 2005, Matsui *et al.* 2005). These mutants show phenotypes of *cardia bifida* with abnormal apico-basal polarity and improper junctional complexes, suggesting improper epithelialization in early cardiomyocytes (Pandur *et al.* 2002, Garriock *et al.* 2005, Matsui *et al.* 2005). Similar phenotypes are reported in N-cadherin blocking studies in chick (Linask *et al.* 1997) and fibronectin mutants in zebrafish and mouse (Glickman & Yelon 2004). Altogether, these suggest that cardiomyocyte coherent migration is regulated by *Wnt-11* through cadherin-mediated signaling and by interaction with extracellular matrix proteins.

Indeed, Wnt-11 signaling requires binding with extracellular matrix proteins, heparan sulfate proteoglycans (HSPG), during mouse kidney morphogenesis (Kispert *et al.* 1996). Moreover, during gastrulation both fish embryos with mutated glypican - an HSPG - and glypican-depleted Xenopus laevis embryos exhibit defects in *Wnt-11* signaling (Ohkawara *et al.* 2003). Taken together, these suggest that *Wnt-11* interaction with extracellular matrix proteins is a preserved mechanism for Wnt-11 signaling between diverse biological contexts.

Although expression of *Wnt-11* in mouse cardiogenic mesoderm has not been clearly demonstrated (Cohen *et al.* 2012), *Wnt-11* may be present in this region and dramatically upregulated upon fusion of the cardiac crescent with formation of the early heart tube from E8.0 onwards (Kispert *et al.* 1996, Terami *et al.* 2004, Cohen *et al.* 2012). Mouse *Wnt-11* knockouts do not show severe phenotype of cardiac bifida; therefore if Wnt-11 is important in early cardiac epithelialization, its function may be redundant with another Wnt, such as Wnt-5a.

Later on in the heart looping stage, *Wnt-11* expression has been detected in the outflow tract (Kispert *et al.* 1996, Zhou *et al.* 2007) with roles in OFT assembly. The results presented in this thesis were the first to show that mouse Wnt-11 gene and protein are active in ventricular myocardium from E8.0 onwards, participating in ventricular myocardium development and maturation in looped and pre-septated heart E10.5-E12.5. We showed that Wnt-11 signaling regulation involves cardiomyocyte N-cadherin and β-catenin protein expression and protein -protein interaction, in line with the previous studies in the cardiogenic mesoderm

of lower vertebrates (Garriock *et al.* 2005). Our finding of active Wnt-11 signaling at this stage of ventricular development was later confirmed by others (Abdul-Ghani *et al.* 2011, Cohen *et al.* 2012). The latter Wnt-11 studies revealed novel roles for Wnt-11 in cardiomyocyte survival and cardiac progenitor maintenance. Cohen *et al.* show an unexpected co-requirement for Wnt-11 and another non-canonical Wnt, Wnt-5a, in regulation of SHF progenitor population size in mice (Cohen *et al.* 2012). Moreover, in the same publication in differentiating mouse embryonic bodies *Wnt-5a* and *Wnt-11* are co-required to promote both FH and SHF fates by their attenuating effect on repressor canonical Wnt, therefore activating progenitor terminal differentiation (Cohen *et al.* 2012) (Fig. 7).

Wnt-11 and cardiac transcription factor regulation

Wnt-11 is essential for heart development, being required for *in vivo* cardiac marker gene expression (Pandur *et al.* 2002, Afouda *et al.* 2008). The first line of evidence was produced by Eisenberg *et al.* from an *in vitro* model system showing the ability of Wnt-11 to trigger cardiomyogenic faith in noncardiac mesoderm of quail embryonic explants (Eisenberg *et al.* 1997, Eisenberg & Eisenberg 1999). Subsequent studies addressed the issue of cardiac "induction" in numerous *in vitro* model systems of embryonic pluripotent cells (Flaherty *et al.* 2008, Flaherty *et al.* 2012), quail mesodermal cells (Eisenberg & Eisenberg 1999, Terami *et al.* 2004), murine ES cells (Ueno *et al.* 2007), but also adult progenitors such as human endothelial progenitor cells (Koyanagi *et al.* 2005b) or murine bone marrow-derived multipotent cells (Flaherty *et al.* 2008, Flaherty *et al.* 2012). Expression of Wnt-11 in all these pluripotent cells triggers expression of major cardiac transcription factors: Nkx2.5, Mef2c, and Gata-4 defining their cardiomyocyte fate.

Our results during early cardiogenesis are in line with this showing impaired cardiac transcription factor expression due to loss of Wnt-11 function in ventricular myocardium. This primary mechanism might be behind the cardiomyopathy seen in our adult mouse *C57Bl6 Wnt-11* knockout study. Adult onset cardiomyopathies have been reported in human patients with haploinsufficiency mutations in these cardiac transcription factors (Costa *et al.* 2013, Li *et al.* 2013)

Wnt-11 and Gata-4

Human *Wnt-11* integrative genomics analysis shows TCF/LEF-binding sites within *Wnt-11* proximal promoter region as well as double Gata-binding sites within intron 2, conserved in other mammalian orthologs (Katoh & Katoh 2005), suggesting that both canonical-Wnt and Gata factors can activate *Wnt-11* expression. The interaction between Gata factors and Wnt-11 is reciprocal since Gata-4/6 are required for *Wnt-11* expression during Xenopus cardiogenesis, and Wnt-11 promotes Gata-4/6 differentiation program in maturing cardiomyocytes (Afouda *et al.* 2008). *Gata* factors link canonical and non-canonical Wnt signaling in cardiogenesis; while canonical Wnt inhibits Gata factors expression, canonical Wnt is also capable of upregulating non-canonical *Wnt-11* activity in *in vitro* and mouse model (Lin *et al.* 2007, Ueno *et al.* 2007) and consequently promoting Gata-induced cardiomyocyte differentiating program. These findings evoke the biphasic roles of canonical Wnt during the cardiogenic program, first promoting the precardiac mesodermal progenitors and later repressing the cardiomyocyte progenitor terminal differentiation (in Zebrafish and ES cells) (Gessert & Kuhl 2010). Although Wnt-11 alone is sufficient to activate Gata-4 cardiomyogenic program in pluripotent cells, synergistic and perhaps sequential signaling by other cardiogenic factors is critical for optimal terminal differentiation of cardiomyocytes (Cohen *et al.* 2012).

Wnt-11 and calcium signaling

During early heart tube formation in Xenopus Wnt-11 signals are required for genesis of the myocardial electrical gradient through L-type calcium channels, a mechanism which is not dependent on connexins or cell shape (Panakova *et al.* 2010). This study revealed a previously unrecognized role for Wnt-11/Ca signaling in establishing a physiological electrical gradient in the plane of the developing cardiac epithelium through modulation of ion channel function (Panakova 2010). Furthermore, this data demonstrated that Wnt-11 is capable of regulating subcellular calcium domains. This data is intriguing in relationship to an avian study showing that calcium signaling has a regulatory role in cardiac precursor cells during their commitment towards cardiomyocyte fate (Linask & Linask 2010).

In avians, Wnt-11 expression has also been detected in conduction system elements (Bond *et al.* 2003), but with no evidence on conduction system function later in heart development or heart disease.

It remains unclear whether the same type of Wnt-11 /Ca signaling is required in mammalian early or later heart formation. In our mouse *Wnt-11* mutants Cx-40 showed a normal expression pattern during ventricular development in cardiac development E10.5-E12.5, in addition to normal action potential and calcium fluxes in isolated embryonic cardiomyocytes (E12.5). No arrhythmias were observed in our mutants during echography investigations.

2.3.2 Wnt-11 during kidney development

Wnt-11 is expressed at the distal tips of the branching ureter throughout embryonic development but it is not essential for induction of nephrogenesis *in vitro* or *in vivo*, compared to other ureter bud-expressed Wnts as *Wnt-6*, *Wnt-7b*, *Wnt-9b* able to induce nephrogenesis in *in vitro* or *in vivo* conditions (Carroll *et al.* 2005). At the tips of the branching ureter *Wnt-11* expression takes place in an autoregulatory feed-back loop with mesenchymal ligand glial-cell derived neurotrophic factor Gdnf and its epithelial cRet tyrosine kinase receptor (Kispert *et al.* 1996, Majumdar *et al.* 2003) (Fig 4). *Gdnf* is decreased in 129 SV *Wnt-11* mutants and the lower number nephron phenotype seen in these mutants seems to correlate to possibly decreased trifurcation type of branching which is observed in later kidney development (Majumdar *et al.* 2003, Costantini 2010). For more information on Wnt signaling in the cortex nephrogenesis, see section 2.2.1.

Optimal ureter bud branching does indeed seem to determine an optimal nephron number. This has important clinical relevance as kidneys with a low number of nephrons are at high risk of developing hypertension since nephrons are naturally gradually lost during the aging process; in addition, it may also lead to childhood end stage renal failure (Cain *et al.* 2010).

Besides ureter branching no other role has been clearly proposed or demonstrated for *Wnt-11* signaling in kidney development, although *Wnt-11* gene activity has been noted, for example, in the medulla around medulla tubules together with Wnts which signal through both canonical and noncanonical Wnt pathways Wnt-4, Wnt-9b and Wnt-7b. So far clear roles in programming kidney tubule diameter and elongation through cell polarity pathways have been demonstrated for Wnt-9b and Wnt-7b in mouse models through coordinated cell migration and orientated cell division check (Merkel *et al.* 2007, Karner *et al.*

2009, Yu *et al.* 2009, Yu 2011). Final nephron endowment is completed during embryogenesis week 36 in humans and postnatal day 5 in mice, although the kidney tubules continue to mature long into the postnatal period. Later Wnt-11 roles during these developmental stages have not been addressed until the *C57Bl6 Wnt-11* knockout model in the present study. Our results from this mice study suggest that Wnt-11 might have an additional role during kidney development, such as regulation of tubule elongation and convolution, with involvement of Wnt-9b signaling, which was severely reduced in these kidney tubules.

3 Outline of the present study

Of the mammalian Wnts, Wnt-11 has mainly been classified as non-canonical, triggering a β-catenin-independent signaling pathway. Wnt-11 has proven to be involved in kidney ureteric tip branching (Kispert *et al.* 1996, Majumdar *et al.* 2003) and heart outflow tract morphogenesis (Kispert *et al.* 1996, Zhou *et al.* 2007). Possible connections between Wnt-11 and cadherin-mediated cell adhesion and migration have been drawn in *in vivo* studies performed on lower vertebrates and quail models (Eisenberg *et al.* 1997, Ulrich *et al.* 2005). *In vitro* investigations have revealed its ability to promote cardiogenesis in several types of progenitor cells (Pandur *et al.* 2002, Terami *et al.* 2004, Koyanagi *et al.* 2005a, Koyanagi *et al.* 2005b, Flaherty *et al.* 2008). The goal of the present study was to reveal novel Wnt-11 roles during heart and kidney development with relevance to human congenital diseases.

Specific aims of this study were:

1. To uncover new roles of Wnt-11 during myocardium development and to study the impact of its loss on heart function.
2. To reveal additional roles of Wnt-11 during tubule morphogenesis and study the consequences of Wnt-11 loss on kidney function.

4 Material and methods

Detailed description of the material and methods used in this study are found in the original article I and manuscripts II-III.

Method	Original article
Mouse line and genotyping	I, II, III
Tissue preparation	I, II, III
Cardiomyocyte primary cell culture	I
Histology staining (Hematoxylin Eosin)	I, II, III
Histology staining (Masson Trichrome)	I, II, III
Histology staining (Pass-Schiff)	II, III
Immunohistochemistry	I, II, III
Morphometry studies	I, II, III
Western Blotting	I
Immunoprecipitation	I
Tissue RNA extraction and real time PCR	I, II
In situ hybridization	I, III
Plasma and urine sampling and analysis	III
Cardiomyocyte cellular electrophysiology	I
Doppler echocardiography	I, II
Myocardial Strain analysis	II
Telemetry	II
Transmission electron microscopy	I, III
Statistical analysis	I, II, III

5 Results

5.1 *Wnt-11* deficiency in *129SV* background leads to primary cardiomyopathy causing heart failure with severe lethality in midgestation (I)

It has previously been shown that *Wnt-11* is strongly expressed in the primitive heart tube OFT segment (Kispert *et al.* 1996), from E8.5-E8.75, and plays an important role in the morphogenesis of this heart segment (Zhou *et al.* 2007). In addition to this, low levels of *Wnt-11* have been reported in the early ventricle myocardium (Eisenberg & Eisenberg 2006). Our *Wnt-11* expression assay shows *Wnt-11* transcripts and protein in ventricular and atrial myocardium in the looped heart at E10.5 and E12.5 (I Fig. 1 and online Supplementary Fig 1, and 2). These findings suggest a specific role for Wnt-11 in myocardium development.

To investigate putative functions of Wnt-11 in myocardium morphogenesis we studied myocardium morphology and function in *Wnt-11*$^{-/-}$ mice in *129Sv* background, with a previously reported severe lethality during *in utero* development (Majumdar *et al.* 2003, Zhou *et al.* 2007). We investigated in more detail the *Wnt-11*$^{-/-}$ lethality curve and noted that these mice were still recovered in normal Mendelian ratio at E12.5, but in less than 50% from the expected ratio at E13.5. Although it has previously been shown that OFT morphogenesis and septation are impaired in *Wnt-11*$^{-/-}$ (Zhou *et al.* 2007), at E12.5 heart septation is not completed; therefore we hypothesize that it cannot explain the severe mid-gestation lethality.

Our close inspection revealed that *Wnt-11*$^{-/-}$ mice did indeed have a myocardium defect in the form of a thinner ventricular wall already at E10.5 (I Fig.). Moreover, this associated with defects in LV and RV specific ventricular myocardium patterning, shown by compact and trabeculated myocardium morphometry analysis at E10.5 and E12.5 (I Fig 2). These were not caused by impaired apoptosis or proliferation processes (data not shown), confirming previous findings (Zhou *et al.* 2007). Signs of heart failure were observed in conjunction with the *Wnt-11*$^{-/-}$ myocardium defects, such as dilated atrial appendices, pleural and pericardial effusions (Supplemental fig 3 online). Later gestation embryos had hypoplastic hearts with thinner compact myocardium and less dense trabeculations with abnormal morphology (Fig. 8).

Furthermore, non-invasive Doppler echocardiography assay on E12.5 embryos *Wnt-11*$^{-/-}$ showed impaired IRT ($p < 0.001$, Table 1 in I and Supplementary Fig. 4 online), indicating abnormal embryonic myocardium relaxation in early diastole with preservation of the systolic function. This has probably as consequence increased arterial and venous pressure, seen as increased pulsatility indices for umbilical artery, descending aorta and ductus venosus ($p < 0.001$, Table 1 in I and Supplementary Fig. 4 online). As increased PIs in umbilical artery and aorta are observed in human placental insufficiency, we analyzed the mutant placenta and found normal histology, excluding placental insufficiency from causes of cardiac failure. Impaired relaxation seemed not to be caused by changes in cardiomyocyte excitability or calcium signaling as isolated ventricular cardiomyocytes from both study groups showed no significant differences in electrophysiology assay *in vitro* investigating action potential, calcium transient amplitude, duration, oscillation rate or pacing (Supplementary Fig. 6 online). In addition, we found that 92% of the E12.5 *Wnt-11*$^{-/-}$ embryos had holosystolic AV valve regurgitation compared to 8% in the control group ($p < 0.001$); however, no defects in AV valvular cushions were noted. This is most probably a functional type of regurgitation due to the observed dilatation of the ventricular chambers (I Fig. 2).

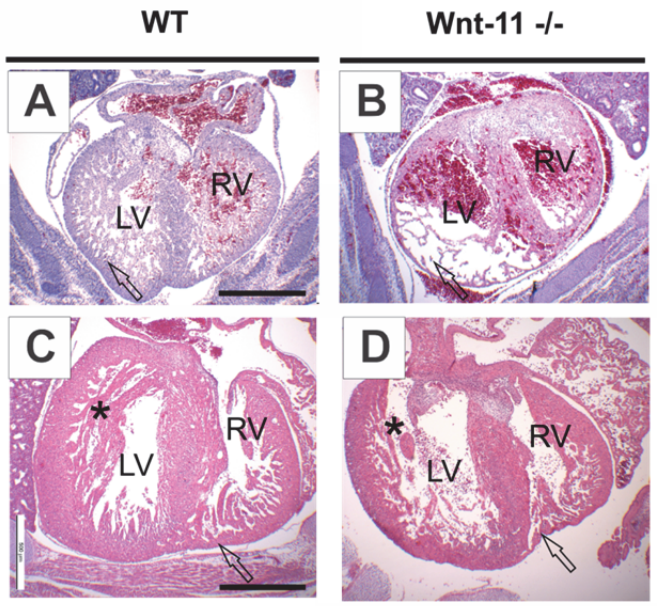

Fig. 8. Histology of later gestation *129 SV Wnt-11* knockout embryos E14.5 (A-B) and E17.5 (C-D). Note the thinner compact myocardium (arrow) and abnormal myocardium trabeculations (star) in both ventricles. Bars 500 µm.

5.2 Loss of *Wnt-11* in ventricular cardiomyocytes is linked to impaired cell adhesion and cytoarchitecture associated with delayed differentiation (I)

During gastrulation, Wnt-11 signaling has been known to regulate cell cohesion type of movement through cadherin cell adhesion molecule (Ulrich *et al.* 2005), while N-cadherin is important for cardiomyocyte adhesion and differentiation (Ong *et al.* 1998, Toyofuku *et al.* 2000, Ferreira-Cornwell *et al.* 2002, Kostetskii *et al.* 2005, Li *et al.* 2005). Given this, we investigated if *Wnt-11*$^{-/-}$ ventricular myocardium defects are caused by abnormalities in cardiomyocyte cell-cell contacts and myofibrillar apparatus.

Immunohistochemistry assay using N-cadherin/β-catenin, phalloidin for F-actin and cardiac α-actinin revealed severe cardiomyocytes cell shape abnormalities in *Wnt-11*$^{-/-}$ (I Fig.). While at E10.5 WT ventricle cardiomyocytes

were cuboidal in shape and regularly aligned in layers, loss of *Wnt-11* caused irregularity in early cardiomyocyte size and shape, with random cellular arrangement in the ventricular compact layer or the emerging trabeculations (Fig. 3 in I). This is even clearer in E12.5 when WT cells become elongated, while *Wnt-11*$^{-/-}$ remained round and poorly organized (I Fig. 3). Cytoskeletal F-actin arrangement was disturbed in compact myocardial and trabeculated myocardial *Wnt-11*$^{-/-}$ cells compared to WT (I Fig. 3 G-H). *Wnt-11*-deficient cells also had altered N-cadherin and β-catenin protein expression, so that co-localization of these two essential cardiomyocyte adhesion molecules was severely randomized. In controls these molecules were well co-localized around the ventricular cardiomyocyte borders at early E10.5 (Figure 3 in I). Abnormal N-cadherin and β-catenin protein expression and protein-protein interaction in early ventricles have also been shown by immunoprecipitation assay: severely reduced N-cadherin/ β-catenin ratio with increased total N-cadherin levels ($p < 0.05$) (I Fig. 4). This aspect of cadherin/catenin dynamics has also been investigated *in vitro* where *Wnt-11*$^{-/-}$ cardiomyocytes showed reduced capacity to form cell clusters compared to controls (Fig. 9), in line with previous observations in gastrulating zebrafish cells (Ulrich *et al.* 2005).

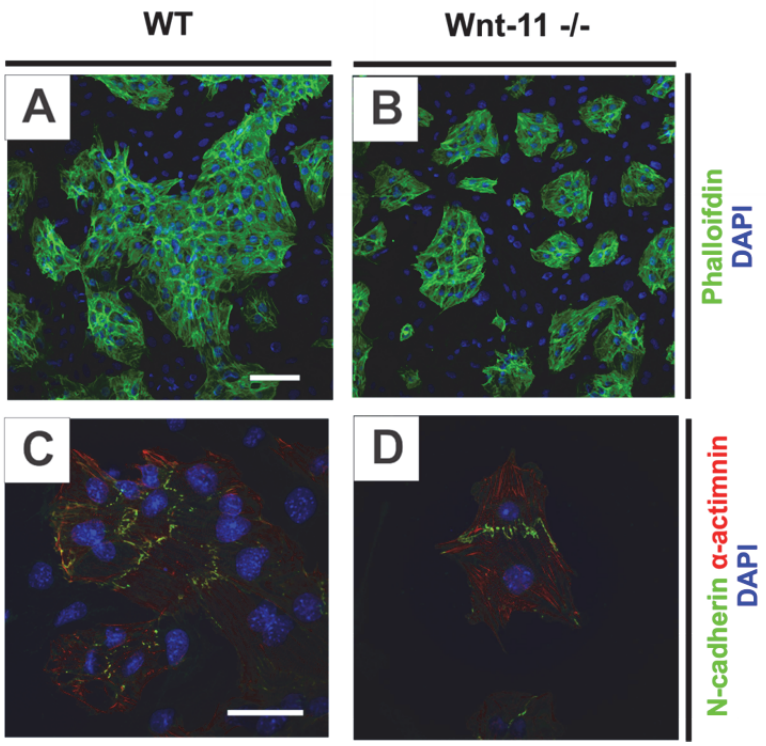

Fig. 9. Confocal images of primary cardiomyocyte cultures from E12.5 WT and *Wnt-11*$^{-/-}$ ventricles. (A-B) Staining for F-actin with Phalloidin (green) and (C-D) N-Cadherin (green) and α-actinin (red). In all images DAPI (blue) for nucleus. Note smaller cluster formation in *Wnt-11*$^{-/-}$ cardiomyocyte cultures. Bars A-B 100 μm, C-D 50μm.

Immunohistochemistry on E12.5 cells shows poor sarcomeric organization in *Wnt-11*$^{-/-}$, as demonstrated by cardiac α-actinin (Supplemental Fig. 7 online). This has been evaluated in detail by our TEM analysis in line with our abnormal cardiomyocyte morphological findings from immunohistochemistry. *Wnt-11*$^{-/-}$ cardiomyocytes are poorly differentiated, as shown by their low myofibrillar content, poor spatial arrangement in immature myofibrillar bundles and immature cell-cell junction ultrastructure (I Fig. 5 and Supplementary Fig. 8 online).

In summary, these observations support the conclusion that Wnt-11 signaling has a specific role in the control of cardiomyocyte architecture in developing myocardium.

5.3 Wnt-11 deficiency abnormalities in ventricular myocardium associate with changes in genes implicated in heart development (I)

The *Gata-4*, *Nkx2.5*, and *Mef2C* genes encode important cardiac transcription factors upstream of other essential signals for cardiogenesis, which include natriuretic proteins ANP and BNP. These are prime candidate targets for Wnt-11 signal transduction in mammalian ventricular development, given that Wnt-11 regulates their expression in cell culture and frog mesoderm explants (Eisenberg & Eisenberg 1999, Pandur *et al.* 2002, Terami *et al.* 2004, Koyanagi *et al.* 2005a, Cohen *et al.* 2008, Flaherty *et al.* 2008). *In situ* hybridization and *qRT-PCR* analysis revealed that the *Gata-4*, *Nkx2.5*, *Mef2C*, and *ANP* (atrial natriuretic peptide) transcripts were reduced in *Wnt-11*-deficient heart compared with controls both at E10.5 and E12.5 (Fig. 6 in I). In contrast, *B-type NP* (*BNP*) expression was induced at E10.5, coinciding with increased phosphorylated-JNK activity in ventricular myocardium, as shown by Western blotting. These latter changes are possibly in response to heart dysfunction (Cameron & Ellmers 2003, Lubbers & Eghtesady 2007).

Level expression changes in heart transcription factors could be part of the pathogenesis mechanism of the *Wnt-11*$^{-/-}$ embryonic heart defects, in line with the findings that a heterozygous knock-out or a hypomorphic allele for any of these genes can lead to similar heart malformations (Bruneau 2008). Our results suggest that these genes important for ventricular myocardium development are modulated by Wnt-11 signaling.

5.4 *Wnt-11* is expressed in late myocardium development and adult *C57Bl6 Wnt-11* knockout mice present CMP phenotype (II)

The next aim was to investigate putative roles of Wnt-11 in late myocardium development; however, *129SV Wnt-11* knockout has severe lethality during midgestation and no mice survive to adulthood. Given that genetic modifiers are known to modulate the penetrance or severity of cardiac disease in the mouse (Barrick *et al.* 2007), we have crossed the *Wnt-11* knockout allele to another mouse genetic background, the *C57Bl6*. Compared to the severe *in utero* lethality observed in the previous *Wnt-11* knockout mouse investigations (Zhou *et al.* 2007, Bergmann 2010, Nagy *et al.* 2010, Cohen *et al.* 2012), the *C57Bl6 Wnt-11*$^{-/-}$ mice from this study show normal viability until the first weeks of postnatal stage (see

viability data in III) when around 50% of the mutant mice die. This suggests that *in utero* lethality of the *Wnt-11* knockout mice caused by the cardiac phenotype is modulated by genetic background.

Histology of 12- to 16-week-old mouse survivor hearts revealed notably enlarged hearts in *C57Bl6 Wnt-11*$^{-/-}$ compared to WT, including a few individuals with a gross cardiomegaly phenotype (1/10) (II Fig 1 C). We noted abnormal asymmetrical thickness in LV ventricular walls with significant LV papillary muscle hypertrophy (II Fig 1 B, E). *Wnt-11*$^{-/-}$ desiccated heart mass relative to total body mass was significantly increased compared to controls ($p < 0.001$, $n = 6$) and in line with this, cardiomyocytes had a larger cross section area as a sign of cellular hypertrophy (II Fig. 1 J). Altogether, these findings suggest that the *Wnt-11*$^{-/-}$ hearts have a hypertrophic type of cardiomyopathy.

In addition to the notable hypertrophy phenotype, we noted ventricular noncompaction segments with thin compact myocardium and abnormal clubbed trabeculations located in the apex and mid-anterior wall of LV and RV (II note apical LV noncompaction Fig. 1F). We investigated capillary formation by endothelial PECAM and noted no significant difference compared to the control group, suggesting normal capillary network formation in mutant myocardium (II Fig. 1 L).

Some inherited hypertrophic cardiomyopathy models involve cardiomyocyte hyperplasia; however, this was not the case in *Wnt-11*$^{-/-}$ neonatal myocardium (II Fig 2). The PCNA signal was weaker in certain areas of *Wnt-11*$^{-/-}$ myocardium, including noncompaction areas. The proliferating cell nuclear antigen (PCNA) is required for DNA replication preceding mitosis as well as DNA nucleotide excision repair, labeling cells that recently completed karyokinesis or cytokinesis. This issue needs further investigation with a specific marker for the mitotic phase (for example, phospho-Histone H3 or bromodeoxyuridine) to elucidate the significance of this finding.

We reported previously that in *129SV* mouse background *Wnt-11* is expressed in the ventricular myocardium of the tubular heart, looped heart and before heart septation is completed at E12.5. In line with this, *Wnt-11*-deficient hearts showed defects in myocardium morphogenesis, leading to heart failure and severe lethality in midgestation. This indicated the importance of Wnt-11 signaling in pre-septated heart early myocardium development. Our *Wnt-11 qRT-PCR* results showed that the *Wnt-11* gene is active as well in late ventricular myocardium, at E16.5 and neonatal stage (II Fig. 3), in line with previous findings in other genetic backgrounds (Abdul-Ghani *et al.* 2011, Cohen *et al.* 2012). This suggests a role

for Wnt-11 signaling in late myocardium development, including after birth in the neonatal stage.

5.5 Phenotypic characterization of Wnt-11$^{-/-}$ hearts - Functional assay telemetry, standard Doppler and 2D strain imaging (STE) (II)

Functional assay included blood pressure measurements by telemetric method, classic global cardiac assay by Doppler echocardiography and segmental tissue Doppler echography by the STE method.

Besides cardiac defects, *C57Bl6 Wnt-11*$^{-/-}$ mice showed glomerulocystic hypoplastic kidneys with mild to moderate loss of renal function (III). Therefore we investigated if *C57Bl6 Wnt-11*$^{-/-}$ mice show increased blood pressure (Lennerz *et al.* 2010, Torres & Harris 2011), which would have explained the cardiac hypertrophic phenotype. Telemetry assay showed no significant differences in systemic blood pressure between the two study groups.

No significant statistical difference between the study groups was shown by standard Doppler echography for systolic or diastolic functional parameters (II Table1). However, there was significant hypertrophy in the left ventricular posterior wall in the male *Wnt-11*$^{-/-}$ group (WT left ventricle posterior wall diastolic thickness 0.75 ± 0.03 mm and systolic 1 ± 0.03 mm versus *Wnt-11*$^{-/-}$ left ventricle posterior diastolic thickness 0.95 ± 0.06 mm, $p < 0.001$, and systolic 1.29 ± 0.06 mm, $p < 0.05$) (II Table 1). For further functional assessment we used Speckle tracking echography (STE), which is a non-Doppler echographic technique. This method allows quantification of regional systolic and diastolic myocardial function by measuring the magnitude and the rate of myocardial deformation relative to its original shape through the measurement of the strain rate (SR) (Hoit 2011, Mondillo *et al.* 2011). We performed an STE assay on *Wnt-11*$^{-/-}$ and *WT* control mice for radial but also longitudinal strain rate, in short and long axis views (see Fig. 4 A). Our results showed notable changes in radial, circumferential and longitudinal SR values in *Wnt-11*$^{-/-}$ ventricular segments compared to WT (see Table 2 and Fig 4). Statistical significance between the study groups was observed for short axis radial SR, which was lower in *Wnt-11*$^{-/-}$ inferolateral wall segment during systole (5.3 ± 1.9 s^{-1} versus WT 9 ± 1.3 s^{-1}, $p < 0.05$) and in *Wnt-11*$^{-/-}$ inferior wall segment during diastole (-5.3 ± 2.3 s^{-1} versus WT -8.8 ± 1.4 s^{-1}, $p < 0.05$ (II Table 2 and Figure 4 B). Short axis circumferential SR showed statistically significantly lower values in *Wnt-11*$^{-/-}$

anterior wall segment during systole (-3.7 ± 0.9 s^{-1} versus WT -7.8 ± 1.2 s^{-1}, $p < 0.03$), while the anterolateral wall segment showed improved circumferential SR values compared to WT during diastole (9 ± 1.7 s^{-1} versus WT 5.6 ± 1.7 s^{-1}, $p < 0.0.5$) (II Table 2 and Figure 4 C). In long axis view, global or segmental longitudinal systolic SR was not changed between the two groups. In contrast, global longitudinal diastolic SR was significantly decreased together with all segments of the anterior wall and apex in *Wnt-11*$^{-/-}$ compared to WT (global longitudinal diastolic SR 3.3 ± 0.6 s^{-1} versus WT 5.3 ± 0.4 s^{-1}, $p < 0.001$) (Table 2 and Figure 4 D).

Taken together, these results suggest impaired relaxation in large segments of the *Wnt-11*$^{-/-}$ LV myocardium anterior and apical wall and impaired contraction in mid-myocardium, near papillary muscle insertion.

5.6 Wnt-11 loss in C57Bl6 background leads to glomerulocystic kidney with abnormal tubular morphology and impaired kidney function (III)

Our study shows that *C57Bl6 Wnt-11* knockout mice developed a hypoplastic kidney with specific pathologic changes such as cortical microcysts, a phenotype with a 100% penetrance (III Figure 2 and supplementary figure 1). Our histology and marker analysis revealed that *C57Bl6 Wnt-11*$^{-/-}$ cysts have glomerular origin, demonstrated by the presence of glomerular rudiments (III Figure 2 and Supplementary figure 1). The kidney phenotype resembles the human glomerulocystic kidney (GCK) (Lennerz *et al.* 2010). The overall parenchymal architecture, a medullary ray consisting of a group of straight tubes of collecting ducts with a fan-like configuration, was disturbed as compared with the controls (III Figure 2 E,G and Figure 5 C-D). The papilla conformation was more convoluted than in the controls (III Figure 2, E, F) and the cell morphology of the terminal collecting ducts, the Bellini ducts, was altered from the typical columnar pattern to a squamous one (III Figure 2, compare D with H, arrow), as also depicted with the AQP2 marker (III Figure 5, compare K, L with I, J). In addition, the luminal diameter of the PTs within the cortical labyrinth had increased (III Figure 2, compare N with J circled tubules).

Besides these 100% penetrant phenotypes, ~25% of the *Wnt-11*$^{-/-}$ mice (NB and adult mice, $n = 25$) had more severely affected kidneys with large glomerular cysts (Figure 2 M, arrow) and tubular origin cysts, as indicated by markers (Supplementary figure 2). Thus, the *Wnt-11* deficiency affects the organization of

the kidney tubules and glomeruli and provides a novel mouse disease model that may prove relevant for human glomerulocystic kidney disease studies.

OPT analysis demonstrated that *Wnt-11*$^{-/-}$ kidneys were smaller compared to WT, with a 34% reduction in pelvic volume ($p < 0.001$) (III Figure 3 A-D and E-F and Supplementary figure 3 A). The *Wnt-11*$^{-/-}$ kidney pelvis is dysmorphic, with severe reduction in dorsoventral diameter (46% smaller compared to WT, $p < 0.05$, Figure 3 B, D, F), while cortico-medullary diameter was less affected (10% smaller than in WT, $p < 0.05$, Figure 3 A, C, E). Moreover, *Wnt-11*$^{-/-}$ kidney tubules had abnormal spatial morphology, with increased convolution (III Figure 3 G-J and Supplementary figure 3 B-B' and C-C'). We conclude that Wnt-11 signaling has an important role in the coordination of the spatial organization of the nephron tubular epithelium in three dimensions,

To evaluate kidney function in *C57Bl6 Wnt-11*$^{-/-}$ adult mice, we performed physiological analysis of serum and urine chemistries. These showed lower creatinine clearance, as 52% relative to WT value ($n = 10$, $p < 0.05$) (III Supplementary figure 4 A and Supplementary tables 1, 2), indicating a significant decrease in glomerular filtration rate (GFR) in *Wnt-11*$^{-/-}$. This is in line with our findings of elevated *Wnt-11*$^{-/-}$ Blood Urea Nitrogen (BUN) relative to plasma Creatinine (Crea) compared to controls ($n = 10$, $p < 0.05$) (III Supplementary figure 4 B, Supplementary tables 1, 2). Altogether, these results indicate a mild-to moderate degree of kidney failure, with preserved tubular function as plasma and urine analytes showed no significant differences between the study groups (III Supplementary table 1, 2)

5.7 Wnt-11 is expressed in tubular epithelial cells from cortex to tip of papilla and throughout kidney organogenesis (III)

It is poorly known whether the *Wnt-11* gene is expressed after birth and/or in the fully mature kidney. To explain the complex phenotype in *Wnt-11*$^{-/-}$ mice we investigated Wnt-11 gene and protein expression in more detail. In NB mice Wnt-11 was detected more intensely in CD epithelial cells, including papillary ducts in adult mice (III Figure 1). Discrete expression was observed in NB and adult proximal tubule (PT) and papillary ducts from NB (III Figure 1). No expression was depicted in the glomerular tuft cells (III Figure 1 A and E glomerulus indicated by dotted line circled areas). The Wnt-11 expression results support the idea that Wnt-11 would serve as a signal that contributes to the spatial organization of the epithelial tubular cells *in utero* but also later.

5.8 Abnormal Wnt-11$^{-/-}$ kidney tubule morphology suggests impaired cell polarity processes (III)

We next addressed in more detail the consequences of *Wnt-11* deficiency on kidney tubule morphology. We counted the number of DAPI+ cells within the tubular cross sections and determined the percentages of tubules with a given number of cells in the tubular cross section.

The analysis showed enlarged tubular cross sections with more DAPI+ cells counts in *Wnt-11* knockouts (III Figure 4, compare C, D, G, H with A, B, E, F, arrow), which was in line with the histological findings (III Figure 2). Around 40% of the newborn *Wnt-11*$^{-/-}$ PTs had 6–8 cells per cross section while in the controls only tubules with 4–5 cells per cross section were seen (III Figure 4I). Notably larger tubules with 6–8 or 9–10 cells per cross section were noted in 60% of the total tubules in the kidneys of adult *Wnt-11*-deficient mice, whereas the controls had 4–5 cells per cross section (III Figure 4I). Similar changes were found in the CD (Figure 4J).

In contrast to the PT and CD changes, the cross sections of the terminal papillary tubules, the Bellini ducts, of the NBs, but not of the adult mice, were narrower than in the controls (Figure 5, compare G, H with E, F). This difference in phenotype can be explained by the fact that Bellini ducts are formed from papillary CD tubules converging in larger Bellini tubules, while PT and CD in the cortex elongate during development to form narrower tubules. The data suggest that Wnt-11 is critical for the process of controlling the luminal diameter of the tubules during the later stages of kidney maturation.

Previous investigations show that tubular circumference is finely regulated during embryogenesis, mainly by processes involving cell polarity pathways (Karner *et al.* 2009, Yu *et al.* 2009); therefore our results suggest that Wnt-11 is connected to spatial and temporal control of tubular luminal diameter. In support of this, the gene encoding *Wnt-9b*, a critical tubular cell polarity factor (Carroll *et al.* 2005, Karner *et al.* 2009, Karner *et al.* 2011), was down-regulated in *Wnt-11*$^{-/-}$ at E16.5 and at birth (III Figure 7, compare E-H, L-N with A-D, I-K, arrow).

5.9 *Wnt-11* deficiency alters cell proliferation and cell survival in the cortex, correlating with changes in nephron progenitor gene expression (III)

To obtain a better view of the cellular and molecular mechanisms of kidney hypoplasia and tubular abnormalities in Wnt-11-deficient mice, we next addressed potential changes in cell proliferation (PCNA, P-H3), apoptosis (TUNEL) and progenitor cell marker genes.

Proliferation (P-H3) of the cortical cells was reduced in *Wnt-11*$^{-/-}$ (III Figure 6, compare B with A, K); however, it did not correlate with the cystogenesis (data not shown). The PCNA marker, also coupled to DNA repair and apoptosis (Brenner *et al.* 2003), depicted an increase in staining in the kidneys of the NB mice, but not at E16.5 (data not shown). Consistent with this, there was TUNEL+ overlapping in some of the PCNA+ cells in the cortex of the *Wnt-11*$^{-/-}$ kidneys (III Figure 6, compare D, F with C, E, arrow; K). No significant differences were noted in the medulla or papilla, except that the distribution of proliferating cells within the tubules was different, especially at the tip of the papilla (III Figure 6 G-J).

The progenitor cells within cortical domain *Six2*, *Hox10* cluster genes and the *Foxd1* gene were altered in *Wnt-11* knockout (III Figure 8 A-L). The failure in Wnt-11 signaling was thus reflected in changes in nephron progenitor and stromal marker expression. The data suggest that Wnt-11 function contributes to the coordination of nephrogenesis alongside Wnt-9b. The decrease in proliferation and increase in apoptosis in the *Wnt-11*$^{-/-}$ cortical progenitor domain may lead to cessation of nephrogenesis via control of the genes that are important for this process.

6 Discussion

6.1 Wnt-11 signaling controls development of the early myocardium and is essential for normal cardiac functional development (I)

Our study points to an additional critical function for Wnt-11 in heart development, namely development of the ventricular myocardium. Wnt-11 is expressed by the pre-septated heart ventricular cardiomyocytes and is important in shaping their spatial organization and differentiation during the development of the four-chamber heart. The ventricular cardiomyocytes in the *Wnt-11*$^{-/-}$ embryos were disorganized in relation to each other; the compact ventricular wall was hypoplastic with poorly developed trabecular processes. It should be noted that septation of the OFT in the mouse embryo becomes complete at E14.5 (Bruneau 2002) and human fetuses with TGA may have undisturbed cardiac and placental functional development (unpublished observations) (Bruneau 2008). Given these, the OFT defect does not fully explain the early lethality of the *Wnt-11*$^{-/-}$ before heart septation is completed; it is therefore likely that the ventricular myocardial developmental defects are the primary factor contributing to the failure of the *Wnt-11*-deficient embryonic heart as both the properly formed compact and trabecular compartments are essential for normal heart function. Altogether, these suggest a critical function for Wnt-11 in controlling ventricular myocardium development.

Our Doppler ultrasound analysis indicated changes in several functional parameters in the *Wnt-11*$^{-/-}$ heart at midgestation, during which the mouse embryo is growing fast and should increase its cardiac output to maintain adequate placental and fetal perfusion. We find that the IRT% was significantly greater in the *Wnt-11*$^{-/-}$ embryos, and this could lead to abnormal filling of the ventricles causing an increase in atrial and venous pressures (Makikallio *et al.* 2005). Indeed, we observed an increase in the pulsatility of the ductus venosus blood velocity waveform and also identified holosystolic AV valve regurgitation in the *Wnt-11*$^{-/-}$ embryonic hearts. As no abnormalities were noted in the developing AV cushions, the regurgitation was most probably functional, caused by dilatation of the ventricles. No structural abnormality was noted in the mutant placenta, excluding it from causes of heart failure.

Our study indicates that Wnt-11 is important for myocardium development and heart function. Previous *in vitro* studies show that increase expression in *Wnt-11* marks the terminal differentiation of the cardiomyocytes and by adding Wnt-11 to progenitor cells increases formation of beating foci (Ueno *et al.* 2007). In line with these and *in vivo Xenopus* studies (Garriock *et al.* 2005, Afouda *et al.* 2008, Cohen *et al.* 2012), our *129Sv Wnt-11*$^{-/-}$ mouse investigation showed poor cardiomyocyte differentiation altogether with low level of cardiac transcription factors essential for this process. The sensitivity of the heart development to the dosage of *Nkx2.5*, *Mef2C* or *Gata-4* was recapitulated in mouse models of human mutations (Pu *et al.* 2004, Jay *et al.* 2005, Bruneau 2008). Among these the decreased activity of the *Gata-4* cardiac transcription factor has a notable significance for the *Wnt-11*$^{-/-}$ heart pathology. Gata-4 and Wnt-11 have reciprocal positive regulatory properties (Afouda *et al.* 2008, Afouda *et al.* 2008, Afouda & Hoppler 2011), share cardiogenic promoting activity (Eisenberg & Eisenberg 1999, Pandur *et al.* 2002, Terami *et al.* 2004, Jay *et al.* 2005, Afouda *et al.* 2008) and are co-required for an optimal cardiomyocyte terminal differentiation.

We showed that Wnt-11 regulates myocardium development by coordinating the co-localized expression of the cell adhesion molecules N-cadherin and β-catenin, which are critical for the spatially specific organization of cardiomyocytes (Linask *et al.* 1997, Zuppinger *et al.* 2000, Sedmera 2005, Hirschy *et al.* 2006, Phillips *et al.* 2007). Wnt-11 is known to control forces of cohesion and cell adhesion in some other systems by regulating endocytosis of another cadherin superfamily member, E-cadherin (Ulrich *et al.* 2005). Similarly, Wnt-11 signaling may control the localization of N-cadherin and β-catenin to specific cellular domains of the embryonic cardiomyocytes. This mechanism promotes cell adhesion and is important for the organization of the cells in a specific orientation during development of the ventricular wall. By affecting cardiomyocyte organization Wnt-11 signaling might also influence processes dependent on it, such as orientation of cell division affecting the myocardium patterning or specific response to a polarized morphogen. In the working myocardium abnormal cardiomyocyte organization may especially affect the structural integrity with direct consequences on force transmission.

In other systems Wnt-11 signaling has been involved in the planar cell polarity (PCP) pathway controlling the convergent extension (CE) movements that take place during gastrulation and during organogenesis, including formation of the early heart (Ulrich *et al.* 2005, Brade *et al.* 2006, Karner *et al.* 2006). Several PCP pathway components regulate heart development, e.g. Scribble and

Vangl2 are known to control outflow tract and ventricular myocardium development, as Wnt-11 is shown to do here. Mutations in *Scribble* and *Vangl2* cause a similar cardiac phenotype to that seen in *Wnt-11* mutants. PCP proteins are thought to operate at the level of a putative frizzled receptor and upstream of the small cytoplasmic GTPases Rac and Rho, which link the PCP pathway to the dynamics of the cytoskeleton. In line with this, we noted a correlation between the changes in cytoskeletal organization and the organization of the cardiomyocytes, similarly to that observed in chick muscle (Gros *et al.* 2008). Hence, as a non-canonical Wnt, Wnt-11 may control the directional movements of the cells in the assembling ventricular wall by modulating cell adhesion in connection with changes in the cytoskeleton.

In summary, Wnt-11 is implicated in heart development, as it controls the differentiation and organization of the cardiomyocytes in the ventricular wall. The reason for the failure of the *Wnt-11*-deficient heart probably lies in a reduced capacity to improve its function, especially the diastole, during the early developmental stages. This is caused primarily by disturbed adhesion between the ventricular cardiomyocytes, mediated by impaired co-localized expression of the N-cadherin/ß-catenin adhesion complex with a simultaneous change in the organization of the cytoskeleton. Wnt-11 is a critical signal for myocardium development and may function as a PCP pathway signal in the ventricular wall, regulating the convergent extension type of cell organization by controlling cellular adhesion.

6.2 Cardiac phenotypes in *Wnt-11* knock out models and their relevance to human inherited cardiac diseases (I and II)

In clinical work human heart malformations are mainly evaluated in the old frame of the segmental model of heart development, with congenital diseases of big arteries and valves separated from congenital diseases of the myocardium (Buckingham *et al.* 2005). However, myocardium shares cellular lineage with all the other heart structures mentioned above, shown by the recent discovery of first and second heart field (SHF) (Bruneau 2008, Sirbu *et al.* 2008, Pandur *et al.* 2013). The *Wnt-11* gene has been shown in our studies and by others to be expressed in early heart tube (Kispert *et al.* 1996, Nagy *et al.* 2010, Abdul-Ghani *et al.* 2011, Cohen *et al.* 2012) and to be important for cardiomyocyte terminal differentiation, especially for the cells derived from SHF (Cohen *et al.* 2012). In

line with these *Wnt-11* mutants shows primary defects in ventricular myocardium but also OFT structures (I, II,(Zhou *et al.* 2007))

We have shown previously that *Wnt-11* loss causes primary defects *in utero* in myocardium maturation and patterning, in pre-septated heart (Nagy *et al.* 2010), and OFT defects in septated heart stages (Zhou *et al.* 2007). Such OFT defects may directly alter hemodynamic conditions, which may secondarily influence myocardium development (Sedmera 2005). This most probably occurs in late gestation when septation is complete, and at birth together with the complex circulatory changes during the transition from fetal to neonatal states (Aiello & Binotto 2007, Blyth *et al.* 2008). The association of these cardiac structural disease and primary cardiomyopathy results in complete lethality of *129SV Wnt-11*$^{-/-}$ mice from midgestation up to neonatal stage.

In contrast, *C57Bl6 Wnt-11*$^{-/-}$ shows improved survival. Our analysis of adult *C57Bl6 Wnt-11*$^{-/-}$ show no OFT defects, which were detected in this background with low penetrance (~10%, n = 10) and not later than neonatal stage. This is in contrast with *129Sv* background where OFT defects have 100% penetrance (Zhou *et al.* 2007). We hypothesize that the cardiomyopathy phenotype in *C57Bl6 Wnt-11*$^{-/-}$ is a primary consequence of abnormal myocardium development and not secondary to other cardiac structural defects.

The myocardium phenotypes observed in *Wnt-11*$^{-/-}$ *C57Bl6* mice show similarities to human cardiomyopathy reports, including overlapping congenital cardiomyopathy phenotypes in human patients (Klaassen *et al.* 2008, Kelley-Hedgepeth *et al.* 2009, Tsai *et al.* 2009). Moreover, each of the cardiomyopathies in *Wnt-11*$^{-/-}$ *C57Bl6*, the hypertrophic and noncompaction forms, are found in human patients as isolated forms which share genetic origin (Klaassen *et al.* 2008, Kelley-Hedgepeth *et al.* 2009, Tsai *et al.* 2009).

Hypertrophic cardiomyopathy is maybe the most common primary form of inherited cardiomyopathies (1:500) and diagnosis must exclude other identifiable causes, such as hypertension (Elliott *et al.* 2008). Ventricular noncompaction has a reported lower prevalence; however, the true prevalence is unclear and it has been postulated that is has been underdiagnosed or even wrongly diagnosed as HCM (Pignatelli *et al.* 2003, Elliott *et al.* 2008). The cardiomyopathy in *Wnt-11*$^{-/-}$ *C57Bl6* mice is not likely to be secondary to a general condition like arterial hypertension, as mean systemic blood pressures did not differ in *Wnt-11*$^{-/-}$ compared to controls.

It has recently been shown that cardiomyocyte hyperplasia does not cease at neonatal stage, but is responsible for heart growth much longer than previously

thought, until adolescence (Mollova *et al.* 2013). It has also has been defined that the rate and pattern of cardiomyocyte proliferation are an important regulator of cardiac compact and trabeculated myocardium architecture, determining the ratio of compact to trabeculated myocardium tissue, which is specifically defined for each ventricular segment and closely related to functional parameters (Sedmera *et al.* 2000, Henderson & Anderson 2009, Sedmera & Thompson 2011, Sedmera 2011). Proliferation was not affected in pre-septated heart myocardium of *Wnt-11*$^{-/-}$ mutants (I and (Cohen *et al.* 2012); however, *C57Bl6 Wnt-11*$^{-/-}$ adult mice ventricular myocardium abnormal architecture may be linked to the abnormal proliferation, and this issue is worthy of further investigation.

Recent discoveries show new insights into ventricular noncompaction pathogenesis, which involves developmental arrest in trabecular maturation and myocardial compaction in a mouse model of human mutation of Notch pathway regulator (Luxan *et al.* 2013). This mouse model presents mostly thin myocardium phenotypes, while in human patients with this mutation either a thin myocardium or hypertrophic myocardium with noncompaction segments has been reported. Follow-up of human cases of neonatal primary hypertrophy reveals the dynamic character of the congenital cardiomyopathy forms during postnatal myocardium development, where the initial isolated neonatal finding of hypertrophic cardiomyopathy evolved to ventricular noncompaction and associated hypertrophy later in life (Betrian Blasco *et al.* 2010). In line with these, our previous findings indicated arrest in trabecular and compact myocardium maturation in *Wnt-11*$^{-/-}$ pre-septated embryonic heart; this also associated with poor cardiomyocyte organization and impaired N-cadherin/β-catenin adhesion (Nagy *et al.* 2010). Similar phenotypes with thinner myocardium and poorly polarized cardiomyocytes in early development have been reported in mouse models of cell polarity pathway core components *Vangl2* and *Scribble*, with resemblance to myocardium noncompaction-like phenotypes in late gestation (Phillips *et al.* 2005, Phillips *et al.* 2007, Henderson & Chaudhry 2011). Moreover, altered cardiomyocyte cell cycling and ventricular noncompaction was associated with ventricular septal defects in a mouse model of human Noonan syndrome which includes an array of cardiomyopathy phenotypes. This mouse model holds a gain of function mutation in tyrosine phosphatase *Src* homology region 2 leading to overactive MAPK- ERK1/2 pathway during cardiac development, with consequent abnormal modulation of involving cell proliferation, differentiation, migration, adhesion and survival (Nakamura *et al.* 2007). *Wnt-11* has been shown to modulate these processes in *in vivo* and *in vitro*

models, suggesting a common link between *Wnt-11*, cell polarity core components and downstream kinase effectors controlling cardiomyocyte proliferation and survival, differentiation and organized migration and adhesion (Nagy *et al.* 2010, Abdul-Ghani *et al.* 2011, Cohen *et al.* 2012, Flaherty *et al.* 2012, Uysal-Onganer & Kypta 2012). It is worth investigating whether any cell polarity core genes are affected in *C57Bl6 Wnt-11*$^{-/-}$ mice, or whether cardiomyocyte polarization is affected *in utero* or during neonatal/postnatal development. It also remains under question whether the array of these hypertrophic changes involves altered gene expression of important cardiac transcription factors involved in cardiac development and maturation but also adult heart hypertrophic response *Gata-4*, *Nkx2.5*, *Mef2C*, *ANP*, *BNP* in line with our previous results (Nagy *et al.* 2010) and others (Cohen *et al.* 2012).

Global cardiac systolic and diastolic function is preserved in *Wnt-11*$^{-/-}$ *C57Bl6* as shown by standard echographics parameters in male and female groups. However, our speckle tracking myocardial strain rate assay detected especially lower ventricular wall longitudinal regional relaxation in the male group in contrast with the female group, which was not statistically significantly different from controls despite similar severity in cardiomyopathy histological phenotypes.

This cardiac regional dysfunction has previously been well correlated with the severity of global diastolic dysfunction in human patients presenting with HCM associated with preserved ejection fraction/systolic global function (Goto *et al.* 2006, Wang & Nagueh 2009, Kasner *et al.* 2010). In these studies lower longitudinal diastolic SR correlated with poor LV filling pressure, and in similar patients lower diastolic SR was found to be significantly decreased, also in myocardial segments without apparent hypertrophy (Goto *et al.* 2006). Although strain rate imaging provides support and additional details to standard echography on diastolic ventricular function, the true clinical value of diastolic strain and strain rate parameters has not yet been defined and it is not ready for standard practice (Hoit 2011). Nevertheless, STE functional evaluation of mouse models of human CMP is extremely useful for understanding the pathogenesis and functional course of the human disease.

An array of identified mutations is available for diagnosing the human inherited cardiomyopathies, although a significant proportion of the “idiopathic” cardiomyopathy human cases have no unidentified genetic cause (Seidman & Seidman 2001, Alcalai *et al.* 2008, Kimura 2010). Our findings show here that the *Wnt-11* gene is required for normal mouse ventricular myocardium development while disturbed *Wnt-11* signaling during ventricular development leads in adult

mouse to noncompaction cardiomyopathy and hypertrophic remodeling of the myocardium, associated with regional myocardium dysfunction. Altogether our data propose *Wnt-11* gene as a new candidate to be investigated in human congenital cardiomyopathy studies.

6.3 Kidney phenotype in *Wnt-11*$^{-/-}$ *C57Bl6* mice suggests novel roles for Wnt-11 in late nephrogenesis (III)

Our study shows that *C57Bl6 Wnt-11* knockout mice developed a hypoplastic kidney, in line with the previous findings from a *129SV Wnt-11* study (Majumdar *et al.* 2003). In the previous study a normal kidney histology was described; in contrast, the kidneys of the *C57Bl6 Wnt-11*$^{-/-}$ had clear pathologic changes with a specific cortical microcystic phenotype with a 100% penetrance (III Figure 2). This suggests that kidney *Wnt-11* knockout phenotype is sensitive to genetic background modulators.

6.3.1 Wnt-11 knockout as a disease model for human glomerulocystic kidney disease studies

Although the clinical manifestations of glomerular cystic kidneys are variable and kidney function can be stable for years, most cases do progress to end-stage kidney failure (Torres & Harris 2009, Bissler *et al.* 2010, Torres & Harris 2011). Our findings with the *Wnt-11* knockout models are consistent with the hypothesis that the genetic background with specific genetic modifiers may modulate kidney diseases that involve cystogenesis based on studies in human and rat model (Woolf *et al.* 2002, Bissler *et al.* 2010, Lennerz *et al.* 2010, O'Meara *et al.* 2012). Hence, *Wnt-11* knockout mice could serve as a model for identifying genetic modifiers of potential significance in relation to the risk of glomerular cystic kidney diseases in humans.

The fact that *C57Bl6 Wnt-11* knockout mice survive to adulthood provided a unique model for studying the roles of Wnt-11 at later stages in the life cycle. *Wnt-11* is expressed in kidney tubular cells and its loss causes abnormal tubule morphology by the impairment of tubular diameter and convolution control. Interestingly, the primary tubular abnormalities in the *Wnt-11*-deficient kidneys did not cause tubular cysts, unlike in the *Wnt-7b/9b* knockouts (Karner *et al.* 2009, Yu *et al.* 2009), but they did secondarily impair the glomerular morphology. Most importantly, this led to glomerular dysfunction and kidney failure. The *Wnt-11*

knockout shows a number of similarities to some forms of human glomerulocystic kidney disease and their mouse models (Woolf *et al.* 2002, Bissler *et al.* 2010, Lennerz *et al.* 2010, O'Meara *et al.* 2012). Among these, two of the well-known human mutation causing polycystic kidney disease associated with glomerular cysts affect the *Hnf-1β* and *Pkd1* genes, which are not expressed in glomerular structures. During embryonic development the *Hnf-1β* gene is strongly expressed in branching ureteric bud that gives rise to CD system and discretely in comma/S-shape body forming the nephron proper, while *Pkd1* is intensely expressed in differentiating PT (Igarashi *et al.* 2005, Ahrabi *et al.* 2010). While *Hnf-1β* mutations cause a specific hypoplastic glomerulocystic kidney phenotype with involvement of cortex glomeruli, *Pkd1* mutations may cause as early manifestation glomerular cysts prior to developing massive tubular cysts (Igarashi *et al.* 2005, Ahrabi *et al.* 2010). Mouse studies show that these genes seem not to be essential in early induction of nephrogenesis but rather in late tubule nephrogenesis, similarly to *Wnt-11*. Altogether, this suggests that any degree of primary morphological defects in the kidney tubules is sufficient to compromise glomerular structure and function secondarily.

6.3.2 Wnt-11 signaling fine-tunes nephrogenesis

Besides possessing abnormal tubule morphology, the *Wnt-11*$^{-/-}$ kidneys were hypoplastic and we found severely decreased expression of *Wnt-9b*, a critical UB signal that triggers nephrogenesis. This raises the possibility that Wnt-11 signaling in ureteric bud tip may regulate *Wnt-9b* expression and suggests a mechanism by which Wnt-11 modulates the nephrogenesis process. Wnt-11-mediated control of *Wnt-9b* expression appears to be reciprocal, since Wnt-11 expression is also reduced in the absence of *Wnt-9b* signaling in the kidney (Carroll *et al.* 2005, Karner *et al.* 2009, Gessert & Kuhl 2010, Karner *et al.* 2011, Uysal-Onganer & Kypta 2012).

Significantly, the *Six2* gene expression was down-regulated in both the *Wnt-9b* and *Wnt-11* knockouts. It has been suggested that the concentration of active Wnt-9b may make a critical contribution to either the self-renewal of the Six2+ progenitor cells or to the induction of their transition to form the nephron epithelium (Hendry *et al.* 2011, Karner *et al.* 2011, Rumballe *et al.* 2011, Little & McMahon 2012, Park *et al.* 2012). Based on this, we propose that Wnt-11 may fine-tune the output of Wnt-9b signaling to coordinate the self-renewal and commitment of the Six2-positive nephron progenitor cells. In conclusion, our

results indicate that Wnt-11 not only serves as an early ureteric bud signal, but also has a later function in fine-tuning nephrogenesis.

Wnt-11 signaling also has a notable influence on the stromal progenitor cells, since the expression of *Hox10* and *Foxd1* was down-regulated in the *Wnt-11*$^{-/-}$ cortex, a finding that points to a role for Wnt-11 as an epithelial signal in controlling the compartmentalization of the kidney cortical mesenchyme (Levinson *et al.* 2005, Yallowitz *et al.* 2011). Moreover, Six2+ cells' self-renewal depends on positive regulatory feedback from Foxd1 (Levinson *et al.* 2005, Hendry *et al.* 2011, Park *et al.* 2012).

Given all these, we consider that Wnt-11 contributes in some way to the segregation and maintenance of the Six2+ progenitor populations. It may exercise its influence on the Six2+ cells through the control of Wnt-9b or via regulation of stromal Hox10-Foxd1 expression, a matter which warrants further investigation.

6.3.3 Wnt-11 and cell polarity pathway control of kidney tubule morphogenesis

Besides these early inductive events, *Wnt-11* is expressed in the collecting tubules in a manner that correlates with *Wnt-9b* expression, a signal that mediates tubule morphogenesis by the PCP pathway (Karner *et al.* 2009). *Wnt-11* knockout increases the luminal diameter of the PT and CD as is the case with the *Wnt-9b* hypomorph (Karner *et al.* 2009). The results presented here demonstrate that the loss of Wnt-11 function affected Wnt-9b expression and the tubular abnormalities caused by *Wnt-11* deficiency correlated with *Wnt-9b* knockout ones, especially the impairment of tubular luminal diameter development.

Besides having an influence on the luminal diameter, *Wnt-11* deficiency also affected the convolution of the tubules, as revealed by OPT. Elongation and expansion of the tubules is critically controlled by the convergent extension (CE) movement and oriented cell divisions (OCD). These processes may dictate the pattern and degree of tubular convolution. The Wnt-11 signal regulates CE movements during gastrulation (Ulrich *et al.* 2005) and also later in some other systems (Wallingford *et al.* 2002, Wallingford & Mitchell 2011). Given this, we speculate that Wnt-11 may control tubulogenesis through a similar function. Taken together, Wnt-11 may be a novel Wnt contributing to kidney tubulogenesis via the PCP pathway, but this remains to be seen.

In summary, *Wnt-11 C57BL6* knockout has provided a unique developmental Wnt model. In the present work we show that besides having a role in UB

branching and the CD derived from the process, Wnt-11 signaling contributes to the spatial organization of the tubular epithelial cells in the nephron, since convolution of the nephron is deregulated in its absence. This study suggests a novel concept worthy of further investigation, namely that Wnt-11 might have some organizational signaling roles operating via the *Six2*, *Wnt-9b*, and *Foxd1/Hox10* genes to coordinate nephrogenesis in general. The finding that impaired *Wnt-11* function leads to glomerular cysts with significant loss of glomerular filtration indicates that the mouse model generated here is useful for gaining a better molecular understanding of the pathogenesis of human glomerulocystic kidney diseases (Woolf *et al.* 2002, Lennerz *et al.* 2010).

6.4 Wnt-11 signaling commonalities between heart and kidney development

Although it is difficult to draw clear commonalities between the roles of Wnt-11 in these two distinct organ developments, this work supports the existing concept in literature that Wnt-11 acts in general as a modulator of convergent extension-like movements during development. Similar to human congenital diseases, both kidney and heart *Wnt-11* knockout phenotypic manifestation depends on genetic background modifiers.

The present study shows specifically that loss of Wnt-11 during mammalian cardiac organogenesis leads to poor organization of cardiomyocytes within the ventricular myocardium, which can result in two possible outcomes: a severe early onset outcome, with heart failure leading to embryonic or neonatal lethality, or to a late onset outcome causing cardiomyopathy. Loss of *Wnt-11* during mammalian kidney organogenesis causes kidney hypoplasia with glomerular cystic formation caused by primary abnormalities in tubule convolution and lumen formation.

The array of data presented in this thesis is in line with previous studies showing that Wnt-11 signaling plays significant roles in mammalian heart and kidney development, with relevance to human congenital disease studies.

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Original articles

I Nagy II, Railo A, Rapila R, Hast T, Sormunen R, Tavi P, Räsänen J & Vainio SJ (2010) Wnt-11 signaling controls ventricular myocardium development by patterning N-cadherin and beta-catenin expression. Cardiovasc Res 85(1): 100–109.

II Nagy II, Szabo Z, Kerkelä R & Vainio SJ (2014) *Wnt-11* deficiency causes primary cardiomyopathy in *C57Bl6* mice. Manuscript.

III Nagy II, Naillat F, Jokela T, Miinalainen I, Sormunen R & Vainio SJ (2014) Wnt-11 signaling contributes to kidney tubular system development during nephrogenesis. Manuscript.

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