Defects in trabecular development contribute to Left Ventricular Noncompaction

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Abstract

Left Ventricular Noncompaction (LVNC) is a genetically heterogeneous disorder the etiology of which is still debated. During fetal development trabecular cardiomyocytes contribute extensively to the working myocardium and the ventricular conduction system. The impact of developmental defects in trabecular myocardium in the etiology of LVNC has been debated. Recently we generated new mouse models of LVNC by the conditional deletion of the key cardiac transcription factor encoding gene *Nkx2-5* in trabecular myocardium at critical steps of trabecular development. These conditional mutant mice recapitulate pathological features similar to those observed in LVNC patients, including a hypertrabeculated left ventricle with deep endocardial recesses, subendocardial fibrosis, conduction defects, strain defects and progressive heart failure. After discussing recent findings describing the respective contribution of trabecular and compact myocardium during ventricular morphogenesis, this review will focus on new data reflecting the link between trabecular development and LVNC.

Keywords Trabeculation · Ventricular noncompaction · Compaction · Lineage tracing · Ventricular conduction system

Introduction

Left ventricular noncompaction (LVNC) is a complex and heterogeneous cardiomyopathy, characterized by hypertrabeculation and deep trabecular recesses in the left ventricle and is the most common cardiomyopathy with a large spectrum of symptoms ranging from normal variants to a pathological phenotype [1]. Patients display a high prevalence of conduction defects. The etiology of LVNC is still debated within the scientific and medical community, in particular the impact of developmental defects of ventricular trabeculae on the occurrence of LVNC [2]. Ventricular trabeculation is a normal, but transient, step during embryonic development [3, 4]. In mammals, trabeculae undergo a necessary compaction step at fetal stages during formation of a competent myocardial wall. Trabeculae also contain the progenitor cells of the ventricular conduction system (VCS), controlling the rapid propagation of the electrical activity in the ventricles [5, 6]. Recently, it has been demonstrated that embryonic trabeculae play an important

role in the formation of the ventricular wall including the VCS, and that failure of their development trigger pathological features of LVNC similar to those observed in symptomatic patients [7]. In this review, we focus on the development and cell fate of ventricular trabeculae, highlighting their potential role in cardiac diseases.

Ventricular Trabeculae

Trabeculae are transient embryonic structures forming finger-like myocardial projections in the lumen of ventricles, giving the heart a sponge like structure [8, 9]. They play a role in oxygenating the myocardium prior to the establishment of the coronary vascularization, and a role in fast conduction by expressing the gap junction protein *Connexin40* (*Cx40*) before the development of the ventricular conduction system (VCS) [10, 11]. Trabeculae are apparent at embryonic day (E) 9.0 in the mouse or at the end of the fourth gestational week in human, when the heart tube consists of an external myocardial layer and an internal endocardial epithelium. These two layers are separated by extracellular matrix known as the cardiac jelly. The first trabeculae appear in the cardiac jelly between endocardial-myocardial contact points and extend radially into the ventricular lumen at the outer curvature of the looped-heart tube [10]. This process, known as trabeculation, occurs in all vertebrates but although diverges between species. Here we discuss mechanisms that have been recently uncovered that provide insight into the molecular and cellular pathways driving trabeculae formation.

Morphogenesis of Embryonic Trabeculae in Mammals

Trabeculation depends on tissue mechanics, cardiac jelly composition, cell behavior, and cell-to-cell signaling between the endocardium and the myocardium [12–14]. In mammals, both oriented cell division and delamination of cardiomyocytes from the early myocardial layer are important early events in the formation of trabeculae. In the mouse, lineage tracing of single cells during trabeculation revealed that, in addition to polarized migration similar to that observed in zebrafish [15], cardiomyocytes undergo perpendicular cell division at E9.0/9.5, leading to the entry of a daughter cell into the cardiac lumen to initiate trabeculation. In this way the two daughter cells exhibit different localizations in the ventricle and are likely to respond to different developmental cues leading to their segregation [16]. During this process N-cadherin reorganization is required for the establishment of the mitotic spindle orientation in order to drive cardiomyocytes delamination and perpendicular division [16].

Recently a new model of trabeculation in mice has been proposed that integrates dynamic endocardial and myocardial cell behaviors with ECM remodeling [17]. The first apparition of trabeculae occurs at E8.0 in mouse embryo, when several endocardial sprouts ingress through the cardiac jelly and toward the myocardium. At this stage, the myocardium consists of a continuous layer of cardiomyocytes at the most outer ventricular surface and discontinuous luminal cardiomyocyte clusters defined as trabecular units. From E8.5 to E9.0, endocardial projections encapsulate these trabecular units and establish points of contact with the compact myocardium. This process individualizes trabecular units and creates multiple matrix rich areas. From E9.0,

trabeculae continue to expand by radially orientated cardiomyocyte proliferation and potentially by recruitment of new cardiomyocytes until the end of ventricular septation at E14.5 in mouse embryos. By this stage, the ventricular endocardium becomes tightly associated with the myocardial cells as the intervening cardiac jelly disappears. The trabecular zone constitutes the most important mass of the ventricular myocardium, whereas the outer compact layer remains only a few cells thick. During trabeculation, the endocardial Neuregulin 1 and NOTCH1 pathways cooperate dynamically to regulate the balance between ECM synthesis and degradation, essential for trabecular architecture and growth [17], and also regulate cardiomyocyte proliferation by promoting the expression of *Bmp10* [18]. Furthermore, the ECM composition during trabecular growth is dynamically controlled by the secreted matrix metalloproteinase ADAMTS1 and its chromatin-based transcriptional repressor Brg1 [19]. In mice mutant for genes in these pathways, higher ECM degradation blocks trabecular growth, whereas the absence of ECM degradation leads to excessive and disorganized trabecular growth contributing to non-compaction cardiomyopathy phenotypes. Nevertheless, how endocardial sprouting is regulated and the cellular and molecular mechanisms that drive the formation of trabecular units remain to be elucidated.

Compact vs Trabecular Cardiomyocytes

Ventricular trabeculation generates two myocardial domains: a trabecular zone adjacent to the endocardium and a sub-epicardial compact zone [4]. Lineage analyses in chick and mouse embryos have shown that compact and trabecular cardiomyocytes are clonally related [20, 21]. However they differ in term of morphogenesis, proliferative capacities, gene expression and contribution to the adult cardiac structures [3]. There is a gradient of cardiomyocyte proliferation across the ventricular wall. Trabecular cardiomyocytes have a lower proliferative rate than those in the compact wall. Indeed, a balance between proliferation and differentiation is critical to both generate a sufficient number of cells and initiate lineage specification [22]. The trabecular zone is characterized by the expression of the genes *Irx3*, *Cx40*, *Nppa*, *Etv1*, *Bmp10*, *Slit2* and *Sema3a* whereas the compact zone is characterized by the expression of *Tbx20*, *Hey2*, *Loxl2* and *N-Myc* [6, 22–28] (Fig. 1A).

Cell Fate of Trabeculae during Ventricular Wall Formation in Mammals

Trabecular Compaction

In mammals, from fetal stages trabeculae are gradually compacted and integrated into the ventricular wall thickness, concomitant with the expansion of the compact zones [3, 4, 29]. This results in the formation of a thick and competent myocardial wall. The compaction step is specific to higher vertebrates since ventricular trabeculae are maintained in the heart of adult fishes and reptiles which retain regenerative capacity [9, 30]. In adult zebrafish heart, *tbx5a* expression is persistent only in the trabecular myocardium and genetic fate mapping of trabecular-*tbx5a*⁺ cells shows that these cells are able to switch their fate and differentiate into

cortical myocardium during both embryonic and adult heart regeneration [31]. This suggests that trabecular myocardium displays high degree of cell plasticity and/or retains proliferative ability.

From E14.5 of mouse development, trabeculae stop extending and start to consolidate at their base and gradually integrate into the ventricular wall thickness, a process that is complete by early postnatal stages. The myocardium gradually compacts inwards the ventricular lumen and from the base to the apex. Finally the trabecular meshwork disappears as the compact zone grows and matures, resulting in a ventricular wall that is largely composed of compact myocardium with a relatively smooth endocardial surface [4]. During ventricular compaction, cardiomyocytes of the trabecular myocardium participate in the formation of the papillary muscles and VCS, whereas endocardial cells associated with the base of trabeculae contribute to the coronary vasculature. In fact during compaction the intertrabecular recesses are compressed to capillaries and endothelial cells arising from the endocardium form intramyocardial coronary vessels [32, 33]. A similar process takes place during neo-vascularization following myocardial infarction in the adult heart [34]. Epicardial-derived cells and cells from the sinus venosus also participate in building the coronary vasculature [35, 36].

The process of compaction is poorly described in the literature and often causes confusion. Certain authors support a dynamic coalescence of ventricular trabeculae, while others argue in favor of a remodeling of the compact zone that undergoes an extensive proliferation resulting in the zipping-up of trabeculae [24]. Interestingly, recent genetic tracing and retrospective clonal analysis in the mouse has suggested that the compaction step may be in part driven by expansion of the fetal compact myocardium into the trabecular layer through its higher proliferative activity. This leads to the formation of a hybrid zone composed of cardiomyocytes derived from both the compact and trabecular zones [24]. Preventing proliferation of cardiomyocytes in the compact zone compromises the formation of the hybrid zone, and results in a thin and non-compacted ventricular myocardium with persistent trabeculae associated with contraction defects. In contrary, preventing proliferation in the trabecular zone does not perturb ventricular compaction. These results are coherent with the higher proliferative activity of the compact zone and the switch from trabecular expansion to compact zone expansion during the compaction step. Nevertheless, this study does not exclude that both expansion of the compact zone and coalescence of intertrabecular recesses are two critical events that drive the compaction process.

Trabecular Specification into Conductive and Contractile Cardiomyocytes

As trabeculae gradually integrate the compact wall, trabecular cardiomyocytes differentiate into contractile, also designed as working, cardiomyocytes and conductive cells. Lineage tracing experiments and retrospective clonal analysis of *Cx40*⁺ cells have demonstrated that the peripheral VCS including Purkinje fibers (PFs), originates from embryonic trabeculae [5]. Ventricular trabeculae contain bipotent myogenic progenitors which segregate progressively into conductive and contractile fates between E10.5 and E14.5; lineage restriction appears almost complete by E16.5. Segregation into a conductive fate is associated with a reduced proliferative capacity compared to contractile cardiomyocytes that exhibit a high proliferative rate until

perinatal stages [37]. Furthermore, the progressive segregation of trabecular cardiomyocytes into cells with a contractile myocardial fate is accompanied by the gradual loss of expression of trabecular markers including *Etv1*, *Irx3*, *Cx40* and *Sema3a*, the expression of which becomes restricted to the VCS at postnatal stages [6, 23, 25, 26, 38] (Fig. 1B).

Indeed, ventricular wall morphogenesis seems to result from a continuous process during which trabecular cells are progressively incorporated into the compact myocardium. This process follows the formation of the ventricular wall from base to apex as demonstrated by the more severe defects observed in the apex region in mutant mice or in human patients suffering from LVNC [39, 40]. Mutations in the NKX2-5 gene have been identified in LVNC patients indicating a role for this important cardiac transcription factor in compaction [41, 42]. In *Nkx2-5* heterozygous mutant mice, noncompaction of the ventricular myocardium is observed in the apex suggesting a late compaction of the apical part, as previously suggested for the development of the coronary vasculature [36]. A similar phenotype is observed in *14-3-3ε*^{-/-} mice that display abnormal coronary vasculature and ventricular noncompaction in the apical region of the heart [43]. These observations, in addition to the early segregation of the central VCS components at the crest of the IVS [44], suggest that ventricular myocardial morphogenesis and VCS development follow the same base to apex axis through embryonic development. This assumption is supported by optical mapping data showing that electrical activity propagates through a base to apex axis in early stages of embryonic development [10, 45].

LVNC: a Poorly Understood Cardiomyopathy

Left ventricular noncompaction (LVNC) is a rare genetic or acquired cardiomyopathy also known as ventricular hypertrabeculation. LVNC is typically characterized by the presence of an excessive lace-like network of trabeculae with deep intertrabecular recesses. However, the anatomical and clinical features are very heterogeneous because the degree of hypertrabeculation is highly variable among patients. Moreover, LVNC occurs in association with several genetic mutations and is often associated with other cardiac diseases, in particular dilated cardiomyopathy [1]. Thus, a large spectrum of clinical conditions varying from asymptomatic to severe pathological forms through to sudden cardiac death are observed. The most prevalent symptoms are conduction defects present in approximately 90% of patients. Around 60% of adult patients develop heart failure and around 13-24% thromboembolism [46]. It is still unclear if LVNC is a separate cardiomyopathy, or an anatomical trait observed in association with other primary cardiomyopathies. Moreover, several asymptomatic adults displaying a normal ejection fraction and no risks of complications such as arrhythmia and stroke have been observed with excessive ventricular trabeculae [2]. Consequently, LVNC prognosis and diagnosis are difficult and result in an underestimated prevalence among the worldwide population. Patients are usually diagnosed when the conditions become symptomatic or when complications occur. One study has estimated a prevalence of 9.2% among children affected by cardiomyopathies. In adults referred for echocardiography, the prevalence is 0.01–0.30% [46].

In congenital forms of LVNC, more than 40 mutated genes have been associated with LVNC [46]. Most of these genes are also associated with other cardiomyopathies and encode for sarcomeric proteins, related binding proteins, and cytoskeletal proteins [47]. However, the evidence of a direct cause between any of these mutations and LVNC remains to be established, illustrating the importance of generating mouse models of LVNC in order to investigate the developmental origin of this misunderstood cardiomyopathy. One challenging point concerns the etiology of LVNC which is largely unknown. It is still unclear whether LVNC results from excessive trabeculae formation and/or a defect in the later compaction processes during ventricular myocardium morphogenesis. Comparative studies, however, support an arrest of myocardial compaction during fetal stages [48]. Several studies using mouse models have argued that the phenotype of ventricular hypertrabeculation as observed in *FKBP12* [49], *MIB1* [50], *Numb* [51] and *Nkx2-5* [52] mutant mice, mostly result from defective cell proliferation and are mainly associated with dysregulation in the NOTCH pathway. Others have supported that defects in cardiomyocyte myofibrillogenesis and polarization during ventricular development identify a common pathogenic pathway of LVNC [53, 54]. However, recent evidence indicates that excessive trabeculation results from defective compaction zone expansion and not from failure of the compaction of pre-existing trabeculae [2, 55]. Inhibiting proliferation of the compact zone by deleting *Yap1*, a key transcription co-factor required for normal cardiomyocyte proliferation, prevents the expansion of the compact zone and results in noncompaction of the ventricular myocardium [24]. Several other mouse models including the ventricular *Nkx2-5*-conditional deletion support this assumption; however, most of these mutants present a very thin compact layer and are embryonic lethal and thus do not fully recapitulate symptomatic features observed in LVNC patients.

Clues from *Nkx2-5*-Trabecular Deficient Mice Support a Developmental Origin of Pathological LVNC

In a recent study, we wanted to directly address the question whether hypertrabeculated myocardium could result from a failure of trabecular development. Defects in ventricular compaction and conduction are common traits observed in patients and in mutant mice carrying mutations in *NKX2-5* gene, encoding a key transcriptional regulator of cardiac development [52, 56–58]. In order to study the temporal and spatial requirement of *Nkx2-5* during trabecular morphogenesis and dissect the role of this gene in the pathogenesis of LVNC, we conditionally inactivated *Nkx2-5* in early or late trabecular myocardium, at timepoints corresponding to trabecular morphogenesis and compaction [7]. *Nkx2-5*-floxed mice were crossed with the *Cx40-CreERT2* mouse line, in which tamoxifen inducible Cre activity is under the transcriptional control of the *Cx40* locus. In *Nkx2-5^{ΔTrabE10}* mutant mice, *Nkx2-5* was deleted at the embryonic stages E10.5-E11.5, when *Cx40* is expressed in developing trabeculae during active trabeculation. In *Nkx2-5^{ΔTrabE14}* mutant mice, *Nkx2-5* was deleted at fetal stages E13.5-14.5, when *Cx40* expression is restricted to trabeculae during their compaction.

Nkx2-5^{ΔTrabE10} and *Nkx2-5^{ΔTrabE14}* adult mutant mice exhibit a hypertrabeculation phenotype with deep intertrabecular recesses associated with subendocardial fibrosis and hypoplasia of the Purkinje fibers (PF), although the compact zone was not reduced. The phenotypes after early and late *Nkx2-5* deletion vary only in their degree of severity. These results demonstrate that disruption of trabecular development contributes to the occurrence of hypertrabeculation (Fig. 2A). By performing inducible lineage tracing of *Cx40*⁺ trabeculae, we have demonstrated that *Cx40*⁺ cells labelled at E10-11 or E13-14, contribute predominantly to generate working cardiomyocytes that extend throughout the ventricular myocardium. Cardiomyocytes generated by *Cx40*⁺ cells labelled at E10-11 are observed deep in the myocardium, while *Cx40*⁺ cells labelled at E13-14 contribute to cardiomyocytes that are restricted to the inner myocardial layer (Fig.1B). The difference of severity in the hypertrabeculated phenotype observed between early and late *Nkx2-5*-deletion is thus correlated with the extent of deletion in the ventricular myocardium rather than resulting from different mechanisms acting independently during trabeculation or trabecular compaction. *Nkx2-5^{ΔTrabE10}* mice displayed defects in papillary muscle compaction, severe PF hypoplasia, numerous endocardial islets, important subendocardial fibrosis and disruption of the molecular boundary between the compact and the trabecular zone in the adult heart. The main difference with previous models is that conditional deletion restricted to the trabecular compartment provokes a less severe phenotype and allows the development of a compact myocardium thick enough for supporting cardiac function and compatible with life. In mutant embryos, hypertrabeculation was associated with a slight upregulation of cardiac proliferation at E14.5 without disturbing the compact-trabecular boundary at this stage. The maintenance of this embryonic compact-trabecular boundary has been observed in other mouse models of noncompaction affecting notch signaling. The glycosyltransferase manic fringe (*MFng*) is a modulator of Notch ligand in ventricular endocardial cells and is expressed from E8.5 and downregulated after E11.5 in mouse embryos. *MFng^{tg}::Tie2-Cre* transgenic mice display forced MFng expression in endocardial cells leading to disruption of the Notch1 activation in ventricular myocardium [59]. Neonate mice exhibit thin compact myocardium and non-compacted trabeculae in both ventricles. In E16.5 mutant embryos, the boundary between compact and trabecular myocardium is maintained since *Hey2* and *Bmp10* (or *Cx40*) expressions remain complementary, despite the presence of deep recesses in the compact myocardium referred as the “intermediate myocardium”.

Finally our results suggest that hypertrabeculation phenotype results primarily from both early cardiac proliferation defects and impaired trabecular coalescence. Thus, trabecular cells are actively involved in the process of compaction. Moreover, the presence of deep endocardial invaginations and transcriptional deregulation of numerous genes involved in the vascular system suggest that ventricular compaction involves an intimate communication between cardiomyocytes and endothelial cells. However, the absence of defects in coronary arteries in contrast to other models shows that noncompaction can be dissociated from coronary artery development. Furthermore, transcriptomic analysis demonstrate no differences in dysregulated pathways between *Nkx2-5^{ΔTrabE10}* and *Nkx2-5^{ΔTrabE14}* adult mutant mice. This suggests that *Nkx2-5* regulates common developmental processes throughout trabecular development. While

previous data favor defects in proliferation of the compact myocardium, our results provide evidence that defects in trabecular morphogenesis lead also to mouse models of LVNC and suggest that trabecular compaction and compact zone expansion are progressive processes.

In the first longitudinal study of a LVNC model we followed-up cardiac function in conditional *Nkx2-5* mutant mice over one year. Our results revealed that *Nkx2-5^{ΔTrabE10}* and *Nkx2-5^{ΔTrabE14}* mutant mice display all the clinical signs of symptomatic LVNC, including conduction defects, progressive cardiac contractility defects with age and in 50% of old mutant mice signs of heart failure, although, importantly, the hypertrabeculation and fibrosis phenotypes remain identical throughout adult life (Fig. 2B). Thus, these mutant mice provide good models for studying LVNC and highlight the multiple roles of *Nkx2-5*, reflecting the heterogeneity of clinical features observed in LVNC patients. Finally, our results support the idea that even if excessive trabeculation is not itself a clinical entity, LVNC may be attributed to a defect in the global and progressive maturation of the ventricular myocardium that could be the primary cause of the LVNC pathology.

Future Directions

Consistent with the intimate relationship between VCS and ventricular wall formation, the maturation state of trabeculae is an important criterion to take in consideration regarding LVNC cardiomyopathy, and not exclusively the persistence of excessive trabeculation in the ventricular cavity. In this case the failure of trabecular cardiomyocytes to adopt a conductive fate would impact on ventricular wall development, especially the formation of the VCS and the appearance of subendocardial fibrosis and intertrabecular recesses. Lineage tracing of *Cx40*+ trabecular cells indicates that the coalescence of trabeculae into the ventricular wall correlates with the progressive restriction of *Cx40* expression to the conduction system. This pattern of expression is similar to that of *Sema3a*, another PFs marker[26]. Furthermore, this lineage restriction is accompanied by a decrease in the number of *Cx40*+ trabecular progenitors, and a progressive integration of *Cx40*-derived cells into the working myocardium. This demonstrates that trabecular development is concomitant with VCS differentiation and that PF hypoplasia is likely to be directly linked to the conduction defects observed in our LVNC mouse models. Thus, disturbing trabecular differentiation may have an important impact in the pathogenicity of hypertrabeculation and correlates with symptomatic forms of this cardiac anomaly. In the future, it would be relevant to further study the impact of defective VCS alone to decipher its role in pathological features of cardiomyopathies.

Compliance with Ethical Standards:

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Figures legend

Fig. 1 Molecular identity and genetic tracing of trabecular cells. **A** Spatiotemporal expression pattern of trabecular markers and compact zone markers in the ventricular myocardium during development. Trabecular markers are downregulated in the compact zone at E14.5 but maintained in trabecular cells (orange shading) that differentiate into contractile and conductive cardiomyocytes. Working cardiomyocytes deriving from trabeculae progressively lose the expression of trabecular markers (black dots). At the adult stage, the expression of trabecular markers *Cx40*, *Sema3a*, *Irx3* and *Etv1* is restricted to Purkinje fibers (PF) expressing also *HCN4* and *CNTN2*. **B** Genetic tracing of trabecular cells in adult mice showing the distribution of trabecular-derived cells (green dots) in transverse sections at the mid ventricle after genetic labelling at E10.5 or E14.5. Labelling at E10.5 shows large contribution of trabecular cells to the working myocardium of the left ventricle (LV) extended toward the compact myocardium, including the left free wall and the papillary muscles. Labelling at E14.5 shows a more restricted distribution in the inner part of the myocardium at a subendocardial level. Labelling at E18.5 shows the restriction of trabecular cells into the PF (red).

Fig. 2 Mouse models of pathological LVNC. **A** Short-axis cine images recorded by magnetic resonance imaging at end-diastole (upper panels). Anatomical structure of opened left ventricle (LV) (mid panels). Immunostaining on transverse sectioned-mid left ventricle of 3 month-old control (CTL), *Nkx2-5* ^{Δ TrabE10} (Δ TrbE10) and *Nkx2-5* ^{Δ TrabE14} (Δ TrbE14) show the morphology of adult hypertrabeculation and deep intertrabecular recesses (white arrows) induced by deletion of *Nkx2-5* in trabecular cardiomyocytes during development. **B** The follow-up of cardiac function shows a correlation of impaired Ejection Fraction with conduction and strain defects in absence of aggravation of the hypertrabeculation phenotype with age. HF: Heart failure. IVS: Interventricular septum. *: papillary muscles. Adapted from Choquet et al. [7]