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Mechanisms of trabecular formation and specification during cardiogenesis

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Abstract

Trabecular morphogenesis is a key morphologic event during cardiogenesis and contributes to the formation of a competent ventricular wall. Lack of trabeculation results in embryonic lethality. The trabecular morphogenesis is a multistep process that includes, but not limited to, trabecular initiation, proliferation/growth, specification and compaction. Although a number of signaling molecules have been implicated in regulating trabeculation, the cellular processes underlying mammalian trabecular formation are not fully understood. Recent works show that the myocardium displays polarity, and oriented cell division and directional migration of the cardiomyocytes in the monolayer myocardium are required for trabecular initiation and formation. Furthermore, perpendicular oriented cell division is an extrinsic asymmetric cell division that contributes to trabecular specification, and is a mechanism that causes the trabecular cardiomyocytes to be distinct from the cardiomyocytes in compact zone. Once the coronary vasculature system starts to function in the embryonic heart, the trabeculae will coalesce with the compact zone to thicken the heart wall, and abnormal compaction will lead to left ventricular non-compaction (LVNC) and heart failure. There are many reviews about compaction and LVNC. In this review, we will focus on the roles of myocardial polarity and oriented cell division in trabecular initiation, formation, and specification.

Keywords

Myocardial polarity; oriented cell division; trabeculation and trabecular specification

1. Trabeculation and compaction

The heart is the first functional organ formed in mammalian embryonic development. During cardiogenesis, cardiac progenitor cells from the cardiac crescent migrate toward the ventral midline to form a linear heart tube with a smooth inner surface[12]. When the heart tube undergoes looping, myocardium along the outer curvature of the tube will grow inward and form sheet-like structures that extend from the myocardium[1,2], which are the newly

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initiated trabeculae[3,4]. As the early embryonic heart does not have a coronary circulatory system to perfuse itself, the sheet-like trabeculae function to increase surface area to facilitate nutrients and oxygen exchange. A lack of trabecular formation will result in less availability of oxygen and nutrients in the myocardial tissue and causes embryonic lethality. Trabecular malformation leads to cardiomyopathy and heart failure[5,6]. Trabeculae are also required for the formation of Purkinje fibers and enable the myocardium to increase its mass in the absence of coronary circulation. Lastly, trabeculae also function to anchor mitral and tricuspid valves via papillary muscles.

During normal cardiac morphogenesis, starting at ~E14.5 in the mouse and about weeks 5–8 of human embryonic life, the coronary plexus gradually forms and delivers blood to the myocardium. The basal portions of the trabeculae will coalesce with the ventricular wall to thicken the compact myocardium, subsequently the inter-trabecular recesses are compressed to capillaries to link to the coronary circulation system[3]. This trabecular zone coalesce with the compact zone is called the trabecular compaction, starting at the base of the heart and progressing toward the apex[3]. Disturbed compaction during development results in left ventricular noncompaction (LVNC)[3,7,8]. About half a million Americans suffer from LVNC[9]. LVNC is morphologically characterized by excessive trabeculation of the left ventricular walls with deep inter-trabecular recesses that communicate with the ventricular cavity but not with the coronary circulation. The hypoperfusion of the sub-endocardium and abnormal relaxation in LVNC might cause heart failure in patients[10,11].

Despite the importance of trabeculation and compaction, the cellular and molecular mechanisms that regulate trabeculation and compaction are still not clear. This review will focus on the cellular and molecular mechanisms underlying trabecular formation and specification.

2. Monolayer myocardium displays transmural polarity.

Cell polarity is defined as asymmetric localization of cellular organelles, transporting lipid vesicles, cytoskeleton and proteins that include signaling receptors, adaptor proteins, and cell fate determinants. Cells establish polarity in response to chemical, electrical, mechanical, or other physical stimuli. Cell polarity is involved in multiple biological processes including directional migration, oriented cell division (OCD), morphogenesis and asymmetric cell division[12–15]. Loss of cell polarity is associated with uncontrolled cell growth and disrupted morphogenesis[16]. The roles of cell polarity in cardiac morphogenesis have recently been gradually revealed. We and other groups previously reported that the epicardium and pro-epicardium displayed apical-basal polarity, and polarity loss by tissue specific deletion of polarity genes causes abnormal epicardial and pro-epicardial development[17–21]. The function of cellular polarity is not fully appreciated in myocardial morphogenesis. The observation that cardiomyocytes of the monolayer of the heart tube migrate inward toward the heart lumen but not outward to the pericardial sac indicates that the myocardial layer displays a transmural polarity. When N-Cadherin, a major component of the adherens junctions of the heart, is disrupted specifically in the heart, the cardiomyocytes migrate randomly with some cells residing in the pericardial sac, which suggests that N-Cadherin is required to establish transmural polarity (Fig. 1). The

myocardial layer's transmural polarity is further demonstrated by the localization of extracellular matrix proteins such as collagen and cardiac jelly, which localize to the lumen side of the myocardial layer (Fig. 2). The extracellular matrix proteins in cardiac jelly might provide essential cues to polarize the cardiomyocytes. Recently several exciting studies show that the monolayer of myocardium displays apical-basal polarity in both zebrafish and mouse[22–24] (Fig. 2). These studies found that polarity complex proteins such as Par3, aPKC and Par6 are asymmetrically localized across the monolayer of the myocardial wall. Apical-basal polarity might be essential for oriented cell division during trabecular morphogenesis in the mouse[23,24], while apical-basal polarity in zebrafish might maintain the epithelial features of the myocardium and might be essential for apical constriction and promoting the cardiomyocyte entry into the myocardium[22].

3. Cardiomyocytes in the myocardial epithelium undergo directional migration and OCD

Tissue morphogenesis depends on the spatiotemporal arrangement of cells during development. The most well-known mechanism that contributes to the final shape of a tissue or organ is cellular intercalation in response to chemotactic cues or morphogens[25]. A less well known but essential mechanism is OCD[25]. OCD is determined by the alignment of the mitotic spindles to body shape[26]. OCD is a polarized cell division and a potential asymmetric cell division that contributes to cellular diversity and differentiation during development[27]. To determine whether OCD occurs during trabecular morphogenesis and trabecular specification, Domian's and my labs used cellular and genetic tools to examine the cellular behaviors and the underlying genetic basis of trabecular initiation. We found that cardiomyocytes undergo OCD based on immunostaining, with about 40% of the mitotic cells aligning their spindle parallel to the myocardial epithelium, and about 40% of the mitotic cells aligning perpendicularly to the myocardium epithelium at about E8.5[23,24]. Furthermore, individual cardiomyocytes genetically labeled prior to trabeculation via the inducible Cre mediated brainbow / multicolor labeling system were traced and the labeled cells/clones were analyzed to reveal that most labeled cells undergo one round of division to yield two cells by 24 hours after tamoxifen induction. In some clones, one daughter cell is at the surface of the compact zone and the other daughter is in the trabecular or inner compact zones, from which we infer that the two daughter cells resulted from a perpendicular division. In some clones, both daughter cells are at the surface of the compact zone, suggesting a parallel division. While in some clones, the labeled cells migrate away from the outer compact zone, suggesting that the labeled cells migrate and exit the surface of myocardium[24]. The *in vivo* single cell labeling further established that the cardiomyocytes in the early myocardial epithelium undergo OCD and directional migration (Fig. 3). These studies show that the trabecular morphogenesis in the mouse is different from zebrafish, in which trabeculae are initiated by directional migration but not OCD[28,29]. Whether OCD and directional migration contribute to trabecular morphogenesis needs further study, which will be discussed below.

4. Cardiomyocytes' directional migration and OCD contribute to trabecular formation.

The observed perpendicular OCD of the cardiomyocytes in the myocardial epithelium can potentially send a daughter cell into cardiac jelly, which might result in trabecular initiation. Another potential mechanism of trabecular initiation is directional migration in which some cardiomyocytes undergo cytoskeleton rearrangement, become elongated, orient perpendicularly to the heart wall, and eventually migrate into the cardiac jelly to result in trabecular initiation. In zebrafish, the trabeculae are initiated by an epithelial-mesenchymal like transition and then migrate into the cardiac lumen to initiate trabeculation[22,28]. Although OCD occurs in the myocardial epithelium of the zebrafish, it does not contribute to trabecular initiation based on observations with variety tools, including time-lapse imaging[22,28,30]. However, time-lapse observation of the cardiomyocyte cellular dynamics during mouse trabecular morphogenesis is technically challenging, and is not feasible at this moment. Instead, single cell lineage tracing was used to infer cellular dynamics during trabecular formation in our study[24]. To determine whether directional migration and perpendicular OCD contribute to trabecular formation in the mouse, cardiomyocytes were labeled at E8.0-E8.5, a stage when the myocardium consists of a single cell layer and trabeculation has not yet initiated. 72 hours after induction, the labeled single cell has undergone several rounds of cell divisions to form a clonal cluster that exhibits specific geometric patterns. Based on the geometric distribution and anatomical annotation of each clone, the clones are categorized into four different patterns (Fig. 4). In the transmural clone, labeled cells are localized to the inner compact and trabecular zones, with one cell of the clone remaining in the outer compact zone. It can be inferred that the transmural clone is likely derived from a perpendicular OCD. In the surface clone, all the cells are in the outer compact layer of the myocardium, and are derived from parallel OCD. In the third clonal type, all the cells are in the inner compact zone and/or trabecular zone, and are defined as an inner clone, and were derived from directional migration. The forth type is the mixed clone, which displays two or more cells in the outer compact zone and some cells in the inner compact or trabecular zones, which might be derived from directional migration and OCD. These results suggest that inner clone, transmural clone and mixed clones contribute to trabecular formation, and directional migration and OCD contribute to trabecular initiation (Fig. 4)[24]. Details of the trabecular formation based single cell lineage tracing studies can be found in the original study[24]. Our study is consistent with previous work using intragenic recombination or virus showing that labeled cardiomyocyte clones grow in the orientation of the transmural axis or the myocardial planar axis[31–34], which might result from perpendicular OCD and parallel division, respectively. Furthermore, our study answers the question whether the trabecular and compact cardiomyocytes specified before trabecular formation. The lineage tracing data show that some clones such as transmural clones and mixed clones contain cardiomyocytes in both compact and trabecular zone, suggesting that the compact cardiomyocytes and trabecular cardiomyocytes might be derived from the same population during cardiogenesis and are not specified before trabecular initiation.

5. Molecular mechanisms that regulate directional migration and trabecular formation.

The machinery that orients the spindle orientation is conserved from nematodes to mammals and involves an evolutionarily conserved adaptor protein LGN that binds lipid-anchored Gai at the cell cortex[35–37]. LGN interacts and recruits NuMA to form a complex at the cell cortex. This complex anchors spindle astral microtubules to the cell cortex and applies a pulling force on those microtubules through associated dynein to orient the mitotic spindle[35,36,38–40]. For the daughter cells to be properly positioned within the tissue, the position of the mitotic spindle must be tightly coordinated with the cortical polarity, and the LGN/NuMA/Gai complex has to interact with the polarity complex Par3/Par6/aPKC via Inscuteable. To establish the correct orientation of the mitotic spindle, cells respond to instructive spatial cues from their local environment to establish asymmetric localization of polarity complex: Par3/Par6/aPKC. This polarity complex binds to LGN/NuMA/Gai to establish the mitotic spindle orientation. Another essential complex required to establish mitotic spindle orientation is the adherens junction. Adherens junctions are the primary sites of epithelial cell attachment, and are essential for maintaining epithelial sheet integrity, apical-basal polarity and cytoskeleton. This complex includes E-Cadherin or N-Cadherin and cellular adaptor proteins such as different catenins and p120[41]. Previously, it was shown that loss of adherens adhesion disrupts the orientation of cell division in different tissues and species[42–46]. Our work shows that Numb Family Proteins are required to stabilize the adherens junctions and establish epicardial polarity. Epicardial cell specific deletion of Numb Family Proteins or β-catenin disrupts the epicardial cell's polarity, randomizes the epicardial cell's mitotic spindle orientation, causes less perpendicular OCD, and fewer epicardial cells entering into the myocardium[17]. Later it was found that cell–cell adhesion proteins such as E-cadherin function as an instructive cue for orientation of cell division by directing interaction with the LGN/NuMA/Gai complex. This mediates the stabilization of cortical associations of astral microtubules at cell–cell adhesions to orient the mitotic spindle [47].

During cardiogenesis, deletion of *Cdh2,* the gene encoding N-Cadherin, which is the major cadherin in cardiomyocytes, causes cardiomyocytes to be loosely associated[48], and the hearts of the knockout have an abnormal morphology and exhibit an absence of trabeculae[24]. The mitotic spindle orientation pattern of the *Cdh2* null cardiomyocytes in the heart is random and significantly different from that of the controls. However, the random mitotic orientation of the *Cdh2* null cardiomyocytes might be due to cardiac growth arrest of the knockout, and whether OCD defect contributes to the trabeculation defects is not clear. To avoid cardiac growth arrest and further study how N-Cadherin regulates trabeculation, *Cdh2* within some individual cells of $Rosa26^{CreERT2}$; $Rosa26^{Conf}$; $Cdh2^{fl/fl}$ mosaic hearts were deleted by inducing Cre activation at E7.75 and the clonal patterns and distributions of *iCdh2* clones examined 72 hours later. The *Cdh2* null clone display more surface and mixed clones, but less transmural and inner clones. In addition, the overall geometric location of *Cdh2* null clones is different from that of the control clones as the *Cdh2* null clones primarily localized to the heart surface and failed to invade as deeply into the heart as control clones, suggesting that N-Cadherin is required for invasion/migration. The reduced

number of transmural, inner and mixed clones, and reduced invasion of all the clones might contribute to the trabeculation defects of the *Cdh2* heart specific knockout and indicates that N-Cadherin regulates OCD and directional migration in a cardiomyocyte autonomous manner[24]. In zebrafish, although OCD does not contribute to trabeculation, the dynamic localization of N-Cadherin is required for cardiomyocyte entry toward the cardiac lumen to initiate trabeculation[49]. Whether N-Cadherin relocalizes during mouse trabeculation is not clear. In zebrafish, apical constriction mediated directional migration does not take place in the absence of Nrg/ErbB signaling, blood flow and cardiac contractility[22]. Whether Nrg1/ErbB2/4 regulates cardiomyocytes entry into myocardium via cardiomyocyte polarity and OCD in the mouse is unknown.

6. Compact and trabecular cardiomyocytes display different expression profiles, which might contribute to the differential maturation of the trabecular and compact cardiomyocytes.

Trabecular cardiomyocytes, which take the major responsibility for pumping at early stages of cardiac development, are more differentiated than compact zone cardiomyocytes[3]. Trabecular and compact cardiomyocytes display different features with trabecular cardiomyocytes exhibiting a lower proliferation rate and being more molecularly mature than cardiomyocytes of the compact zone[50]. These zones can also be distinguished by the differential expression of many genes. For example, *p57*, *Irx3, BMP10,* Sphingosine 1-phosphate receptor-1 and *Cx40* are highly expressed in the trabecular zone, while *Tbx20*, *Hey2,* and *N-Myc* are highly expressed in the compact zone[3,24,51–55]. While these genes are specifically expressed in trabecular or compact cardiomyocytes at certain stages, the functions of each gene in the two zones are not clearly elucidated so far.

We adapted an RNAscope ISH/IFS system, in which single mRNAs and proteins can be detected simultaneously, to determine that BMP10 and Hey2 are expressed in trabecular zone and compact zone, respectively[24]. BMP10 is required to regulate cardiomyocytes proliferation via inhibiting the expression of p57, a major cell cycle inhibitor in the embryonic heart, to regulate cardiomyocyte proliferation[53]. Therefore, the finding that BMP10 is expressed in the trabecular zone, where cardiomyocytes proliferate at a slower rate comparing to cardiomyocytes in the compact zone, is surprising and suggests that differential BMP10 expression is not responsible for the differential proliferation rates between the compact and trabecular zones. The mechanism for how BMP10 regulates cardiomyocyte proliferation will need further study.

The asymmetric expression of *Hey2* might contribute to the different features of trabecular and compact cardiomyocytes. Recent work using the heart specific *Hey2* knockout shows that *Hey2* plays a role in cardiomyocyte specification or maturation. Cardiac specific *Hey2* knockout hearts display ectopic atrial gene expression[56,57]. *Hey2* null cardiomyocytes displayed abnormal mitochondria, abnormal accumulation of glycogen particles, disorganized myofibrils, and increased expression of β-MHC and ANF genes [58], indicating defects in differentiation and maturation. These defects in *Hey2* null cardiomyocytes might be the cause of the dilated left ventricular chamber with markedly diminished fractional

shortening of the left ventricle observed in these mice [58]. These studies indicate that *Hey2* might be involved in cardiomyocyte maturation during heart development and trabecular specification, specifically to repress cardiomyocyte maturation. This is supported by the observation that *Hey2* deletion in cardiomyocytes promotes cardiomyocyte maturation and is consistent with the observation that compact cardiomyocytes are less mature than cardiomyocytes in the trabecular zone.

7. OCD contributes to trabecular specification via a mechanism of extrinsic asymmetric cell division

The differential expression profiles of trabecular and compact markers might contribute to their distinct differentiation and maturation as discussed above; however, the underlying mechanism of how the markers are differentially expressed is unknown [3,51,52]. One potential mechanism contributing to the differential expression of *Hey2* and *Bmp10* in trabecular and compact cardiomyocytes would be the asymmetric distribution of *Hey2* and *Bmp10* in cardiomyocytes that undergo perpendicular OCD. To determine whether perpendicular OCD contributes to regional specification, E9.5 heart sections were stained with acetylated α-Tubulin and p120 (an adherens junction-associated protein that marks the membrane) and hybridized with probes to *Bmp10* or *Hey2* mRNA[24]. The number of signal dots or signal intensity in dividing cells were counted and the ratio between the two domains of the dividing cell at telophase or the two daughter cells after the division was calculated. We found that the mitotic cells undergoing perpendicular divisions did not display asymmetric distribution of *Hey2*. Our work showed that asymmetric expression of *Hey2* and *Bmp10* occurs after but not before cytokinesis in perpendicular OCD, and that their asymmetric distributions are due to the differences in geometric location between the two daughter cells. In parallel divisions, asymmetric distribution of Hey2 and BMP10 is not observed. The daughter cell closer to cardiac jelly in perpendicular oriented cell division displays less *Hey2* and more *BMP10* compared to the other daughter cell that is relatively closer to the surface of the heart, indicating that potential instructive cues for trabecular regional specification might lie in the cardiac jelly and endocardium (Fig. 5).

8. Future directions.

With the essential functions of trabeculae well established but the mechanisms of trabecular formation and compaction unclear, there are many questions that need to be further studied. For instance, are the cardiomyocytes that undergo OCD or directional migration are predetermined before trabecular initiation? Many signaling pathways or molecules, including NRG1/ErbB2,4[6,59,60], Notch signaling[61], Hand2[62], YAP[63,64], DAAM1[65], and Numb Family Proteins[66,67] regulate trabecular initiation. Recent work shows that signaling pathway such as Notch1 and NRG1/ErbB2 regulates the expression level of Hey2, but not the asymmetric distribution between the compact and trabecular zones[55]. The signaling pathways regulate the asymmetric distribution of *Hey2* or other genes with differential expression between the two zones remains unknown. Also unknown is whether the expression of different trabecular markers are regulated by the same signaling pathways. Furthermore, in zebrafish, apical constriction or cardiomyocyte depolarization does not take

place in the absence of Nrg and ErbB signaling or blood flow and cardiac contractility. Whether Nrg1/ErbB2/4 regulate cardiomyocytes polarity to control their entry into myocardium in the mouse is unknown. With the genetic tools such as, *Hey2*^{CreERT2}, which is specifically active in compact zone at early embryonic stage, and *Nppa*^{CreERT2}, which is specifically active in trabecular cardiomyocytes[68], to separate trabecular and compact cells being available, more definitive experiments can be designed to reveal the mechanisms of trabeculation and compaction in the future.

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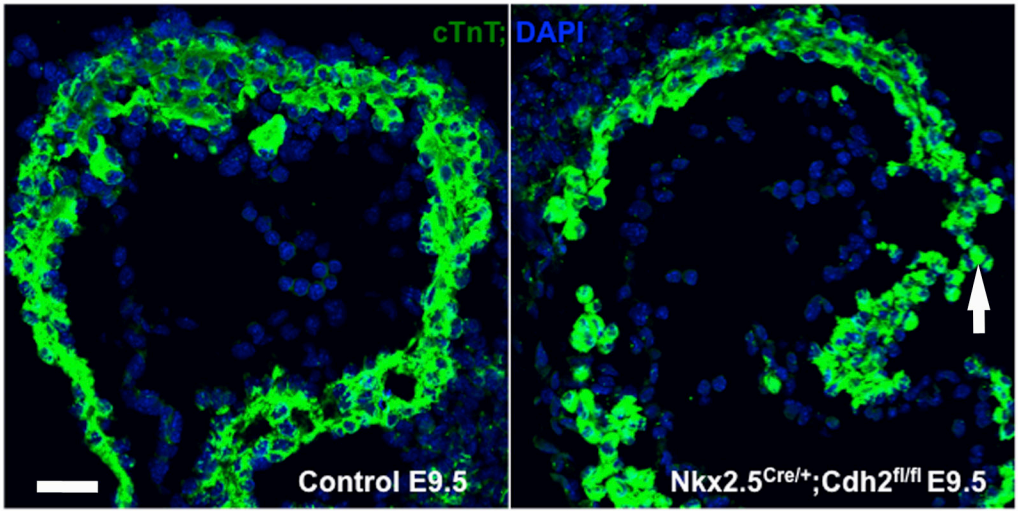

Figure 1. N-Cadherin is required to establish the polarity of the monolayer myocardium.

In the control heart at E9.5, cardiomyocytes migrate inward toward the heart lumen, while some cardiomyocytes in the *Nkx2.5*$^{Cre/ERT2}$*; Cdh2*$^{fl/f}$ migrate outward to the pericardial sac indicated by while arrow. Scale bar is 20 μm.

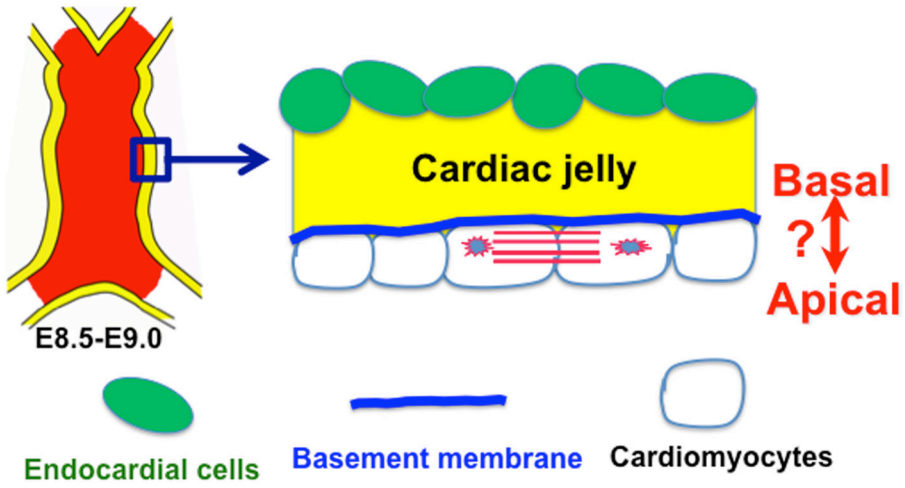

Figure 2. The monolayer of myocardium displays apical-basal polarity.

Mouse embryonic heart tube at E9.5-E9.0 has a smooth inner surface and contains a monolayer myocardium and a monolayer endocardium. The monolayer myocardium displays transmural polarity with basement membrane inside the heart lumen. ?: The orientation of apical-basal polarity shows some controversy, as a group says the domain that abuts the heart lumen is the apical domain or basal domain by another group.

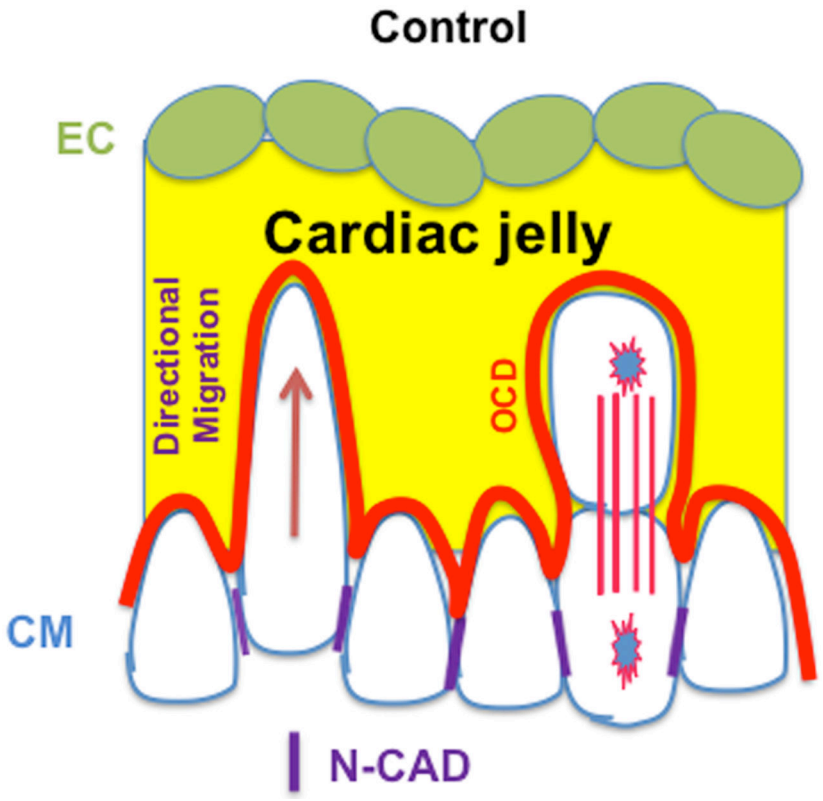

Figure 3. Directional migration and OCD contribute to trabecular initiation.

Both directional migration and OCD contribute to trabecular initiation, and N-Cadherin might be essential to establish the apical-basal polarity for directional migration and OCD. This figure is adapted from a published figure[24].

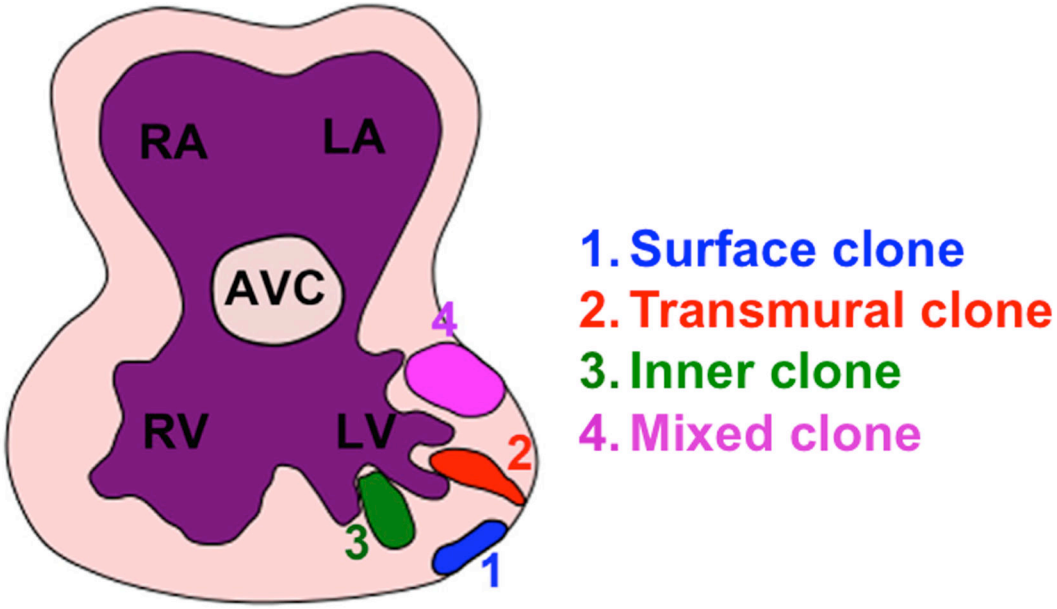

Figure 4. The transmural, inner and mixed clones contribute to trabecular formation.
Cells of surface clones localize to the surface of the myocardium. Cells of inner, transmural and mixed clones contribute to the formation of trabeculae and inner compact zone. This figure is adapted from a published figure[24].

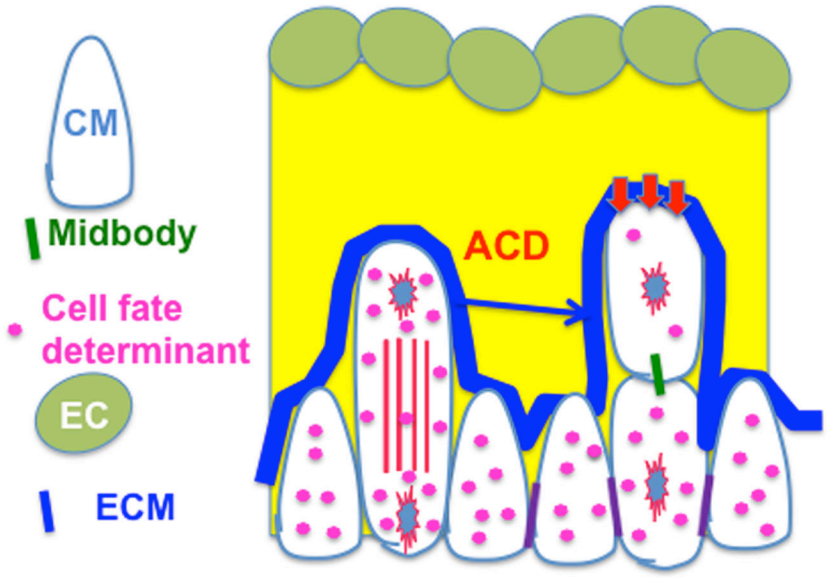

Figure 5. Perpendicular OCD is an extrinsic asymmetric cell division.

Cell fate determinants are symmetrically distributed to a dividing cell at telophase, but asymmetrically between the two daughter cells that are still linked by midbody, indicating the extrinsic asymmetric cell division. This figure is adapted from a published figure[24].